

Age structure, growth and longevity in the common toad, *Rhinella arenarum*, from Argentina

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Abstract. Age structure, growth and longevity was determined in the common toad, *Rhinella arenarum*, from a suburban pond located in the Pampa plains, central Argentina during two breeding seasons, in 2000 and 2008 by using skeletochronology, which relies on the analysis of the annual lines of arrested growth (LAGs) in bones. Both females and males were captured in 2008, while only males were recorded in 2000. Females were significantly larger than males. Mean population age was 2.4 ± 0.9 years in 2000. In 2008, the difference in age was not significant between the sexes (Males: 3.0 ± 0.7 , $n = 21$; Females: 2.6 ± 0.9 , $n = 12$), neither between males in 2000 and 2008. The longevity in males of 2000 was 6 LAGs and exceeded that of males (5 LAGs) and females (4 LAGs) in 2008. Von Bertalanffy curves showed that the growth coefficient in the males of 2000 ($K = 2.97 \pm 0.47$) was almost double that of females ($K = 1.21 \pm 0.10$) and males ($K = 1.01 \pm 0.14$) of 2008. Males and females *Rhinella arenarum* show different morphological and life history traits and the year of sampling can significantly influence the estimation of the studied parameters such as age at maturity and growth rates.

Keywords. Demographic traits, age, *Rhinella arenarum* toad, skeletochronology, von Bertalanffy model.

INTRODUCTION

The common South American toad, *Rhinella [Bufo] arenarum*, belongs to the family Bufonidae. This toad has a wide distribution in South America and it is found in Argentina, Bolivia, Brazil, Uruguay and Paraguay. It has been assessed at Least Concern (LC) according to IUCN Red List of Threatened Species (IUCN, 2015). Adults normally congregate in large breeding groups at lentic water bodies. *R. arenarum* inhabits in a wide range of habitats, including coastal environments, subtropical

or tropical forests, and rural or urban areas. The habitat diversity and large population size make this toad a particularly conspicuous species. Although ecology, the feeding strategy and habitat preference of this anuran have been recently investigated (Sanabria et al., 2007; Quiroga et al., 2009; Bionda et al., 2011a; 2012; 2013), the demographic life-history traits still remain largely unknown (but see Echeverria and Filipello, 1990).

Regarding amphibians and reptiles, skeletochronology is a widely used method for retrospective age estimation of individuals with unknown recapture history

(Hemelaari, 1988; Smirina, 1994; Sinsch, 2015), and an excellent tool to assess population age structure. Skeletochronology relies on the analysis of annual growth zones alternating with dense lines of arrested growth (LAGs) in bones. LAG formation is considered to be mainly due to a genetically controlled based circannual rhythm, which is synchronized with and reinforced by seasonal cycles such as hibernation and occasionally aestivation in temperate zone species (Kleinenberg and Smirina, 1969; Castanet et al., 1993; Smirina, 1994; Sinsch et al., 2007). This study focusses on the dynamics of age structure and related life history parameters in a single population of *R. arenarum* breeding in a temporary pond in 2000 and 2008. Members of this population have annual hibernation and short-term aestivation periods. Aims of this study were (1) to report the size and age at sexual maturity, the longevity and the age structure in the common toad, *Rhinella arenarum*, and (2) to test for the presence of sexual dimorphism with respect to age- and size-related life-history traits and their variability between the study years.

MATERIALS AND METHODS

Study area

The study area belongs to the Pampa plains of central Argentina. The area is characterized by moderately undulating plains and a temperate climate with an annual mean temperature of 23°C in January and 6°C in July and with mean annual temperature of 18°C. Rainy seasons alternate with dry ones, with rains typically starting in October and continuing through the warm months until March with a mean annual rainfall of 800-1000 mm (Gatica et al., 2012). The study pond was located in the Campus of National University of Rio Cuarto (33°06'S, 64°25'W; 465 m a.s.l., pond size 966 m²). This is an area with permanent and temporary ponds which are surrounded by a small strip of forest.

