

Body size, age and growth pattern of *Physalaemus fernandezae* (Anura: Leiuperidae) of Argentina

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Abstract. We studied body size, age structure, age at maturity, longevity, and growth pattern of *Physalaemus fernandezae* from a population living at the Reserva Natural Punta Lara, Buenos Aires, Argentina by skeletochronological methods. Furthermore, we evaluated the sexual size dimorphism of this species in relation to age at maturity and growth rate. We also discussed the chronological formation of the Lines of Arrested Growth (LAGs) in relation to the reproductive activity of *P. fernandezae*. Body size was sexually dimorphic; females were significantly, on average, larger and heavier regardless of age, than males. Out of 91 samples that were processed, 65 (36 male, 22 female, 7 juveniles) sections showed well-defined LAGs in the periosteal bone. The minimum number of LAGs counted was 2 for males and 3 for females. We did not find sexual differences in longevity. Thus, the difference in potential reproductive lifespan between sexes was of 1 year. Although the mean age was higher in females, it did not differ significantly between sexes. There was a strong positive and significant correlation between snout-vent length and body mass. The growth coefficients (K), estimated by the von Bertalanffy growth equation, were higher in males than in females for both size and body mass. The results indicate that females, with lower growth rate than males, took longer time to reach the minimum maturation size, and thus, were older and larger at first reproduction. We assumed that the number of LAGs observed in *P. fernandezae* is equivalent to only one period of decreasing growth found each year immediately prior to the winter reproductive season. We suggest that this decrease in growth rate may occur between May and June, due to the decrease of temperatures, but also in combination with the scarcity of precipitation.

Key words: body size, age, growth, skeletochronology, *Physalaemus fernandezae*.

Introduction

Body size is possibly the most fundamental trait related to fitness of an animal. This is due to its close association with longevity, fecundity, metabolic rate and tolerance to environmental stress (Calder 1984, Schmidt-Nielsen 1984). Several factors can contribute to variation in adult body size in amphibians, both between and within populations. Differences in body size and/or shape between sexes within a population, commonly known as sexual size dimorphism (SSD), might be produced under the driving force of sexual selection (Shine 1988) and/or might be the result of ecological differences between sexes (Slatkin 1984, Shine 1989).

In amphibians, post-metamorphic terrestrial growth represents between 90 % - 99.9 % of total growth, until average adult size is attained (Werner 1986). Furthermore, this growth exhibits

an indeterminate pattern with an exponential period that decreases considerably after the onset of reproduction, with the attainment of an asymptotic size (Hemelaar 1988, Halliday & Tejedo 1995). Thus, the variation in terrestrial growth rates and age at maturity, as one of the main proximate mechanisms determining intersex adult size variability, has been widely studied in amphibians and reptiles (Monnet & Cherry 2002, Üzümlü 2009, Sinsch et al. 2010, Casale 2011).

For age determination in amphibians, skeletochronology is a good alternative tool to mark-recapture studies, which are very time-consuming. It is based on the presence of cyclic and annular bone growth, which can be visualized in cross-sections of the bone (Castanet 1982, Castanet & Smirina 1990). This method of counting the number of LAGs (lines of arrested growth) in cross-sections of phalanges, obtained by toe clipping, is the most commonly used tool for evaluating the

age structure of amphibians and reptiles, providing age estimation through nonlethal means (Castanet & Smirina 1990).

The neotropical genus *Physalaemus* Fitzinger 1826 is currently composed of 45 small terrestrial frog species occurring from Venezuela and the plains of southeastern Colombia to Argentina (Frost 2011). The “whistling dwarf frog” *Physalaemus fernandezae* (Müller 1926, see Fig. 1) is the most austral species of the genus, occurring in the departments of Canelones, Florida, and San José in southern Uruguay, and the Buenos Aires province in Argentina (Barrio 1965, Gallardo 1965, Núñez et al. 2004). It is the only frog from Buenos Aires whose reproductive period occurs mainly during the winter and with a second short period in the summer (Barrio 1964, 1965, Gallardo 1965). The IUCN set the conservation status of *P. fernandezae* as Least Concern, in view of its relatively wide distribution and presumed large population (Lavilla & Langone 2004).



Figure 1. A male of the “whistling dwarf frog” *Physalaemus fernandezae*.

