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Chronology of the LAGs formation and body growth in *Argenteohyla siemersi* from northeastern Argentina

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Abstract

We studied the chronology of LAG formation and validated the method for estimate age in *Argenteohyla siemersi* inhabiting the humid Chaco of Argentina. In addition, we determined whether well-expressed growth marks are formed in *A. siemersi* when raised during one year in laboratory, to determine whether formation is genetically or environmentally based. Individuals increased 84.51% in BM and 52.47% in SVL after one year of growth in the laboratory. Two out of eight individuals that survived at the end of the experiment showed well-expressed growth marks. Overall, we confirm the reliability of the skeletochronology in estimating age of this specie and reinforce the hypothesis that, the LAG formation is ultimately caused by a general intrinsic (genetic) control.

Key words: Skeletochronology, Lines of arrested growth, Growth, *Argenteohyla siemersi*, Argentina.

Running title: Chronology of Growth Marks in *Argenteohyla siemersi*.

Introduction

Determination of accurate ages and growth rate of individuals has been one of the most important interest of evolutionary biologist, since the knowledge of the age structure of a population is an important tool for studies of demography and conservation status of any specie (Halliday & Tejedo 1995). Different methods of age determination have been implemented, however, skeletochronology (the technique based on counting Lines of Arrested Growth, LAGs, in the bone tissue) has become a standard procedure to estimate the age in a large number of vertebrates including amphibians (Castanet 1982, Castanet & Smirina 1990). Skeletochronology was first applied in amphibians from the temperate region, where the climatic conditions impose a cessation of activity during winter, and demonstrated the presence of deposited growth marks deposited in the bone tissue and its annual chronology (Castanet & Smirina 1990, review in Sinsch 2015). At present the increase of studies regarding aging and growth patterns in tropical and subtropical anurans (Kumbar & Pancharatna 2002, Marangoni et al. 2009, 2012, 2018a,b; Cajade et al. 2013, Attademo et al. 2014, Bionda et al. 2015, Quiroga et al. 2015, Stănescu et al. 2016), support the hypothesis of Castanet et al. (1993) that LAG formation is genetically based, with an annual rhythm, and which could be reinforced the seasonal cycle. Despite these additional studies of LAGs formation in subtropical and tropical anurans, before ages can be assigned confidently we need to confirm the chronology of LAG formation (Marangoni et al. 2009). This is not always confirmed and most studies assume the annual chronology by comparison with other related species, but exceptions to the "one LAG per year" rule, related to a double cycle of annual activity (hibernation and aestivation), have been reported (Caetano et al. 1985, Caetano 1990, Caetano & Lecalir Jr. 1999, Olgun et al. 2005, Iturra-Cid et al. 2010).

The monospecific genus of hylid frogs *Argenteohyla* Trueb 1970 is included in the tribe Lophiohylini (Miranda-Ribeiro 1926), which represents the monophyletic group known as "Casque-headed Frogs" (Faivovich et al. 2005). *Argenteohyla siemersi* (Mertens 1937) is characterized by having a heavily ossified skull, and the skin of the dorsal surfaces of the skull partially co-ossified with the underlying bone (Trueb 1970). These characteristics are related to its secretive life, remaining the great part of the time inside of bromeliads, and to its anti-predator mechanisms, as were recently demonstrated (Cajade et al. 2017). The distribution range of *A. siemersi* includes, from north to south, the phytogeographic provinces of Chaco, Espinal and Pampeana (Cabrera 1976), being present in Argentina, Paraguay and Uruguay (Trueb 1970). According to IUCN *A. siemersi* is listed as "Endangered" (Lavilla et al. 2004). The little time to observe *A. siemersi* in activity is during its explosive reproduction. Most of the information for this species comes from taxonomy-related research (Barrio 1966, Trueb 1970, De Sá 1983, Williams & Bosso 1994, Morand & Hernando 1996, Céspedez 2000). Additional studies where done in relation to investment (Diminich & Zaracho 2008), reproductive activity and calling behaviour, as well as the tadpole redescription (Cajade et al. 2010), age, body size and growth (Cajade et al. 2013), and multiple anti-predator mechanisms (Cajade et al. 2017). In addition, our recently study in *A. siemersi* has shown that well-expressed growth marks are formed in this specie (Cajade et al. 2013) and assume the general "one LAG per year" rule, but it has never been confirmed. Furthermore, our previous study did not allow us to determine the causes LAGs formation. Thus, the main goal of this study was to confirm the chronology of LAG formation and valid method for estimating age in *A. siemersi*. A secondary goal was determining whether well-expressed lines of arrested growth are formed in *A. siemersi* in laboratory, without the effects of highly variable of the environment. This would help to determine whether formation is genetically or environmentally based.

