Life-History Traits of Three Syntopic Species of the South American Redbelly Toads (Anura: Bufonidae: *Melanophryniscus*) from the Atlantic Forest of Argentina

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Abstract.—Amphibians from Atlantic Forests of South America are one of the most threatened vertebrates of the world, mainly due to the habitat loss and infectious diseases. With the goal to improve the knowledge of the life-history traits of the amphibian that inhabit these regions, and thus, to aid conservation and management-decision making, our main goals were to describe the reproductive activity pattern and analyze the interspecific variation in body size and the reproductive traits of three species of redbelly toads (*Melanophryniscus*) of the Atlantic Forest of Argentina. We also analyzed age structure, whether Sexual Size Dimorphism (SSD) exists, and if this dimorphism could be explained by proximate mechanisms such as differences in growth patterns. The *Melanophryniscus* we studied bred during two or three consecutive days, in several explosive events that occurred between April 2009 and October 2012. These events were determined by a trade-off between the air and water temperature, and the level of the water bodies. We observed spatial segregation among the three species we studied when they reproduced synchronously and in sympatry and with the presence of multiple clutches. We found inter-specific differences in body size. We recorded male-biased sex ratio and SSD in all three species of *Melanophryniscus* studied. We also found significant interspecific differences in age-related parameters following the differences in body size. Species were not sexually dimorphic by age. We also did not find covariation between body size and reproductive traits. These new insights allow us to predict the responses of *Melanphryniscus* we studied to the impact of the destruction of their habitat and global warming.

Key Words.—age; body size; Paranaense Forest; reproduction; skeletochronology

Introduction

Amphibians are considered the most threatened class of vertebrates in the world, with about 32.4% of species either globally extinct or threatened with extinction (Critically Endangered, Endangered, and Vulnerable), representing 2,030 species (Hoffmann et al. 2010; International Union for Conservation of Nature [IUCN] 2011). Direct pressure from human activities such as habitat loss and pollution, as well as indirect factors associated or reinforced by global warming, such as exposure to the fungal disease chytridiomycosis, have been suggested as major causes of decline (Frost et al. 2006; Pounds et al. 2006; IUCN 2011). The most of threatened amphibians are found in Tropical Rainforests including the Atlantic Forests of South America (IUCN 2011). Deforestation is the main driver of the loss of tropical and subtropical forests, with the consequent loss of richness and altered composition of the anuran community (Gangenova et al. 2018). Bridging the existing gap in knowledge of the life history of the amphibians inhabiting these regions becomes a main goal for conservation biologists to understand how they will respond to threats, and to aid conservation- and management-decision making.

Atlantic Forest of Argentina (or Paranaense Forest; AFA, hereafter) is an ecoregion with a complex topography, a great diversity of microhabitats, and high humidity (Galindo-Leal and Gusmão Câmara 2003). The high precipitation regime allows the presence of numerous water bodies, such as streams, and permanent and temporary ponds. The dense vegetation cover limits evaporation and water bodies have a long hydroperiod mitigating the impact of climate warming on amphibians (Duarte et al. 2012). The variability in microhabitats, ponds, and streams present in the AFA, and the lower risk of desiccation for the species of amphibians that live there, favors specializations and the persistence of rare species, resulting in one of the most diverse ecoregions of amphibians (Vaira et al. 2012).

In the AFA, approximately 60 species of anurans have been recorded, which are 34.2% of the total number of species in the country (Vaira et al. 2012). Moreover, this ecoregion is host to eight endemic species such as: Kurupi Treefrog (*Boana curupi*), *B. stellae* (no common name), Snub-nosed Frog (*Scinax perereca*), Green Treefrog (*Trachycephalus dibernardoi*), *Proceratophrys bigibossa* (no common name), Schmidt's Spinythumb Frog (*Crossodactylus schmidti*), Krauczuk's Redbelly Toad (*Melanophryniscus krauczuki*), and the Ocellated Treefrog (*Itapotihyla langsdorffii*; Vaira et al. 2012).

Despite the importance that the AFA has for the overall biodiversity of the region, it is one of the most degraded ecoregions in South America (Galindo-Leal and Gusmão Câmara 2003). This represents the last and most threatened refuge of the Atlantic Forest in the continent (only 7% of its original extension persists), and has almost disappeared in countries such as Paraguay and Brazil due to anthropogenic activities (Galindo-Leal and Gusmão Câmara 2003; Holtz and Placci 2003; Placci and Di Bitetti 2006). Within Argentina, the original surface of this rainforest in the Misiones province (Fig. 1) was approximately 29,000 km^2, while only 9,000 km^2 (32%) of the original surface remained at the beginning of the 21st Century, with deforestation rates of 150–200 km^2/y (Holtz and Placci 2003). Using the most optimistic estimates by these authors, only about 6,300 km^2 exist today (21.7% of the original area). One of the main causes of this degradation in Brazil and Argentina is the replacement of native forests by monoculture tree plantations (mainly pines, *Pinus* spp.; Zurita 2019), which has reduced the richness and altered the composition of the anuran community, and the body size of individuals in populations due to this habitat conversion (Gangenova et al. 2018, 2020). In particular, the canopy-protected subtropical amphibians that inhabit the AFA have become more vulnerable to global warming because of the ongoing deforestation (Duarte et al. 2012). These impacts might even change the distribution of suitable areas, increasing the risk of extinction for many redbelly toads (*Melanophryniscus*; Zank et al. 2014), and a wide variety of other taxa. Given the high degree of threats affecting the AFA due to the advancement of the agricultural and farming frontier, habitat replacement, and recent fires and consequential desiccation, the existing gap in the knowledge of the life-history traits of many species, and of *Melanophryniscus* species in particular, is worrying. The aim of our study was to increase the ecological knowledge of the amphibians inhabiting this ecoregion.