The main contributions to knowledge on *P. fernandezae* have been works related to osteology (Lobo 1992), larval features (Alcalde et al. 2006), natural history and taxonomy (Barrio 1953, 1964, 1965, Gallardo 1965, Nascimento et al. 2005, Lobo 1996; Tomatis et al. 2009). However, population and ecological aspects have not yet been investigated. The aims of this study were to present the first data on age structure, age at maturity, longevity, and growth pattern by use of skeletochronology. Furthermore, we determined whether sexual size dimorphism in body size exists in *P. fernandezae*, and evaluated it in relation to age at maturity and growth rate. We also discussed the chronological formation of the LAGs in relation to reproductive activity pattern.

Materials and methods

Study site

Field work was carried out in “Reserva Natural Punta Lara”, Buenos Aires, Argentina (34° 47' 53.3" S 58° 00' 46.41" W). The study site is located near the coast of Río de La Plata and is characterized by a pampean grassland with a moderate livestock activity. The area belongs to the Pampeana phytogeographic province (Cabrera & Willink 1973) and the ecoregion of Pampa (Burkart et al. 1999) characterized by grassland vegetation composed primarily by species of the genera *Stipa* sp., *Poa* sp., *Panicum* sp., and *Paspalum* sp. The weather is warm-temperate, with an annual mean temperature ranging from 13 °C to 17 °C, and an annual rainfall that reach between 600 and 1200 mm throughout the year (Cabrera & Willink 1973).

Collection Methods and Individual Measurements

In 2006, nine pitfall traps were installed at “Reserva Natural Punta Lara” as required by another study (Agostini et al. 2011). Pitfall traps consisted of plastic buckets (capacity of 20 L) with 1 L of 10% formalin, which have been dug into the ground so that their rims are at ground level (Campbell & Christman 1982). Pitfall traps were placed five meters apart along three parallel transects of 15 meters in length each containing three traps. Traps were opened between July 1st and August 1st (within the reproductive season of *P. fernandezae*), and were checked every 15 days. All anurans were collected and kept in a 70% alcohol solution. 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 (Ohaus, explorer-pro EP 214C).

Sex and maturity of individual was detected by examination of gonads and external nuptial features. Male maturity was determined by testes size, presence of dark vocal sacs and nuptial excrescences. In females, the maturity of the ova was determined by degree of pigmentation (Crump, 1974). Examined specimens are housed in the herpetological Collection of the Museum of La Plata, Argentina (Voucher specimens: MLP.A.5264 - MLP.A.5354). In addition, due to lack of recently metamorphosed individuals in the pitfall traps of 2006, we used the data of BM and SVL of froglets (n = 20), stage 46 (Gosner 1960), from ten foam nests collected on July 11th 2010 by DB to make a more precise adjustment to the von Bertalanffy (1938) growth model (see below). The foam nests were kept together in the laboratory in plastic pools filled with dechlorinated tap water until tadpoles reached metamorphosis, between the 7th and 12th September 2010. Room temperature was kept roughly constant (range 24–26 °C) and the photoperiod followed a daily cycle of L16:D8. Tadpoles were fed with boiled lettuce and commercial fish food *ad libitum* every two days.

Skeletochronology

We used 91 (37 male, 27 female, 7 juveniles, 20 froglets) clipped digits of *P. fernandezae* for this skeletochronological study. Laboratory protocols followed the standard methods of skeletochronology (e.g. Smirina 1972). We selected the third phalanx of each animal, which were washed in water for 30 min and then decalcified in 5% ni-

tric acid for 30–45 min. Afterwards, they were washed in running tap water for 5 min and washed overnight in distilled water. Then, the phalanxes were frozen (Tissue-Tek, Sakura Finetex Europe B.V., Denmark, frozen section medium) and cross-sectioned at 16 μ m using a cryomicrotome. Sections were stained for 3–6 h at room temperature with Ehrlich's hematoxyline (details in Tejedo et al. 1997). From each phalange, 15 to 20 transverse sections were mounted in aqueous synthetic resin (Aquatex®, Merck KgaA, Germany) on a glass microscope slide. We used a Nikon FX-35 A, mounted on Nikon UFX-II microscope, to take digital images of those diaphysis sections in which the size of the medullar cavity was at its minimum and that of bone at its maximum. Cross sections were viewed 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 micrometer. First, we recorded the presence/absence of the line of metamorphosis (LM) and of lines of arrested growth (LAGs). Age was determined using two observers (FM and RC) who counted LAG independently to prevent any bias, considering the outer perimeter of the bone as a LAG (Patón et al. 1991). In those toads 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). Secondly, we discriminated annual growth marks (LAGs *sensu stricto*) from non annual ones (irregular interruptions during inactivity periods), using the method described in Sinsch et al. (2007). The age of maturity was defined as the lowest age recorded in a reproductive toad from this population. Finally, we measured the longest and shortest perpendicular axes of bone perimeter of two diaphyseal sections *per specimen*, following the methods of Hemelaar (1985). We determined the average diameter of bone by square rooting the product of the two axis measurements.