Materials and methods

On 5 November 2014, 11 tadpoles (41 Gosner Stage) of *Argenteohyla siemersi* were captured in a temporary pond on at Riachuelito (27°33'45.80"S; 58°34'48.67"W), Department of San Luis del Palmar, Corrientes province, Argentina. Tadpoles were kept separately in plastic trays filled with dechlorinated tap water until tadpoles reached the metamorphosis (defined as the emergence of the first forelimb; Gosner stage 42). Each metamorph was raised separately in 3-liter plastic box containing a substrate of humid tissue paper and a piece of plastic pipe with water for shelter. Animals were maintained at controlled environmental conditions for one year (19 November 2014 – 19 November 2015). Room temperature was kept roughly constant (range 20–24 ºC) and the photoperiod varied from 10 to 14 daylight hours, following a natural cycle. Metamorphs were fed with natural collected grasshoppers, dragonflies, caterpillars and crickets. The animals were offered only one item, and the same amount (e.g. one or two grasshoppers, depending of their size) each time they were fed, to ensure that all animals were in equal environmental conditions, including their feeding behaviour. The snout–vent length (SVL) of the metamorphs was measured using a digital caliper 0.01 mm, and body weight (BM) using an Ohaus® Traveler Scale TA320 electronic balance (precision 0.01 g). All measurements were recorded once per week. After one year, on 19 November 2015, we clipped the third digit of the right forefoot and preserved in 70 % alcohol for age determination by skeletochronology. Skeletochronology is a non-lethal andd widely-used method, where the growth periods appear as broad bands of tissue separated by narrower lines, or annuli, which mark periods of reduced growth (Halliday & Verrel 1988). We followed the standard methods in skeletochronology (e.g., Smirina 1972, Castanet & Smirina 1990), with minor modifications proposed by Marangoni (2006). Clipped digits were washed in water for 30 minutes and then decalcified in 5% nitric acid for 1-3 hours. The bone samples were then dehydrated, paraffin-embedded, sectioned using a rotation microtome (Arcano RMT-30) at 14–16 µm, and stained with Harris haematoxylin. We took digital images of those cross-sections where the size of the medullar cavity was at its minimum and the periosteum was at its maximum, using a Leica DM500 optic microscope and software Leica LEAD Technologies Inc.V1.01. Cross-sections were observed and measured using the computer package Image-Pro Plus version 4.5 (Media Cybernetics 1993–2001; Silver Spring, MD, USA), and calibrated using a standard micrometre. Two independent observers (FM and JMP) recorded the presence/absence of the line of metamorphosis and counted the LAGs. In those individuals with no remnant of the line of metamorphosis we estimated the degree of resorption by osteometrical analysis (Sagor et al. 1998, Tomašević et al. 2008). We distinguished annual growth marks (i.e., LAGs sensu stricto) from non-annual ones (i.e., irregular interruptions during short periods of inactivity), using the method described in Sinsch et al. (2007). We first tested the data for normality and homoscedasticity using Shapiro-Wilk and Levene tests, and chose the statistical tests accordingly. We computed von Bertalanffy growth model (von Bertalanffy 1938) following Beverton & Holt (1957): $SVLt = SVLmax \cdot (1-e-k \cdot (t- t0))$, where SVLt is the expected or average SVL at time (or age) t, SVLmax is the asymptotic average SVL, k is the growth rate coefficient and t0 is the time or age when the average SVL was zero. A high value of k indicates that SVLmax will be achieved soon. All tests were done using the statistical package Statistica 6.0 (Statsoft Inc., USA 2001). We used a significance level of $\alpha = 0.05$.

