ANIMAL SCIENCE

Body size, age and growth in males populations of *Boana pulchella* (Anura, Hylidae)

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Abstract: Age determination in amphibians is crucial to the investigation of life-history traits. In this context, we studied, for the first time, the life-history traits of *Boana pulchella* from a sample (63 adult males) of three populations (Las Acequias, Río Cuarto, Alejandro Roca) in central Argentina using the skeletochronological method. All adults of *B. pulchella* studied showed recognizable bone structures that allowed age determination. The average snout-vent length of sexually mature males was 43.39 mm. The maximum observed longevity was 5 years (5 Las Acequias, 4 Río Cuarto, 3 Alejandro Roca) and minimum age at sexual maturity was 2 years (same in the three populations), with mean of 2.96 years. Body size and age were positively correlated (except in Alejandro Roca populations). The growth patterns, estimated by the von Bertalanffy growth equation, do not show differences between populations, and the growth rate decrease after the attainment of sexual maturity. In conclusion, the determination of the individual age of the different populations of *B. pulchella* allowed us for the first time to establish the characteristics of the life history of the species, important for future comparisons whit others populations and future conservation biology studies.

Key words: Treefrog, *Boana pulchella*, life-history traits, skeletochronology.

INTRODUCTION

In the biological studies of amphibians, accurate age determination of individuals has been one of the most important subjects (Misawa & Matsui 1999), since the accurately assessed individual age permits estimate longevity, growth, age at sexual maturity and growth rate, very important in long-lived animals such as amphibians (Castellano et al. 1999), fundamental information about the characteristics of the life story. To evaluate age of amphibian populations, skeletochronology is one of the most widely used techniques, and studies the presence of growth layers in the long bones by counting the lines of arrested growth (LAGs) (Sinsch 2015).

Life-history traits are vitally important to individuals, and the variation in any one of these characteristics can have a significant effect on reproduction, overall fitness and survival of an individual (Stearns 1992, Chamberlain 2011). Many authors have used age and body size of individuals to quantify life-history traits, and to understand the evolutionary life history and ecology of amphibians (Morrison & Hero 2003, Liao et al. 2010, Liao & Lu 2011, Gül et al. 2011, 2014, Özdemir et al. 2012, Altunışık et al. 2013, Huang et al. 2013, Gümüş & Üzüm 2015, Zivari & Kami 2017, Erismis 2018).

In amphibians, body size is easier to measure than the number of LAG, hence, many studies assess whether body size is a good estimator of age (López et al. 2017). It is widely

assumed that amphibians show indeterminate growth and therefore there are a strong positive and significant correlation between body size and age (Duellman & Trueb 1994, Halliday & Verrell 1988, Gümüş & Üzüm 2015), and several studies have demonstrated it (Liao & Lu 2010a, Ashkavandi et al. 2012, Altunişik & Özdemİr 2013, Otero et al. 2017a, b, Baraquet et al. 2018a, b), therefore then size variation has to be adjusted for age variation (Cvetkovic et al. 2009, Liao & Lu 2012). However, other studies provided evidence against it, for example, Pancharatna & Deshpande (2003) in studies of *Limnonectes limnocharis* show poor correlation between the number of LAGs and the body size; Leclair et al. (2000) did not find significant correlation between age and size in males and females of *Rana sylvatica*, like that Pancharatna & Deshpande (2003) in studies of *Barbarophryne brongersmai*. This suggests that body size may not be a reliable criterion for age in some species, because as growth rate prior to the age of first breeding appears to be a far more significant source of variance in body size than age (Halliday & VerrelI 1988).

The common tree frog, *Boana pulchella* (Figure 1), is a widely distributed amphibian species, occurring from southeastern Brazil, Uruguay, southeastern Paraguay and eastern, central and northern Argentina (Cei 1980, Frost 2020). They use a wide variety of habitats, forests, grasslands, and flooded savannahs; it breeds in permanent ponds and flooded grasslands, to infields, gardens, small lakes, streams and human settlements and it has been classified as LC (Least Concern) in the IUCN Red List of Threatened Animals (Salas et al. 1998, IUCN 2020, M. Baraquet, personal communication). According to characters of external morphology, bioacoustic and molecular, this taxon is the sister species of the *B. cordobae* (Faivovich 2004).

