

Age structure, growth pattern, sexual maturity, and longevity of *Leptodactylus latrans* (Anura: Leptodactylidae) in temperate wetlands

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Abstract. We present the first data on age structure, growth pattern, and lines of arrested growth (LAG) for *Leptodactylus latrans* in temperate wetlands. Based on these data, we estimate LAG periodicity, age, size at sexual maturity and longevity for this species. We also tested for differences of these parameters between sexes. The age was determined through skeletochronology. Female maturity was determined by presence of differentiated ova, while male maturity was assessed through histological analysis to evaluate spermatozoid production. To establish whether this species marks one LAG per year, eight individuals were kept one year in captivity. For each specimen, LAG was compared for different phalanges of the same toe clipped at start and end of captivity. *Leptodactylus latrans* marked one LAG per year, indicating a growth rhythm adjusted to a seasonal environment and mainly driven by genetic factors. Longevity was five years for both sexes and frogs reached sexual maturity during the first year, exhibiting a reproductive lifespan of four years. Sexual maturity was related to a minimal size of 60 mm or a body mass of around 33 g. There was no difference in either size or growth pattern between sexes. The von Bertalanffy growth model showed that *L. latrans* grows fast after metamorphosis and their growth rate strongly decreases at about three years, probably due to the increased allocation of energy to reproduction. The high growth rates and early sexual maturation of *L. latrans* would allow an elevated rate of population renewal.

Keywords: Criolla frog, growth curve, life-history traits, reproduction, skeletochronology.

Introduction

Skeletochronology is a technique to determine age in vertebrates. It allows visualizing the lines of arrested growth (LAG) in bones and estimating the age through nonlethal means. In ectotherms, the annual growth is cyclic, with alternating periods of rapid and slow growth (Castanet and Smirina, 1990; Kyriakopoulou-Sklavounou, Stylianou and Tsiora, 2007). In diaphyseal cross-sections of long bones, the period of rapid growth appears as a broad layer and the period of slow growth (hibernation) is expressed as a narrow line, which is the LAG (Castanet and Smirina, 1990; Smirina,

1994; Ento and Matsui, 2002; Kyriakopoulou-Sklavounou, Stylianou and Tsiora, 2007). Most but not all amphibians typically have one ring per year (Kumbar and Pancharatna, 2001; Marangoni et al., 2011; Sinsch, 2015), corresponding in temperate regions to decelerated growth during winter (Smirina, 1994; Ento and Matsui, 2002; Castanet et al., 2003; Kyriakopoulou-Sklavounou, Stylianou and Tsiora, 2007). In some anurans, instead of a single LAG, the yearly growth mark may consist of multiple lines interrupted by very short growth events (Olgun et al., 2005; Iturra-Cid, Ortiz and Ibagüengoytía, 2010). Differences in the number of LAGs per year were also reported for different populations of the same species and related to environmental and climatic variables (Olgun et al., 2005; Sinsch, Oromi and Sanuy, 2007). Thus, one LAG per year is not a universal rule, and periodicity of LAG formation should be investigated before assigning age through skeletochronology in a given species or population.

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In amphibians, body size is easier to measure than the number of LAG. Hence, several studies assessed whether body size is a good estimator of age. Although this correlation was confirmed for some species (Leclair, Leclair and Gallant, 2005; Iturra-Cid, Ortiz and Ibargüengoytía, 2010), in others body size was a poor indicator (Khonsue, Matsui and Misawa, 2000; Ento and Matsui, 2002). Alternatively, growth models are widely used to study growth patterns in amphibians and allow comparisons between sexes, populations, and species along altitudinal and latitudinal gradients (Iturra-Cid, Ortiz and Ibargüengoytía, 2010; Marangoni et al., 2011; Hsu et al., 2014; Sinsch and Dehling, 2017). Longevity and age to attain sexual maturity are crucial for understanding species demography. Tropical species tend to mature earlier, while temperate ones live longer and have a longer reproductive lifespan (Sinsch, 2015; Sinsch and Dehling, 2017). But these life-history traits vary among and within species, or between sexes (Kumbar and Pancharatna, 2001; Hsu et al., 2014; Sinsch, 2015; Sinsch and Dehling, 2017). Therefore, life-history traits like age at sexual maturity, longevity, reproductive lifespan, growth pattern, and sexual differences still cannot be unambiguously inferred in tropical amphibian species by considering data from temperate species.

