



# Reproductive biology of the nest building vizcacheras frog *Leptodactylus bufonius* (Amphibia, Anura, Leptodactylidae), including a description of unusual courtship behaviour

Gabriel Faggioni<sup>1</sup>, Franco Souza<sup>1</sup>, Masao Uetanabaro<sup>1</sup>, Paulo Landgref-Filho<sup>2</sup>, Joe Furman<sup>3</sup>  
& Cynthia Prado<sup>1,4</sup>

<sup>1</sup>Programa de Pós-Graduação em Ecologia e Conservação, Universidade Federal de Mato Grosso do Sul, Campo Grande, Brasil

<sup>2</sup>Campo Grande, Brasil

<sup>3</sup>Houston, USA

<sup>4</sup>Departamento de Morfologia e Fisiologia Animal, Universidade Estadual Paulista, Jaboticabal, Brasil

We describe the reproductive biology and sexual size dimorphism of a population of the vizcacheras frog *Leptodactylus bufonius* in the Brazilian Chaco. Reproduction takes place during the rainy months (September–March). During courtship, females emit reciprocal calls and both sexes perform vibratory movements of the body; the latter is described for the first time in anurans. Amplexus and oviposition occurred inside subterranean chambers. The temperature in closed chambers was lower than outside chambers, which may aid in reducing desiccation risks of eggs and tadpoles. Females were larger than males, but males had longer heads and shorter tibias, which may be related to digging. The study reinforces the importance of ongoing discoveries on anuran natural history.

**Keywords:** Chaco, natural history, sexual size dimorphism, subterranean chamber, vibratory movements

## INTRODUCTION

The genus *Leptodactylus* Fitzinger, 1826, comprises 74 species distributed from southern Texas to Argentina, including Caribbean islands (Frost, 2015). Frogs in this genus exhibit reproductive modes ranging from aquatic nests, such as in species of the *L. latrans* group, to terrestrial nests found in the *L. fuscus* group (Heyer, 1969; Prado et al., 2002). Males in the *L. fuscus* group build chambers in moist soil near streams or ponds, where eggs are deposited in foam nests produced during amplexus (Crump, 1995; Giaretta & Kokubum, 2003; Lucas et al., 2008). The subterranean chambers and foam nests prevent eggs and larvae from physical damage and desiccation during periods of prolonged drought (Downie, 1984; Caldwell & Lopez, 1989). Species in the genus *Leptodactylus* also display complex courtship behaviours. For example, females can emit reciprocal calls to communicate with males prior to amplexus (e.g., *L. fragilis*: Bernal & Ron, 2004; *L. syphax*: Silva et al., 2008; *L. troglodytes*: Kokubum et al., 2009). Physical contact between the sexes prior to mating might aid in males guiding females to an oviposition site (Martins,

1988; Haddad & Giaretta, 1999; Haddad & Sawaya, 2000; Lucas et al., 2008; Kokubum et al., 2009).

Species in the *L. fuscus* group reproduce in subterranean chambers which may vary in size, shape, presence of tunnels and number of compartments (Crump, 1995; Martins, 1988; Giaretta & Kokubum, 2003; Arzabe & Prado, 2006; Kokubum et al., 2009). The vizcacheras frog *L. bufonius* Boulenger, 1894, belongs to this group (Heyer, 1969; Ponssa, 2008; de Sá et al., 2014), and is a Chacoan species distributed from southern Bolivia to northern Argentina, Paraguay, and the states of Mato Grosso and Mato Grosso do Sul in central Brazil (Souza et al., 2010; Pansonato et al., 2011; Frost, 2015). Males of *L. bufonius* build subterranean chambers using their heads, and after spawning females may close the chamber opening possibly for moisture maintenance and thermal isolation (Crump, 1995; Reading & Jofré, 2003).

Although *L. bufonius* is listed as Least Concern in the IUCN red list (Heyer et al., 2004), the Chaco is severely threatened by human activities (Bucher & Huszar, 1999; Souza et al., 2010). Most information on the Chaco herpetofauna is limited to Paraguay and Argentina (Leynaud & Bucher, 1999; Brusquetti & Lavilla, 2006),

