

# Skeletochronological Study of Age, Longevity and Growth in a Population of *Rana nigromaculata* (Amphibia: Anura) in Sichuan, China

Min MAO<sup>1,2</sup>, Yan HUANG<sup>1,2</sup>, Zhiping MI<sup>1,2</sup>, Yanhong LIU<sup>1,2</sup> and Caiquan ZHOU<sup>1,2\*</sup>

<sup>1</sup> Key Laboratory of Southwest China Wildlife Resources Conservation (Ministry of Education), China West Normal University, Nanchong 637009, Sichuan, China

<sup>2</sup> Institute of Rare Animals and Plants, China West Normal University, Nanchong 637009, Sichuan, China

**Abstract** A skeletochronological study was conducted for the age, longevity and growth of a *Rana nigromaculata* population in northeastern Sichuan, China. Of 139 specimens, 119 (68 males and 51 females) exhibited distinct arrested growth lines in phalanges. Age at first reproduction was found to be two years of age for males and three for females. Maximum longevity was estimated to be at least six years in males and seven years in females. Average age did not differ between males and females. Breeding females were significantly older than breeding males. Females were larger in body size and heavier in body mass than males. Age was not correlated with body size and body mass in males, but a significant correlation was found among age, body size and body mass in females. The growth curve appeared significantly different between the sexes, with a higher growth rate throughout life and a larger asymptotic size in females. Moreover, analysis of the growth model indicated that the population studied was relatively stable.

**Keywords** age structure, *Rana nigromaculata*, population, skeletochronology

## 1. Introduction

Anthropogenic alteration of the environment has arguably propelled the Earth into its sixth mass extinction event and amphibians are at the forefront (Rohr *et al.*, 2008). Amphibian population declines at regional and global scales have recently been the focus of attention across the scientific community (Blaustein *et al.*, 1994; Carrier and Beebee, 2003; Wake and Vredenburg, 2008; Rovito and Masucci, 2009). More than 32% of amphibian species are threatened and more than 43% are experiencing some form of population decline (Stuart *et al.*, 2004). Investigations related to monitoring and conservation of amphibian populations have increased within the last decade (Young *et al.*, 2001). Conservation based on the biological understanding of a species' ecology including its life history traits can be largely promoted (Eaton *et al.*, 2005).

Age structure, body size and growth patterns are important life history traits in amphibians. The exact estimate of individual ages gives us access into

understanding the life history traits across animal groups (Morrison and Hero, 2003). Determination of amphibians' ages by skeletochronology method has already been used in a variety of species and described in detail by many workers (Mina, 1974; Hemelaar, 1981, 1983, 1988; Bastien and Leclair, 1992; Cherry and Francillon-Vieillot, 1992). It is known that analysis of cross sections of phalanges on amphibians showed concentric rings, which were called as year rings due to corresponding to years of growth. Each year ring is considered to consist of a growth ring and a dark line named as the line of arrest of growth (LAG) resulting from its limitation on the growth of growth ring (Hemelaar and Vangelder, 1980; Hemelaar, 1981; Kulkarni and Pancharatna, 1996). Skeletochronology has recently experienced an increase in use for determining the ages and growth for most amphibians, including those in temperate (Hemelaar, 1988; Miaud and Guyétant, 1999; Lu *et al.*, 2006; Guarino and Erismis, 2008; Kyriakopoulou-Sklavounou *et al.*, 2008; Chen *et al.*, 2011; Liao, 2011), tropical (Guarino *et al.*, 1998; Khonsue *et al.*, 2000; Pancharatna and Deshpande, 2003) and subtropical regions (Guarino *et al.*, 1998; Lai *et al.*, 2005; Liao and Lu, 2010a, b, c; Li *et al.*, 2010; Liao *et al.*, 2010; Liao *et al.*, 2011; Yang *et al.*, 2011).