Melanophryniscus is a putative monophyletic taxon (Graybeal and Cannatella 1995), the sister group of remaining bufonid toads (Frost et al. 2006; Jetz and Pyron 2018; Dubois et al. 2021) and is currently represented by 31 species traditionally arranged in three phenetic groups (see Deforel et al. 2021). They are distributed in southern Brazil, southern Bolivia, Paraguay, Uruguay, and central and northern Argentina (Zank et al. 2014). Of 31 species, 11 have been recorded for Argentina, and three of them inhabit the AFA, in the Misiones Province (Baldo and Basso 2004; Vaira et al. 2012). They are Uruguay Redbelly Toad (*Melanophryniscus atroluteus*, from the *M. stelzneri* group), Rivera Redbelly Toad (*M.* aff. *devincenzii*, from the *M. tumifrons* group), and Krauczuk's Redbelly Toad (*M. krauczuki*, unassigned to any group, see Deforel et al. 2021; Fig. 2). Although these species are partially syntopic and usually reproductively synchronic in the study area (see below), they can be diagnosed by their color patterns, external morphology, and advertisement calls. There is significant variability in the body sizes
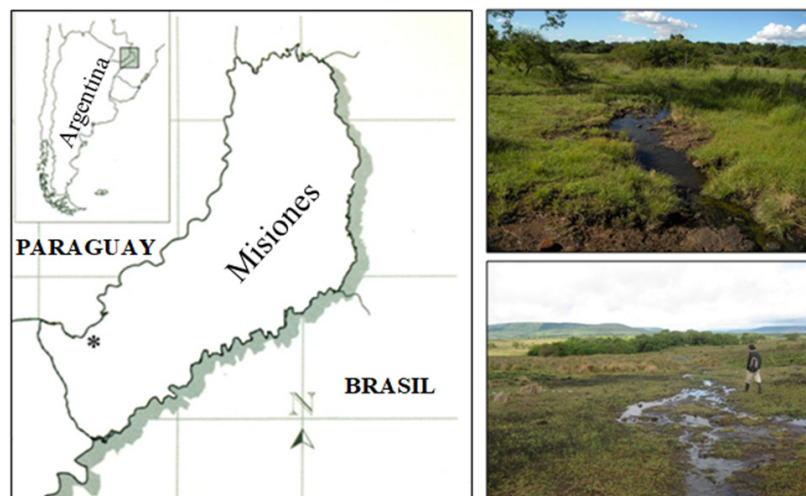

Figure 1. (Left) Location of the population and (Right) two characteristic semi-permanent streams used by the three species of *Melanophryniscus* to reproduce that we studied. (Photographed by Federico Marangoni).

Figure 2. Dorsal (A, C and E) and ventral (B, D and F) views of (A,B) Uruguay Redbelly Toad (*Melanophryniscus atroluteus*), (C, D) Rivera Redbelly Toad (*M.* aff. *devincenzii*), and (E,F) Krauczuk's Redbelly Toad (*M. krauczuki*). (Photographed by Diego Baldo). (G) Rivera Redbelly Toad spawning and sticking the clutch on the basaltic soil in the wet border of the stream, (F) female showing unken-reflex behavior (Photographed by Federico Marangoni).

at inter and intraspecific levels, with temperature and precipitation as the main factors responsible for the observed size clines (Bidau et al. 2011). Furthermore, the body size variation has implications on the reproductive traits of *Melanophryniscus*, which are explosive breeders with a strong association between reproductive activity and rainfall (Vaira 2005; Goldberg et al. 2006).

Here we studied the reproductive activity patterns and interspecific variation in body size and reproductive traits of three species of *Melanophryniscus* genus of AFA. We also analyzed age structure, whether Sexual Size Dimorphism (SSD) exists, and if this dimorphism could be explained by proximate mechanisms such as differences in growth patterns. This will increase our knowledge of the life-history traits of these amphibians, which help predict the responses of these species to the impact of the destruction of their habitat and global warming, and provide information to evaluate species management and conservation strategies.

MATERIALS AND METHODS

Study site.—The study was conducted at Ñu Pyahú (27°29'25.00"S, 55°40'7.30"W), 30 km northeast of Posadas city, Misiones Province, Argentina (Fig. 1). This site is located within the southernmost landscape unit of AFA (District of Champs or District of Champs and Shrubs; Cabrera and Willink 1980; Olson et al. 2001; Brown and Pacheco 2006). This region, with mean temperatures of 25° C in summer and 15.6° C in winter, and average annual precipitation ranging 1,400–2,400 mm (Giraudo et al. 2003), is characterized by Savannas, diverse types of grasslands, Urunday (*Astronium balansae*) Forest, wetlands, and groves of Yatay Palm, *Butia yatay* (Giraudo et al. 2003; Carbonell 2008). It is a landscape transition that contacts with Humid Chaco, the Esteros del Iberá, and Espinal in the Corrientes province (Cabrera 1971; Cabrera and Willink 1980).

TABLE 1. Snout-to-vent length (SVL), body mass (BM), right hindlimb length (HLR), head width (HW), tibia length (TL), arm length (AL), clutch mass (CM), clutch size (CS), egg size (ES), and relative clutch mass (RCM) of male and female of Uruguay Redbelly Toad (*Melanophryniscus atroluteus*), Rivera Redbelly Toad (*M.* aff. *devincenzii*) and Krauczuk's Redbelly Toad (*M. krauczuki*). The abbreviation SDI = sexual dimorphism index. The asterisks (*) compare sexual size dimorphism (SSD) within species (* = $P < 0.001$, ns = not significant). Different lowercase letters mean significant differences in reproductive traits between species using a Scheffe´ Post Hoc Multiple Comparison Test at $\alpha = 0.05$. All values are means ± 1 standard deviation with sample size in parentheses.

Trait	*M. atroluteus* (290)			*M.* aff. *devincenzii* (97)			*M. krauczuki* (123)		
	Male	Female	SDI	Male	Female	SDI	Male	Female	SDI
Body									
SVL	23.63 ± 1.18 (148)	25.76 ± 1.41 (66)*	1.09	23.49 ± 1.16 (67)	27.45 ± 1.54 (30)*	1,17	20.88 ± 1.21 (94)	23.52 ± 1.44 (23)*	1.13
BM	1.02 ± 0.16 (204)	1.36 ± 0.29 (86)*	1.34	0.92 ± 0.14 (67)	1.4 ± 0.27 (30)*	1.52	0.67 ± 0.07 (77)	1.07 ± 0.30 (29)*	1.58
HLR	25.74 ± 1.32 (145)	27.42 ± 1.45 (66)*	1.06	27.4 ± 1.46 (45)	30.07 ± 1.44 (22)*	1.10	23.62 ± 1.51 (76)	24.39 ± 1.03 (23)ns	1.03
HW	6.89 ± 0.35 (148)	7.33 ± 0.34 (66)*	1.06	7.34 ± 0.31 (46)	8.15 ± 0.43 (22)*	1.11	6.64 ± 0.26 (77)	7.05 ± 0.29 (23)*	1.08
TL	7.94 ± 0.33 (148)	8.44 ± 0.43 (66)*	1.06	8.33 ± 0.36 (46)	9.1 ± 0.33 (22)*	1.09	7.16 ± 0.33 (77)	7.68 ± 0.27 (23)*	1.07
AL	5.98 ± 0.33 (148)	6.28 ± 0.40 (66)*	1.05	6.52 ± 0.39 (46)	7.25 ± 0.35 (22)*	1.11	5.7 ± 0.35 (77)	6.23 ± 0.30 (23)*	1.09
Reproductive									
CM		0.32 ± 0.13 (38)a			0.64 ± 0.22 (13)b			0.37 ± 0.13 (21)a	
CS		131.1 ± 71.47 (41)a			217 ± 84.69 (20)b			196 ± 64.42 (20)b	
ES		1.4 ± 0.16 (15)a			1.72 ± 0.16 (17)b			1.51 ± 017 (20)b	
RCM		0.28 ± 0.11 (38)a			0.48 ± 018 (13)b			0.39 ± 0.15 (21)b	