Data collection

A total of 159 *R. arenarum* were collected during two breeding seasons, 2000 and 2008 (105 males and two juveniles in 2000; 39 males and 15 females in 2008) from September to December, the period of major breeding activity of this species in this region (Bionda et al., 2011a; 2012; 2013). The individuals were hand-captured during surveys at the pond shore, mostly after rainfall. We measured the snout-vent length (SVL; mm) of each individual with a Vernier caliber (0.01 mm precision). The sex was determined using external secondary sexual characters: presence of vocal sacs and nuptial pads, and coloration (males with brownish or greenish back; females' greyish or light brown back, scattered with large dark or dark brown blotches). Then a toe of the forelimb was clipped at the level of the penultimate

phalanx and stored in 70% ethanol until being processed for skeletochronological analysis. The toe clip was used as a batch mark to prevent resampling (Bionda et al., 2011b; 2013). After biometric measurement and toe-clipping, we released the animals at the sampling site.

Laboratory procedures followed the standard methods of skeletochronology (Sinsch et al., 2001): (1) decalcification of bones (5-10% formic acid, 24 h), (2) fixation in Bouin's solution (for sample of 2000) or formol 4% (for sample of 2008) (at least 12 h), (3) HistoresinTM (JUNG) (for sample of 2000) or paraffin embedding (for sample of 2008), (4) cross sectioning of the diaphysis at 8-10 µm using a JUNG RM2055 (for sample of 2000) and an ARCANO rotation (for sample of 2008) microtome, (5) staining with 0.05% cresylviolet (5-10 min, sample collected in 2000) or with Ehrlich's haematoxylin (2 min, sample collected in 2008), (6) light microscopic count of the number of lines of arrested growth (= LAG) using an Olympus BX 50 (for sample of 2000) and a Zeiss Axiophot-Axiolab (for sample of 2008), (7) documenting the most informative cross sections with a AxiocamHRC Zeiss digital camera using Axio Vision 4.3. The number of lines of arrested growth (LAGs) in each section was counted in the periosteal part of the bone by at least two authors (SK, US, CB, AM). The criteria for incomplete, faint or double lines, as well as LAGs potentially lost due to endosteal resorption were applied according to Tsiora and Kyriakopoulou-Sklavounou (2002), Guarino et al. (2011). Consequently, we define age as the number of LAGs counted. The adult sample used for skeletochronology was 105 males collected in 2000, and 21 males and 12 females collected in 2008.

Growth rate was estimated using the von Bertalanffy's (1938) model (e.g., Üzüm and Olgun, 2009; Liao and Lu, 2010; Guarino et al., 2011). We used the following equation: $SVL_t = SVL_{max} - (SVL_{max} - SVL_{met}) e^{-K(t - t_{met})}$, where SVL_t = average SVL at age t , SVL_{max} = average maximum SVL, SVL_{met} = average SVL at metamorphosis (fixed to 11.5 mm according to Bionda, 2011), t = number of growing season experienced (age), t_{met} = proportion of the growing season until metamorphosis (age at metamorphosis), and K = growth coefficient (shape of the growth curve). The von Bertalanffy growth model was fitted to the empiric age-size data using the least square procedure (e.g., Cogălniceanu and Miaud, 2002; Cicek et al., 2011). Estimates of SVL_{max} and K are given with the corresponding 95% confidence interval.

The following demographic variables were calculated according to Leskovar et al. (2006): (1) age at maturity: the minimum number of LAGs counted in breeding individuals; (2) longevity: the maximum number of LAGs counted in reproductive individuals; (3) potential reproductive lifespan: the difference between longevity and age at maturity; (4) size at maturity: the average snout-vent length of all first breeders with the minimum number of LAGs; (5) modal lifespan: median of age distribution.

Data analysis

The significance level used in all tests was $P < 0.05$. Descriptive statistics are given as mean \pm standard deviation,

but as some subsets of age and SVL data differed from a normal distribution, we used the non-parametric Mann-Whitney U-test to test for significant differences between the two sexes and between years. Growth parameters were statistically compared based on the range of the $CI_{95\%}$ interval. Tests were performed using the statistical packages STATISTICA 6.0 (Statsoft Inc., USA 2001) and STAGRAPHICS Centurion XVI (Statpoint, Inc., 2014).

