



First record of growth rings for 11 native subtropical anuran species of South America

AMANDA J.C. BRUM¹, LUIZA LOEBENS¹, MAURÍCIO B. DOS SANTOS³ and SONIA Z. CECHIN^{1,2}

¹Animal Biodiversity Postgraduate Program, Federal University of Santa Maria, 1000 Roraima Avenue, Camobi, 97105-900 Santa Maria, RS, Brazil

²Department of Ecology and Evolution, Federal University of Santa Maria, 1000 Roraima Avenue, Camobi, 97105-900 Santa Maria, RS, Brazil

³Toxicological Biochemistry Postgraduate Program, Federal University of Santa Maria, 1000 Roraima Avenue, Camobi, 97105-900 Santa Maria, RS, Brazil

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Abstract: Skeletochronology is the most accurate method to estimate a population age structure. The methodology is based on the analysis of secondary bone tissue in order to count growth rings. This study aimed to provide initial data, allowing researchers to further work out in the age of individuals and populations, sampling evidence of the presence of growth rings in 11 native species (representing nine families) of a subtropical region of southern Brazil. Four bone samples of each specimen were used to perform the skeletochronological analysis: the penultimate phalanges of the 3rd and 4th fingers, the humerus, and the femur. The presence of growth rings was confirmed in the periosteal layer of the bones of all analyzed species. In comparison with phalanges, growth rings of humeri and femora are more irregular and less distinguishable. This is the first record of growth rings to the native species herein analysed. The skeletochronology was proved to be an effective tool in determining the age of anuran amphibians from a subtropical region, since this environment presents well defined climatic seasonality.

Key words: Bone chronology, frogs, lines of arrested growth, southern Brazil.

INTRODUCTION

The study of amphibians' population age structure is crucial to understanding population dynamics and species natural history, since it provides information regarding growth rate, sexual maturity, longevity, and reproductive lifespan (Li et al. 2010). The analysis of bone tissue to estimate age (known as skeletochronology) has been used in both fossil (Botha and Chinsamy 2000, Sander

2000, Tütken et al. 2004, Botha-Brink et al. 2018) and extant tetrapods, such as 'reptiles' (Avens et al. 2009, Goshe et al. 2010), birds (Ricqlès et al. 2003), and amphibians (Socha and Ogielska 2010, Cajade et al. 2013, Bionda et al. 2015, Sun et al. 2016, Kumbar and Lad 2017, Tessa et al. 2017). Skeletochronology is based on the presence of growth rings (lines of arrested growth — LAGs) in transverse sections of the bones, in which broad lines represent the growth period and narrow lines represent a growth pause. Thus, each narrow line characterizes one growth year, allowing the

Correspondence to: Amanda Jamille Carvalho Brum
E-mail: amanda.c.brum94@gmail.com
ORCID: <https://orcid.org/0000-0002-4507-8624>

estimation of the age of individuals (Castanet and Smirina 1990).

The formation of annual growth marks in anurans from temperate zones occurs due to seasonal climatic variation, since growth is delayed along the winter (Castanet et al. 2003). According to Castanet et al. (1993), annual formation of LAGs has a genetic basis and under natural conditions their formation is synchronized with the climatic seasonality. It is noteworthy that age estimation by LAGs counting is effective only if the species' growth pattern is annual (Gibbons and McCarthy 1983). However, although some studies point that anurans from environments with low seasonal climatic variation usually present an irregular pattern of LAGs formation (Kusrini and Alford 2006), other studies indicate that species from tropical regions may also have evident LAGs (Rebouças et al. 2018).

Southern Brazil has a subtropical climate and resembles temperate regions concerning seasonal temperature variation (Overbeck et al. 2007). The heterogeneous environment in southern Brazil hosts an anuran fauna distributed in nine recognized families: Alsodidae, Bufonidae, Centronelidae, Hylidae, Hylodidae, Leptodactylidae, Microhylidae, Odontophrynidae, Phyllomedusidae (Santos et al. 2014). However, studies of subtropical herpetofauna are mainly focused on investigating the communities' structure (Eterovick et al. 2005), and describing the species distribution patterns (Rosset et al. 2006). Therefore, studies involving skeletochronology of native species from South American subtropical regions are scarce (Echeverria and Filipello 1990, Marangoni et al. 2009, Iturra-Cid et al. 2010, Cajade et al. 2013, Caldart et al. 2019). Thus, we investigated the presence of LAGs in anurans representing all families (11 native species) of southern Brazil.