Leptodactylus latrans is widely distributed in the Neotropics (Heyer et al., 2010). Individuals are relatively large (120–140 mm) and supposedly exhibit sexual size dimorphism (Gallardo, 1987), but with males larger than females, an unusual pattern in amphibians (Monnet and Cherry, 2002). They use a wide variety of habitats, from wet grasslands to highly modified agroecosystems, occurring in habitats with tropical to temperate climates and from the sea level to 1400 m a.s.l. (Heyer et al., 2010). In temperate wetlands, they exhibit an extended foraging and reproductive activity throughout the year, but with an increase during the spring and mainly in September (López, Scarabotti and

Ghirardi, 2011; Sánchez et al., 2013). Juvenile recruitment occurs mainly in the summer from December to March (López, Scarabotti and Ghirardi, 2011), but the age structure of its populations is still unknown. This species has economic importance because it is eaten by local people, mainly along the fluvial littoral of Argentina, and other South American countries (Heyer et al., 2010; Ghirardi and López, 2017). Here, we present data on age structure, growth pattern, LAG marking periodicity, age and size at sexual maturity, and longevity.

Materials and methods

Study area

The Middle Paraná River floodplain is a complex system of islands with alluvial forests, wetlands, secondary rivers, streams, and lagoons that covers 20 000 km² of north-eastern Argentina (Drago et al., 2003). In this region, climate is damp and mesothermal. The warmer months are November to March, with a mean annual temperature of 18.4°C. Annual precipitation is about 1000 mm, and occurs mainly from October to April (Peretti, 1996). The activity of amphibians is concentrated in the warm and rainy season (López, Scarabotti and Ghirardi, 2011).

Sampling methods and individual measurement

Amphibians were sampled monthly between August 2004 and September 2005 on six ponds along the Middle Paraná River, near Santa Fe city, Santa Fe Province, Argentina. Five of the sites were on Sirgadero Island (31°42′17.2″S; 60°40′02.0″W, DATUM = WGS 84; 31°38′43.8″S, 60°40′24.2″W; 31°38′55.1″S, 60°40′38.1″W; 31°38′46.5″S, 60°40′44.9″W; and 31°38′50.2″S, 60°41′00.8″W), and one was in the Natural Reserve of Universidad Nacional del Litoral (31°38′18.7″S; 60°40′08.1″W). Sampling sites were less than 8 km apart within the Crevasse Splays and Levees of the Santa Fe and Coronda river channels (Marchetti et al., 2013), and interconnect during annual floods. Thus, we considered specimens as members of one population. On each pond, five pit fall traps were placed 2 m from the water edge, and remained open for ten days per month. Pitfall traps consisted of one 20-l plastic buckets with a damp sponge at the bottom to avoid dehydration (Angulo et al., 2006). Anurans collected in pitfall traps were euthanized in situ through a solution of benzocaine (Angulo et al., 2006). In the laboratory, we measured the snout-vent length (SVL) to the nearest 0.01 mm, using a digital caliper, and body mass (BM) to the nearest 0.001 g, using an electronic scale (Sartorius MC1, Lab. LC 2200 S, Germany).

To test if *L. latrans* has one LAG per year, we captured eight individuals on December 2013 in a permanent pond in

Monte Vera, Santa Fe Province, Argentina (31°29'25.72''S; 60°40'1.15''W). We took frogs to the laboratory and clipped the first and second phalange of the second toe of the left foot. Frogs were kept from December 2013 to January 2015. During captivity, frogs were fed once a week with a mix of *Tenebrio molitor* larvae and arthropods. We cut the third phalange of the second toe of the left foot of frogs after one year of captivity and used skeletochronology methods to determine the periodicity of LAG formation. Subsequently, frogs were kept in laboratory for two weeks, and then released in the site of their capture. Collected specimens are deposited in the collection of the National Institute of Limnology (INALI: CONICET-UNL; Appendix).

