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A skeletochronological estimate of age in a population of the Siberian Wood Frog, *Rana amurensis*, from northeastern China

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Abstract. I used skeletochronology to estimate age structure of the Siberian Wood Frog, *Rana amurensis,* from northeastern China. Average age did not differ significantly between males and females. Age at sexual maturity in both males and females was 1 year. For both sexes the maximum age observed was 4 years. Average body size differed significantly between the sexes, with males being larger than females. A significantly and positively correlation between body size and age was found within each sex in the population. When the effect of age was controlled, males also had larger size than females, suggesting that sexual selection for larger males might improve male mating success.

Keywords. Siberian Wood Frog, skeletochronology, age, body size.

INTRODUCTION

The Siberian Wood Frog, *Rana amurensis,* is widely distributed in west and east Siberia, the Russian Far East, Korea, northern and central Mongolia, and northeastern China (Terent'ev and Chernov, 1965; Kuzmin, 1999; Zhao and Adler, 1993). According to IUCN's evaluation *R. amurensis* cannot be regarded as a declining species (Kuzmin et al., 2008). This species occurs most frequently in open, wet places such as wet meadows, swamps, overgrown shores of lakes, riverbanks, and open areas in forests with abundant vegetation and arboreal debris (Zhao and Zhao, 1994). Breeding activity occurs in shallow lakes, ponds, large puddles and swamps with stagnant water (Fei, 1999). Hibernation mainly occurs from early October to April-May (Zhao and Adler, 1993). Reproduction takes place from March-April (usually May elsewhere), whereas in cold northern areas the breeding season may extend until mid-July (Fei, 1999). So far, information about demographic data on the species remains largely unknown.

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Seasonal climate variation affects the physiological activities of amphibians (i.e. bone growth) in temperate regions, resulting in formation of line of arrested growth (LAG) (Smirina, 1972). Skeletochronology, i.e., recording the periods of arrested growth in long bones like the phalanges (Castanet & Smirina, 1990), is an effective tool and has been widely used to evaluate age and growth of amphibian populations in natural conditions. This method has also applied to many anurans species from widespread regions (temperate, Hemelaar, 1988; Miaud et al., 1999; Lu et al., 2006; Matthews and Miaud, 2007; Guarino and Erismis, 2008; Ma et al., 2009; tropical, Khonsue et al., 2000; Pancharatna and Deshpande, 2003; subtropical, Lai et al., 2005; Liao and Lu, 2010a, b, c; Liao et al., 2011; Li et al., 2010).

Here, I used skeletochronology to assess individual age, growth and longevity the Siberian Wood Frog, *Rana amurensis*, from northeastern China where these demographic parameters were unavailable. My aims were to compare age structure, body size and growth between males and females and gain insight into the mechanisms determining sexual size dimorphism.

MATERIAL AND METHODS

The population was located in a lake of Shamajie town in Zalantun City (47°58'N, 122°46'E), at an altitude of 300 m a.s.l., in inner Mongolia Autonomous region of northeastern China, where all individuals hibernated in holes at the bottom of the lake in groups. Being in a temperate region, the study area has a strongly seasonal climate. Monthly average temperature ranges between -25.7 °C and 22.8 °C. Annual average temperature is 2.4 °C and annual total precipitation is 480 mm. The typical vegetation around the lake includes Zhang child pine, *Pinus sylvestris*, spruce, *Picea asperata*, mountain willow, *Populus davidiana*, Yuehua Tree, *Betula dahurica* and black birch, *B. pendula*.

All frogs were captured in holes of the lake from mid-December 2009 to early January 2010 when they were in hibernation. Adult specimens were sexed by their secondary sexual characteristics (nuptial pads in male, evidently swollen belly due to egg masses in female). In the absence of nuptial pads and swollen belly individuals were classed as juveniles. Body size was measured using a caliper to the nearest 0.1 mm. The third phalanx of longest toe of the right hind limb was clipped, and stored in 10% neutral buffered formalin for skeletochronology. In order to minimize disturbance of frogs during hibernation, we quickly collected data and released the individuals at the points of capture.

Each individual digit was cleaned of surrounding tissues of the phalanx, and then put in 5% nitric acid to decalcify for 48 h. Decalcified digits were stained for 200 min in Harris's haematoxylin. Subsequently, stained bones were dehydrated through successive ethanol stages of 70, 80, 95, and 100% for approximately 1 h in each concentration. Phalanges were then processed for paraffin embedding in small blocks. Cross-sections (13 μm) thick were obtained by means of rotary microtome, and the phalanx with the smallest medullar cavity was selected and mounted on glass slides. I observed the sections though a light microscope and photographed the best of them using a Motic BA300 digital camera mounted on a Moticam2006 light microscope at × 400 magnifications. The analysis of lines of arrested growth (LAGs) was performed by two persons (WB Liao and ZP Mi) with previous experience of the technique. I confirmed the endosteal resorption of LAGs in this species based on the presence of the Kastschenko Line (KL; the interface between the endosteal and periosteal zones; Rozenblut and Ogielska 2005).