Many studies have been conducted about *B. pulchella*, concern to systematic position (Duellman et al. 1997, Faivovich et al. 2004, Faivovich et al. 2005, Dubois 2017), cytogenetic (Ananias et al. 2004, Baraquet et al 2013a, Ferro et al. 2018), morphology (Barrio 1965, Cei 1980, Carezzano et al. 2015), tadpoles (Cei 1980, Kher 1987, Kolenc et al. 2008); bioacoustics (Barrio

Figure 1. Adult male *Boana pulchella* from Las Acequias (Córdoba, Argentina). (Photographed by Mariana Baraquet).

1965, Salas et al. 1998, Baraquet et al. 2007, 2013b, Ziegler et al. 2011); diet (Maneyro & da Rosa 2004, Antoniazzi et al. 2013) and ecotoxicology (e.g. Lajmanovich et al. 2005, De Arcaute et al. 2014, Natale et al. 2018, Barreto et al. 2020). However, despite being a widely studied species and one of the most widespread amphibians in South America surprisingly for this species the individual age has been poorly studied. So far only one study relied on skeletochronology (Brum et al. 2019) reporting the first record of growth rings for *B. pulchella*, although there are no data available about the life-history traits of the species.

So, this research is the first study on life-history traits of *B. pulchella* and the aims of our study were: (1) examine the applicability of skeletochronological method for the species; (2) determine the age of individuals of *B. pulchella* species from the count of lines of arrested growth (LAGs); (3) evaluate the correlation between age and SVL and (4) estimate the age and size at sexual maturity, potential reproductive lifespan, longevity and rate of growth to species, on the basis of individual ages estimated.

MATERIALS AND METHODS

Data were collected between September 2007 and May 2010 in three localities in Córdoba province, Argentina: 1) Alejandro Roca (n = 17, 33° 21' 06'' S, 63° 42 '10'' W, altitude 206 m a.s.l), 2) Las Acequias (n = 19, 33º 15' 26.16'' S, 63° 55' 15.10'' W, altitude 269 m a.s.l), 3) Río Cuarto (n = 27, 33° 06' 40.78'' S, 64° 18' 16.88'' W, altitude 420 m a.s.l).

All specimens were captured by hand, newly metamorphosed frogs while emerging from ponds and adult males emitting their advertisement call. The specimens were allocated in a scientific collection (ECO-EA

202-203, Depósito de Colecciones Húmedas, Departamento de Ciencias Naturales, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto). We measured the snout-vent length (SVL) of each individual using a digital caliper with a resolution of 0.01 mm and then we clipped the longest right phalanx of hind limb of each frog and preserved it in 70% ethanol until being processed for skeletochronological analysis. Our study was authorized by Environmental Secretary of Córdoba Government (A01/2013), and the Ethical Committee of Investigation of the Universidad Nacional de Río Cuarto (file number 38/11).

Skeletochronological studies followed the protocol of Castanet et al. (1993) and Sinsch et al. (2001): (1) fixation in formol 4% (at least 12 h), (2) decalcification of bones (10% formic acid, 24 h), (3) paraffin embedding, (4) cross sectioning of the diaphysis 10-12 μm by a rotary microtome Leica® RM2125 RTS, (5) staining with Ehrlich's haematoxylin (2 min), (6) light microscopic count of the number of lines of arrested growth (LAG´s), using a light microscope Zeiss Axiophot-Axiolab (100X), (7) documenting the most informative cross sections with a digital camera Axiocam ERc 5s, software ZEN 2.3 lite. 4.3.

Individual age was estimated following Sinsch (2015) for identification and interpretation LAGs. We examine of at least 10 cross sections per individuals, and the number of LAGs in each section was counted in the periosteal part of the bone independently by two observers (MB and MO). We assessed endosteal resorption based on the presence of the Kastschenko line (KL; the interface between the endosteal and periosteal zones; Rozenblut & Ogielska 2005) and confirmed endosteal resorption following the protocol of Lai et al. (2005). Irregularities such as double and false lines were identified following Liao & Lu (2010a).

We estimated life-history traits following Otero et al. (2017a, b): (1) age at sexual maturity: minimum number of LAGs counted in breeding individuals; (2) longevity: maximum number of LAGs counted in adults; (3) potential reproductive lifespan: difference between longevity and age at sexual maturity; (4) SVL at sexual maturity: average SVL of all first breeders with the minimum number of LAGs; and (5) modal lifespan: mode of age distribution.