Age estimation

We used the first and second phalange of the fourth toe of the left foot of frogs collected during 2004 and 2005 to estimate the age structure of *L. latrans*. Furthermore, we used the phalanges of the eight frogs kept in captivity during one year to determine the periodicity of the LAG formation. In both cases, the phalanges were fixed in 10% formalin and preserved in 70% ethanol. The clipped phalanges were washed in tap water for half an hour, decalcified in 5% nitric acid for six hours, and rinsed in tap water overnight. Phalanges were successively dehydrated by a series of 70%, 95%, and 100% ethanol, infiltrated with Pathoclear® and embedded in paraffin. Ten transverse sections (5 µm thick) were cut for each toe using a Reichert rotating microtome, stained with haematoxylin and eosin (Ghirardi et al., 2011), and placed in a single microscope slide per specimen. Those sections with the smallest bone cavity and thicker cortical bone (5–10 per slide) were examined to count the number of LAGs using an optical microscope (Leica DM 2500) with a camera.

Sexual maturity analysis

All specimens with one or more LAGs and 102 specimens of 0 LAG were sexed using analysis of gonads under stereoscope microscope and/or secondary sexual characteristics (e.g., nuptial pads, vocal sac). Specimens with 0 LAG, without development of secondary sexual characteristics or with non-distinguishable gonads ($n = 48$), were considered as juveniles with undetermined sex and were excluded from sexual maturity analysis. We examined gonads of 81 female frogs under stereoscope microscope. Ovary maturity was assessed by the presence of differentiated ova (mature ovary: MO), while an undifferentiated mass was recorded as immature ovary (IO; Antoniazzi et al., 2014). In males, we processed the left testis of 23 specimens for routine histology to assess spermatozoid production. Testes were successively dehydrated by 70%, 95% and 100% ethanol, infiltrated with Pathoclear® and embedded in paraffin. Transverse sections (5 µm thick) were cut using a Reichert rotating microtome and stained with haematoxylin and erythrosine (Ghirardi et al., 2011). Cuts were examined using an optical microscope (Leica DM 2500) coupled with a camera. For sexual maturity analysis, we choose males and females with 0 to 5 LAGs and from every month. For each sex, the age of maturity was defined as the lowest age of a reproductive specimen.

Statistical analyses

We tested for a correlation between BM and SVL using a Pearson product-moment correlation. As they were highly positively correlated ($t_{(229)} = 39.557$; $r = 0.93$; $P < 0.001$) we used only SVL in models henceforth. We tested sexual size dimorphism with a Linear Model using SVL as response variable and sex and LAGs as predictor variables, and we tested for interaction. Difference between sexes in age at maturity was tested using a Generalized Linear Model, with Poisson distribution, using LAG as response variable and sex of mature specimens as predictor variable. To test if there was a difference in size among age classes (LAG), we used a Linear Model with Tukey Honestly Significant Difference (HSD) post-hoc test using SVL as response variable and LAG as predictor variable. Model assumptions were verified graphically. We analysed the growth pattern for all specimens and for each sex with the nonlinear von Bertalanffy growth model proposed by Beverton (1954) and Beverton and Holt (1957):