Collection methods and individual measuring.—We collected 510 mature *Melanophryniscus* from fall 2009 to spring 2012, in 20 reproductive events that occurred immediately after heavy rains (*M. atroluteus*, n = 290; *M.* aff. *devincenzii*, n = 97; and *M. krauczuki*, n = 123; Table 1, Appendix). We collected either calling males or pairs in amplexus during chorusing mornings between 0900 and 1300 to ensure that all individuals were mature. We separated toads by species and sex, placed them in independent plastic bags (60 × 40 cm), and brought them to the laboratory. We recorded the following measurements based on Duellman (1970): snout-vent length (SVL); right hindlimb length (HLR); head width (HW); tibia length (TL); and arm length (AL) using a digital caliper (0.1 mm precision). We measured HLR by placing each toad on laminated graph paper (± 1 mm). We measured body mass (BM) to the nearest 0.0001 g, using an electronic balance (Denver Instrument Tp-214; Denver Instrument Company, Goettingen, Germany). We clipped the third toe of the right hind limb and stored them in 70% ethanol at room temperature for age estimation through skeletochronology (see below). We released all individuals back into their original ponds within 24–48 h after their capture. The sampling scheme we followed was the general guidelines proposed by the Ministerio de Ecología y Recursos Naurales Renovables, Misiones Province, Argentina. We obtained air and water temperatures from two temperature data loggers (HOBO Pendant; Onset Corporation, Bourne City, Massachusetts, USA), which were deployed by Ayala (2011) from February 2010 to January 2012, in a similar environment located 27 km (straight line) from the study site. The other logger was deployed by us in the study site for water temperatures during same period. The loggers recorded temperatures every 15–30 min and from this, we calculated the mean monthly temperatures (Appendix). We obtained rainfall (monthly means from 2009–2011) from the meteorological station at the Estación Experimental Agropecuaria Cerro Azul, Instituto Nacional de Tecnología Agropecuaria (INTA), Cerro Azul, Misiones Province located 24 km (straight line) from our study site (Appendix).

Measurement of reproductive traits.—We collected amplecting pairs from fall 2009 to spring 2012. We separated pairs by species and sex in plastic bags (60 × 40 cm), and brought them to the laboratory. We randomly assigned pairs to plastic containers (35 × 25 × 10 cm) filled with 2 L of well water, where they quickly went back into amplexus. We also placed rocks or grass

inside the containers so that amplecting pairs of *M.* aff. *devincenzii* and *M. atroluteus* were able to attach their clutches to the substrate. We photographed the resulting egg clutches using a digital Coolpix S10 camera (Nikon Corporation, Minato City, Tokyo, Japan), mounted on a Nikon C-DS magnifying glass, and obtained counts of the number of eggs in each clutch (clutch size, CS) using Image-Pro Plus 4.5 (Media Cybernetics, Silver Spring, Maryland, USA). For each gravid female, we obtained clutch mass (CM) by subtracting her mass after oviposition from her mass prior to it (Tejedo 1992). We used relative clutch mass (RCM) as an estimate of reproductive effort, calculated as the ratio of clutch mass to female body mass obtained after oviposition (Shine 1992; Bonnet et al. 2003). We photographed a random sample of about 30 eggs from each clutch with a digital Coolpix S10 camera, mounted on a Nikon C-DS magnifying glass to calculate the eggs size (ES). We measured the longest and shortest perpendicular axes of 15 eggs per sample to the nearest 0.01 mm using Image-Pro Plus 1.1 (Media Cybernetics) and we took the square root of the product of the two axis measurements (Marangoni et al. 2008, 2019). Egg size (ES) was always measured at Gosner stages 10 and 11 (Gosner 1960). We returned all eggs from each clutch to their original ponds within 24–48 h after the capture of adults.

Skeletochronology.—Skeletochronology is a non-lethal, widely used method to estimate age in amphibians by counting the number of lines of arrested growth (LAGs) in cross sections of long bone cortices and teeth (Sinsch et al. 2015, and references therein). The growth periods appear as broad bands of tissue, separated by narrower lines or annuli, which mark periods of reduced growth (Halliday and Verrel 1988). We followed the standard methods in skeletochronology (e.g., Smirina 1972; Castanet and Smirina 1990), with minor modifications proposed by Marangoni (2006). We washed clipped digits in water for 30 minutes, decalcified samples in 5% nitric acid for 1–3 h, dehydrated and paraffin-embedded the samples, sectioned them using a rotation microtome at 14–16 μm, and finally stained sections with Harris hematoxylin. 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 high-resolution camera (Sony SSC-DC50AP; Sony Group Corporation, Minato City, Tokyo, Japan) attached to a Olympus BX50 microscope (Olypus Corporation, Shinjuku City, Tokyo, Japan). We observed and measured cross-sections using the computer package Image-Pro Plus version 4.5 (Media Cybernetics) and calibrated sections using a standard micrometer. Independently, we 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). Annuli (sensu Peabody 1958) were easily distinguishable from actual LAGs. They always stained weaker than true LAGs and were often broader, as previously described by Leclair et al. (2005) and Sinsch et al. (2007) in temperate species. We computed the bone size following the methods of Hemelaar (1985) and we measured the longest and shortest perpendicular axes of each LAG in each of two diaphyseal sections per specimen examined. Afterwards, we multiplied together axis measurements and calculated the square root of the product (average diameter of each LAG). This procedure was done for the LAGs in each of two diaphyseal sections per specimen. We computed the following age-related parameters for each sex (*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 breeding 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 patterns.—We used nonlinear estimation in STATISTICA 8 statistical package (StatSoft Inc., Tulsa, Oklahoma, USA) to compute von Bertalanffy (1938) growth model following Beverton and Holt (1957):

$$SVL_t = SVL_{max} (1-e^{-k (t-t_0)})$$

where SVL_t is the expected or average SVL at time (or age) t, SVL_{max} is the asymptotic average SVL, k is the growth rate coefficient and t_0 is the time or age when the average SVL was zero. We fitted von Bertalanffy (1938) growth model and estimated growth parameters (VBGPs) by nonlinear Least Squares Regression. We considered two estimated VBGPs to be significantly different at the 0.05 level when their 95% confidence intervals (CI) did not overlap. We used between seven and 10 juveniles (0 LAGs) of each species to make a more precise adjustment to the von Bertalanffy (1938) growth model. We used measurements of SVL at metamorphosis from three localities to fit the von Bertalanffy growth model (Coordinates, mean (n): *M. atroluteus* 29°00.6´26.51”S, 56° 55´56.90”W, 6.78 mm, n = 26; *M.* aff. *devincenzii* 27°33’31.9”S, 55°42’56.1”W, 7.59 mm, n = 6; and *M. krauczuki* 27°29’25’’S, 55°40’06’’W, 6.17 mm, n = 7. We housed metamorphs of *M.* aff. *devincenzii* and *M. krauczuki* in the

Herpetological Collection of Laboratorio de Genética Evolutiva (LGE), Instituto de Biología Subtropical, Posadas, Misiones, Argentina, under acronyms LGE 7118 and LGE 24967, 24968, 24969, 24970, 24971, 24972. We preserved *M. atroluteus* in the Laboratorio de Investigación en Diversidad, Ecología y Conservación de Vertebrados (LABIDECOV, Universidad Nacional del Nordeste), Corrientes, Corrientes Province, under acronym LABIDECOV-14.