Growth curves were estimated according to von Bertalanffy's equation (von Bertalanffy 1938) as used in several studies in amphibians (e.g., Hsu et al. 2014, Liao et al. 2016, Otero et al. 2017a, b, Baraquet et al. 2018a): $SVL_t = SVL_{max} - (SVL_{max} - SVL_{met}) e^{-K(t-tmet)}$, where SVL_t = average SVL at age t (mm); SVL_{max} = maximum asymptotic SVL (mm); SVL_{met} = average SVL at metamorphosis (mm); t = number of growing seasons experienced (age in years); t_{met} = proportion of the growing season until metamorphosis (age at metamorphosis fixed to 0.4 years according to Otero et al. (2017a) and Baraquet et al. (2018a) for your sister species, B. cordobae); and K = growth coefficient.

Descriptive statistics for SVL and age, was calculated used Shapiro-Wilk tests to analyzed normality of distribution. Because the number of females per population is low, we used only data from males for analysis of

B. pulchella (n = 63). Pearson's correlation coefficient was used to determine relationships between SVL and age.

All tests were performed using Statistica 6.0/W software package (Statsoft Inc., USA) and Statgraphics Centurion XVI.I. All probabilities were two-tailed, and the significant level was set at a = 0.05. Means were given ± SD.

RESULTS

Skeletochronology

Sixty-seventh phalangeal cross-sections belonging to 63 males and 4 newly metamorphosed were carefully examined to estimate the individual age. All adult of B. pulchella studied showed recognizable bone structures that allowed age determination. In newly metamorphosed individuals, sections had a large medullar cavity and no LAGs. In all phalangeal cross sections showed a series of narrow concentric hematoxylinophilic rings, interpreted as LAG, separated by wide growth layers (Figure 2).

Endosteal resorption was present in 44.07% of the sample, and well-defined Kastschenko lines were observed but never caused any problem in age determination (Figure 2). Double and false LAGs were observed, but we counted the double line as one LAGs and false weren't accounted for (Figure 2b).

Body size and age

Mean and standard deviation of SVL for newly metamorphosed frogs (SVLmet) was 22.81 ± 2.23 mm (range, 20.87-24.74 mm). SVL of sexually mature males varies between 31.59 mm and 51.47 mm, and the average SVL was calculated as 43.39 mm ± 3.70.

Mean age of adult males of *B. pulchella* was 2.96 ± 0.76 years. The maximum observed lifespan (longevity) was 5 years, potential reproductive lifespan was 3 years and modal age was also 3 years (45.9%). Minimum age at sexual maturity was 2 year, and the smallest sexually mature male showed a SVL of 31.59 mm (Figure 3).

Pearson correlation showed that the SVL was significantly positively correlated with age (r = 0.5779, P < 0.000). An overlap of body length among the age classes was observed, however, in most individuals the larger body size was related to old older age.
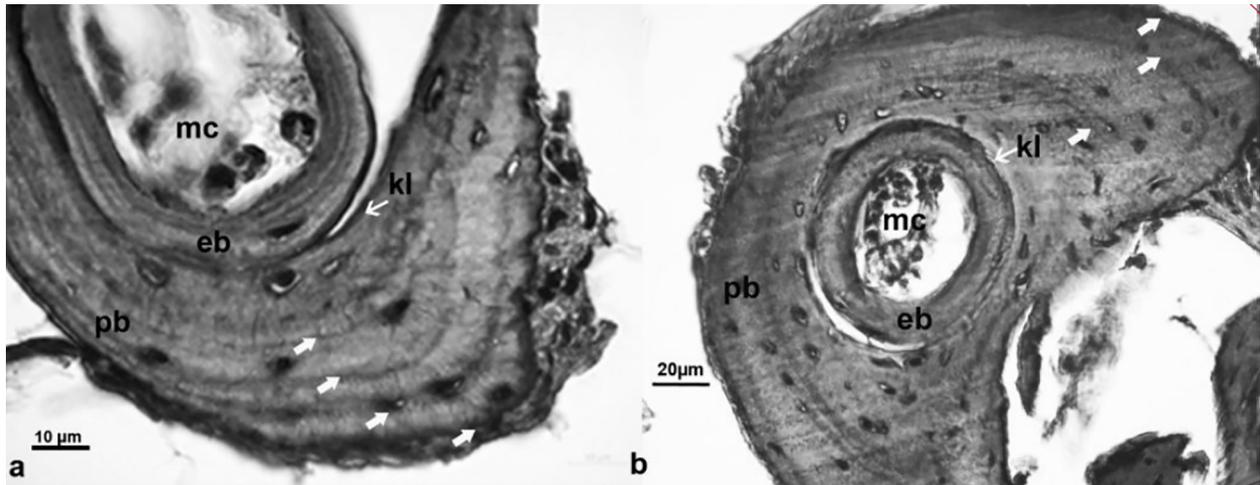
Figure 2. Examples of phalangeal cross-sections (10 µm thick) of *Boana pulchella*. (a) Male, SVL: 47.65 mm; 4 LAGs. (b) Male, SVL: 45.84 mm; 3 LAGs. White Arrows = lines of arrested growth (LAGs), in b 1 double LAGs; mc= Medullar Cavity; pb = Periosteal Bone; eb= Endosteal Bone; kl = Kastschenko Line. (Photographed by Mariana Baraquet)
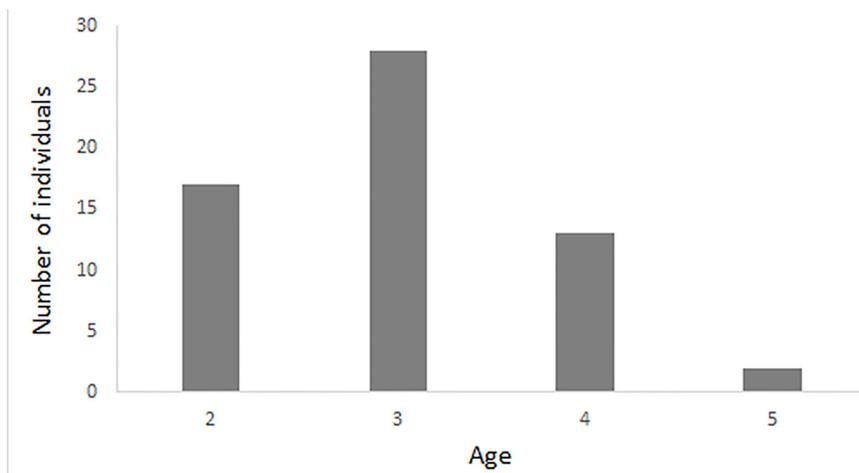
Figure 3. Age frequency distributions of male of *Boana pulchella*.