Sexual size dimorphism was described by SDI index (Lovich and Gibbons, 1992). The equation form was: SDI = (mean length of the larger sex/mean length of the smaller sex) ±1 (SDI = 0 when both sexes are of similar size, SDI > 0 when females are larger than males, SDI < 0 when males are larger than females).

I assessed interaction between age and sex using general linear models (GLMs) treating SVL as a dependent variable, and age and sex as fixed factors. I used Student's *t*-test to compare differences in body size and age between males and females and Kolmogorov-Smirnov test to identify age structure between the sexes. The relationship between body size and age was analyzed using linear regression. I also conducted ANCOVAs treating SVL as a dependent variable, with age as covariate to see significance of differences in body size between sexes when the effects of age were controlled. All values given are shown as mean ± SD, and the level of significance was $P < 0.05$.

RESULTS

I captured 92 individuals (45 males, 46 females and 1 juvenile) in this population. Of 91 adult specimens, all individuals exhibited clear Lines of arrested growth (LAGs) in their cross sections phalanges (Fig. 1). An endosteal resorption of LAG that completely eroded the first (innermost) periosteal LAG was observed in one female and one year was

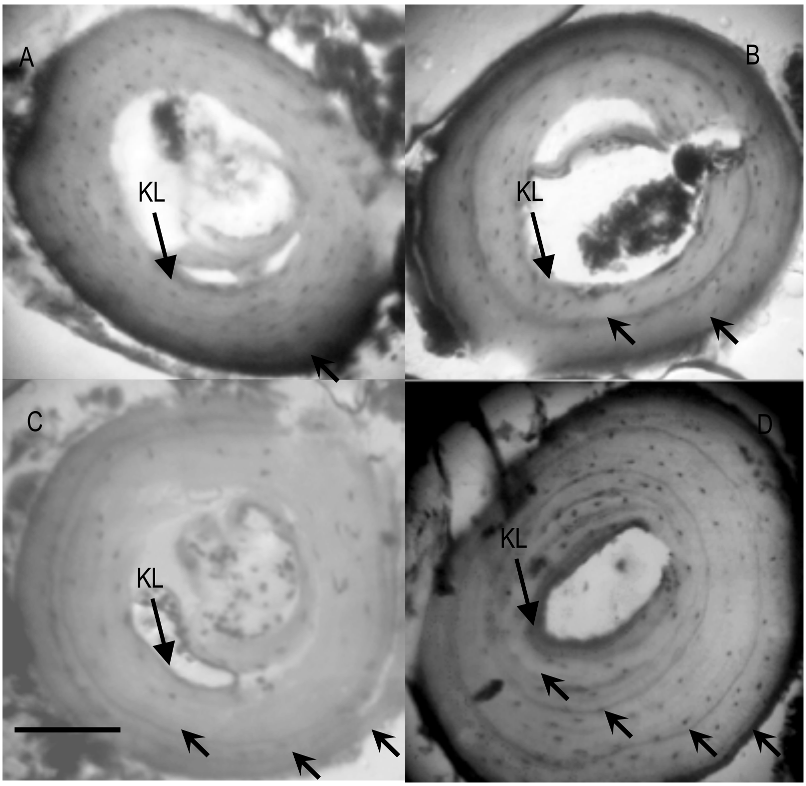

Fig. 1. Four hematoxylin-stained cross sections of the phalangeal bone (A: a 1-yr old male; B: a 2-yr old female; C: a 3-yr old female; D: a 4-yr old male) in adult Wood Frog, *Rana amurensis,* from northeastern China. Arrows indicate the lines of arrested growth (LAG). KL represents Kastschenko Line, the interface between the endosteal and periosteal zones. Scale bar: 150 μm.

added as her true age. Very close space of hematoxylinophilic lines (double LAGs) and false lines were not observed.

I found some individuals that had reached sexual maturity before entering hibernation in the year they metamorphosed. The smallest sexually mature males had a mean SVL of 42.5 mm. The smallest gravid females had a mean SVL of 39.5 mm. I found only one immature individual that was 1 year old and had SVL of 29.8 mm, far away from the average minimum for sexually mature 1-year-old frogs (42.5 mm).

Adult age ranged from 1 to 4 years in both males and females (Fig. 2). Age distribution did not differ significantly between males and females (Kolmogorov-Smirnov test: D = 0.35, P = 1.00). Average age did not differ significantly between males and females (males, 1.8 ± 1.0 yr; females, 1.6 ± 0.8 mm; Student's t-tests: t = 1.04, P = 0.30).