Statistical analyses.—We log-transformed all variables to achieve normality, and we made all analyses with type III General Linear Mixed Models using the STATISTICA 8.0 statistical package (StatSoft Inc.). We used multi- and univariate Analyses of Variance (ANOVA) to test for differences in the six body size variables measured among species and between the sexes within species. We used Linear Regressions to test the association between female size and clutch characteristics. We then used residual analysis to compare female size-adjusted clutch characteristics across species and sexes. We analyzed the trade-off between clutch size and egg size by computing residual scores from the linear regression of CS and ES to female SVL. We carried out MANCOVA and ANCOVAs, using female weight as a covariate to remove the effect of female size when we analyzed the species differences in reproductive traits. We compared means using post-hoc Scheffé Multiple Comparison Test (Scheffé 1953) at α = 0.05. We also assessed the Sexual Size Dimorphism (SSD) for each body measurement using the Sexual Dimorphism Index (SDI), following Lovich and Gibbons (1992): SDI = mean size$_{larger sex}$/mean size$_{smaller sex}$, with the result arbitrarily defined as positive when females are larger than males, and negative when males are larger.

Results

Seasonal reproductive activity pattern.—During this study, the streams where the three species of *Melanophryniscus* breed were filled with autumn rains at the end of March, and they dried completely between late November and early December (Appendix). These rain-filled, shallow, semi-permanent streams cause the formation of numerous small temporary ponds due to the basaltic topography with steep slopes along their course. The breeding activity occurred after moderate or heavy rains in the middle-autumn (April), associated with a decrease in air and water temperature, and ended in mid-spring (late September-October), associated with an increase in temperature (Appendix). Throughout this period, the three species breed in explosive events determined by rains, along two or three consecutive days. The daily air temperature in the spawning events throughout the reproductive period varied within 23.7°–16.2° C and the water temperature within 25.2°–19.2° C in April and May, respectively. Breeding activity was observed between 0900–1700. Once the reproductive period was over, we observed occasional isolated male calling during rainy days in November; however, we never registered chorus, females, or amplectant pairs from November to April (Appendix).

Field observation.—When we arrived at the reproductive sites, we observed spatial segregation among the three species studied when they reproduce synchronously and in sympatry at the study site. Thus, we observed *M. krauczuki* and *M.* aff. *devincenzii* in the upper parts of the streams, while separated by several meters of distance we found *M. atroluteus*, in the lower parts of the stream, or each of them occurring alternately separate along the stream. We observed chorusing behavior in every reproductive event, and it was very common to see males in active defense movements of calling sites or eventually searching females. Males outnombered females in the reproductive sites (sex-ratio mean ± standard deviation: 2.63 ± 1.07 for *M. atroluteus*, 2.27 ± 0.48 for *M.* aff. *devincenzii*, and 2.60 ± 0.44 for *M. krauczuki*), and this difference increased as more individuals participated in reproductive events (Appendix). In these situations, it was possible to see males of *M. krauczuki* clasping other males in occasional encounters or that intruder male attempts to separate other male in amplexus, while fighting or calling interaction in active defense of calling site usually, were observed in *M. atroluteus*. Field observations allowed us to see how the spawning occurs in different sites depending on the species and in multiple clutches (spreading several egg masses at different time intervals and sites). *Melanophryniscus* aff. *devincenzii* and *M. krauczuki* usually stuck the clutch to the basaltic soil or the rocks where the stream has fast water flow speed (Fig. 1) and occasionally to the submerged grass or under cattle manure. *Melanophryniscus atroluteus* usually stuck the clutch to the submerged grass in small holes caused by the trampling of cattle, where the slope of the streams is soft, and the water fills the holes. We observed other amphibian species such as Hensel's Swamp Frog (*Psedupaludicola falcipes*) and Rio Grande Dwarf Frog (*Physalaemus riograndensis*) reproducing synchronously at the same sites throughout most of the breeding season of studied *Melanophryniscus* species. In addition to these two species, once the spring rains have begun, we have found other species synchronously at the study site, such as Dumeril's Striped Frog (*Leptodactylus gracilis*), Rapids Frog (*Limnomedusa macroglossa*), Barker Frog (*Physalaemus cuvieri*), *Physalaemus* aff. *albonotatus* (no common name), and *Rhinella azarai* (no common name).
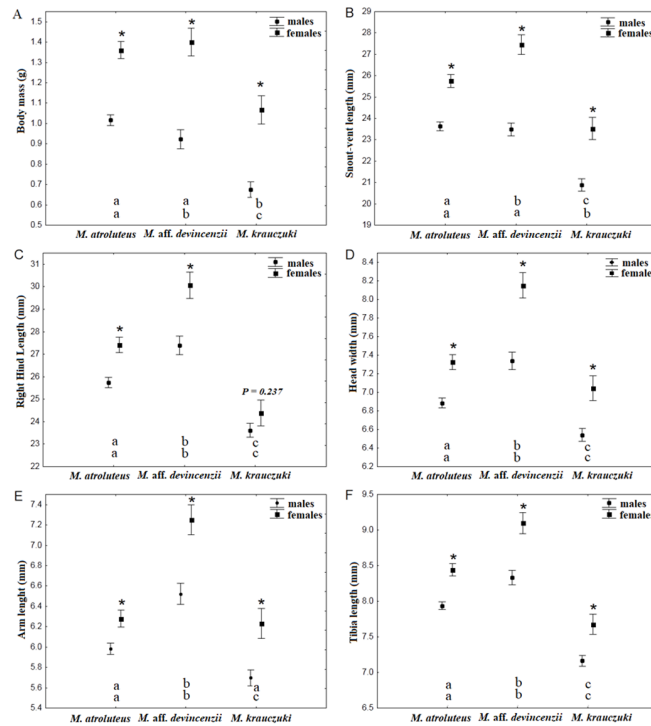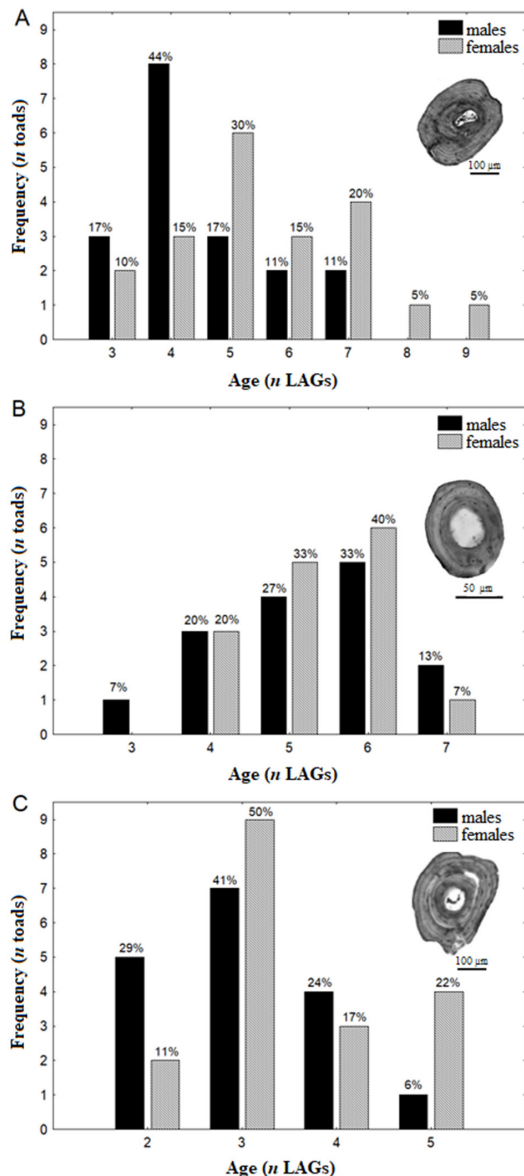
FIGURE 3. Body size-ajusted varied greatly among Uruguay Redbelly Toad (*Melanophryniscus atroluteus*), Rivera Redbelly Toad (*M. aff. devincenzii*), and Krauczuk's Redbelly Toad (*M. krauczuki*) and between sexes within species. (A) snout-to-vent length (SVL), (B) body mass, (C) right hind length, (D) head width, (E) arm length, (F) tibia length. Different lowercase letters mean significant differences using a Scheffe´s Post Hoc Multiple Comparison Test at α = 0.05. Letters compare differences among species, males (upper) and females (lower). The asterisks (*) compare sexual size dimorphism (SSD) within species (*P* < 0.001). The values of B-F represent least squares means ± 1 standard error, where the SVL was used as a covariate.