RESULTS

Body length

The average SVL of males sampled was 99.4 ± 8.8 mm (range 76.1–117.7, $n = 105$) in 2000 and 101.5 ± 7.1 mm (range 89.4–112.5, $n = 39$) in 2008. In the females, the average SVL was 108.6 ± 9.6 mm (range 82.6–123.3, $n = 15$) in 2008. The SVL of the individuals in 2008 differed significantly between sexes (Mann-Whitney, $U = 194.5$; $P < 0.05$), but there was no difference in SVL between the males in the two sampled years (Mann-Whitney, $U = 10.4$; $P = 0.51$). The size distribution of males and females did not differ from a normal distribution (Shapiro-Wilk test: males $W = 0.977$, $P = 0.37$, females $W = 0.882$, $P = 0.11$; Fig. 1).

Age structure

All adults studied showed well-defined lines of arrested growth (LAGs) in the periosteal bone layer, which allowed assessing the individual age (Fig. 2). Endosteal resorption was present in 33% of the sample (e.g., Fig. 2D), but the resorption did not hamper age determination because the first LAG was never completely reabsorbed. In many cases the outer most lines were closely adjacent, but at the insertion site of the phalangeal ligament it was possible to discern the peripheral LAGs and to reliably count them (Fig. 2C). Double LAGs (two very closely adjacent growth marks) were rarely observed and were counted as a single LAG (Figs. 2A, B).

The demographic life history traits are summarized in Table 1. Mean age was 2.4 ± 0.9 LAGs (= years) in the male sample of 2000, whereas that of the male and female sample of 2008 was 3.0 ± 0.7 and 2.6 ± 0.9 LAGs, respectively (Fig. 3). The modal age did not significantly differ between the sexes in 2008 ($U = 128.5$; $P = 0.93$), and did neither between males in 2000 and 2008 ($U = 25.0$; $P = 0.46$). The modal age was 2 LAGs in males in 2000, and 3 LAGs in males and 2 LAGs in females in 2008 (Table 1). The minimum number of

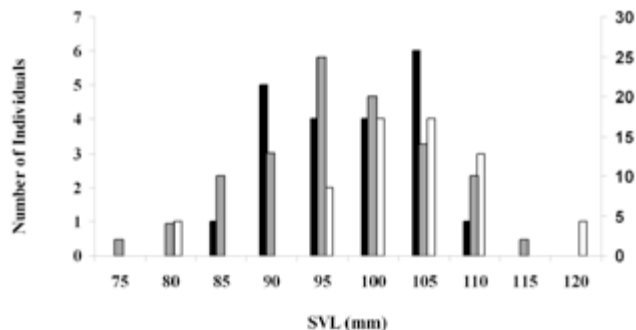


Fig. 1. Snout-vent length distribution (5 mm classes) of *R. arenarum*. Males sampled in 2000 (grey bars, secondary vertical axis), males (filled bars) and females (empty bars) sampled in 2008.

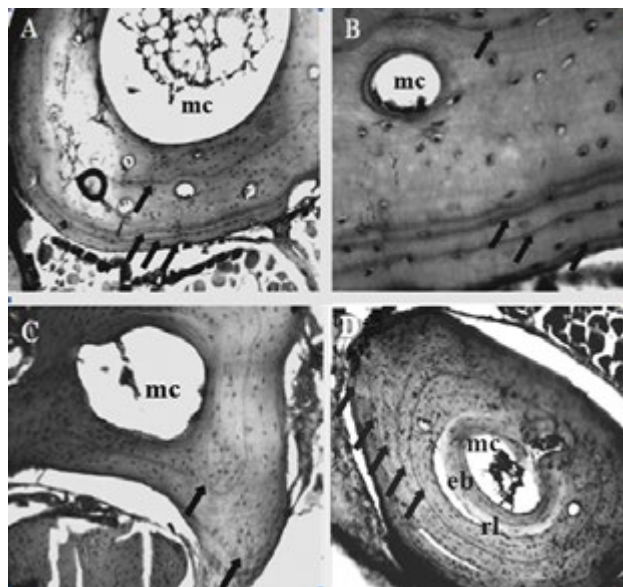
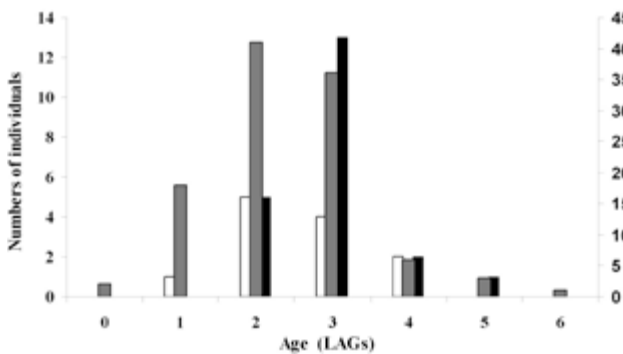