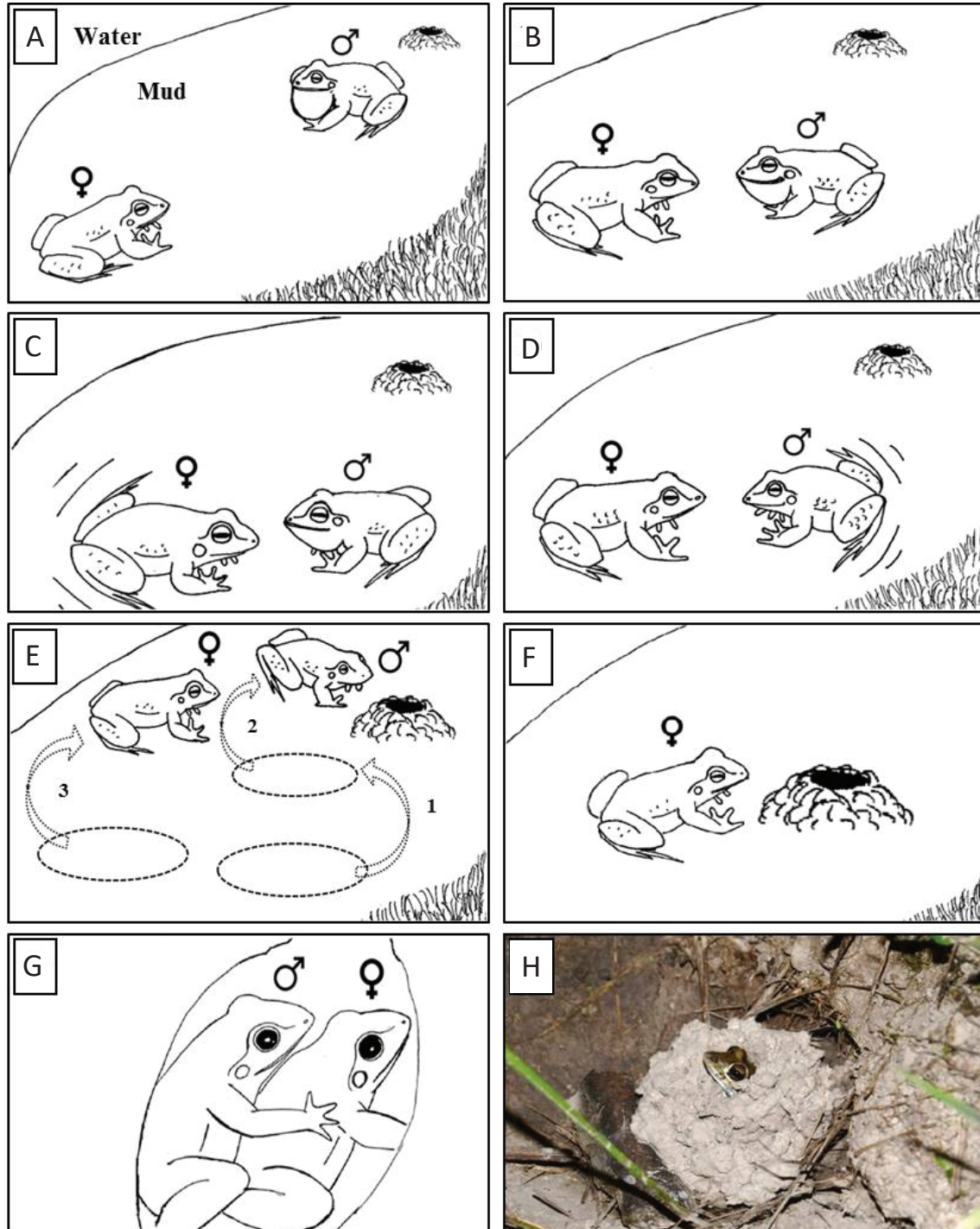
Correspondence: Gabriel Paganini Faggioni (faggioni@hotmail.com)

and studies on the natural history of anurans from this area are still lacking (but see Souza et al. 2010). Here, we describe the reproductive biology of *L. bufonius* in this area, including the first description of the complete courtship behaviour. We also report on sexual size dimorphism (SSD) and its relationship with chamber construction.

## MATERIALS AND METHODS

### Study area and data collection

This study was conducted in central Brazil, southwestern Mato Grosso do Sul. With a flat topography, semi-arid climate and distinct vegetation, the Chaco is a biogeographic province occupying approximately



**Fig. 1.** Complete sequence of courtship in *Leptodactylus bufonius*. (A) The ♂ emits advertisement calls next to a subterranean chamber and the ♀ moves close to the pond. (B) After approaching the ♂, the ♀ emits a reciprocal call, stimulating the ♂ to emit a courtship call. (C) The ♀ performs rapid vibratory movements. These movements begin at the anterior part of her body and move in a wave-like manner ending at the posterior part. (D) The ♂ performs the same vibratory movements and (E) jumps toward the chamber entrance followed by the ♀. (F) The ♂ enters the chamber and the ♀ follows. (G) Amplexus and oviposition occurs inside the chamber. (H) A female *L. bufonius* frog with her head protruding from the mud chamber. Vibratory movements of the body performed by both sexes in steps (C) and (D) are described here for the first time.