The Dark spotted frog, *Rana nigromaculata* is a

\* Corresponding author: Prof. Caiquan ZHOU, from China West Normal University, Nanchong, Sichuan, China, with his research focusing on resource ecology and conservation biology of terrestrial vertebrates.  
E-mail: Drcqzhou1@163.com

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common species in temperate East Asia. It occurs across much of eastern and northeastern China, the Amur River valley in Russia, the Korean Peninsula and most parts of Japan, and is widely distributed at relatively low altitude, not being found above 2200 m (Zhao and Adler, 1993; Fei, 1999; Zhao, 1999; Yang *et al.*, 2004). It has been found across a variety of habitats, including river pools, channels, lakes, ponds, swamps, ditches and rice fields (Zhao and Adler, 1993; Zhao and Zhao, 1994). Hibernation generally starts from September–November and ends in February–May of the following year when mean air temperature is about 13°C (Fei *et al.*, 2005; Liao *et al.*, 2010). This frog is considered an explosive breeder based on a short breeding season of 25–35 days from late April to mid May (Liao *et al.*, 2010; Khonsue *et al.*, 2001a). Many cases such as habitat destruction, anthropogenic impact and increasing ultraviolet radiation may be considered responsible for some parts of amphibians decline (Bradford, 1991; Sherman and Morton, 1993; Blaustein, 1994; Pechmann and Wilbur, 1994). Moreover, non-native species invasion (Hayes and Jennings, 1986; Bradford *et al.*, 1993; Knapp and Matthews, 2000), along with biological factors such as interaction between species occupying the same ecological niche (Khonsue *et al.*, 2001a), could also be taken as contributors to the decline of amphibians. But evidence for chief reasons of amphibian population decline still remains largely unavailable. Therefore, from the study of life history traits of *R. nigromaculata*, we can understand the population fluctuations and then provide important data for its conservation.

We used skeletochronology to study life history traits in a *R. nigromaculata* population in northeastern Sichuan, China, with the following aims: 1) to examine the differences in age, body size, longevity and growth between males and females; 2) to analyze age structure of the population to gain insight into whether *R. nigromaculata* is declining; and 3) to provide data for species conservation.

## 2. Materials and Methods

The dark spotted frogs were captured in the Yingxi Town of Nanchong (30°50'N, 106°07'E, 338 m a.s.l.) in northeastern Sichuan, China and were used for anatomical experiments conducted by the China West Normal University in mid-April 2010. Necessary samplings were made after the anatomical experiments. A total of 139 individuals were sexed based on the presence of secondary sexual characteristics, such as external vocal sacs along the neck and the presence of

nuptial pads on the inner surface of the first digit of the forelimb (Khonsue *et al.*, 2001a, b; Fei *et al.*, 2005). Subsequently, for each individual, snout-vent length (SVL) was measured using an electronic caliper (to the nearest 0.01 mm) and body mass was recorded using an electronic scale (to the nearest 0.01 g). The third phalange (the longest one) of the left forelimb were excised and stored in 10% formalin for skeletochronological analysis. Afterwards, all specimens were preserved in 10% formalin to retain the integrity of all their organs.

To determine the age of all individuals, we adopted the prevailing technique of skeletochronology by counting the lines of arrested growth (LAGs) in the cross-sections of phalanges (Castanet and Smirna, 1990). The surrounding tissues of phalanges were cleaned, washed in running water, and decalcified for 48 h in 5% nitric acid. We washed all samples in running water for 24 h to remove all traces of the decalcifying agent, and stained the samples using Harris' haematoxylin for 3 h. Subsequently, the stained bones were dehydrated by running them step by step through 70% ethanol for 1 h, 80% ethanol for 1 h, 95% eosin-alcohol dye liquor for 3 min, 95% ethanol for 1 h, 100% ethanol for 30 min, 1:1 xylene-alcohol mixture for 20 min, 100% xylene for 30 min and paraffin for about 100 min in the thermostat (60 °C) until the tissues were infiltrated completely. Then, the impregnated phalanges were embedded in paraffin prior to sectioning using standard histological techniques. Samples were transversely sectioned (13 µm thick) using a rotary microtome, and series of sections with minimum medullar cavity and thickest cortical bone from each individual were mounted on microscope slides. At least five mid-diaphyseal sections were examined for LAGs with a LEITZ dialux 40 microscope, and the best sections were photographed using a Motic BA300 digital camera mounted on a Moticam 2006 light microscope at × 400 magnifications. Sections were independently examined by two observers. When the numbers of LAGs counted by the two observers did not agree, the sections were examined again until a consensus was reached. In order to guarantee the accuracy of estimation, the month of capture was taken into consideration. We did regard the incomplete line at the outer periosteal margin as a LAG because we collected the individuals in mid April, when the formation of newest ring has not yet completed. Based on the suggestions of Castanet and Smirna (1990), we confirmed the first LAG endosteal resorption of bone by comparing the diameter of LAG-1 of adults with that of the outer periosteal margin of juveniles.