$$E[SVL|t] = L_{\infty}(1 - e^{-K(t-t_0)})$$

where $E[SVL|t]$ is the expected or average length at time (or age) t , L_{∞} is the asymptotic average length (SVL_{asympt}), K is a Brody growth coefficient, and t_0 is a modelling artefact that represent the time or age when the average length was zero. The von Bertalanffy model for body length data is commonly used to estimate size–age relationships in species with asymptotic growth (see Ogle, 2017). Starting values of von Bertalanffy growth model were estimated using a polynomial regression. To fit the model to the average growth curve, we used least squares. We estimated the parameters L_{∞} , K , and t_0 from our age estimates and SVL measurements. Confidence Intervals for parameters were constructed through bootstrap using 1000 iterations. Since the calculation of K is very sensitive to the size distribution of age class LAG 0 (Marangoni et al., 2011), we only include 36 recently metamorphosed individuals from January 2005 for this age class. We considered as significant the observed differences between sample estimates of growth curve parameters only if their 97.5% bootstrapped confidence intervals did not overlap. We considered the maximum age achieved by males and females (longevity) as the maximum number of LAGs. Analyses were performed using the packages Hmisc (Harrell et al., 2016), car (Fox and Weisberg, 2011), multcomp (Hothorn et al., 2008), FSA (Ogle, 2017), and nlstools (Baty et al., 2005) for R version 3.3.3 (R Core Team, 2017); scripts and data were deposited in FigShare (López, 2017).

Results

We analysed 231 *Leptodactylus latrans*, of which 94 were males, 89 females, and 48 juveniles with undetermined sex (table 1; figs 1 and 2). There was no difference in size between

Table 1. Mean with standard deviation (SD) and range of *L. latrans* body mass (BM) and snout-vent length (SVL). The number of specimens is indicated in parentheses in the first column.

	BM mean \pm SD (g)	BM range (g)	SVL mean \pm SD (mm)	SVL range (mm)
All specimens (231)	43.12 \pm 54.64	0.38-202.55	56.28 \pm 29.58	15.16-120
Males (94)	58.74 \pm 64.51	0.5-202.55	65.41 \pm 28.84	29.16-120
Males with spermatozooids (20)	123.43 \pm 62.95	33-202.55	93.82 \pm 17.82	62.75-120
Females (89)	48.76 \pm 46.80	1.42-147.15	63.59 \pm 26.72	25.86-106.2
Females with mature ova (40)	88.34 \pm 34.84	19.45-147.15	87.05 \pm 12.82	54.49-106.2
Unsexed juveniles (48)	2.05 \pm 1.73	0.38-12.43	24.85 \pm 4.87	15.16-45.58

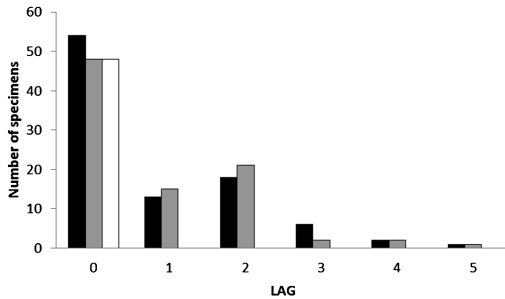


Figure 1. Age structure of the population of *L. latrans*. Number of males (black bars), females (grey bars), and juveniles with undetermined sex (white bars) per LAG.

sexes ($F_{(1, 181)} = 0.51$; $P = 0.47$) and nor interaction between predictor variables sex and LAG ($F_{(1, 179)} = 1.53$; $P = 0.21$). Individuals ranged from 0 to 5 LAG and there was no difference between sexes in age at maturity ($\chi^2 = 0.95$; $P = 0.32$). The maximum longevity recorded for both sexes was 5 years (fig. 1). Without considering juveniles (0 LAG), the age class with most specimens was 2 LAG.

We found females from every age with mature ova throughout the year (fig. 3), but more frequently in frogs with more than one LAG (fig. 4). Only five out of 44 zero LAG females had mature ovaries. Size range of mature females of zero LAG was: SVL = 54.49-92.52 mm, BM = 20.98-94.77 g (table 1). In males, spermatozoid production was observed in every analysed specimen from one to five LAG (analysed specimens of one LAG: $n = 5$; of two LAGs: $n = 7$; of three LAGs: $n = 3$; of four LAGs: $n = 2$; of five LAGs: $n = 1$), but only in two out of five specimens of zero LAG. Size of the two mature males of zero LAG was: SVL = 62.75 mm, BM = 33.78 g; and, SVL =

67.85 mm, BM = 33.03 g. Males with spermatozooids occurred throughout the year. Potential reproductive lifespan was 4 years for both sexes.