1,000,000 km<sup>2</sup> across Argentina, Paraguay, Bolivia and Brazil, characterised by high levels of endemism (Prado et al., 1992; Prado, 1993; Leynaud & Bucher, 1999; Bucher & Huszar, 1999; Brusquetti & Lavilla, 2006; Pennington et al., 2000; Morrone, 2006). The climate is represented by a hot rainy season from October to April and a dry season from May to September; temperatures can range between 49°C and close to 0°C (Pennington et al., 2000; Alvares et al. 2013).

The study was conducted at one focal natural temporary pond (21°42'39"S, 57°43'16"W) and in surrounding temporary ponds located in the municipality of Porto Murtinho between December 2009 and April 2010 (excluding February 2010). The focal pond was surrounded by grass and mud, with small termite mounds limited to the flooded area. We made four field trips, each lasting eight days (32 observation days in total). Daily fieldwork began at 1800 hours and stopped when the species ceased vocalisation activity around 0100 hours. Complementary observations were also made during the day.

#### Reproductive activity and subterranean chambers

For behavioural observations we used focal-animal sampling, all occurrences sampling and sequence sampling (Altmann, 1974). The description of courtship behaviour was based on videos recorded by J. Furman on 24 February 1996 in the Paraguayan Chaco, near the city of Filadelfia, approximately 240 km from the Brazilian studied site at Porto Murtinho. The reproductive period of *L. bufonius* was determined based on observations of calling males and the presence of clutches inside the chambers as well as based on the presence of mature oocytes in ovaries (Prado & Haddad, 2005), examined by making a ventral incision in collected and fixed specimens as described below.

To describe the subterranean chambers, we used a measuring tape (0.1 cm) to take six measurements: external diameter of the nest base, smallest and largest

diameters of the opening, external height of the nest, depth of spawning compartment and total height (i.e. the sum of the external height of the nest and depth of spawning compartment). The internal and external temperatures of the chambers were obtained using a thermometer to the nearest 0.1°C. The temperature of the closed chambers was obtained by drilling small holes that allowed the passage of a thermometer into the chamber. To determine whether internal chamber temperatures were lower than external environmental temperatures, both temperatures were measured during the hottest hour of the day (between 1300 and 1330 hours) on the same day for each chamber.

#### Sexual Size Dimorphism (SSD)

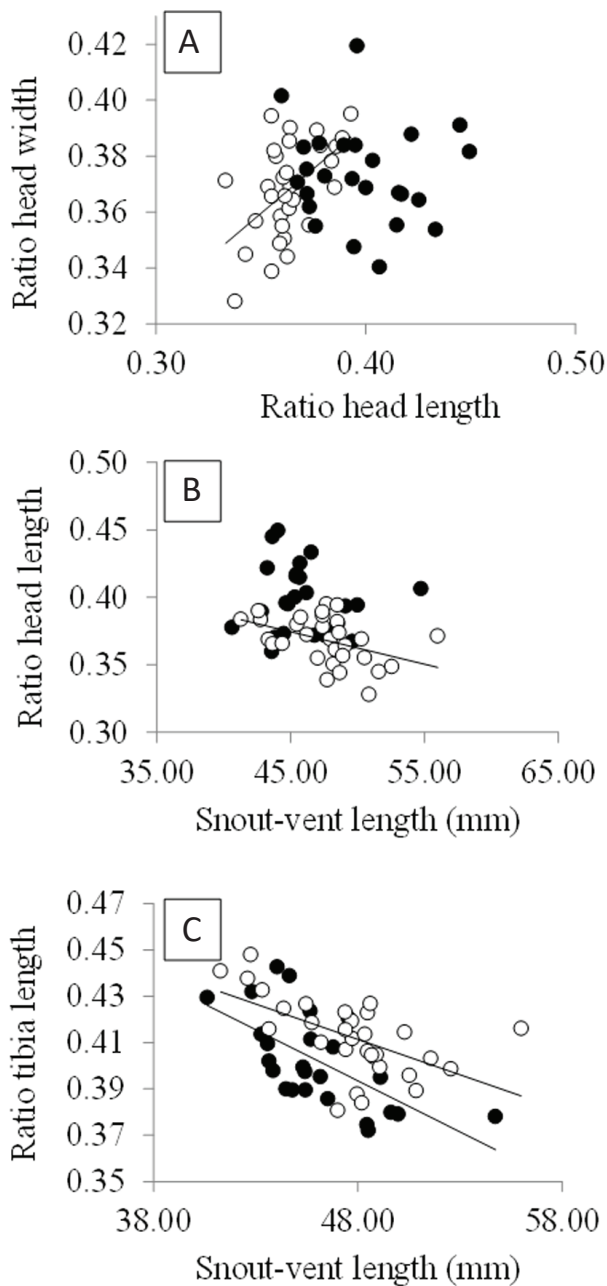
For SSD analyses, we merged data from 36 specimens from the same locality deposited at the Coleção Zoológica de Referência da Universidade Federal de Mato Grosso do Sul (ZUFMS), Campo Grande, Mato Grosso do Sul state, Brazil, adding to 20 individuals collected in October 2008, February, September and December 2009, and January 2010. Individuals were euthanised with 10% Lidocaine, fixed in 10% formalin, preserved in 70% ethanol (McDiarmid, 1994) and deposited in the ZUFMS collection. Individuals were euthanised because we were conducting studies of reproductive effort based on gonad mass that will be published elsewhere. We measured specimens' body mass with a digital balance (to 0.1 g) and used a digital calliper to measure four morphometric characters to the nearest 0.01 mm: snout-vent length (SVL), head width (HW), head length (HL) and tibia length (TL). We separated males from females based on gonads and external characters.