Demographic life history traits

We characterized the studied population of *P. fernandezae* by the measuring five sex-specific life history variables (*sensu* 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) median lifespan: median of age distribution; (5) size at maturity: the average snout-vent length of all first breeders with the minimum number of LAGs.

Growth Estimation

We estimated growth by applying the von Bertalanffy (1938) growth equation, which has been adapted to amphibian growth studies (Miaud et al. 2000, 2007, Ūzūm 2009), according to Miaud et al. $S_t = S_m - (S_m - S_0) e^{-k(t-t_0)}$, where t = number of growing seasons experienced (age); t_0 = age at metamorphosis (proportion of the growing season already elapsed at metamorphosis); S_t = average body size after having experienced t growing seasons; S_m = average maximal body size; S_0 = average body size at metamorphosis; K = growth coefficient, defining the shape of the growth curve.

Reproductive activity

With the aim to analyze the chronological formation of the LAGs in relation to reproductive activity pattern and climatic variables, we described the reproductive activity in the studied population based on a fortnightly monitoring made between January 2005 and December 2007, where we registered calling activity and presence of foam nests. Additionally, for this period, climatic data (temperature and daily rainfall), were obtained from the meteorological station at the Centro de Información Meteorológica del Servicio Meteorológico Nacional, Comando de Regiones Aéreas de la Fuerza Aérea Argentina, located 8 km from the study site, and was used to characterize the environment during reproductive activity.

Statistical analysis

The normality of distributions of analyzed variables was verified with the Liliefors and Shapiro-Wilk tests. Parametric Student's t test was used for comparing pairs of means when the data were normally distributed, and the Mann-Whitney nonparametric U test for comparing ranges otherwise. We carried out ANCOVAs using age as a covariate to account for the effect of age on SVL and BM. We also estimate the sexual size dimorphism with the Lovich & Gibbons (1992) sexual dimorphism index (SDI): $SDI = (\text{size of larger sex} / \text{size of smaller sex}) \pm 1$. +1 if males are larger or -1 if females are larger, and arbitrarily defined as positive when females are larger than males and negative in the contrary. We used linear regressions to test the association between body size (SVL and BM) and age, and diameter of bone. The von Bertalanffy growth model was fitted to the average growth curve using the least square procedure. All tests were done with the statistical package STATISTICA 6.0 (Statsoft Inc., USA 2001) and a significance level of $\alpha = 0.05$ was used.

Results

Body size was sexually dimorphic, with females being significantly larger (SVL: $t = 7.464$, $df = 62$, $P < 0.0001$; BM: $t = 5.647$, $df = 62$, $P < 0.0001$), in average SVL and BM than males (SVL, females: Mean = 22.29 mm, Min. = 20.18 mm, Max. = 24.02 mm, SD = 1.15 mm, $n = 27$; SVL, males: Mean = 20.49 mm, Min. = 18.16 mm, Max. = 22.72 mm, SD = 0.77 mm, $n = 37$; BM, females: Mean = 1.17 g, Min. = 0.66 g, Max. = 1.64 g, SD = 0.29 g, $n = 27$; BM, males: Mean = 0.87 g, Min. = 0.66 g, Max. = 1.08 g, SD = 0.12 g, $n = 37$). ANCOVAs show that, regardless of age, females are bigger than males (SVL: $F_{1, 55} = 38.273$, $P < 0.0001$; BM: $F_{1, 55} = 23.533$, $P < 0.0001$). The sexual dimorphism index (SDI) in body size was 0.088 (SVL) and 0.345 (BM). In age classes 3 to 6 where sample size allowed estimation (age = number of LAGs observed, see discussion), variation in SDI was: (age class/SDI)

