

Age, body size and clutch size of *Rana kunyuensis*, a subtropical frog native to China

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Age, body size and clutch size are important demographic traits directly related to the life history strategy of a species, but little is known about these parameters in *Rana kunyuensis*, a frog endemic to China. In the present study, we investigated body size, age structure and clutch size of this species in a population from Mt. Kunyu. Age at maturity of males and females was 1 year and 2 years, respectively. The maximum age of males and females was 4 and 5 years, respectively. Females were significantly larger than males. Our results revealed a positive correlation between age and body size; however, body size was not a good indicator of age because there was an extensive overlap in body size among age classes. Clutch size was also positively correlated with body size. These data provide the first detailed life history information for *Rana kunyuensis*.

Key words: age structure, body size, clutch size, *Rana kunyuensis*, skeletochronology

Body size, age and clutch size are three important demographic traits of animal populations that are key components to understanding the life history of a species. Among amphibians there is often a positive relationship between individual age and body size (Duellman & Trueb, 1985; Halliday & Verrell, 1988; Kyriakopoulou-Sklavounou et al., 2008; Liao & Lu, 2010), and body size and fecundity (Cummins, 1986; Wogel et al., 2002; Tomašević et al., 2008). For amphibians, age can be assessed non-lethally using skeletochronology (Castanet & Smirina, 1990; Rozenblut & Ogielska, 2005; Chen & Lu, 2011; Chen et al., 2011).

Rana kunyuensis, distinguished from *R. chensinensis* based on unique morphological features (Lu et al., 2000; Lu & Li, 2002), is only distributed on Mt. Kunyu, located near Shandong, China (Fei et al., 2010). Individuals start to breed at the end of February when the water temperature is above 8°C, although some male frogs may grasp females during hibernation in anticipation of breeding (Li et al., 2006). The breeding period lasts for 2–3 weeks (personal observation), and thus the species

can be viewed as an explosive breeder (Wells, 1977). Females lay 200–1400 eggs before daybreak in the lentic area and swag of streams or ponds (Li et al., 2006). The egg incubation period is about 49 days (Sun et al., 2003). Adult frogs hibernate at the bottom of the river from October to February (Li et al., 2006).

Demographic studies on Chinese anurans are limited (Lu et al., 2006; Liao, 2009). In this study, we document the age structure, body size and clutch size of *R. kunyuensis* from Mt. Kunyu. Our main objectives were to (i) provide basic information about the demography of this little-known subtropical frog, and (ii) investigate the relationship between clutch size and body size.

We conducted our field work in 2011 at Mt. Kunyu (37°10'–37°20' N, 121°37'–121°56' E, 112 m a.s.l.), in the northeastern province of Shandong, China. Our 3 km study plot was situated along a stream (37°30'N, 121°74'E, 110–150 m altitude) surrounded by temperate broad-leaved mixed forests. The mean annual temperature is 12.5 °C, and annual precipitation is 984 mm.

All specimens (total 72 frogs, 40 males and 32 females, Table 1) were collected in the day during the breeding season. Frogs were caught by hand in their spawning habitats. We identified frogs with nuptial pads on foredigits as sexually mature males, and those with well-developed oocytes (easily visible through the skin of the abdomen) as sexually mature females. Snout-vent lengths (SVL) of flattened frogs were measured to the nearest millimetre using calipers. The longest phalange of the left hindlimb was clipped and preserved in 10% formalin for histological analysis. Amplexed pairs were taken to the lab to determine clutch sizes. After clutches were laid, we counted the individual number of eggs in each clutch. All frogs were released at the site of capture after laying eggs.

Age was estimated following Ma & Lu (2009). Phalanges were cleaned of surrounding tissue, washed in running water for 12 hours and decalcified for 48 hours in 5% nitric acid, followed by washing of all samples in running tap water for 24 hours to remove all traces of the decalcifying agent. We then stained the decalcified digits for 80 min using 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.