Interspecific variation in body size.— *Melanophryniscus krauczuki* was the smallest species for all morphometric variables measured (Table 1, Fig. 3). A MANOVA on SVL, BM, HLR, HW, TL and AL, showed significant effects of species (Wilk's λ = 0.228, $F_{12,732}$ = 66.57, *P* < 0.001) and sex (Wilk's λ = 0.424, $F_{18,1036}$ = 20.33, *P* < 0.001). There were significant effects of species on the six variables measured (Table 2). The significant differences among the three species studied were mainteined in size-ajusted BM, HLR, HW, AL, and TL (post-hoc comparisons, Scheffé range test, *P* < 0.001 in all comparison; Fig. 3), whereas the BM and SVL were not significantly different between *M. atroluteus* and *M.* aff. *devincenzii* (post-hoc comparisons, Scheffé range test, BM: *P* = 0.074; SVL: *P* = 0.061; Fig. 3).

Sexual size dimorphism.—The six variables measured showed significant effects of sex nested within species (Table 2). All variables measured were sexually dimorphic, except for HLR in *M. krauczuki* (*P* = 0.237; Table 1, Fig. 3). Females were significantly larger and heavier than males, having relatively wider heads and longer limbs, arms, and tibia (Table 1, Fig. 3). Regardless of age, females are larger than males (*M. atroluteus*: SVL: $F_{1, 35}$ = 27.79, *P* < 0.001; BM: $F_{1, 35}$ = 34.90, *P* < 0.001; *M.* aff. *devincenzii*: SVL: $F_{1,27}$ = 73.63, *P* < 0.001; BM: $F_{1, 27}$ = 64.23, *P* < 0.001; *M. krauczuki*: SVL: $F_{1, 32}$ = 58.19, *P* < 0.001; BM: $F_{1, 32}$ = 57.46, *P* < 0.001). The SDI also showed sexual size dimorphism: females were significantly larger than males in all six parameters measured (Table 1). The sexual dimorphism

TABLE 2. Univariate Analysis of Variance *F*-values for snout-to-vent length (SVL), body mass (BM), right hindlimb length (HLR), head width (HW), tibia length (TL) and arm length (AL) of the three species of *Melanophryniscus* studied. The abbreviation df = degrees of freedom and significant *P* values show with an asterisk (*) at *P* < 0.001.

Source	SVL		BM		HLR		HW		TL		LB	
	df	F	df	F	df	F	df	F	df	F	df	F
Species	2	141.01*	2	105.04*	2	186.5*	2	131.67*	2	224.09*	2	114.5*
Sex (species)	3	126.76*	3	122.93*	3	38.38*	3	67.75*	3	63.26*	3	41.34*
Error	405		504		371		376		376			

FIGURE 4. Age structure of the (A) Uruguay Redbelly Toad (*Melanophryniscus atroluteus*), (B) Rivera Redbelly Toad (*M.* aff. *devincenzii*), and (C) Krauczuk's Redbelly Toad (*M. krauczuki*). Embedded cross sections of phalanges stained with Ehrlich's hematoxylin of (A) *M. atroluteus* (female, 25.94 mm SVL; 5 y), (B) *M.* aff. *devincenzii* (male, 24.12 mm SVL; 4 y), and (C) *M. krauczuki* (males, 20.76 mm SVL; 3 y).

index was much higher for BM despite the fact that we used data from females weighed after oviposition. The bone perimeter, however, was not sexually dimorphic (sex nested within specie: $F_{3,95} = 0.64$, $P = 0.589$)

Age-related parameters.—We found 103 sections (38 *M. atroluteus*, 30 *M.* aff. *devincenzii*, 35 *M. krauczuki*) that showed recognizable bone structures, which 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 to assess individual age (Fig. 4). Endosteal resorption never prevented the age estimation. The line of metamorphosis was visible in 72.5% of the samples. Due to endosteal remodeling in the remaining specimens, the metamorphosis line was not visible and the inner 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. Species had a significant effect on mean age ($F_{2,97} = 29.74$, $P < 0.001$). *Melanophryniscus krauczuki*, the smallest-bodied species (Table 1, Fig. 3), attained maturity earlier and had a shorter longevity and lifespan than *M. atroluteus* and *M.* aff. *devincenzii* (Table 3). Mean age of *M. krauczuki* also differed significantly with the remaining species (post-hoc comparisons, Scheffé range test, $P < 0.001$). The minimum number of LAGs counted in reproductive individuals was 2 in *M. krauczuki* and 3 in *M. atroluteus* and *M.* aff. *devincenzii*, while the mean longevity was 8, 7, 5 (*M. atroluteus*, *M.* aff. *devincenzii*, and *M. krauczuki*, respectively; Table 3, Fig. 4). We did not find significant effects of sex (nested within species) on mean age ($F_{3,97} = 2.66$, $P = 0.053$). Within species, age at maturity and longevity were sexual dimorphic only in *M.* aff. *devincenzii* and *M. atroluteus*, respectively (Table 3, Fig. 4). Age showed a positive relationship with SVL, but were not significant for any species (*M. atroluteus*: $P = 0.059$, n = 38; *M.* aff. *devincenzii*: $P = 0.509$, n = 30; *M. krauczuki*: $P = 0.167$, n = 35). SVLmax was significantly higher, whereas the growth rate coefficient was lower, in females than males of the three *Melanophryniscus* species (Table 4). SVLmax estimated for the model did not show differences with the SVL measured in any sexes in the three species (Table 1 and 5).