MATERIALS AND METHODS

Skeletochronology was applied in species of wide distribution in southern Brazil (Paraná, Santa

Catarina, and Rio Grande do Sul states). According to Koppen's climate classification, the region's climate is considered humid subtropical (Cfa-Cfb), with well-defined temperature seasonality (Alvares et al. 2013). All sampled species have seasonal activity pattern linked to the warm season (see Sá and Gerhau 1983, Kwet and Miranda 2001, Kaefer et al. 2007, Both et al. 2008, Narvaes and Rodrigues 2009, Machado et al. 2014, Caldart et al. 2016): Alsodidae — *Limnomedusa macroglossa* (Duméril and Bibron 1841); Centronelidae — *Vitreorana uranoscopa* (Müller 1924); Bufonidae — *Rhinella fernandezae* (Gallardo 1957) and *Melanophryniscus atroluteus* (Miranda-Ribeiro 1920); Hylidae — *Boana pulchella* (Duméril and Bibron 1841); Hylodidae — *Crossodactylus schmidti* (Gallardo 1961); Leptodactylidae — *Leptodactylus fuscus* (Schneider 1799) and *Physalaemus cuvieri* (Fitzinger 1826); Microhylidae — *Elachistocleis bicolor* (Guérin-Méneville 1838); Odontophrynidae — *Odontophrynus americanus* (Duméril and Bibron 1841); Phyllomedusidae — *Phyllomedusa iheringii* (Boulenger 1885). All specimens used were already deceased and deposited in the herpetological collection of the Santa Maria Federal University (ZUFMS — Appendix).

Three specimens of each species were examined. The following measurements were made for each specimen: body mass (BM), using a balance (0.01-g precision) and snout-vent length (SVL), using a digital caliper to the nearest 0.01 mm. The specimens were dissected to extract bone samples from penultimate phalanges of the 3rd and 4th fingers of the foot, and long bones (humerus and femur). As a standardization procedure, all bone samples were collected from the limbs on the right side of the specimens.

After extraction, the bone samples were dehydrated on an increasingly series of alcohol (70–100%) for one hour for each step. Then, they were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for one week. Thereafter, the bones were processed in historesin, transversely sectioned in a Leica RM 2245 rotary microtome, and stained

using toluidine blue for ten minutes (Caputo et al. 2010). After stained, the samples were dried in kiln for one day. For each specimen, four sections from the middle region of the bone diaphysis were taken. Slices were independently analyzed using a Zeiss Axio Scope A1 microscope with an Axiocam MRc 5 digital camera by at least two different authors. To identify the occurrence of bone resorption in long bones, the LAGs counting was comparatively analyzed considering phalanges as references. The bone resorption was also determined based on the absence of remaining cartilage tissue from the larval stage (line of metamorphosis) (Rozenblut and Ogielska 2005).

RESULTS, DISCUSSION AND CONCLUSIONS

Transverse sections of phalanges and long bones of all species exhibited LAGs. Although the rings

were not uniformly clear in all bones, LAGs were observed in the periosteal layer of the bones of all analyzed species. Phalanges and long bones often had the same number of LAGs. However, the phalanges allowed a better identification of LAGs than long bones and considering long bones, identification of LAGs was better in the femur than in the humerus. Likewise, regarding the phalanges, the identification of LAGs was better in the phalanges of the 4th finger than in phalanges of the 3rd finger.

The number of LAGs identified in the studied species was (see Table I): *M. atroluteus* (2-3; Fig. 1b), *C. schmidtii* (1-3; Fig. 1g), *B. pulchella* (2-4; Fig. 1e), *R. fernandezae*, *Phyl. iheringii*, and *Phys. cuvieri* (3-4; Fig. 1c-f-i), *Li. macroglossa* (3-5; Fig. 1a), *O. americanus* (2-5; Fig. 1k), *Le. fuscus* (1-

TABLE I
Number of lines of arrested growth (LAGs), snout-vent length (SVL), and body mass (BM) of anurans from Subtropical climate.