Body size varied between frogs of different ages ($F_{(5, 225)} = 137.76$; $P < 0.001$; Tukey HSD: 0 LAG differed from the rest of the age classes with $P < 0.001$; 1 LAG from 2 LAG with $P = 0.04$; 1 LAG from 3 LAG with $P < 0.01$). The asymptotic body length (SVL_{asympt}) was 98.71 mm with a Brody growth coefficient (K) of 1.45 (table 2; fig. 5). We observed one additional LAG after one year for the eight frogs kept in captivity (table 3).

Discussion

Leptodactylus latrans displayed one LAG per year, indicating a growth rhythm that tracked a seasonal environment. The formation of lines of arrested growth is ultimately caused by a genetically based circannual rhythm, which under natural conditions becomes synchronized with, and reinforced by, the seasonal cycle (Castanet et al., 1993). This hypothesis has been increasingly supported by studies on different species in different climates (e.g., Leskovar et al., 2006; Sinsch, Oromi and Sanuy, 2007; Marangoni et al., 2009, 2011).

Longevity of *L. latrans* was five years for both sexes. This is similar to longevity in several anurans from different families and regions (e.g., Kumbar and Pancharatna, 2001; Kyriakopoulou-Sklavounou, Stylianou and Tsiora, 2007; Marangoni et al., 2011) and from that of anurans that occur in the same latitudes as *L. latrans* studied here (see Sinsch, 2015 for a review) or species of the same genus (six

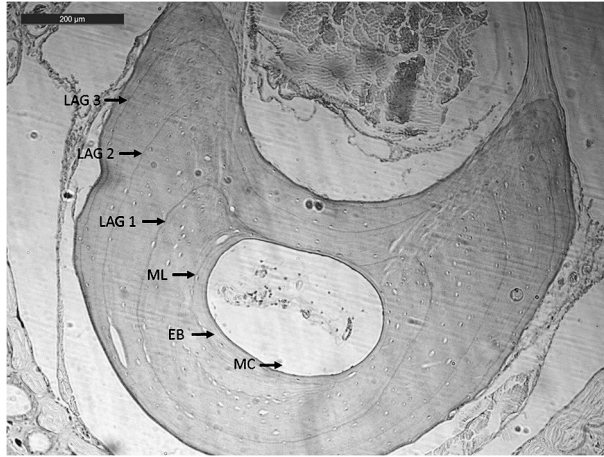


Figure 2. Transverse sections of a phalange of the fourth toe of the left foot of a specimen of *L. latrans*. MC: medullar cavity; EB: endosteal bone; ML: line of metamorphosis; LAG: line of arrested growth.

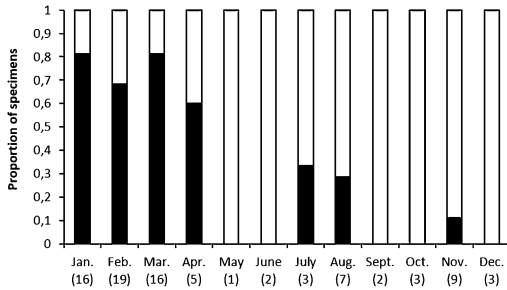


Figure 3. Proportion of females with immature ovaries (black bars) and with mature ovaries (white bars) throughout the year. The number of frogs is shown in parentheses: month (*n*).