The age of each frog was established by two authors who independently counted the number of (complete and partially resorbed) lines of arrested growth (LAGs) present in the bone sections. We assumed that the number of LAGs corresponded to the age of an individual because *R. kunyuensis* lives in a climate with strong seasonal cycles (Morrison et al., 2004; Lai et al., 2005). By choosing sections with small marrow cavities and by considering the Kastschenko line (KL, the division line between the endosteal and periosteal zones; Rozenblut & Ogielska, 2005), we tried to avoid underestimating numbers of LAGs due to complete or partial endosteal resorption of periosteal LAGs (Castanet et al., 1993).

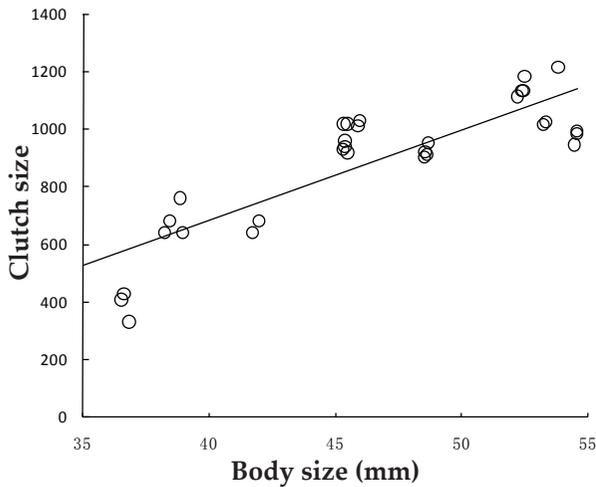


Fig. 1. Correlation between clutch size and body size (snout-vent length) in breeding *Rana kunyuensis* from Mt. Kunyun, China.

Student's *t* tests were used to test for differences in mean age and body size between males and females. Analyses of covariance (ANCOVA) were used to investigate whether size difference between the sexes was caused by individual age. Chi-square tests were used to compare differences in age distributions between the sexes. Spearman correlations were used to assess the relationship between body size and age and between clutch size and body size. All statistical analyses were run with SPSS 13.0 (SPSS Inc. 2004). Probabilities were two-tailed and all values are given as the mean±standard deviation (SD).

Age at sexual maturity of males and females was 1 and 2 years, respectively (Table 1). Males and females possessed a maximum of 4 and 5 LAGs, respectively. Mean age did not differ significantly between the sexes (Student's *t*-test: $t_{70}=1.61$, $P=0.112$). The age distributions of males and females differed significantly (males, $\chi^2=31.8$, $df=3$, $P<0.001$; females, $\chi^2=9$, $df=3$, $P=0.029$).

SVL was significantly greater in females than in males (Student's *t*-test: $t_{70}=2.504$, $P=0.015$), even when individual age (ANCOVA: $F_{1,69}=329.826$, $P<0.001$) was controlled for ($F_{1,69}=5.954$, $P=0.017$). SVL ranged between 30.2 and 54.6 mm (Table 1). There was a positive correlation between age and body size in both sexes (males, $r_s=0.832$, $n=40$, $P<0.001$; females, $r_s=0.937$, $n=32$, $P<0.001$). SVL ranges of males and females overlapped

between age classes (Table 1). Clutch size ranged from 330 to 1214 eggs per female (876 ± 229 , $n=32$ females), with a positive correlation between clutch size and body size ($r_s=0.784$, $n=32$, $P<0.001$; Fig. 1).

Skeletochronology has been widely used to evaluate age and growth in amphibians (Castanet & Smirina, 1990; Guarino & Erismis, 2008; Liao & Lu, 2010; Chen & Lu, 2011). As shown in studies from closely related species (*R. temporaria*, Miaud et al., 1999; *R. chensinensis*, Lu et al., 2006; and *R. amurensis*, Chen et al., 2011), the age of *R. kunyuensis* can be easily estimated by counting the clear LAGs in phalangeal cross-sections.