Interspecific variation in reproductive traits.—We obtained oviposition in the laboratory in 82 amplecting pairs (*M. atroluteus*: n = 41, *M.* aff. *devincenzii*: n = 20, and *M. krauczuki*: n = 21). Using the female size as a covariate, there were significant effects of species on reproductive traits (CM, ES, CS and RCM; Wilk's λ = 0.417, $F_{6,80} = 7.324$, $P < 0.001$). All reproductive traits measured differed significantly among species (CM: $F_{2,68} = 10.85$, $P < 0.001$; CS: $F_{2,77} = 10.61$, $P < 0.001$; ES: $F_{2,48} = 14.12$, $P < 0.001$; RCM: $F_{2,68} = 10.85$, $P < 0.001$). We found an effect of female size, when used as a covariate, only in the interspecific analyses of the RCM ($F_{1,68} = 14.57$, $P < 0.001$). *Melanophryniscus* aff. *devincenzii*, the largest-bodied species (Table 1, Fig. 3), showed the highest values of reproductive traits (Table 1, Fig. 3); however, this species did not show significant differences with *M.*

TABLE 3. Life-history traits of male and female Uruguay Redbelly Toad (*Melanophryniscus atroluteus*), Rivera Redbelly Toad (*M. aff. devincenzii*), and Krauczuk's Redbelly Toad (*M. krauczuki*). For LAG (lines of arrested growth) and size at AM (age at maturity of the youngest first breeders, in mm), values are means ± standard error, and maximum size (in mm) is at age in LAGs [in brackets]. Abbreviaitons are PRLS = potential reproductive lifespan (in years), ML= median lifespan (in years), and n = sample size.

Species/Sex	n	LAGs	Mode [Frequency]	ML	AM [LAGs]	Longevity [LAGs]	PRLS	Size at AM [n]	Maximum Size
M. atroluteus									
Males	18	4.56 ± 1.25	4(8)	4	3	7	4	22.74 ± 1.48 [3]	24.64 [4]
Females	20	5.55 ± 1.61	5(6)	5	3	9	6	25.31 ± 1.04 [2]	27.96 [7]
M. aff. devincenzii									
Males	15	5.27 ± 1.16	6(5)	5	3	7	4	22.25 [1]	26.16 [6]
Females	15	5.33 ± 0.90	6(6)	5	4	7	3	26.41 ± 0.95 [3]	29.7 [7]
M. krauczuki									
Males	17	3.06 ± 0.9	3(7)	3	2	5	3	20.87 ± 0.4 [5]	21.94 [4]
Females	18	3.5 ± 0.99	3(9)	3	2	5	3	23.3 ± 0.06 [2]	25.65[5]

krauczuki in mean CS, ES and RCM (Scheffé post hoc multiple comparison, Table 1).

Reproductive traits vs. female size and age.— Overall there were no significant relationships between reproductive variables and female size and age, except between ES and SVL, where the ES negatively correlated with SVL *in M. atroluteus* (Table 5). In *M. krauczuki* the ES showed a positive relationship with SVL (Table 5). Moreover, CM showed a significant positive relationship with CS (*M. atroluteus*: r^2 = 0.348, $P < 0.001$, n = 38; *M. aff. devincenzii*: r^2 = 0.421, $P < 0.05$, n = 13; *M. krauczuki*: r^2 = 0.356, $P < 0.01$, n = 20).

DISCUSSION

Seasonal reproductive activity pattern.—The balance between local temperature and water availability in the *Melanophryniscus* populations studied determined the beginning and length of the reproductive period. Reproduction began in association with a decrease in air and water temperature (April), after heavy autumn rains increased the streams flow in March. While the last reproductive event was recorded when both temperatures began to increase (October), one month before the streams dried completely between November and December. The beginning of the reproduction period, associated with a decrease in the temperatures and the preference of the

TABLE 4. Estimated von Bertalanffy growth parameters (VBGPs) of Uruguay Redbelly Toad (*Melanophryniscus atroluteus*), Rivera Redbelly Toad (*M. aff. devincenzii*), and Krauczuk's Redbelly Toad (*M. krauczuki*). Abbreviations are R = Coefficient of determination with sample size (n) in parentheses, S_m = average maximal body size, S_0 = average juvenile body size (0 LAGs), K = growth coefficient, defining the shape of the growth curve, SE = standard error, and CI = 95% confidence interval.

Species / Sex	R (n)	S_m (SE) CI	S_0 (SE) CI	K (SE) CI
M. atroluteus				
Male	0.995 (28)	23.22 (0.29) 22.62–23.82	7.66 (0.26) 7.12–8.2	0.82 (0.19) 0.38–1.25
Female	0.992 (30)	25.33 (0.28) 24.76–25.91	7.74 (0.36) 6.92–8.39	0.75 (0.21) 0.41–1.31
M. aff. devincenzii				
Male	0.995 (25)	23.94 (0.35) 23.22–24.67	7.66 (0.26) 7.12–8.19	0.85 (0.22) 0.39–1.30
Female	0.994 (25)	27.8 (0.64) 26.54–29.21	7.82 (0.35) 6.93–8.39	0.77 (0.28) 0.19–1.35
M. krauczuki				
Male	0.996 (27)	20.58 (0.18) 0.21–20.95	6.03 (30.8) 5.63–6.44	0.88 (0.23) 0.42–1.15
Female	0.992 (28)	23.40 (0.31) 22.76–24.03	6.45 (0.34) 5.34–6.73	0.71(0.21) 0.37–1.09

TABLE 5. Relationships between reproductive variables: clutch mass (CM), clutch size (CS), egg size (ES), and female size: snout-vent length (SVL) and body mass (BM), in Uruguay Redbelly Toad (*Melanophryniscus atroluteus*), Rivera Redbelly Toad (*M.* aff. *devincenzii*), and Krauczuk's Redbelly Toad (*M. krauczuki*). All variables were log-transformed. *P* values: * < 0.05, ** < 0.01, ns = not significant.