Growth estimation was conducted by non-linear

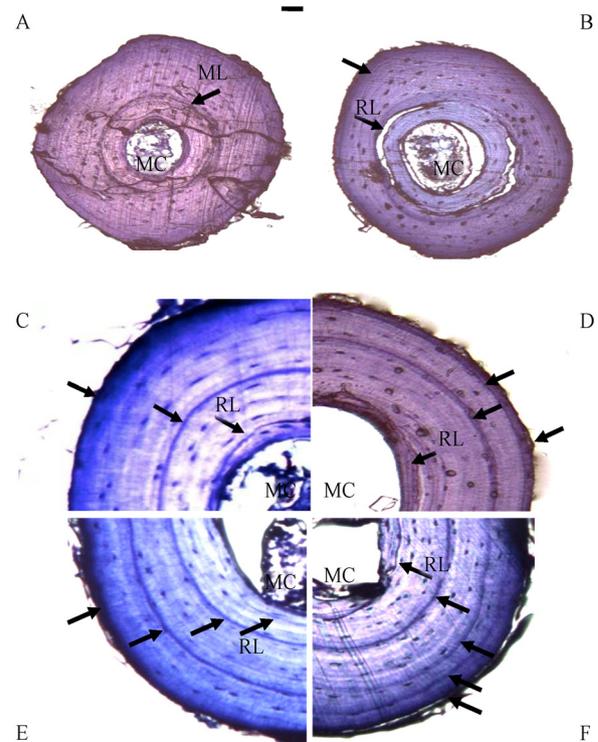
regression in software SPSS 13.0 using von Bertalanffy's function which has been used for other anurans (Cogalniceanu and Miaud, 2003; Lu *et al.*, 2006; Liao *et al.*, 2010). The von Bertalanffy's function  $S_t = S_{\max} (1 - e^{-kt+b})$ , where  $S_t$  is SVL (mm) at time  $t$  (yr);  $S_{\max}$  is the asymptotic size; and  $k$  is a growth coefficient and  $b$  is a constant. The growth rate can be calculated as:  $R = ds/dt = k (S_{\max} - S_t)$ , where  $R$  may be maximum while  $S_t$  is minimum, i. e., SVL at metamorphosis (Liao *et al.*, 2010).

### 3. Results

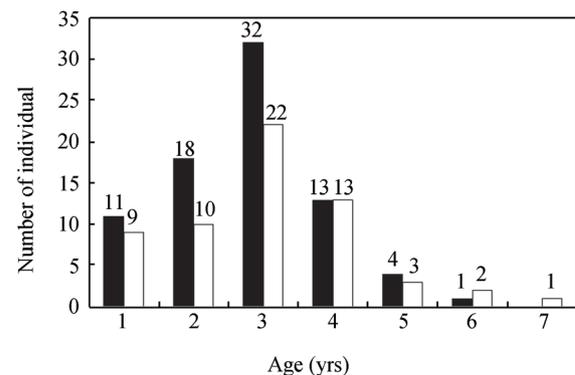
**3.1 Age estimation** Phalangeal cross-sections of *R. nigromaculata* showed a series of narrow concentric haematoxylinophilic lines (Figure 1). Sometimes, one LAG was assumed to be lost due to endosteal resorption, which usually resulted in underestimation of individual age. Therefore, before determining the age of individuals, we compared the diameter of the first visible LAG in adults with that of the outer periosteal margin in juveniles. The first arrested growth line (LAG-1) was partly destroyed by the resorption process in older individuals, while no LAG was reabsorbed completely in our samplings.