3/0.093, 4/0.085, 5/0.006, 6/0.072 for SVL, and 3/0.342, 4/0.264, 5/0.329, 6/0.351 for BM. The mean body size \pm SD of juveniles, considered as individuals without external sex characters, unknown sex, no LAGs present (and thus, assumed that they were in their first growth period), was Mean = 11.91 mm, SD = 1.55 mm, $n = 7$ (SVL), and Mean 0.18 g, SD = 0.08 g, $n = 7$ (BM). Juveniles were significantly smaller than adults and bigger than froglets, as revealed by a Scheffe' post hoc multiple comparison test at $\alpha = 0.05$ ($P = 0.0005$, minimum value in all paired comparisons).

Out of 91 samples that were processed, 65 (36 male, 22 female, 7 juveniles) sections showed recognizable bone structures that allowed age determination. In these sections, well-defined lines of arrested growth (LAGs) were found in the periosteal bone and were relatively easy to count in order to assess individual age (Fig. 2). Endosteal resorption never prevented the age estimation. The line of metamorphosis was visible in 72.5 % of the samples. But even in those specimens in which it had been replaced during the formation of endosteal bone, the first LAG was never completely removed. Annuli (Peabody 1958) were easily distinguishable from actual LAGs. They always stained more faintly and were often broader, as previously described by Leclair et al. (2005) and Sinsch et al. (2007) in species inhabiting temperate regions. Mean diameter of bone in micra was Mean 158.15, SD = 13.26 (males), Mean = 154.3, SD = 15.59 (females) and Mean = 129.85, SD = 15.81 (juveniles). The demographic life history traits of *P. fernandae* are summarized in Table 1 and the age structure of adults in Fig. 3. Females were older and bigger at maturity than males. The minimum number of LAGs counted in reproductive individuals was 2 for males and 3 for females. We did not find sexual differences in longevity. Thus, the difference in the Potential Reproductive Lifespan (PRLS) between sexes was 1 year. The mean age was higher in females, but did not differ significantly between sexes (Mann-Whitney U-test, $Z = 1.537$, $P = 0.157$).

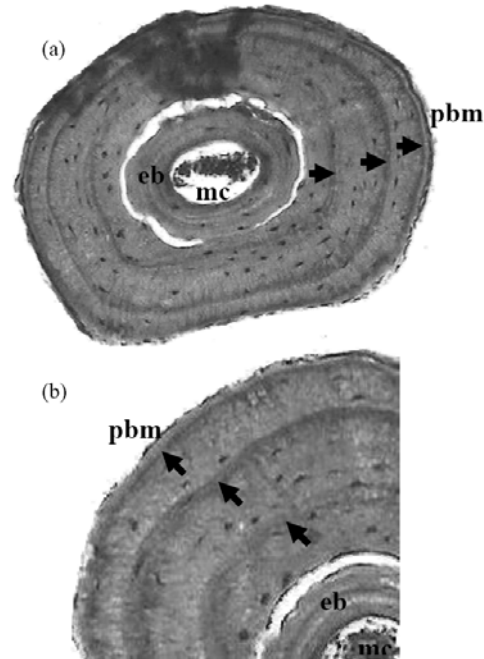


Figure 2. Cross sections of phalanges stained with Ehrlich's hematoxylin of adult females from the Reserva Natural Punta Lara population. Three LAGs are visible in the periosteal bone (indicated by arrows) in two females of 21.38 mm SVL (a) and 22.68 mm SVL (b). mc: medullar cavity, eb: endosteal bone, pbm: periosteal bone margin.

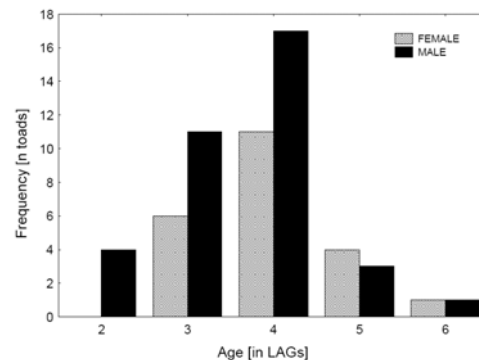


Figure 3. Age distribution of *Physalaemus fernandae*.