Reproductive traits (y) vs. body size	*Melanophryniscus atroluteus*			*Melanophryniscus* aff. *devincenzii*			*Melanophryniscus krauczuki*		
	n	Equation	r^2	n	Equation	r^2	n	Equation	r^2
Clutch mass									
SVL	32	y = 2.349 - 1.122 x	0.015 ns	13	y = 12.944 − 4.046 x	0.250 ns	17	y = -1.272 + 0.063 x	0.0001 ns
BM	38	y = -1.225 - 0.072 x	< 0.001 ns	13	y = -0.396 − 0.385 x	0.032 ns	21	y = -1.083 − 0.323 x	0.030 ns
Age	20	y = -0.334 - 0.174 x	0.194 ns	—	—	—	16	y = -0.711 − 0.107 x	0.079 ns
Clutch Size									
SVL	33	y = 4.749 - 0.0051 x	< 0.001 ns	20	y = 12.857 − 2.282 x	0.097 ns	16	y = 0.184 + 1.582 x	0.035 ns
BM	41	y = 4.590 + 0.659 x	0.025 ns	20	y = 5.488 − 0.584 x	0.066 ns	20	y = 5.201 − 0.197 x	0.009 ns
Age	20	y = 4.491 − 0.014 x	0.001 ns	7	y = 5.415 − 0.005 x	0.0001 ns	15	y = 5.070 + 0.018 x	0.001 ns
Eggs Size									
SVL	8	y = 5.024 − 1.483 x	0.723 **	17	y = -1.886 + 0.732 x	0.156 ns	16	y = -2.929 + 1.065 x	0.301*
BM	15	y = 0.331 − 0.001 x	< 0.001 ns	17	y = 0.483 + 0.189 x	0.120 ns	20	y = 0.414 + 0.101 x	0.035 ns
Age	—	—	—	6	y = 0.018 + 0.086 x	0.491 ns	15	y = 0.464 − 0.009 x	0.007 ns

coldest months of the year, seems counterintuitive in *Melanophryniscus* species of the AFA. These species, however, select very similar ranges of air and water temperatures as observed in other species of the genus from different ecoregions that breed in different months and seasons of the year (Kwet et al. 2005; Cairo et al. 2008; Zank et al. 2014; Duré et al. 2015; Pereira and Maneyro 2018). Fluctuations in the weather condition between the day and the night in local populations of Yungas Redbelly Toad (*M. rubriventris*) also played an essential role in reproductive activity; they preferred warm and humid conditions during nocturnal time (Vaira 2005; Pereyra et al. 2016), while water temperature and pond level predict whether or not a pond is selected to breed (Goldberg et al. 2006). Thus, considering the great variety and usually unpredictable water bodies where *Melanophryniscus* species breed (lentic or lotic; ephemeral, temporal or permanent: Goldberg et al. 2006, 2022; Baldo et al. 2014; Pereira and Maneyro 2016), the balance between air and water temperatures, and the level of the bodies water, becomes crucial to ensure the breeding success. We suggest that in the environments where species of *Melanophryniscus* breed, the local air and water temperature are the most determining constraints of the breeding success in addition to the relevance of the extent and frequency of rainfall.

Despite that our fieldwork was done entirely during daylight hours (between 0900 and 1700), reproductive activity during evening and midnight also was previously registered in the three species studied (pers. obs.). In particular, we observed the presence of amplexus and spawning in *M. atroluteus* and *M. krauczuki* in the same site of the present study during the night, as well as in other sites in central and northern AFA, where it also was registered in *M.* aff. *devincenzii*. These observations are consistent with recent studies showing that nocturnal activity in the genus *Melanophryniscus* is a common phenomenon (Santos and Grant 2011; Sanabria et al. 2014; Pereyra et al. 2016; Pereira and Maneyro 2018). We consider that further studies with specific designs are necessary for the species that inhabit the AFA, to evaluate possible advantages of prolonging reproductive activity at night time (e.g., increase of their fitness in unpredictable environments: Goldberg et al. 2022) and its relationship with a possible buffering mechanism against the effects of climate change (Duarte et al. 2012; Zank et al. 2014) or as a defense against predators (Brodie et al. 1991). In addition, the diel pattern might be explained in the phylogenetic history of the genus, rather than in current functions and selective pressures (Santos and Grant 2011).

Field observations.—We observed spatial segregation among the three species studied when they reproduce synchronously and in sympatry at our study site. Amplectant pairs of the three species spreaded

several egg masses to different sites in the streams or ephemeral holes. As suggested in other species of the genus, it might be explained as a strategy to avoid competition for spawning sites, as a strategy that enhances egg survivorship in unpredictable environment with high rate of desiccation (Bustos Singer and Gutierrez 1997; Goldberg et al. 2006; Cairo et al. 2008), and even to avoid aggressive intra- and interspecific interactions (male-male calling interaction, direct fighting, false amplexus, attempt to dislodge others males in amplexus). Despite the possible pressures that can explain the observed patterns, it is relevant to point out that females do not extrude all eggs in a single clutch, and the number of spawning events carried out by a single female can vary greatly between and within each species (Bustos Singer and Gutierrez 1997; Goldberg et al. 2006; Cairo et al. 2008; Pereyra et al. 2016; Pereira and Maneyro 2018). Without this consideration, we could make erroneous conclusions when evaluating interspecific differences in reproductive traits, and the correlations of them with body size and/or age within species of *Melanophryniscus*.

Intra- and interspecific interactions and spawning strategies described above are well documented in explosive breeders, where sex ratios in populations are male-biased (Wells 1977), which also usually involves the presence of sexual size dimorphism (SSD; Blanckenhorn 2000; Monnet and Cherry 2002). Both male-biased sex ratios and SSD were present in the three species of *Melanophryniscus* studied. Males always outnumbered females in all reproductive events and all variables measured were sexually dimorphic, where the females were significantly larger than the males. Similar sex-ratios were observed in several other species of *Melanophryniscus* (Bustos Singer and Gutiérrez 1997; Goldberg et al. 2006; Duré et al. 2015; Goldberg et al. 2022; Piñeiro etl a. 2022) and a male-biased sex ratio due to differential mortality between sexes was suggested in Yungas Redbelly Toad (*M. rubriventris*; Vaira 2000; Quinzio 2003). SSD and females larger than males have also been found in *M. rubriventris* (Quinzio 2003), the Ventania Redbelly Toad (*M. diabolicus*; Cairo et al. 2008; Zank et al. 2014), Darwin's Redbelly Toad (*M. montevidensis*; Pereira and Maneyro 2018), and Corrientes Redbelly Toad (*M. cupreuscapularis*; Schaefer 2007, but see Duré and Kehr 2006). We suggest that the SSD observed in our study may be developed by the strong male-male interactions and by the sexual selection for small males, due to the advantages that small size has on the fitness in amphibians with explosive breeding (Blanckenhorn 2000).