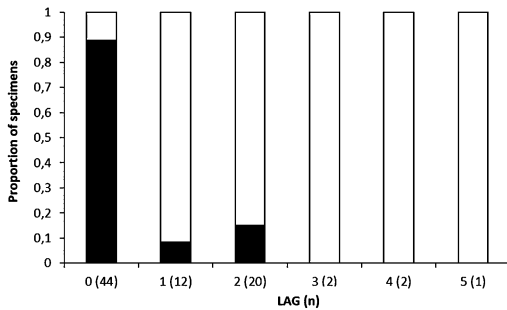


Figure 4. Proportion of females with immature ovaries (black bars) and with mature ovaries (white bars) in specimens of zero to five lines of arrested grow (LAG). The number of frogs is shown in parentheses: LAG (*n*).

years for *L. latinasus* and *L. mystacinus*; Atademo et al., 2014). However, taking into account the large geographical range of *L. latrans*, future studies should evaluate if species

have an inter-population variation following a tropical-temperate climate gradient. Additionally, considering that *L. latrans* occurs from the sea level to 1400 m a.s.l. (Heyer et al., 2010), it would be interesting to evaluate if their populations follow the wide-spread growth pattern in amphibians with a later maturation and higher longevity in high altitude populations (Shang and Lu, 2012; Hsu et al., 2014). Indeed, Sinsch and Dehling (2017) recently proposed that latitudinal effects on demographic life-history traits are not detectable in tropical anurans, while altitudinal effects are limited to a slight size reduction at higher elevations.

We found no sexual size dimorphism. Gallardo (1987) proposed that males of *L. latrans* were larger than females. Sexual size dimorphism is common in anurans and it has been attributed to sexual selection and differences in life-history strategies (Monnet and Cherry, 2002; Quiroga, Sanabria and Marangoni, 2014). When claimed as a result of sexual selection, males are usually smaller than females, contrary to the pattern previously recorded in *L. latrans* (Gallardo, 1987). Sexual size dimorphism has also been explained in terms of differences in age structure between sexes in breeding populations (Monnet and Cherry, 2002), and the lack of size dimorphism in *L. latrans* could be the

Table 2. Estimated parameters of von Bertalanffy growth model for *L. latrans* that best fitted the data, with *t*-based confidence interval at 2.5% and 97.5%: $E[SVL|t] = L_{\infty}(1 - e^{-K(LAG-t_0)})$.

	Parameter value	Std. error	<i>t</i> value	<i>P</i> -value	95% CI
SVL _{asympt}	98.71	2.171	45.48	<2e-16	94.42-103
<i>K</i>	1.45	0.17	8.65	7.29e-15	1.12-1.79
<i>t</i> ₀	-0.2	0.02	-8.62	8.68e-15	-0.25--0.15

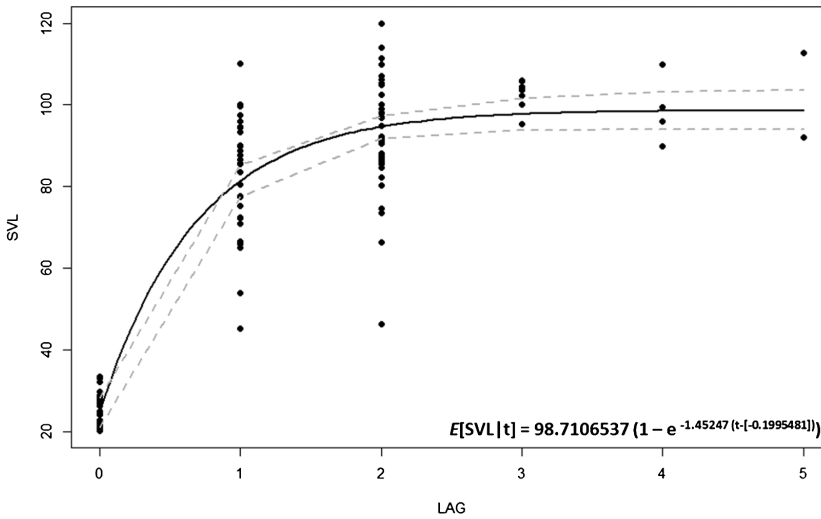


Figure 5. von Bertalanffy growth curve with 36 recently metamorphosed 0 LAG individuals from January 2005 and all specimens (males and females) with 1 to 5 LAG (95% confidence intervals in grey dashed lines).

Table 3. Number of lines of arrested grow (LAG) at the beginning (*t*₀: December 2004) and at the end of the experiment (*t*₁: December 2005), for the eight *L. latrans* specimens kept in captivity.