Table 1. Features of populations life history traits of *Physalaemus fernandae*. AM = age at maturity of the youngest first breeders; PRLS = potential reproductive lifespan; size = snout-vent length.

Sex	N	Mean \pm SE [LAGs]	Mode [Frequency]	Median Lifespan [yr]	AM [LAGs]	Longevity [LAGs]	PRLS [yr]	Size at AM \pm SE [mm]	Maximum Size (age) [mm]
Males	36	3.61 \pm 0.15	4(17)	4	2	6	4	20.36 \pm 0.27	21.72 (5)
Females	22	4 \pm 0.174	4(11)	4	3	6	3	22.09 \pm 0.38	24.04 (4)

The body size of *P. fernandezae* was poorly correlated with age and diameter of bone in both sexes, while BM was strongly positive and significant correlation with SVL. Positive, but not significant correlation between body size and diameter of bone was recorded in both sexes. A positive correlation between age and body size was recorded in males, but it was negative for females (Table 2). Thus, smaller females were not necessarily the youngest.

The R^2 -values (female: $R^2 = 0.957$, male: $R^2 = 0.956$ for SVL; and female: $R^2 = 0.907$, male: $R^2 = 0.931$ for BM), when growth (age in LAGs vs body size) was estimated by applying the von Bertalanffy (1938) growth equation, and asymptotic standard errors of the estimated parameters (Sm and K) indicate that the model fits quite well (Fig. 4). Average body size at metamorphosis S_0 (SVL: Mean 9.97 mm, SD = 0.97 mm, BM: Mean = 0.1 g, SD = 0.02 g) was taken from twenty individuals recently metamorphosed, as described above. The predicted maximal body size (Sm) was always larger than measured average values. Females had larger average maximal body size than males (female, Sm: Mean = 26.55 mm, SD = 2.56 mm; male, Sm = Mean = 23.18 mm, SD = 1.18 mm for SVL, and female, Sm: Mean = 1.67 g, SD = 0.49 g, male, Sm: Mean = 1.09 g, SD = 0.12 g for BM). The growth coefficient (K), which determines how fast the body size approaches its maximum value, was higher in males than in females, for both size and body mass (SVL, females: Mean = 0.32, SD = 0.09; males: Mean = 0.43, SD = 0.09; BM, females: Mean = 0.26, SD = 0.14; males Mean = 0.41, SD = 0.11) (Fig. 4).

Based on the monitoring data we registered two reproductive periods per year for *P. fernandezae*. They were characterized by one main breeding period in winter-spring associated with a significant decrease in temperature and moderated rains, and a short period in summer-autumn associated with high temperatures and heavy rains (Fig. 5).

Discussion

We observed sexual size dimorphism for both in SVL and BM in *Physalaemus fernandezae*, with females being, on average, larger and heavier than males. The SSD and body sizes observed in this study were in agreement with the ones previously reported by Barrio (1964). Furthermore, SSD

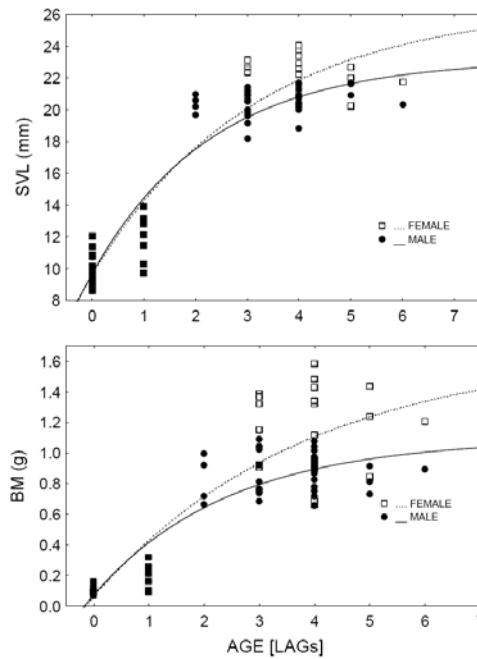


Figure 4. Growth curves for snout-vent length and body mass in males and females of *Physalaemus fernandezae*. Growth curves were fitted to von Bertalanffy's growth equation.