**3.2 Age structure** The age of *R. nigromaculata* from Yingxi Town in Nanchong, Sichuan, China ranged from one to seven years in females and one to six years in males (Figure 2). Age at the first reproduction was estimated to be two years in males and three years in females based on the maturity characteristic (e. g., mature spermatozoa found in the tests and eggs found in the ovaries). Longevity was estimated to be at least six and seven years in males and females, respectively. Age distributions between males and females differed significantly (Kolmogorov-Smirnov test:  $D = 0.24$ ,  $P < 0.01$ ), with a concentrated distribution in male individuals with two and three LAGs (58.33% of total male individuals) and in females with three and four LAGs (50.63% of total female individuals). Average age did not differ significantly between males and females (Student's  $t$ -test:  $t = 1.06$ ,  $P = 0.29$ ). Breeding females were significantly older than males (females,  $3.71 \pm 0.15$  years; males,  $3.09 \pm 0.11$  years; Student's  $t$ -test:  $t = 3.34$ ,  $P < 0.01$ ).

**3.3 Body size and body mass** Females were significantly larger in SVL and heavier in body mass than males (Table 1). ANCOVAs with age as the covariate showed that age was one of the factors affecting sexual difference in body size and body mass (body size,  $F = 6.16$ ,  $P < 0.001$ ; body



**Figure 1** Phalangeal bone cross-section of *R. nigromaculata* from Yingxi Town, Nanchong, Sichuan, China. A: Sub-adult, showing one ML; B: Male, showing one LAG (arrow) and one RL; C: Male, showing two LAGs (arrows); D: Female, showing two LAGs and one outer edge of bone (arrows); E: Male, showing three LAGs (arrows); and F: Male, showing three LAGs and one outer edge of bone (arrows). MC: Marrow cavity; ML: Metamorphosed line; RL: Resorption line. Scale bar = 50  $\mu$ m.



**Figure 2** Age composition distribution of males (close bars) and females (open bars) from the *R. nigromaculata* population in Sichuan, China.

mass,  $F = 4.24$ ,  $P = 0.001$ ). This sexual-size dimorphism was obvious in age class one (individuals with one LAG), three and four in particularly (Table 2; Mann-Whitney  $U$ -test:  $P < 0.05$ ). Moreover, the same case about sexual-mass dimorphism was in age class one and four (Table 2; Mann-Whitney  $U$ -test:  $P < 0.05$ ). Age was not correlated with body size and body mass in males (Pearson's correlation coefficients: body size,  $r_s = 0.14$ ,  $n = 124$ ,  $P = 0.14$ ; body mass,  $r_s = 0.07$ ,  $n = 124$ ,  $P = 0.48$ ), but a

**Table 1** Comparison of body size (SVL), body mass and age of *R. nigromaculata* between the sexes. Values are mean ± SE, with the number of individuals in parentheses.

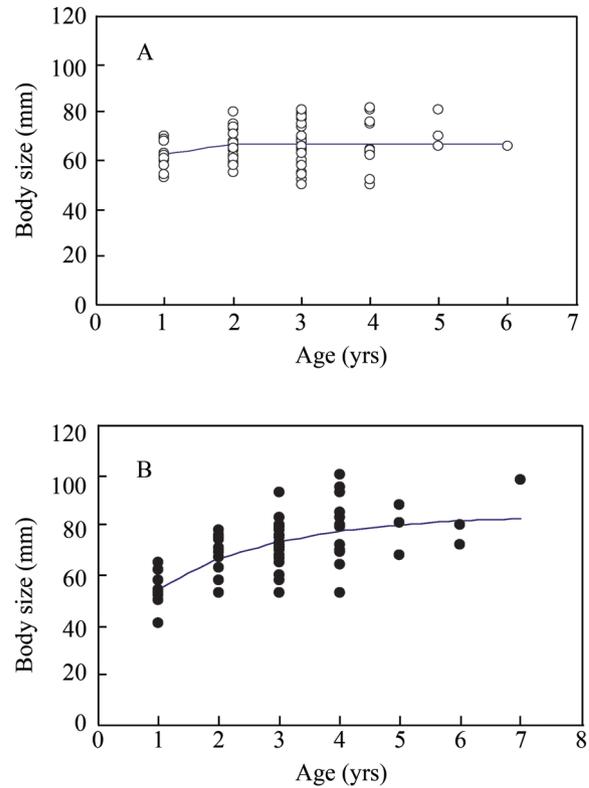
Sex	Body size (mm)	Body mass (g)	Age (yrs)
Female (n = 60)	71.20 ± 1.68	25.84 ± 1.28	3.02 ± 0.17
Male (n = 79)	66.00 ± 0.88	22.55 ± 0.82	2.80 ± 0.13
<i>t</i>	2.91	2.26	1.06
<i>P</i>	0.008	0.033	0.293