Temperature	Photoperiod	<i>n</i>	LAG <i>t</i> ₀	LAG <i>t</i> ₁
25 ± 1°C	12 h light /	1	0	1
	12 h dark	3	1	2
Room	natural	1	0	1
		2	1	2
22 ± 1°C	natural	1	1	2

result of the absence of age structure between sexes in the studied population.

Leptodactylus latrans grows fast after metamorphosis and their growth rate strongly decreases at about three years, probably due to the increased allocation of energy to reproduction (Hemelaar, 1988; Marangoni et al., 2009, 2011), since sexual maturation is typically correlated with an abrupt decline in somatic growth in anurans (Jørgensen, 1992). As in other anu-

ran species (Kumbar and Pancharatna, 2001; Lauck, 2005; Yilmaz, 2005), especially tropical ones (Sinsch and Dehling, 2017), many individuals of *L. latrans* reach sexual maturity during their first year. Despite the lack of difference between sexes in age at maturity, all males older than one year were sexually mature, while we found small one and two-year old females that had not mature ovaries yet. Somatic growth may approach zero concurrently with the initiation of the ovarian cycle and vitellogenesis. The comparatively marginal energetic cost of spermatogenesis entails a less tightly coupling between sexual maturation and reduction of somatic growth (Jørgensen, 1986, 1992). This difference in energy investment between males and females should be determining the delayed breeding of some 1 and 2 LAG females.

Most adult *L. latrans* undergo at least two reproductive seasons, while older frogs (five

LAGs) potentially have four reproductive seasons in temperate wetlands populations (López, Scarabotti and Ghirardi, 2011; Sánchez et al., 2013). Early maturation has been observed in tropical anurans (Sinsch and Dehling, 2017) and leads to a reproductive lifespan that extends for most of frog postmetamorphic life. The early maturity described in *L. latrans* is also found in the congeneric and sympatric *L. latinasus* (Marangoni pers. comm.), although this pattern would not be the only one found in species of this genus (Attademo et al., 2014). The sexual maturity of *Leptodactylus latrans* was related to the achievement of a minimal size (SVL ~ 60 mm or BM ~ 33 g) more than an age, and coincides with the one observed for other anurans (Kumbar and Pancharatna, 2001; Lauck, 2005; Yilmaz, 2005). *Leptodactylus latrans* mainly occurs in regions with tropical climate (Heyer et al., 2010), where reproduction may occur year-round, which may explain the continued gametogenesis. Continuous gametogenesis, together with early maturation and fast growing, are life-history traits commonly exhibited by tropical anuran species (Duellman and Trueb, 1986; Morrison and Hero, 2003; Sinsch and Dehling, 2017). Notwithstanding, the temperate and seasonal climate of the Middle Paraná River (Peretti, 1996) determine the reproductive seasonality observed in other populations of *L. latrans* (Sanchez et al., 2011; López, Scarabotti and Ghirardi, 2011). Finally, the high growth rates and early sexual maturation of *L. latrans* would allow an elevated rate of population renewal and resilience (Morrison and Hero, 2003), an important characteristic for a species whose populations are locally pressured by hunting (Heyer et al., 2010; Ghirardi and López, 2017).

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in accordance with animal care guidelines of Regional and National Fauna Administrators, Argentina. Financial support was provided by SECTel (Research Project, code number 2040-013-12), by the National Council of Scientific and Technical Research of Argentina (Consejo Nacional de Investigaciones Científicas y Técnicas -CONICET-) to Antoniazzi C.E., Ghirardi R. and López J.A., and by National Interuniversity Council (Consejo Interuniversitario Nacional) to Llanes R. E.

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Appendix. Catalogue numbers of specimens of *Leptodactylus latrans* collected in this study

2542; 2542-A - 2542-G; 2552; 2558; 2558-A; 2605; 2605-A; 2605-B; 2605-D; 2605-E; 2620; 2620-A - 2620-C; 2620-

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