Results

Eight out of eleven metamorphs of *A. siemersi* kept in the laboratory conditions survived at the end of the experiment (one year later). Morphological traits increased significantly compared to the corresponding values at the beginning of the experiment (Table 1). The mean body mass and SVL at metamorphosis was: Mean = 0.81 g, SD = 0.1 and, Mean = 21.84 mm, SD = 0.81, n = 11 , whereas the body mass and SVL reached at the end of the experiment was: Mean = 5.23 g, SD = 2.64, Mean = 45.95 mm, SD = 4.67, n = 8, body mass (BM) and snout-vent length (SVL) respectively. This represented an increase of 84.51% in BM and 52.47% in SVL after one year of growth in the laboratory. The size of the marrow cavity, metamorphosis line, line of arrested growth and diameter of bone perimeter are summarized in the Table 1. Endosteal resorption was observed only in four cases but did not prevent age estimation. The line of metamorphosis was visible in all specimens, whereas in only two individuals was observed the first LAG (Fig. 1). In addition, after ten months of growth in the laboratory, one of the frog (#9), in which the first well-defined LAG was found in the periosteal bone, presented dark vocal sacs and produced advertisement calls. The R^2-values (BM: 0.770, SVL: 0.905) and asymptotic standard errors of the estimated parameters indicate that the relation between age and SVL fitted von Bertalanffy's growth model (Fig. 1). The predicted asymptotic average BM (BM_{max}: Mean = 10.14 g, SE = 4.78; t_0: Mean = 0.38 g, SE = 0.18; K: Mean = 0.07 g, SE = 0.05, n = 11) and SVL (SVL_{max}: Mean 52.95 mm, SE = 2.20; t_0: Mean = 20. 39 mm, SE = 0.74; K: Mean = 0.07 mm, SE = 0.05, n = 11) were both slightly larger than the measured average values (Table 1).

Discussion

During the 1980s and 1990s several studies suggested that the cyclical growth marks can be absent or very reduced and diffuse in anurans populations living in less continental climates, like tropical or subtropical, where resting periods may not occur (Henández & Seva 1985, Esteban 1990, Esteban et al. 1996 1999, Castanet et al. 1993). However, increasing number of studies confirmed the presence of well-defined LAGs in amphibians living in tropical and subtropical regions, where growth is not constrained by highly variable environment conditions (Kumbar & Pancharatna 2002, Marangoni et al. 2009, 2012, 2018a,b; Attademo et al. 2014; Quiroga et al. 2015, Stănescu et al. 2016; Bionda et al. 2018). In *A. siemersi* this also was already confirmed for our previous (Cajade et al. 2013) and present study. But, before the present study it was still not clear whether these growth marks are formed annually and whether they can be regarded as year rings to determine the age of these subtropical amphibian species. For example, supplementary nonannual LAGs have been observed occasionally (e.g., double LAGs or annuli lines, Tejedo et al. 1997, Sinsch et al. 2007). Thus, before ages can be assigned confidently we need to compare the estimated age based on skeletochronology with the known age of the individuals, and thus, the chronology of LAG formation can be confirmed (Marangoni et al. 2009). Two alternatives are possible to establish this correspondence. (1) using the phalanges of marked individuals at known ages, i.e. at metamorphosis, and apply the method again later when them are recaptured in the field, or after a year of captivity or (2) counting the number of LAGs in individual of unknow age and then, observe the additional LAGs formed after one or two years, when them are recaptured. Our study reports the first evidence of the correspondence between an observed pattern of bone growth and actual age in individuals of *A. siemersi* raised in laboratory conditions for one year (one LAG per year), and furthermore allowed us to confirm the reliability of the skeletochronology in estimating age of this specie. Respect to the low percentage of individuals who presented growth marks (2 out of 8), we think that in spite of the LAGs are genetically determined, they are not formed exactely at one year. We are sure that it could vary more or less a few weeks or some months even. Thus, taking account that we used the skeletochronology when the metamorphs reached exactely 1 year old, we suggest that it could be the explanation to the few individuals with LAGs found.

