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Age and body size of the toad *Bombina maxima* in a subtropical high-altitude population

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We used skeletochronology to determine age and body size of large-webbed bell toads (*Bombina maxima*) from a subtropical, high-altitude population in southwestern China. Mean age did not differ between the sexes. Minimum age at sexual maturity was two years for both sexes, and maximum longevity was six years for males and five years for females. Males were larger than females, also when accounting for the effect of age. A significant relationship between age and body size was only found for males. Sex showed no interaction with age, and age-size relationships did not differ between the sexes. These results suggest that longevity is an important factor accounting for differences in body size between the sexes.

Key words: age-size relationship, age structure, China, longevity, skeletochronology

It is well documented that life-history traits such as age and body size in amphibians are influenced by elevation. Skeletochronology, which uses bone cross-sections for age determination, is an important method to describe individual growth trajectories without the necessity for mark-recapture data over long periods (Hasumi, 2010). This technique has been successfully used recently for age determination in a variety of Chinese anurans (temperate zone, Lu et al., 2006; Liao, 2011; subtropical zone, Lai et al., 2005; Liao & Lu, 2010a, b; Liao et al., 2010; Liao & Lu, 2011; Liao et al., 2011; Liao & Lu, 2012; Chen et al., 2012). The large-webbed bell toad (*Bombina maxima*) is endemic to Sichuan, Yunnan and Guizhou in China, with an altitudinal range between 2000 and 3300 m a.s.l. (Fei & Meng, 2005). Its natural habitats are swamps, marshes, springs, arable land, canals and ditches. Adults emerge from hibernation in spring and breed in aquatic sites from early May until early June (Fei & Ye, 2001). To obtain more detailed information on life-history traits for *B. maxima* we used skeletochronology to obtain data on age and body size at a population near Lugu Lake, Sichuan Province.

The field study was conducted in the vicinity of Lugu Lake, 2685 m a.s.l., southwestern China (100.82°E, 27.72°N). The lake is located in the northwest of the Yunnan plateau in the centre of Ningliang Yi, China and forms the

border between Ningliang County of Yunnan Province and Yanyuan County of Sichuan Province. We captured a total of 69 individuals (40 males and 29 females) by hand at night near rivulets during the breeding season of 2012. We sexed these individuals by secondary sexual characteristics (nuptial pads in adult males and ova in adult females). Following capture, we measured body size (snout-vent length, SVL) of each individual to the nearest 0.02 mm using vernier callipers. We clipped the second toe from the right limb at the articulation proximal to the third phalanx. We fixed the clipped toes in 10% neutral buffered formalin until sectioned.

We conducted all skeletochronological procedures according to Liao & Lu (2010a), staining bone tissues before embedding in paraffin. The muscles and skin of the selected digits were removed and washed in water for 2 hours. They were decalcified in 3% nitric acid for 3 hours and then washed in running tap water overnight. Decalcified digits were stained for 200 min in Harris's haematoxylin. Subsequently, these stained bones were dehydrated through successive ethanol stages of 70, 80, 95 and 100% for approximately 1 hour in each concentration. Phalanges were then processed for paraffin embedding in small blocks. Cross-sections (13µm thick) with the smallest medullar cavity and the thickest cortical bone were selected and mounted on glass slides. We inspected the sections through a Moticam2006 light microscope and photographed them using a Motic BA300 digital camera at ×400 magnification. The analysis of lines of arrested growth (LAGs) was independently performed by two people with previous experience (H.Q. Zhu & L. Jin), and the results were compared until consensus was reached. Endosteal resorption in anurans affects the accuracy of counting LAGs (Hemelaar & Van Gelder, 1980), and can be confirmed by observing the presence of the Kastschenko Line (KL; the interface between the endosteal and periosteal zones; Rozenblut & Ogielska 2005). The complete resorption of the innermost LAG is also confirmed based on the difference in the diameter between LAGs and KL (Liao & Lu, 2010a).

Statistical tests were performed using SPSS v.17.0. We used Student's *t* - tests to compare a difference in body size and age between males and females and Kolmogorov-Smirnov tests to identify age structure between the sexes.