Growth patterns

The von Bertalanffy (1938) growth model correctly fitted the age/body-length relationship, with $r = 0.93$ and percentage of variance explained (VE) = 86% (Figure 4). The estimated asymptotic SVL was 47.48 ± 1.48 mm, with a confidence interval between 44.52 and 50.45 mm, and was less than the maximum SVL recorded in this study (51.47 mm). The growth coefficient K was 0.75 ± 0.12 with the confidence intervals between 0.5 and 1.01. We observed a decrease in growth rate was observed from the second to third year of age, just after the attainment of sexual maturity, 2 years (Figure 4).

Analyses for each population: Las Acequias, Alejandro Roca, Río Cuarto

SVL, age, demographic variables and parameters growth of adult males of *B. pulchella* for each locality are shown in Table I.

SVL was significantly and positively correlated with age in *B. pulchella* in two of the three sites (RC: $r = 0.5123$, P < 0.0063; LA: $r = 0.6781$, P < 0.0028) but not in the Alejandro Roca population ($r = 0.0494$, P = 0.8613). Minimum age at sexual maturity and modal age was the same in all three populations (Table I), and minimum SVL at sexual maturity was slightly larger in Las Acequias populations.
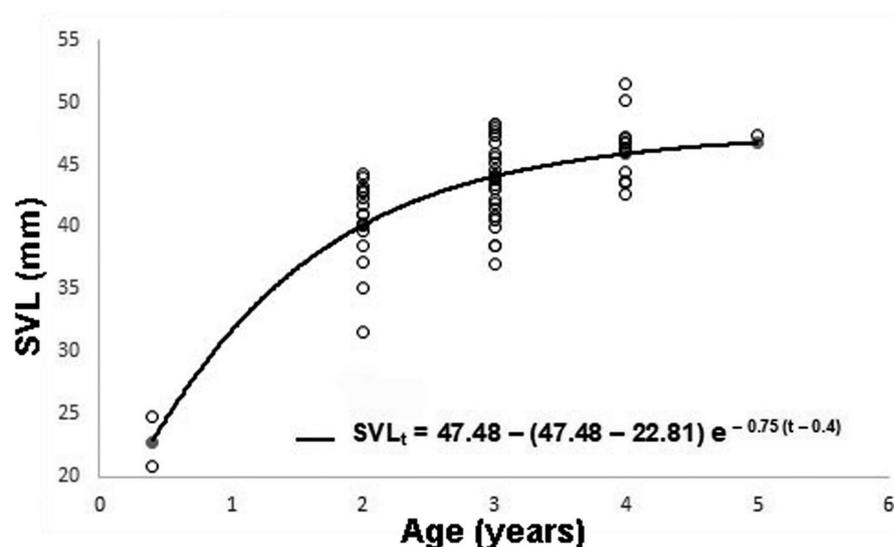
Figure 4. Relationship between snout-vent length (SVL) and age classes in *B. pulchella*. Lines represent the von Bertalanffy´s growth model. Also shown are the Von Bertalanffy´s equations ($SVL_t = SVL_{max} - (SVL_{max} - SVL_{met}) e^{-K (t - tmet)}$) with their respective value.