There was significant difference in average body size between males and females, with males having larger size than females (males, 45.9 ± 5.8 mm; females, 41.5 ± 5.3 mm; Student's t-tests: t = 3.79, P < 0.001). The sexual dimorphism index (SDI) with body size was –0.106. There was also a significant difference in body size between females and males in both 1 and 2 age groups (Table 1; all P < 0.05). The ANCOVAs analysis revealed that the difference in body size between the sexes remained significant ($F_{1,91}$ = 18.28, r^2 = 0.57, P < 0.001) when the effect of age was controlled ($F_{1,91}$ = 89.18, P < 0.001). Average body size in *R. amurensis* males was 60.6 ± 2.9 mm and that of females was 67.7 ± 4.6 mm.

Linear regression analysis showed that significant relationships between age and body size were found within each sex (Fig. 3; males, body size = 4.45 age + 38.12, $F_{1,44}$ = 49.92, r = 0.73, P < 0.001; females, body size = 4.61 age + 34.32, $F_{1,45}$ = 38.00, r = 0.68, P < 0.001). The ANCOVA analysis revealed a non-significant interaction between sex and age ($F_{1,91}$ = 1.28, P = 0.21), and age-size relationships between the sexes do not differ in slope, suggesting that males and females have a similar growth pattern.

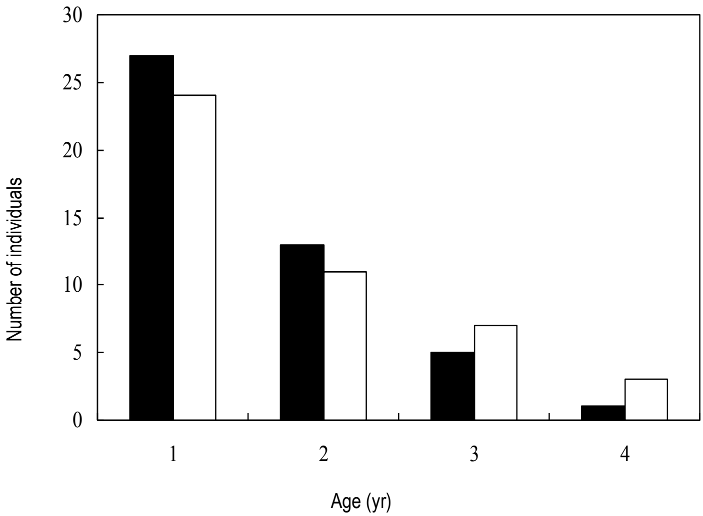

Fig. 2. Adult age structure (male, open bars; female, close bars) in the Siberian Wood Frog, *Rana amurensis,* from northeastern China.

Table 1. Difference in body size (SVL, mm) within each age group in the the Siberian Wood Frog, *Rana amurensis,* from northeastern China. Values in descending order are mean ± SD, range and sample size.

Age class	Females	Males	Z	P
1	39.5 ± 3.9 30.3-47.6 n = 27	42.5 ± 4.0 31.3-47.7 n = 24	2.83	0.005
2	41.7 ± 2.5 37.7-46.0 n = 13	46.9 ± 4.3 38.7-52.3 n = 11	2.75	0.006
3	48.8.0 ± 5.2 42.3-55.0 n = 5	52.0 ± 4.0 46.7-59.1 n = 7	0.73	0.47
4	58.4 n = 1	55.2 ± 3.9 51.2-58.9 n = 3		

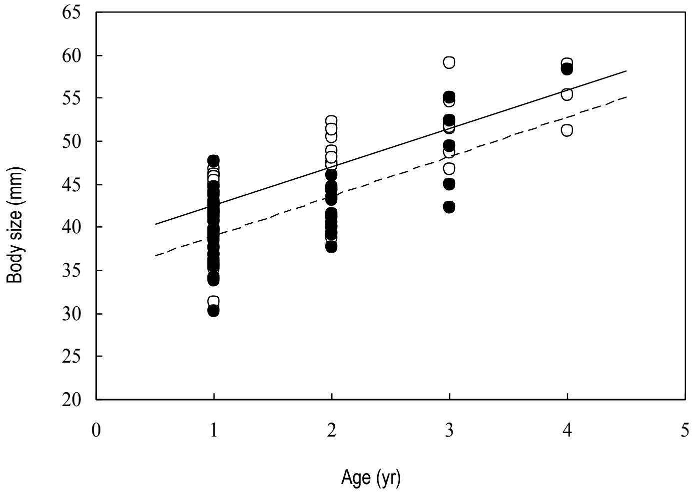

Fig. 3. The significant positively correlation between age and body size (male, open circles; female, close circles) in the Siberian Wood Frog, *Rana amurensis,* from northeastern China.