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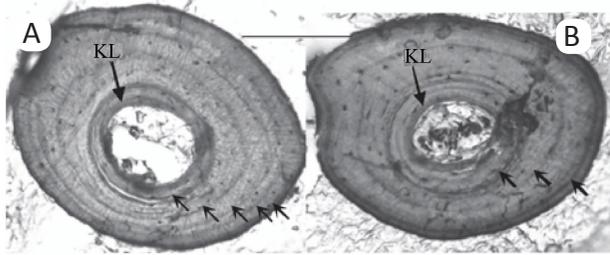


Fig. 1. Two examples (A: 5-yr old male; B: 3-yr old female) of stained cross-sections of the phalangeal bone in the large-webbed bell toad (*Bombina maxima*). Arrows indicate the lines of arrested growth (LAG). KL represents Kastschenko Line, the division line between endosteal and periosteal zones. Scale bar: 100 μ m.

We also compared body size between sexes with analysis of covariance (ANCOVA) using age as the covariate. We assessed interaction between age and sex using general linear models (GLMs), treating body size as a dependent variable and age and sex as fixed factors. The relationship between body size and age was analyzed using linear regression. All values given are mean \pm SD, and the level of significance was $p < 0.05$.

LAGs appeared in periosteal tissue separated from the endosteal tissue by a Kastschenko Line (Fig. 1). Double LAGs were very rare. Endosteal lamellar bone, filling the medullar cavity, showed a smaller or similar number of LAGs, chronologically equivalent to LAGs of the periosteal bone. These endosteal LAGs were not used for age estimation. Part of the periosteal bone was actually resorbed, and the resorption process was enhanced with increasing age. We found that complete resorption of the innermost LAG occurred in one female, so one year was added to estimate her true age.

Minimum age at sexual maturity was two years in both sexes. Maximum longevity reached six years for males and five years for females (Fig. 2). The average age of males and females was 3.5 ± 1.1 years and 3.2 ± 0.8 years, respectively. Age distributions did not differ significantly between the sexes (Kolmogorov-Smirnov test: $D = 0.90$,

$p = 0.39$), and the mean age was significantly different ($t = 1.83$, $p = 0.07$). We have no data on the SVL of one female. Males were significantly larger than females (males: mean \pm SD = 56.0 ± 5.0 mm, range 41.1–63.1, $n = 40$; females: 52.0 ± 5.9 mm, range 39.4–60.8, $n = 28$; $t = 2.94$, $df = 66$, $p = 0.005$, Table 1). The difference in body size between the sexes remained significant when removing the effect of age (ANCOVA: $F_{1,66} = 8.67$, $p = 0.004$). A significant relationship between age and body size was found for males, but not for females (Fig. 3). The GLMs revealed a non-significant interaction between sex and age ($F_{1,66} = 0.22$, $p = 0.88$). Age-size relationships between the sexes did not differ in slope, suggesting that males and females had a similar growth pattern.

The use of phalanges allowed the estimate of age and growth without sacrificing the study animals. The technique could be successfully applied for *B. maxima* due to a clear arrest of growth during hibernation. We found complete endosteal resorption occurring in one individual according to the presence of the Kastschenko Line. In rare cases, a double line may be mistaken for two LAGs, resulting in an overestimation of the age of the sample being examined, or a LAG near the outer edge of the bone may simply be indistinct and overlooked (Özdemir et al., 2012). We did not find double lines and false lines in the present study (when present, false lines are fainter than true LAGs and do not form a complete ring around the bone section; they might result from injury or reduced food supply).

Growth trajectory models can elucidate the determinants of life-history traits such as age at sexual maturity and sexual size dimorphism (SSD) (Shine, 1979; Halliday & Tejedo, 1995; Hasumi, 2010). Minimum age at sexual maturity was the same for males and females. This has previously been observed in other anurans (*Hyla annectans chuanxiensis*: Liao & Lu, 2010b; *Rana nigromaculata*: Liao et al., 2010; *Rana amurensis*: Liao, 2011; *Rana limnocharis*: Liao et al., 2011; *Bufo andrewsi*: Liao & Lu, 2012), although in some species males can also reach sexual maturity one year earlier than females (*Rana*

Table 1. Body size (snout–vent length in mm) of male and female *Bombina maxima* for each age class using Mann–Whitney U - test, expressed as mean \pm SD (range and sample size in brackets).