Fig. 2. Examples of cross sections of phalanges of adult *R. arenarum*. (A) Female, 4 LAGs, SVL = 113.9 mm; (B) female, 4 LAGs, SVL = 113.9 mm; (C) female, 2 LAGs, SVL = 100.7 mm; (D) male, 5 LAGs, SVL = 96.8 mm. Arrows indicate line of arrested growth (LAG). eb: endosteal bone. mc: medullary cavity. rl: resorption line.

LAGs counted in the reproductive individuals was 1 LAG for males in 2000 ($n = 18$ individuals). In 2008, the age at sexual maturity was 2 LAGs both in males ($n = 5$) and females ($n = 2$). The longevity and potential reproductive lifespan were higher in males in 2000 (Table 1). Size at sexual maturity of males was not significantly different between 2000 and 2008 (Mann-Whitney, $U = 15.6$; $P = 0.39$), but reached during the first year of life in the individuals collected in 2000, whereas the individuals from 2008 needed two years to achieve the same size. Females were significantly larger at maturity than males (Mann-Whitney, $U = 9$; $P < 0.05$).

Table 1. Demographic life history traits of *R. arenarum*.

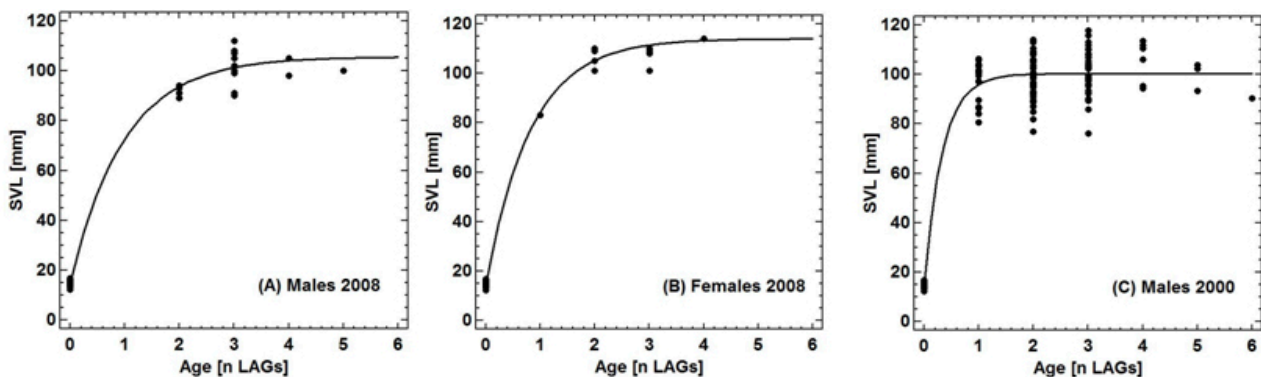
Sex	N	Age mean (LAGs)	Mode (frequency)	Age at sexual maturity (LAGs)	Longevity (LAGs)	Potential reproductive lifespan (years)	SVL at sexual maturity (mm)
Males 2000	105	2.4 ± 0.9 (1-6)	2 (39.1%)	1	6	5	96.1 ± 8.3
Males 2008	21	3.0 ± 0.7 (2-5)	3 (65%)	2	5	3	91.8 ± 2.2
Females 2008	12	2.6 ± 0.9 (1-4)	2 (41.6%)	2	4	2	106.8 ± 3.7

**Fig. 3.** Age distribution (in LAGs) of *R. arenarum*. Males sampled in 2000 (grey bars, secondary vertical axis), males (filled bars) and females (empty bars) sampled in 2008.