In relation to growth patterns, interpopulation comparisons showed that the von Bertalanffy (1938) growth model correctly fitted the age/body-length relationship in the three populations (r = 0.93, 0.96, 0.88 and percentage of variance explained (VE) = 86%, 92%, 78%, respectively to RC, LA, AR).

Alejandro Roca population showed smaller asymptotic size (SVLmax) but a greater growth coefficient (K), indicating that individuals of this population reach their maximum SVL more rapidly than the others populations. For populations of Río Cuarto and Las Acequias no differences were observed in SVLmax, but K was lower in the Río Cuarto population. However, these differences were not statistically significant because the 95% confidence intervals of SVLmax and K overlapped in all populations (Table I).

DISCUSSION

Over the past 30 years, skeletochronology has been widely used in studies of anuran populations, as it has proved to be a reliable determinant of the individual age of amphibians

(e.g. Halliday & Verrell 1988, Sinsch et al. 2001, Quiroga et al. 2015, Yakin & Tok 2015, Oromi et al. 2016, Otero et al. 2017a, b).

The presence of growth layers in bone tissue and the counting of lines of arrested growth (LAGs) serve to evaluate age in *Boana pulchella*, as showed by Brum et al. (2019). Historically, age estimation has long been attempted through the analysis of size class distribution (e.g. SVL measurements) which is not a reliable method, or mark-recapture records, method extremely laborious and much slower collecting of data in natural populations (Halliday & Verrell 1988).

Although Brum et al. (2019) provide the first record of growth rings to species and the first data on age, they only analyzed three individuals. Whereby, the present study provides the first life-history traits data of *B. pulchella*. In relation to the ecology of growth and age to sexual maturity there are many examples in other species among them studies carried out in its sister species, *Boana cordobae* (Baraquet et al. 2018a, Otero et al. 2017a). In both species average age is similar, modal age (3 years) and age at sexual maturity (2 years) is the same, although in *B. cordobae* the smallest sexually mature male showed a SVL

Table I. SVL, age, demographic variables and parameters growth of adult males of *B. pulchella* from three populations. Means ± standard deviation. Also shown are the maximum body length (SVL$_{max)}$, the growth coefficient (K) and the confidence interval (CI). In addition, metamorphs individuals were also included in all estimates.

Variables	Alejandro Roca (n = 17) 206 m a.s.l	Las Acequias (n = 19) 269 m a.s.l	Río Cuarto (n = 27) 420 m a.s.l
SVL	40.26 ± 2.39	46.34 ± 2.97	43.27 ± 3.27
Age (years)	2.60 ± 0.51	3.29 ± 0.85	2.96 ± 0.76
Longevity	3	5	4
Age at sexual maturity	2	2	2
Potential reproductive	1	3	2
Modal age (%)	3 (60%)	3 (41.17%)	3 (44.44%)
SVL$_{max}$ (CI$_{95\%}$)	40.29 ± 1.17 (49.97-53,13)	48.24 ± 2.6 (42.76-53.73)	48.61 ± 2.94 (42.57-54.64)
K (CI$_{95\%}$)	0.88 ± 0.11 (0.66-1.15)	0.79 ± 0.13 (0.52-1.05)	0.58 ± 0.15 (0.26-0.91)

(41.7 mm and 38.9 mm) greater; in addition, SVL and longevity were also greater. This difference is consistent with differences in body size between species, *B. cordobae* is slightly larger than *B. pulchella* (Cei 1980, Gallardo 1987, M.B. Baraquet, unpublished data), and furthermore because for *B. cordobae* the studies includes populations along altitudinal gradient (930 to 2,310 m), and it is known that in amphibians high-elevation populations produce larger and older individuals than their low-elevation counterparts (Miaud et al. 2000, Morrison & Hero 2003, Lu et al. 2006, Hsu et al. 2014, Altunışık & Özdemir 2015), and that age at sexual maturity and longevity are greater at high elevations (e.g., Morrison & Hero 2003, Zhang & Lu 2012, Hsu et al. 2014).