significant correlation between age and body size, as well as age and body mass was found in females (body size,  $r_s = 0.59$ ,  $n = 60$ ,  $P < 0.001$ ; body mass,  $r_s = 0.58$ ,  $n = 60$ ,  $P < 0.001$ ).

**3.4 Growth** Figure 3 shows a growth curve with a von Bertalanffy's function from males and females. Up to the age of two years, the growth curve appeared similar in both sexes. But beyond this age, the growth curve differed significantly between the sexes. Average growth rate (*R*) in females was  $6.29 \pm 0.84$  and that of males was  $0.30 \pm 0.44$ . The growth rate in females was significantly higher than that in males (Student's *t*-test:  $t = 6.74$ ,  $P < 0.001$ ). Additionally, asymptotic size in males ( $66.63 \pm 1.10$  mm) was smaller than that in females ( $83.73 \pm 7.05$  mm).

**4. Discussion**

Among methods of age determination which have been established, the analysis of bone structure using skeletochronology method seems to be most appropriate to assess age and growth for amphibians (Castanet *et al.*, 1977; Francillon, 1979; Hemelaar and Vangelder, 1980; Gittins *et al.*, 1982; Halliday and Verrell, 1988; Hemelaar, 1988; Khonsue *et al.*, 2000; Khonsue *et al.*, 2001a, b; Guarino *et al.*, 2003; Liao *et al.*, 2010; Liao and Lu, 2011; Liao and Lu, 2012). LAGs of amphibians were



**Figure 3** Growth curves estimated by von Bertalanffy's growth model of *R. nigromaculata* (A: Male, open circles; B: Female, close circles) from Yingxi Town in Nanchong, Sichuan, China. Female:  $S_i = 83.73(1 - e^{(-0.52i - 0.54)})$ ; Male:  $S_i = 66.63(1 - e^{(-17.49i + 14.74)})$ .

considered to form in correspondence with environmental changes, such as fluctuations in climate or variations in the availability and quality of food (Guarino *et al.*, 1998; Khonsue *et al.*, 2000; Liao *et al.*, 2010). Moreover, LAGs of species living in the subdesert were known to be related to a long aestivation (Barbault *et al.*, 1979). In addition to the environmental factors, growth remarks in

**Table 2** Comparison of body size (SVL) and body mass between sexes for each age class of *R. nigromaculata*. Mean ± SE are given with sample sizes of each age class in parentheses.

Age (yrs)	Body size (mm)				Body mass (g)			
	Female	Male	<i>z</i>	<i>P</i>	Female	Male	<i>z</i>	<i>P</i>
1	54.12 ± 2.34 (n = 9)	62.36 ± 1.79 (n = 11)	2.47	0.013	13.20 ± 1.35 (n = 9)	20.57 ± 1.87 (n = 11)	2.7	0.007
2	68.40 ± 2.60 (n = 10)	66.79 ± 1.57 (n = 18)	0.82	0.414	24.71 ± 2.00 (n = 10)	21.99 ± 1.30 (n = 18)	1.39	0.164
3	73.03 ± 2.22 (n = 22)	66.19 ± 1.46 (n = 32)	2.55	0.011	26.61 ± 1.86 (n = 22)	23.47 ± 1.56 (n = 32)	1.39	0.164
4	77.48 ± 3.81 (n = 13)	66.25 ± 2.72 (n = 13)	2.29	0.022	31.13 ± 2.83 (n = 13)	21.90 ± 7.04 (n = 13)	2.72	0.011
5	79.00 ± 5.86 (n = 3)	70.75 ± 3.54 (n = 4)	1.26	0.208	34.11 ± 2.62 (n = 3)	26.64 ± 2.86 (n = 4)	1.77	0.077
6	76.00 ± 4.00 (n = 2)	66 (n = 1)	1.23	0.221	29.22 ± 6.60 (n = 2)	16.74 (n = 1)	1.23	0.221
7	98 (n = 1)				40.35 (n = 1)			