The individuals studied here came from an area, coinciding with the phytogeographic province of Humid Chaco (Carnevali 2003), characterized by the presence of numerous temporary and semi-permanent ponds. The climatelacks a pronounced dry season, although periods of rain shortages occur every 4–6 years. Mean annual temperature is 21.5°C and the mean annual precipitation is 1500 mm (Carnevali 1994, a more detailed description of the area in Cajade et al. 2010). *A. siemersi* is an explosive breeder, with a reproductive period from July to November (Barrio 1966, Céspedez 2000, Cajade et al. 2010). The breeding activity of the population of present study occurred immediately after the first heavy rains at the beginning of spring, and is characterized by a very short breeding activity of three days (Cajade et al. 2010). After this period, it is possible to found it active into its refuge in bromeliads or tree holes, and we suggest that *A. siemersi* has no a marked hibernation or aestivation period in nature. This, together with the presence of well-expressed growth marks in individuals raised in laboratory conditions, where growth was not constrained by environmental conditions (Fig. 1), supports the hypothesis that, for this species inhabiting a not highly seasonal subtropical climate, the LAG formation is ultimately caused by a general intrinsic (genetic) control (Sinsch et al. 2007).

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Table and figure caption:

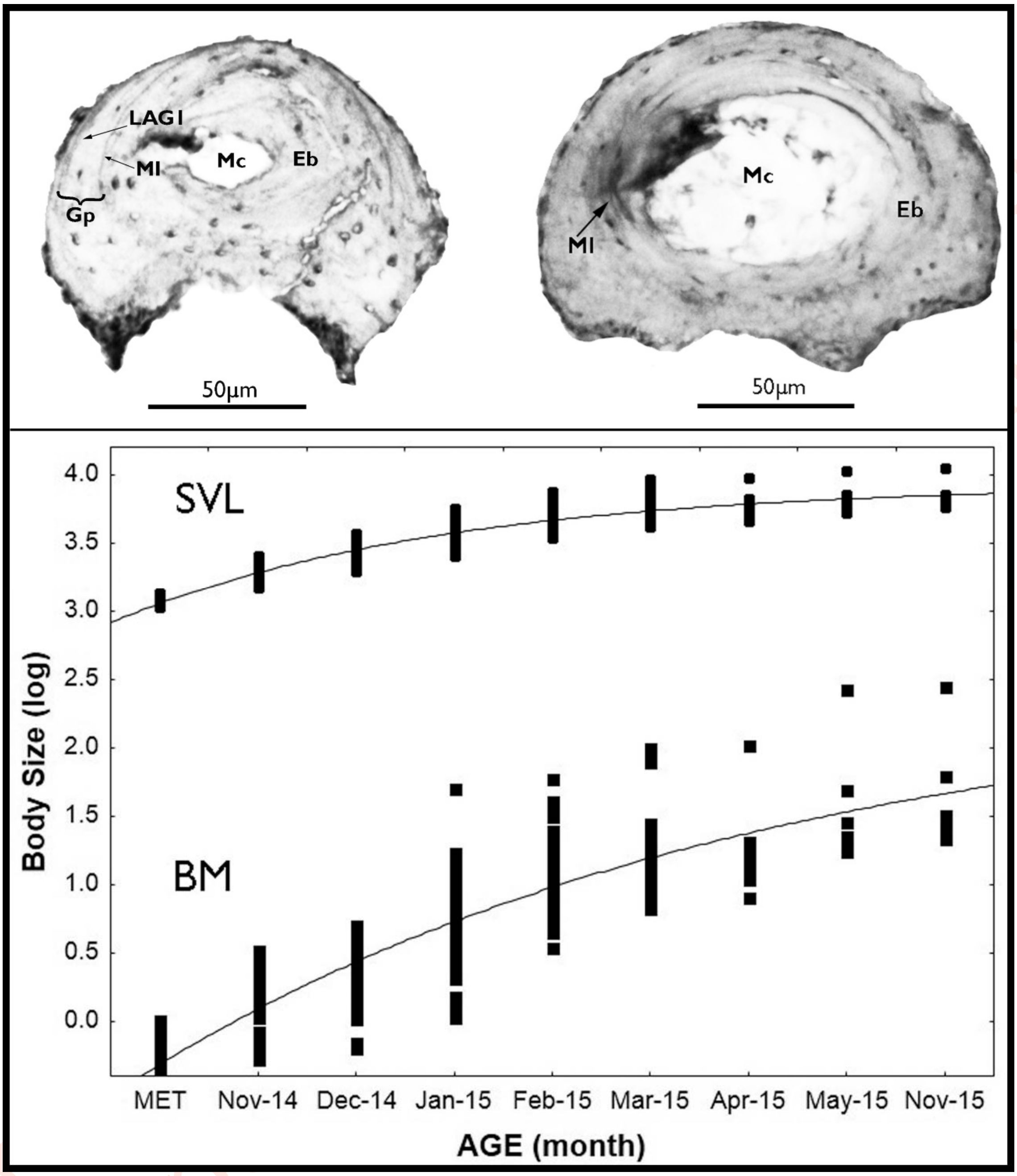
Table 1: Body size at the beginning (metamorphosis) and at the end of the experiment (one year) of *Argenteohyla siemersi*. SVL = snout-vent length, BM = body mass, Mc = marrow cavity, Ml = metamorphosis line, LAG = line of arrested growth, PER = diameter of bone perimeter. * = dead before one year of the experiment.