Sex-specific growth pattern

Growth curves of males and females sampled in 2008 using the von Bertalanffy's model (Fig. 4) did not differ significantly with respect to the growth coefficient ($K_{\text{males}} = 1.01$, $CI_{95\%}: 0.73-1.30$; $K_{\text{females}} = 1.21$, $CI_{95\%}: 1.00-1.42$; $P > 0.05$).

A marked decrease of growth rate was observed from the 2nd to the 3rd LAG which is after the presumed attainment of sexual maturity. The growth coefficient of males was significantly greater in 2000 than in 2008 ($K = 2.97$, $CI_{95\%}: 2.04-3.91$; $P < 0.05$). The maximum asymptotic SVL in 2008 differed significantly between males ($SVL_{\text{max}} = 105.6$ mm, $CI_{95\%}: 101.0-110.2$) and females ($SVL_{\text{max}} = 114.0$ mm, $CI_{95\%}: 110.6-117.4$; $P < 0.05$), i.e. SVL was marginally female-biased. In contrast, modeled maximum SVL of males did not vary significantly between 2000 and 2008 ($SVL_{\text{max}} = 100.3$ mm, $CI_{95\%}: 98.5-102.0$; $P < 0.05$). For all samples, the maximum asymptotic SVL was slightly greater than measured average SVL in this study (males 2000 = 99.4 ± 8.8 ; males 2008 = 101.5 ± 7.1 mm; females 2008 = 108.6 ± 9.6 mm). Models were in good agreement with empirical values (R^2 : males₂₀₀₀ = 0.94, males₂₀₀₈ = 0.99, females₂₀₀₈ = 0.99).

**Fig. 4.** Age-size relationship in males (A) and females (B) sampled in 2008, and in males sampled in 2000 (C). Lines represent the von Bertalanffy models of growth: (A) $SVL [mm] = 105.63 \text{ mm} - (105.63 \text{ mm} - 114.5 \text{ mm}) * e^{(-1.01387 * \text{LAGs})}$; (B) $SVL [mm] = 114.0 \text{ mm} - (114.0 \text{ mm} - 114.5 \text{ mm}) * e^{(-1.2106 * \text{LAGs})}$; (C) $SVL [mm] = 100.28 \text{ mm} - (100.28 \text{ mm} - 114.5 \text{ mm}) * e^{(-2.97484 * \text{LAGs})}$.

DISCUSSION

Skeletochronological age assessment is an essential tool for investigations on demographic traits (e.g., Sinsch et al., 2007; Sinsch, 2015). Moreover, it is a non-lethal destructive technique that can be performed on the phalanges, without sacrificing the animals (Guarino et al., 2008). LAG formation is considered to be mainly due to a genetically controlled based circannual rhythm (Alcobendas and Castanet, 2000; Morrison et al., 2004; Miaud et al., 2007; Marangoni et al., 2009; 2012; Sinsch, 2015). Several studies confirmed the formation of one LAG per year, equivalent to the number of hibernations of each individual, as the most common observed pattern of LAG formation in palaeartic, tropical and subtropical amphibian species (Smirina, 1994; Guarino et al., 2008; Marangoni et al., 2012), but precision decreases considerably in individuals older than 8 years (Sinsch, 2015). Moreover, the annual periodicity of LAG formation is more pronounced when the climate of the sampling area is characterized by a marked seasonal variation (Guarino et al., 2011), as is the case in *R. arenarum*. This toad species reproduces, when winter ends, therefore the last (outermost) LAG and the perimeter of the phalange were very close together due to the absence of substantial bone growth. Aestivation does not seem to have physiological effects on growth, as no typical growth marks (Sinsch et al., 2007) for interrupted summer growth were found. We found no evidence that local individuals reproduce twice a year, a potential cause of double line formation (Díaz Paniagua and Mateo, 1999; Marangoni et al., 2012). The seasonal ovary cycle of *R. arenarum* leans support for a single reproductive period per year, the preovulatory oogenesis begins between July and August, the ovulatory period with mature oocytes extends from September to November, and a post reproductive period occurs between December and June (Medina et al., 2004). Consequently, the low incidence of double lines (5% in the total sample) has probably other causes, for example, an interruption of the hibernation (Sinsch et al., 2007).