Besides, the estimated asymptotic SVL and the growth coefficient K was higher in males of B. cordobae than in B. pulchella, but this difference was not statistically significant because the confidence interval of the two species overlap. Like many anurans, *B. pulchella´*s slowed down

after sexual maturity (Liao & Lu 2010b, Guarino et al. 2011, Baraquet et al. 2018a, Otero et al. 2017a, b), growth rate was high until sexual maturity, after which decreased between the 2nd and 3rd year. It is a general life history strategy for ectothermic organisms that individuals tend to devote more energy into growth before sexual maturity to reach larger body sizes. When they reach maturity, growth slows down and more energy was devoted into reproduction (Iturra-Cid et al. 2010, Sinsch et al. 2010, Hsu et al. 2014).

Besides, the estimated asymptotic SVL and the growth coefficient K was higher in males of *B. cordobae* than in *B. pulchella*, but this difference was not statistically significant because the confidence interval of the two species overlap. Like many anurans, *B. pulchella´*s slowed down after sexual maturity (Liao & Lu 2010b, Guarino et al. 2011, Baraquet et al. 2018a, Otero et al. 2017a, b), growth rate was high until sexual maturity, after which decreased between the 2nd and 3rd year. Age and size at maturity often to be in direct conflict, as optimal time to mature is

the one that maximizes the fecundity benefits of reaching a large size while minimizing the cost of delaying reproduction (Dmitriew 2011). As our results show, in ectothermic organisms it is a general life history strategy that individuals tend to devote more energy into growth before sexual maturity to reach larger body sizes. When they reach maturity, growth slows down and more energy was devoted into reproduction (Iturra-Cid et al. 2010, Sinsch et al. 2010, Hsu et al. 2014); because if an increase growth rate requires additional energy and materials, less energy is available for reproduction (Arendt 1997).

Body size has long been considered one of the most important traits of an organism because it influences nearly every aspect of its life history (Peters 1983). In adult amphibian is determined by three factors: size at metamorphosis, growth rate, and age (Liao & Lu 2012).

The oldest individual in this study (5 LAGs) is also the largest (SVL 51.47 mm). It is widely assumed that amphibians show indeterminate growth and therefore their body size increases with age (Duellman & Trueb 1994, Halliday & Verrell 1988, Gümüş & Üzüm 2015). However, estimation of age from techniques such as skeletochronology has shown that in many species this correlation is weak or non-existent due to a high variability of within year size (Halliday & Verrell 1988), therefore, size cannot be considered a predictor of age, and this correlation must be analyzed for each particular species (Esteban et al. 2002). The present study has shown that the SVL and age are positively correlated, as has been reported in other species (Ashkavandi et al. 2012, Altunişik & Özdemir 2013, Otero et al. 2017a, b, Baraquet et al. 2018a, b). However, body size is not age specific, because there is a large overlap in size between the age classes.

Our study revealed that the estimated average age and longevity of *B. pulchella* were,

respectively, 2.96 and 5 years. The individuals reached sexual maturity within two years, and the modal age was three years. Brum et al. (2019) showed for this same species an average age of 3 and longevity of 4 years. The observed difference in longevity may be due to small sample size analyzed by these authors.

In our samples, we observed double and false LAGs, consistent with the description made by Liao & Lu (2010a): fainter LAGs that do not complete the bone section, called false LAGs and very closely spaced hematoxylinophilic lines, called double LAGs. These double LAGs are caused by unpredictable ecological factors such as abnormal climate impacts (e.g. high temperatures or dry conditions), diseases, or variations in food availability (Castanet et al. 1993, Guarino & Erismis 2008).

In conclusion, determining individual age of different populations of *B. pulchella* allowed for the first time establishes life-history traits of the species. In this way, basic data was obtained to allow comparisons to be made in the future with other populations of this species that are found in different geographical areas from its wide distribution. Also, since the declination of amphibian populations is evident and the lack of knowledge population biology make that cannot be distinguished the causes of that declines, the knowledge of relevant data such as life-history traits could be helpful for future conservation biology studies.

Acknowledgments

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MB and FEP designed the study; MB and PRG extracted the samples; MB and MO performed laboratory protocols following standard skeletochronology methods; MB, FEP and PRG performed the statistics analyses; MB, FEP, MO, PRG, NES and ALM discussed the results and wrote the article.