where females are larger than males, is commonly described for other species of this genus, such as, *P. cuvieri*, and *P. lisei* (Kwet & Di-Bernardo 1999), *P. signifer* (Wogel et al. 2002), *P. henselii* (Barrio 1964), *P. rupestris* (Nascimento et al. 2001), many other anuran species (approx. 90%), and more than 60% in urodele species (Shine 1979, Monnet & Cherry 2002). However, whether the existence of sexual size dimorphism could be explained by differences in age at maturity and/or in post-metamorphic patterns of growth (Monnet & Cherry 2002), has never been studied in *P. fernandezae*. In amphibians, post-metamorphic growth exhibits an indeterminate pattern with an exponential period that decreases considerably after the onset of reproduction where it reaches an asymptotic size (Hemelaar 1988, Halliday & Tejedo 1995). This determines that differences in age at maturity (Halliday & Verell 1988), age structure, post-metamorphic growth rate (Hemelaar 1988), or even sex-specific differences in mortality rate caused by differential predation pressure between sexes (Howard 1981), seem to be the main proximate determinants of adult size variability observed (Monnet & Cherry 2002, Hasumi 2010,

Table 2. Relationships between body size (SVL and BM), age, and diameter of bone (bone size) in both sexes of *Phyllomedusa fernandezae*. All variables were log-transformed. *P* values: * < 0.001, ** < 0.0001.

	Females			Males		
	<i>n</i>	Equation	<i>r</i> ²	<i>n</i>	Equation	<i>r</i> ²
SVL vs BM	26	$y = -3.136 + 0.193 x$	0.583**	37	$y = -0.749 + 0.079 x$	0.264*
<i>Body size vs age</i>						
SVL	22	$y = 23.241 - 0.274 x$	0.038 ns	36	$y = 19.661 + 0.231 x$	0.07 ns
BM	22	$y = 1.216 - 0.019 x$	0.003 ns	36	$y = 0.840 + 0.009 x$	0.004 ns
<i>Body size vs bone size</i>						
SVL	26	$y = 62.647 + 4.123 x$	0.087 ns	37	$y = 15.487 + 6.962 x$	0.054 ns
BM	26	$y = 135.021 + 16.654 x$	0.09 ns	37	$y = 140.937 + 19.717 x$	0.011 ns

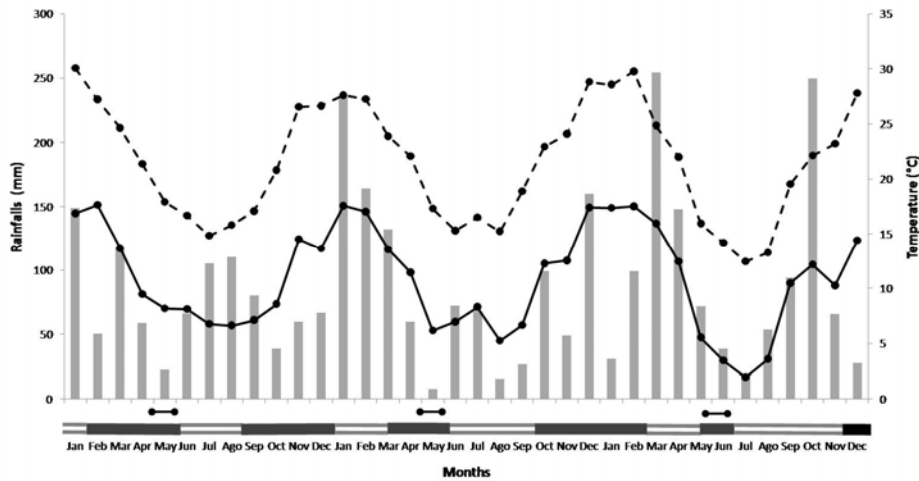


Figure 5. Monthly accumulated rainfall in mm (vertical bars) and daily average of monthly temperatures, maximum and minimum (lines), during 2005 to 2007. Reproductive pattern of *P. fernandezae* is indicated in the horizontal bar: white segments represent periods of breeding activity and dark segments represent periods without breeding activity. Possible periods of LAG formation are indicated with a dark line from starting and ending with dark circles.