Sexual size dimorphism and age-related parameters.—Other non-mutually exclusive factors could be acting together to determine the SSD in *Melanophryniscus* of AFA (see Bidau et al. 2011). SSD might also account for differences in age at maturity, age structure, post- metamorphic growth rate, or even sex-specific differences in mortality rate (Howard 1981; Halliday and Verell 1988; Hemelaar 1988; Monnet and Cherry 2002). The present study is the first skeletochronological analysis made in *Melanophryniscus* species of AFA. We found significant interspecific differences in age-related parameters following the differences in body size. Thus, the smallest-bodied species, *M. krauczuki*, had a lower mean age, was younger at maturity, and had shorter longevity than *M. atroluteus* and *M.* aff. *devincenzii*; however, within species, we did not find sexual dimorphism in the mean age of any of the studied species. Age at maturity was sexually dimorphic only in *M.* aff. *devincenzii*, whereas the longevity showed sexual differences only in *M. atroluteus*. On the other hand, lower growth rate (k) and higher estimated average maximal body size (Sm) were observed in females of the three species. Similar results were found in adults of *M. rubriventris* (3–10 y old), where the mean age was not sexually dimorphic (Quinzio 2003). Conversely, older and larger females than males were found in the Maldonada Redbelly Toad (*Melanophryniscus moreirae*) from the Serra da Mantiqueira plateau in southeastern Brazil (Jeckel et al. 2015). We suggest that the differences in body size observed between the sexes at the intraspecific level cannot be attributed to differences in age structure; nevertheless, differences in growth rate found (females growing slower to reach larger sizes) and other environmental (Bidau et al. 2011; Zank et al. 2014) or genetic factors (Baldo et al. 2012), may also be relevant for understanding the evolution and maintenance of the SSD of the studied *Melanophryniscus* species.

Effects of the variation in body size and SSD on reproductive traits.—We might expect changes in reproductive traits parallel with the body size differences observed among species of *Melanophryniscus* due to positive covariation between female size and reproductive performance (e.g., Berven 1988). In addition, this also was proposed to explain the SSD in favor of the females (Shine 1979). In our study, *M.* aff. *devincenzii*, the largest-bodied species, showed the highest values of reproductive traits; however, this species does not show significant differences with the smallest-bodied species (*M. krauczuki*) in mean CS, ES, and RCM. Furthermore, overall we did not observe covariation between reproductive traits and body size. In addition, our study is the first to reveal that female age does not affect reproductive traits in species of *Melanophryniscus*. The absence of correlation between female body size and fecundity or egg size also was

observed in *M. diabolicus* (Cairo et al. 2008), *M. montevidensis* (Pereira and Maneyro 2018), and *M. cupreuscapularis* (Schaefer 2007). We suggest that the SSD observed in the three species of *Melanophryniscus* of the AFA cannot be explained by differences in reproductive traits or correlated effect of age on them.

Lastly, our study described the reproductive activity pattern and interspecific variation in body size and reproductive traits of three species of *Melanophryniscus* genus of AFA. Furthermore, it is the first skeletochronological analysis made in *Melanophryniscus* species of AFA providing relevant ecological aspects, and the first information available about the possible mechanisms underlying the observed phenotypic variation of the three species analyzed. Thus, based on the fact that thermal tolerance in ectotherms depends on body mass (Leiva 2019), the differences in growth rate and SVLmax observed between species and sexes in *Melanophryniscus* will allow us to predict responses to the impact of habitat destruction and global warming. These new insights should be taken into account when evaluating future management and conservation strategies, for these particular species of high conservation concern at the country level, in one of the most degraded ecoregions in South America.

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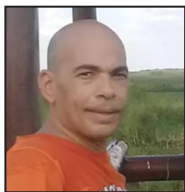
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APPENDIX. Monthly sample size of Uruguay Redbelly Toads (*Melanophryniscus atroluteus*), Rivera Redbelly Toads (*M.* aff. *devincenzii*), and Krauczuk's Redbelly Toads (*M. krauczuki*) along 12 reproductive events from May 2009 to October 2012 (14 May, 30 May, 3 September 2009; 23 and 26 April, 19 May, 2 September 2010; 22 June 2011, 14 April, 14 and 16 August, and 3 October 2012). Temperature and rainfall were log-transformed. The abbreviation SR = sex ratio (males/females). The arrow indicates the months when the stream channels, the reproductive site of the three species, fill with water. The remaining months were dry.
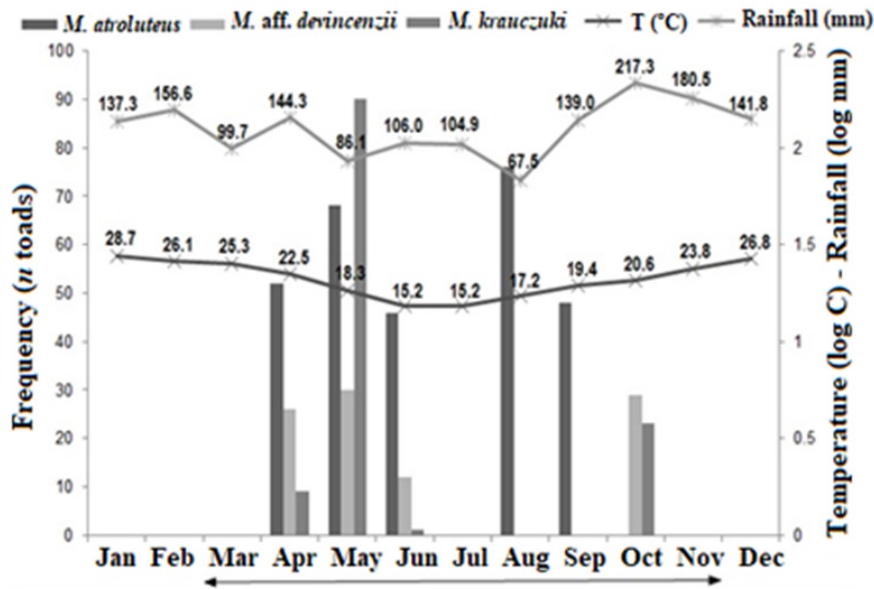
Specie/ Month	M. atroluteus (n = 290)			M. aff. devincenzii (n = 97)			M. krauczuki (n = 123)		
	Male	Female	Σ(SR)	Male	Female	Σ(SR)	Male	Female	Σ(SR)
Jan	0	0	0	0	0	0	0	0	0
Feb	0	0	0	0	0	0	0	0	0
Mar	0	0	0	0	0	0	0	0	0
Apr	39	13	52 (3.00)	19	7	26(2.71)	9	0	9
May	45	23	68(1.96)	19	11	30(1.73)	67	23	90(2.91)
Jun	37	9	46(4.11)	8	4	12(2.0)	1	0	1
Jul	0	0	0	0	0	0	0	0	0
Aug	56	20	76(2.8)	0	0	0	0	0	0
Sep	27	21	48(1.29)	0	0	0	0	0	0
Oct	0	0	0	21	8	29(2.63)	16	7	23(2.29)
Nov	0	0	0	0	0	0	0	0	0
Dec	0	0	0	0	0	0	0	0	0
	Σ=204	Σ=86	X(SR)=2.63	Σ=67	Σ=30	X(SR)=2.27	Σ=93	Σ=30	X(SR)=2.60