Age (yrs)	Females	Males	Z	p
2	48.4 \pm 7.8 (39.4–53.5 $n = 6$)	53.1 \pm 9.0 41.1–63.9 $n = 5$	1.10	0.27
3	52.1 \pm 5.1 37.7–46.0 $n = 12$	54.4 \pm 4.5 45.3–61.9 $n = 16$	1.25	0.21
4	54.0 \pm 5.6 46.1–60.8 $n = 9$	58.0 \pm 3.0 49.6–60.5 $n = 9$	1.19	0.23
5	51.90 $n = 1$	57.5 \pm 3.7 50.9–63.1 $n = 8$		
6		59.9 \pm 0.9 59.5–60.5 $n = 2$		

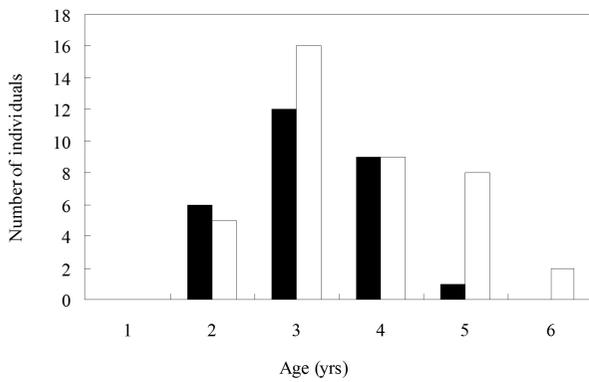


Fig. 2. Age structure (male, close bars; female, open bars) in the large-webbed bell toad (*Bombina maxima*).

temporaria, Miaud et al., 1999, *Amolops mantzorum*, Liao & Lu, 2010a). Previous studies have shown that females can attain a higher longevity than males (*Rana swinhoana*, Lai et al., 2005; *Rana nigromaculata*, Liao et al., 2010; *B. andrewsi*, Liao & Lu, 2012), whereas in our study the oldest individual was a male.

The male-biased SSD found for *B. maxima* is rather unusual for anurans (Shine, 1979; Monnet & Cherry, 2002; for another example see *R. amurensis*: Liao, 2011), while it is a regular occurrence in urodeles (*Triturus vulgaris*: Halliday & Verrell, 1988; *Ommatotriton ophryticus*: Kutrup et al., 2005; *Mertensiella caucasica*: Üzümlü, 2009). Sexual size dimorphism results from differences in age structure and growth rates between the sexes (Khonsue et al., 2001, Monnet & Cherry, 2002, Lu et al., 2006). In our study, age was a major factor affecting SSD, due to males being significantly older than females. Age is positively correlated with size in most anurans (Liao & Lu, 2010a; Liao et al., 2010; Liao & Lu, 2011; Liao, 2011). For *B. maxima* there was a significant relationship between age and body size in males, but not in females. Differential age-size interactions for males and females have been reported previously (Gibbons & McCarthy, 1984; Leclair & Castanet, 1987; Cherry & Francillon, 1992).

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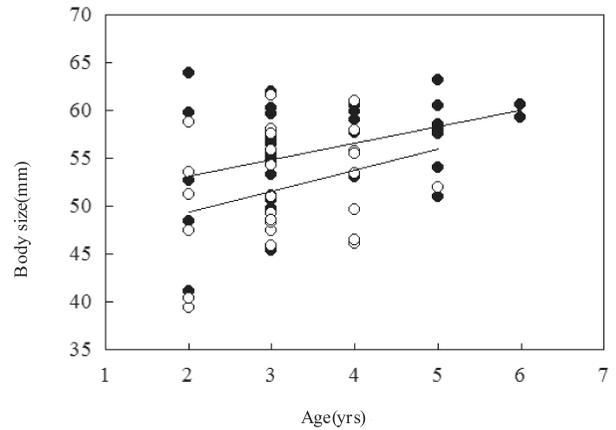


Fig. 3. Relationships between age (x-axis: yrs) and body size (y-axis: mm) for male (solid circles: $y=1.73x+49.63$; $r^2=0.38*0.38$, $p=0.013$) and female (open circles: $y=2.22x+44.79$; $r^2=0.31*0.31$, $p=0.12$) large-webbed bell toads (*Bombina maxima*).

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