Figure 1: Upper part: Cross-sections of the third toe of two individual of *Argenteohyla siemersi* reared in laboratory conditions for one year: Left: Individual #7 with the first LAG visible (BM = 3.81 g; SVL = 44.07 mm). Rigth: Individual #5 without LAG (BM = 5.43 g; SVL = 47.23 mm). First LAG and metamorphosis line (Ml) indicated by arrows, GP = first period of bone growth, Mc = marrow cavity, Eb = endosteal bone. Lower part: Growth curves and estimated parameters from von Bertalanffy equations for body growth (SVL and BM) of *Argenteohyla siemersi*.

Table 1.

Individual	Body Size (Initial – Final)		Bone Size (µm)			
	SVL (mm)	BM (g)	Mc	Ml	LAG1	PER
1	24.37 - 43.88	1.27 - 4.25	40.12	80.75	--	127.91
2	26.81 - *	1.18 - *	--	--	--	--
3	23.86 - 43.01	0.93 - 3.89	55.43	77.89	--	114.00
4	26.7 - 44.84	1.44 - 4.53	61.88	83.91	--	126.62
5	28.79 - 47.23	1.68 - 5.43	61.8	87.42	--	119.26
6	25.16 - 44.33	1.03 - 3.98	73.04	108.02	--	153.53
7	25.01 - 44.07	1.05 - 3.81	23.06	61.73	92.53	112.57
8	25.76 - *	1.38 - *	--	--	--	--
9	26.29 - 57.05	1.35 - 11.51	64.92	76.91	134.39	125.35
10	25.08 - 43.21	1.22 - 3.86	50.27	78.04	--	132.41
11	24.57 - *	1.33 - *	--	--	--	--

359 Figure 1.


LAG1
Ml
Mc
Eb
Gp
50µm
Mc
Ml
Eb
50µm
SVL
BM
Body Size (log)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
MET
Nov-14
Dec-14
Jan-15
Feb-15
Mar-15
Apr-15
May-15
Nov-15
AGE (month)