DISCUSSION.

Skeletochronology has been widely used to estimate age in amphibians (Castanet, 2002), and it can also estimate accurately age structure in *R. amurensis.* For temperate species, growth-marker formation is thought to be a result of seasonal climate changes because the changes will affect bone growth, resulting in an active growth period during the warm season followed by reduced or arrested bone growth during the cold season (Smirina, 1972). The narrow hematoxylinophilic line, called line of arrested growth (LAGs) in bone sections of *R. amurensis* are likely formed as a consequence of its prolonged period of hibernation.

Age at maturity was estimated at 1 year in males and females, suggesting that individuals would spend more time in reproduction. This pattern is similar to the short-living anuran species (*R. chensinensis,* Lu et al., 2006, *R. epeirotica,* Tsiora and Kyriakopoulou-Sklavounou, 2002, *R. nigromaculata,* Liao et al., 2010, *R. limnocharis,* Liao et al., 2011, *Hyla annectans chuanxiensis,* Liao and Lu, 2010b; *Bufo andrewsi,* Liao and Lu, 2011a). However, some studies suggest that males reach sexual maturity one year earlier than females (*R. temporaria,* Miaud et al., 1999, *Amolops mantzorum*, Liao and Lu, 2010a, *Bufo andrewsi*, Liao, 2009). In the present study, the maximum age was estimated at 4 years within each sex. Contrary to this, the maximum age was determined as 5-11 years old in other regions (Kuzmin, 1999; Zhao and Adler, 1993; Chen and Lu, 2011). I found illegal collections actually existing from studied locality. Moreover, mass mortality in hibernacula may occur in study population due to climate condition history (Kuzmin and Maslova, 2003).

Sexual size dimorphism is observed in amphibians (Shine, 1979), and females are often larger than males in most anurans (Monnet and Cherry, 2002). In this study, I found that *R. amurensis* males have larger body size than females. This is in agreement with previous studies for difference in body size between sexes in the Caudata *Triturus vulgaris* (Halliday and Verrell, 1988), *Ommatotriton ophryticus* (Kutrup et al., 2005) and *Mertensiella caucasica* (Üzüm, 2009). Several studies on anurans suggest that sexual size dimorphism results from differences in age structures and growth rates before attainment of maturity between the sexes (Khonsue et al., 2001; Monnet and Cherry, 2002; Lu et al., 2006). For *R. amurensis,* ANCOVA revealed age being a major factor affecting sexual size dimorphism because of males having older age than females. However, growth rate did not affect sexual differences in body size due to a similar growth rate for both sexes. Moreover, sexual selection for body size is one such factor where increased male body size is an important determinant of male mating success (Üzüm, 2009).

As in most anurans (Ryser, 1996; Lu et al., 2006; Liao and Lu, 2010a; Liao et al., 2010; Liao and Lu, 2011b), the age of adult frogs is positively correlated with their size within each sex. However, in other anurans a positive correlation may be true for only one sex (Gibbons and McCarthy, 1984; Leclair and Castanet, 1987; Cherry and Francillon, 1992).

Age, body size and growth rate changes with altitude or latitude in most anurans, individuals from high-altitude or latitude populations having later age at sexual mature, longer longevity, slower growth rate and larger body size than low-altitude or latitude populations due to lower temperature and smaller food availability in high altitude or latitude (Miaud et al., 1999; Lu et al., 2006; Matthews and Miaud, 2007; Liao and Lu, 2010a). For *R. amurensis*, age at sexual mature, longevity and body size will increase with increasing altitude or latitude (Ishchenko, 1996; Zhao and Adler, 1993; Kuzmin et al., 2008). Chen and Lu (2011) found that the maximum age of males and females at the 900-m population was 6 and 7 years, and 5 and 7 years at the 500-m population. This pattern suggests that lower temperature and smaller food availability from cold region may increase longevity and size. However, age at first reproduction in *R. amurensis* was 2 years for males and 3 years for females in both populations at different altitude. The finding may be related to small samplings in both populations.

Although this species has been threatened by general habitat loss (such as the construction of dams on large rivers) and the drainage and pollution of breeding pools

(Kuzmin et al., 2008), no historical information is available about *R. amurensis* population in northeastern China. The 2-3 year as the dominant class in *R. amurensis* is consistent with that displayed by two populations in northeastern China (Chen and Lu, 2011). Moreover, there is significant over harvesting of this species for food, especially in northeastern China where illegal collection has increased since the 2000s. In this population, I did not find older and larger individuals. Therefore, conservation attention should be taken to restore the habitats and reduce human disturbance. This study provides the baseline information on demography in *R. amurensis* and will be useful for their conservation.

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