Family	Species	LAGs		SVL (mm)		BM (g)	
		Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Alsodidae	<i>Limnomedusa macroglossa</i>	3.33 ± 0.58	3–5	54.49 ± 2.71	52.34–57.53	19.53 ± 7.46	16.00–28.10
Bufonidae	<i>Melanophryniscus atroluteus</i>	3.00 ± 0.00	2–3	21.73 ± 0.89	20.86–22.63	1.73 ± 0.21	1.50–1.90
	<i>Rhinella fernandezae</i>	3.67 ± 0.58	3–4	57.41 ± 6.84	45.84–62.25	15.93 ± 4.47	12.00–20.80
Centronelidae	<i>Vitreorana uranoscopa</i>	3.33 ± 1.53	1–5	23.36 ± 0.85	22.76–24.82	0.42 ± 0.14	0.25–0.50
Hylidae	<i>Boana pulchella</i>	3.00 ± 1.00	2–4	36.94 ± 5.65	32.94–44.20	3.47 ± 1.37	1.90–4.40
Hylodidae	<i>Crossodactylus schmidtii</i>	2.33 ± 0.58	1–3	26.28 ± 0.95	25.61–29.61	2.00 ± 0.52	2.00–2.90
Leptodactylidae	<i>Leptodactylus fuscus</i>	3.33 ± 2.08	1–5	44.69 ± 0.81	44.11–46.47	9.00 ± 2.26	7.40–12.25
	<i>Physalaemus cuvieri</i>	3.33 ± 0.58	3–4	29.66 ± 0.66	29.04–30.35	2.57 ± 0.74	2.00–3.40
Mycrohylidae	<i>Elachistocleis bicolor</i>	5.00 ± 1.73	2–7	31.17 ± 2.67	29.43–34.24	3.40 ± 1.15	2.50–4.70
	<i>Odontophrynidae americanus</i>	3.00 ± 1.73	2–5	39.79 ± 9.19	31.37–49.49	10.77 ± 5.52	7.00–17.10
Phyllomedusidae	<i>Phyllomedusa iheringii</i>	3.33 ± 0.58	3–4	50.28 ± 2.52	48.5–53.20	7.50 ± 2.12	6.00–9.00