Sinsch et al. 2010). In the studied population of *P. fernandezae*, females reached maturity at 3 years of age, one year later than males, but they were also bigger at that point. In addition, the growth coefficient (K), which determines how fast the body size approaches its maximum value, was lower in females than in males, for both in SVL and BM. Thus, the females of *P. fernandezae* took longer to reach the minimum size at which maturation may occur (Hemelaar 1988), and were older and bigger at first reproduction than males. An adaptive explanation for this delayed reproduction, lower growth rate, and consequently the attainment of larger sizes, may be the way individuals allocate resources to growth and reproduction (Roff 1992, Stearns 1992). Our results suggest that the delayed maturity of females will allow them to allocate the stored energy to somatic growth during one more year than males, thus reaching sexual maturity at

larger sizes.

This larger body size of females of *P. fernandezae* may also be related to fecundity where selection favors large females with a higher adaptive advantage by increasing egg production (Gibbons & McCarthy 1986), which is positively correlated with body size in amphibians (Kuramoto 1978, Kaplan & Salthe 1979, Tejedo 1992, Marangoni et al. 2008). Although larger adult size obtained by delayed sexual maturity results in increased clutch size, it may also be associated with costs such as increased juvenile and adult mortality (Howard 1981). Thus, an alternate explanation of the differences in adult age distribution and body size, as observed in *P. fernandezae*, is that of differences in sex-dependent reproductive costs, including predation risk, even the predation risk on calling males, who begin their strong reproductive investment (mating calls) one year earlier than fe-

males. However, our results did not support this hypothesis, as there was no difference in longevity between sexes.

We found no significant relationships between age (estimated by LAGs), size (measured by either SVL or BM) and bone size. Our results are consistent with previous studies, casting doubt on the hypothesis that size and age are significantly and positively related in anuran amphibians (reviewed by Cherry & Francillon-Vieillot 1992, Halliday & Verrel 1998, but see Tomašević et al. 2010 and Chen et al. 2011).

Reproductive activity of *P. fernandezae* was markedly bimodal per year, characterized by a reproductive period in winter-spring and another in summer-autumn, as documented by other authors (Barrio 1964, 1965, Gallardo 1965) (Fig. 5). Similar reproductive periods were also documented for *P. henselii* by Barrio (1964, but see Maneyro et al. 2008), who also reports the reproduction in sympatry of both species, in Uruguay. However, this pattern is atypical in comparison to other species of the genus *Physalaemus* which have mainly one reproductive period per year, in the wet/warm season, as concluded from several reports (Barrio 1965, Cardoso 1981, Giaretta & Menin 2004, Camargo et al. 2005, Brasileiro & Martins 2006, Rodrigues et al. 2004). LAG formation 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, Kumbar & Pancharatna 2002, Morrison et al. 2004, Marangoni et al. 2009). One LAG per year, equivalent to the number of hibernations of each individual, represents the most common observed pattern of LAG formation in palaeartic, tropical and subtropical amphibian species (Castanet et al. 1993, Smirina 1994, Morrison et al. 2004, Marangoni et al. 2009, Andreone et al. 2011). However, two instead of one LAG per year (double lines; Castanet & Smirina 1990, Castanet et al. 1993), 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). Since reproductive activity of *P. fernandezae* was bimodal each year (Fig. 5), we would expect a supplementary growth mark with the likelihood of a double annual growth cycle; nevertheless, in our study we did not record double-lines. Thus, we assumed that the number of LAGs observed in *P. fernandezae* is equivalent to only one period of decreas-

ing growth found each year and they are formed as a result of the combinations of two main climate factors (see below), that reinforce the genetically based rhythm, giving a direct estimation of individual age.

At the study site, the period of unfavorable climatic conditions for the majority of amphibian species is represented by cold temperatures occurring in winter; however, this is not the case for *P. fernandezae*. Our data from the monitoring and pitfall traps showed, in agreement with reports by Barrio (1964) that this species mainly reproduces in winter-spring, with a weaker activity in summer-autumn. Then the question, what is the period of decreasing growth of *P. fernandezae* in which the LAGs are formed? All individuals collected in pitfall traps between July 1st and August 1st showed that the outermost LAG and the perimeter of the phalange were very close together with an extremely thin thickness of bone tissue between them, which have become almost indistinguishable (Fig. 2), suggesting a recent period of decreasing growth and the consequent formation of a LAG. Based on these observations, we suggest that the LAG formation in *P. fernandezae* occurs in the period immediately prior to winter reproduction, which could vary between May and June due to the decrease of temperatures combined with the scarcity of precipitations (Fig. 5). However, more studies are needed to test hypotheses about the chronology of LAG formation in *P. fernandezae*.

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