Endosteal resorption of periosteal LAGs in amphibian bones can lead to underestimation (Leclair, 1990; Guarino et al., 1995), while double/false LAGs can lead to overestimation (Hemelaar & Van Gelder, 1980) of individual age. We are confident that our skeletochronological estimates of individual age in *R. kunyuensis* are reliable given that the study species is not very long-lived, and endosteal resorption is rather a problem in long-lived species (Wagner et al., 2011). No double or false LAGs were observed, presumably because of the strongly seasonal environment of *R. kunyuensis* (Kyriakopoulou-Sklavounou et al., 2008; Liao et al., 2010).

Our results show that females were older and larger than males, following the common pattern observed in most amphibians (Monnet & Cherry, 2002; Yilmaz et al., 2005; Chen & Lu, 2011). This sexual difference could be explained by life history theory, which predicts a trade-off of sex-specific energy allocation between current growth and future reproduction (Shine, 1979; Stearns, 1989; Williams, 1996). To maximize lifetime reproductive success, females should invest more energy into somatic growth, which will allow them to delay sexual maturity and thus reach a larger final body size and attain higher fecundity in the future (Miaud et al., 1999; Monnet & Cherry, 2002; Morrison & Hero, 2003). The fact that larger female *R. kunyuensis* had higher clutch sizes than smaller females supported this prediction. Another explanation is that selective pressures upon males are likely to target their early maturation to reduce their probability of death before reaching minimum reproductive size (Stearns, 2000). That males reach sexual maturity earlier than females is commonly observed (Miaud et al., 1999; Eaton et al., 2005; Liao et al., 2010).

Table 1. Snout-vent length (SVL) per age category of breeding *Rana kunyuensis* of different ages from Mt. Kunyun, China. Age was estimated by counting lines of arrested growth (LAGs) in cross-sections of frog phalanges.

LAGs	SVL (mm)							
	Male (n=40)				Female (n=32)			
	n	Mean	SD	Range	n	Mean	SD	Range
1	2	31.0	1.2	30.2–31.9	-	-	-	-
2	8	37.8	0.8	36.7–38.6	8	37.4	1.5	34.5–39.0
3	25	44.4	2.6	39.7–47.3	14	45.9	2.2	41.7–48.7
4	5	48.0	1.5	46.8–49.8	8	53.1	0.9	52.2–54.6
5	-	-	-	-	2	54.2	0.5	53.8–54.6

Body size of adult amphibians is determined by several proximate factors including size at metamorphosis, growth rate and growth duration (Miaud et al., 1999; Morrison & Hero, 2003; Lu et al., 2006; Ma et al., 2009). Because size at metamorphosis is similar between the sexes (9 mm, Li et al., 2006), we assume that growth rate is a major contributor to sexual size dimorphism in *R. kunyuensis*, since the size difference between males and females still existed when the effect of age was controlled for. This result is consistent with results for other anurans (*R. nigromaculata*, Khonsue et al., 2001; *Bufo hemiophrys*, Eaton et al., 2005; *R. swinhoana*, Lai et al., 2005; *Hyla annectans chuanxiensis*, Liao & Lu, 2010).

In adult amphibians, there is a positive but weak correlation between body size and age (Duellman & Trueb, 1985; Halliday & Verrell, 1988), although for some species only one sex shows a size-age correlation (Leclair & Castanet, 1987; Kutrup et al., 2005), and in some amphibians there is no correlation between body size and age (Wake & Castanet, 1995). Similar to other species (*R. ridibunda*, Kyriakopoulou-Sklavounou et al., 2008 and *R. kukunoris*, Chen et al., 2011), *R. kunyuensis* showed a positive correlation between body size and age for both sexes. However, body size of the species cannot be considered as a reliable predictor of age because there was significant overlap in the body size distributions of individuals of different age classes in sexually mature individuals (Halliday & Verrell, 1988; Rysler, 1996; Lu et al., 2006; Chen et al., 2011).

Although clutch size may exhibit considerable variation among females in a single population (Berven, 1988; Lemckert & Shine, 1993), female body size is positively correlated with fecundity in many amphibians (Cummins, 1986; Lemckert & Shine, 1993; Wogel et al., 2002; Tomašević et al., 2008). Our results showed that larger female *R. kunyuensis* had higher fecundity than smaller females.

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