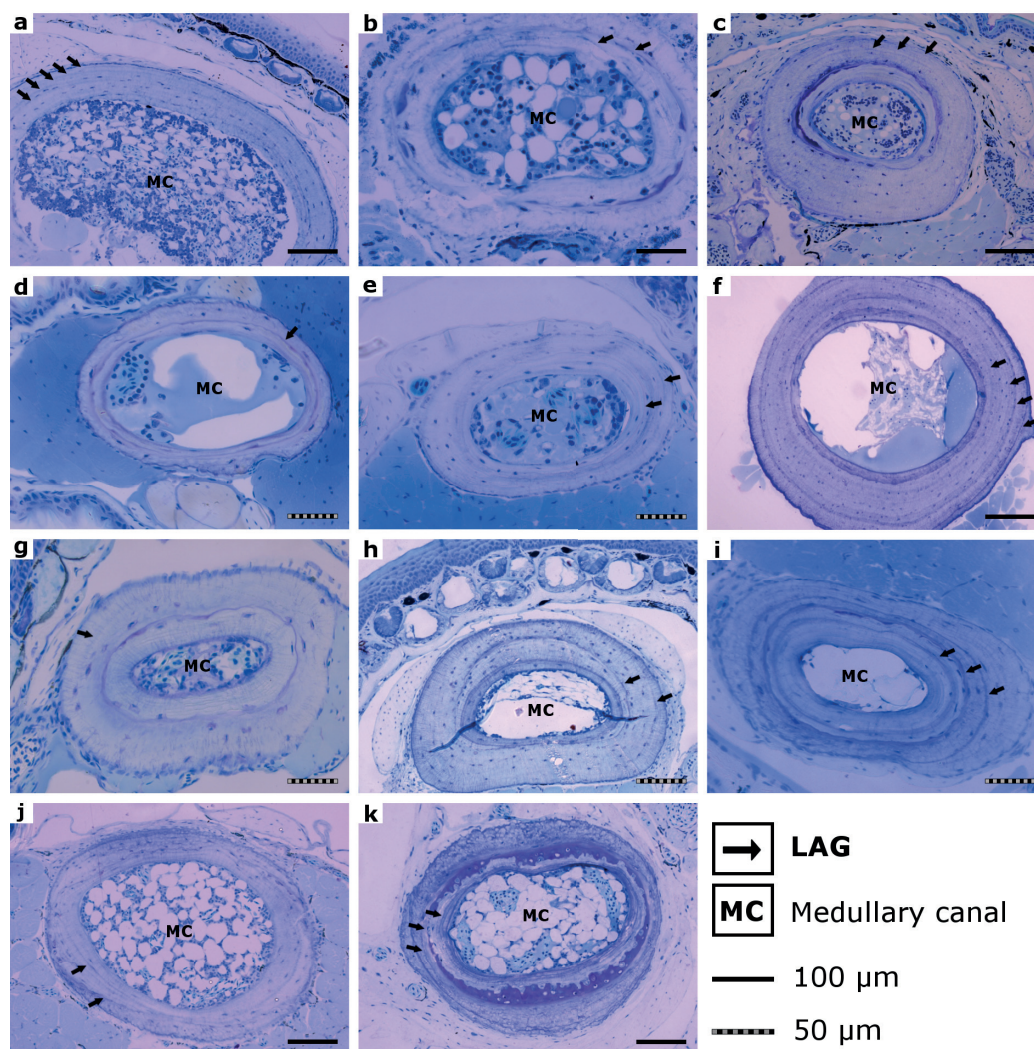


Figure 1 - Cross-sections of the falanges showing the LAGs in (a) *Limnomedusa macroglossa*; (b) *Melanophryniscus atroluteus*; (c) *Rhinella fernandezae*; (d) *Vitreorana uranoscopa*; (e) *Boana pulchella*; (f) *Phyllomedusa iheringii*; (g) *Crossodactylus schmidtii*; (h) *Leptodactylus fuscus*; (i) *Physalaemus cuvieri*; (j) *Elachistocleis bicolor*; and (k) *Odontophrynus americanus*.

5; Fig. 1h), *V. uranoscopa* (1-5; Fig. 1d), and *E. bicolor* (2-7; Fig. 1j).

Skeletochronology was effective for both phalanges and long bones, since all matched the corresponding number of LAGs for each specimen studied. Furthermore, skeletochronology can be a non-lethal procedure if performed using the specimens' phalanges solely (Sinsch et al. 2007, Ginnan et al. 2014, Sinsch 2015, Hudson et al. 2017), since using long bones demands the euthanized of the specimens. Besides, LAGs are more evident

in phalanges than in long bones, thus, phalanges are the most suitable bones for determining age in anurans (Kumbar and Pancharatna 2001). Another advantage of the method is the possibility of analyzing specimens from museum collections, without having to capture new specimens (see Sinsch 2015).

Because of the low sample of specimens per species herein analyzed, we consider this study as an exploratory work, providing evidence that this method is effective in estimating the age of

subtropical climate anurans of southern Brazil. Additionally, this contribution presents the first record of LAGs to 11 native species of southern Brazil. Thereby, we encourage the development of new studies regarding anurans species from tropical and subtropical regions, to better understand the evolution of ecological patterns associated to this environment, and their significance in a broader view concerning anuran amphibians, one of the most ecologically endangered group of vertebrates worldwide.

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AUTHOR CONTRIBUTIONS

AJCB, LL, MBS, and SZC designed the study; AJCB and MBS extracted the samples; AJCB and LL performed the histological protocol; ACJB and LL performed the analyses; AJCB, LL, MBS, and SZC discussed the results; AJCB, LL, MBS, and SZC wrote the article.

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APPENDIX

Voucher individuals analyzed in this study housed in the herpetological collection of the Federal University of Santa Maria (ZUFMS):

Crossodactylus schmidtii (n = 3): BRAZIL: Rio Grande do Sul: Derrubadas, ZUFMS 4682, 5200, 5209.

Elachistocleis bicolor (n = 3): BRAZIL: Rio Grande do Sul: Santa Maria, ZUFMS 0046, 0879; Manoel Viana: ZUFMS 2466.

Boana pulchella (n = 3): BRAZIL: Rio Grande do Sul: Itaara, ZUFMS 4123; Vacaria: ZUFMS 6077; Santana do Livramento, 8473.

Leptodactylus fuscus (n = 3): BRAZIL: Rio Grande do Sul: Ibarama, ZUFMS 3784; São Borja, ZUFMS 5016, 5017.

Limnomedusa macroglossa (n = 3): BRAZIL: Rio Grande do Sul: Aceguá, ZUFMS 5109; Santa Maria, ZUFMS 8456; Santana do Livramento, ZUFMS 8493.

Melanophryniscus atroluteus (n = 3): BRAZIL: Rio Grande do Sul: São Borja, ZUFMS 4984, 4985, 4990.

Odontophrynus americanus (n = 3): BRAZIL: Rio Grande do Sul: Santa Maria, ZUFMS 2762; Dom Feliciano, ZUFMS 3055; São Francisco de Assis, ZUFMS 4462.

Phyllomedusa iheringii (n = 3): BRAZIL: Rio Grande do Sul: Santa Maria, ZUFMS 3051; Caçapava do Sul, ZUFMS 3370; São Sepé, ZUFMS 8082.

Physalaemus cuvieri (n = 3): BRAZIL: Rio Grande do Sul: Santa Maria, ZUFMS 0054, 0238, 1679.

Rhinella fernandezae (n = 3): BRAZIL: Rio Grande do Sul: Arroio Passo do Lava-Pé, ZUFMS 4089; São Borja, ZUFMS 8527, 8528.

Vitreorana uranoscopa (n = 3): BRAZIL: Rio Grande do Sul: Derrubadas, ZUFMS 4571, 4572, 4584.