Sexual size dimorphism (SSD)

Sexual size dimorphism is observed in 90% of the 589 amphibian species surveyed by Shine (1979). In our study as well in those of Echeverria and Filipello (1990) and Bionda et al. (2011a), the body size in *R. arenarum* is female-biased. If adjusted for age (e.g., Ma and Lu, 2009; Guarino et al., 2010; Liao et al., 2015; Ma et al., 2015), female-biased SSD still remains significant, but at a marginally significant level. In agreement with Echeverria and Filipello (1990) size at maturity of males is about

90-100 mm and of females about 100 - 110 mm, indicating that SVL increment during the adult period is very low. Since male and female growth rate are statistically indistinguishable, female-biased SSD may be the result of sex-specific differences in the threshold size for attaining sexual maturity. As in *Bufo bufo* the age of maturity is variable, while a threshold size is mandatory for attaining maturity (Hemelaar, 1988).

The fact that the females are larger than males has been referenced to an increase of egg production, either to produce larger eggs, or to lay more eggs (Cummins, 1986). In *R. arenarum* females the number of eggs was independent of body size, but female mass and mass loss after spawning were positively related indicating that condition rather than size determines egg production (Bionda et al., 2011a).

Currently, SSD in anurans is thought to be largely a function of distinct life-history strategies of males and females (Monnet and Cherry, 2002). As juvenile growth is the main determinant of adult size (Sinsch et al., 2010), females often delay maturity up to one year compared with the males and allocate the energy to somatic growth, thus reaching sexual maturity at larger sizes (Marangoni et al., 2012). Alternatively, females may show significantly increased pre- or post-maturity growth rates compared with males (Ma and Lu, 2009; Guarino et al., 2008; 2011; Liao and Lu, 2012). In agreement with Echeverria and Filipello (1990), our study demonstrates that age at maturity and growth rate k did not differ significantly between the sexes, but the size threshold for attaining sexual maturity of females is greater (Table 1). Therefore, we assume that females continue to grow a few months longer than males at the same rate. Since the temporal unit detectable with skeletochronology is one year and additional female's growth period a few months, prolonged female growth cannot be detected by a difference in LAG number. Growth rate can vary considerably among years (males in 2000 vs males in 2008) indicating that k is probably influenced by local environmental factors. In contrast, maximum size is almost invariable (this study, Echeverria and Filipello, 1990). Adult size variation in *R. arenarum* is about the same in all age classes, rendering an age assessment based on size classes unreliable as in most other anurans (e.g., Guarino et al., 2010; Li et al., 2013).

Demographic life history parameters

Early maturity often implies a short lifespan, while late maturity can be frequently observed in long-lived amphibians species (Smirina, 1994; Guarino et al., 2010; Oromi et al., 2012). In our study population, minimum

age at maturity of *R. arenarum* varied between 1 and 2 years, while longevity was male-biased (6 vs 4 years). Echeverria and Filipello (1990) found a minimum age at maturity of 2 years, and a greater longevity in females (7 years) than in males (5 years) in the Buenos Aires region. The different longevity observed in the present study is probably due to the small sample size. Evidence for a possible trade-off between age at maturity and longevity requires a larger dataset from a larger number of populations. The age distribution (Fig. 3) demonstrates that most individuals do not reproduce more than once or twice in a lifetime being age at maturity 1-2 LAGs and the percentage of individuals with more than 4 LAGs only 10.9%. In females, short longevity is possibly associated with breeding effort as shown in first-breeding *Rana temporaria* females which show a higher annual mortality compared to males (Guarino et al., 2008).

It remains unclear whether the delay of maturity from one to two years in *R. arenarum* in 2008 reflects an adaptive response to variable environmental conditions, such as climate. For example, some variance in levels of average annual rainfall and temperature has been evidenced in the last decade in the study region (Bionda, 2011) and, in general, climatic variability can affect the development and reproductive performance of amphibians (Beebee, 1986; Carey and Bryant, 1995; Blaustein and Kiesecker, 2002; Collins and Crump, 2009; López-Alcaide and Macip-Ríos, 2011). The variation of temperature and precipitation are expected to affect activity patterns, microhabitat use, and thermoregulation and mediate modifications in individual growth rate, reproductive effort, and age structure.

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