bone could also be explained due to intrinsic (genetic) control, but little evidence is available (Kleinenberg and Smirina, 1969; Smirina, 1972; Marangoni *et al.*, 2009). This pattern can be the case in the population of *R. nigromaculata* we studied. Osteometrical analysis indicated the existence of endosteal resorption, which may result in the underestimation of the true age in amphibians (Castanet and Smirina, 1990). In our study, the results from the comparison between the two diameters indicated that LAG-1 was partly destroyed by the resorption process in older individuals, while complete LAGs resorption was not yet observed in our samplings.

Sexual maturity of females is delayed compared to males, possibly due to the need of females for reserved energy for the purpose of supporting gonad and embryo development (Gurarino *et al.*, 1998; Duellman and Trueb, 1994; Tsiora and Kyriakopoulou-Sklavounou, 2002; Degenhardt *et al.*, 2005). In the population of *R. nigromaculata* from the site we studied, age at the first reproduction was 2 years in males, while 3 years in females. Similarly, *R. nigromaculata* males in Japan was observed to start breeding at two years and females at three years of age (Khonsue *et al.*, 2001a). The pattern that adult males seem to be younger than females has been reported previously in anurans species (Ento and Matsui, 2002; Liao *et al.*, 2010; Liao and Lu, 2010a, c). Our findings indicated that females had a longer lifespan than males, as observed in most other amphibians (Morrison *et al.*, 2004; Lai *et al.*, 2005; Liao and Lu, 2010a, c; Kutrup *et al.*, 2011). Differential longevity between the sexes may be explained due to more conspicuous behavior exhibited by males such as loud choruses of males to attract females and more frequent activity to fight or to approach females, which make males detected by predators more easily (Shirose *et al.*, 1993; Tsiora and Kyriakopoulou-Sklavounou, 2002). Longevity of *R. nigromaculata* from Yingxi Town in Nanchong seemed to be longer than that from southwestern China and Japan. Food consumption and the degree of interspecific competition may be considered to bring about interpopulation difference in age and size properties of *R. nigromaculata* (Khonsue *et al.*, 2001a).

As observed in most amphibians, *R. nigromaculata* is sexually dimorphic, with females being larger than males (Shine, 1979; Monnet and Cherry, 2002). In this study, females were significantly larger and heavier than males. Body size of an adult animal is a function of growth rate beyond the maturation period and the duration of growth. In our findings, the growth rate of males decreases soon after maturation while females continue to grow for a

longer time. As a result, delayed maturation, along with higher  $k$  value for females could be explained as main contributors to larger female size. ANCOVA showed that age was a factor resulting in sexual size dimorphism in *R. nigromaculata*. Variations in growth rate could be expected caused by fluctuations in the quality and availability of food, habitat, climate and predator stress (Tsiora and Kyriakopoulou-Sklavounou, 2002). All the individuals were captured in the breeding season, so the heavy clutches of eggs gestated by females may be regarded as the main contributor to heavier females. Moreover, larger body size of females may also be considered to make some contribution to heavier females, but little evidence is available.

Previous studies have indicated that SVL and age show a positive correlation within each sex of *R. limnocharis* (Liao *et al.*, 2010), *R. perezi* (Esteban *et al.*, 1996), *R. ridibunda* (Kyriakopoulou-Sklavounou *et al.*, 2008), and *Hyla arborea* (Kyriakopoulou-Sklavounou and Grumiro, 2002). In our study, the correlation was non-significant neither between age and SVL, nor between age and body mass in males, but a significant correlation was found between age and SVL, along with age and body mass in females, as observed previously in the same species which showed positive correlations between body size and age (*R. temporaria*: Gibbons and McCarthy, 1984).

Our results showed a normal distribution in age structure for both sexes and the concentrated distribution of males was on age classes two and three, while the same case occurred in females on age classes three and four. This modal age indicated a predominance of young individuals. Meanwhile, analysis of age structure indicated that the studied population was relatively stable according to the assumption that the age distribution decreases with age in a stable population (Hemelaar, 1988).

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