To analyse SSD, we compared mean values of SVL, HW, HL and TL in males and females using Student's *t*-test (Zar, 1999). To remove the influence of body size and to reveal SSD differences in body shape, we also compared the body proportions HW/SVL (RHW), HL/SVL (RHL) and TL/SVL (RTL) between males and females. We

**Table 1.** Mean±SD and *p*-values from *t*-tests for comparisons between snout-vent length (SVL), head width (HW), head length (HL), tibia length (TL), ratio head width/SVL (RHW), ratio head length/SVL (RHL), ratio tibia length/SVL (RTL) and body mass (BM) of *Leptodactylus bufonius* males and females sampled in the municipality of Porto Murtinho, State of Mato Grosso do Sul, central Brazil. Females' body mass without ovary mass (BM no.ov.) was also compared with males' body mass.

<i>Leptodactylus bufonius</i> (n=56)					
Characteristics	Males (n=25)		Females (n=31)		Males vs. females <i>p</i> -value
	Mean±SD	Range	Mean±SD	Range	
SVL (mm)	46.03±2.91	40.60–54.74	47.66±3.11	41.28–55.99	0.05
BM (g)	11.00±2.60	7.40–19.40	13.70±2.70	8.40–19.40	<0.01
BM no.ov. (g)	-	-	12.80±2.40	8.20–18.50	0.01
HW (mm)	17.15±0.80	15.63–18.73	17.29±0.96	15.50–18.87	0.55
HL (mm)	18.31±1.52	15.35–22.25	17.52±1.09	15.84–20.79	0.03
TL (mm)	18.48±0.90	17.35–20.70	19.63±1.06	17.90–23.30	<0.01
RHW	0.37±0.02	0.34–0.42	0.36±0.01	0.33–0.39	0.01
RHL	0.40±0.02	0.36–0.45	0.37±0.02	0.33–0.40	<0.01
RTL	0.40±0.02	0.37–0.44	0.41±0.02	0.38–0.45	0.05

used a one-tailed t-test to compare HL between sexes because we expected longer heads in males, due to their chamber construction behaviour. Two-tailed t-tests were used for the remaining traits. We further investigated morphological differences related to male nest construction through linear regressions of the measured traits with SVL. All statistical tests were conducted using R v.3.0.3 (R Development Core Team, 2014).



**Fig. 2.** Linear regressions for morphological traits of male (black circles) and female (white circles) *Leptodactylus bufonius* sampled in the municipality of Porto Murinho, State of Mato Grosso do Sul, central Brazil. (A) Ratio head length (HL/SVL) vs. ratio head width (HW/SVL); (B) snout-vent length vs. ratio head length; (C) snout-vent length vs. ratio tibia length. Regression equations and statistics are described in Table 2.

## RESULTS

### Reproductive activity

Females bearing mature oocytes were found in October 2008, February, September and December 2009 and January 2010. Calling males and foam nests in chambers or tadpoles in ponds were found in December 2009, January and March 2010. The volume of water in the focal pond increased continuously during the rainy season. By February 2010, it was no longer possible to distinguish the study pond from the surrounding temporary ponds. The water began to decrease in March 2010, and the pond was completely dry in the following month. Calling activity decreased in March and ceased during April, when the pond was completely dry.

Males of *L. bufonius* began to vocalise around 1830 hours and remained active until around 0100 hours. The maximum number of males observed calling around the pond in a single night was 10. In general, males called exposed at the edge of the pond, close to termite mounds and mud heaps. On five occasions, we recorded males calling inside cylindrical cavities in mud heaps (probably crab burrows) with room for only one individual. On the subsequent days, these cavities were empty.

### Courtship, spawning and subterranean chambers

Courtship behaviour of *L. bufonius* occurred before individuals entered the subterranean chambers. The general steps identified for the courtship of the species are summarised in Fig. 1; for a summary video see <<https://www.youtube.com/watch?v=nksIsRc05oY>>. Based on the full video, the detailed courtship behaviour observed for a pair of *L. bufonius* is described as follows. At 2200, a female approached until she was approximately 3 cm from the male who was emitting a courtship call. The female started emitting reciprocal calls in antiphony with the male's courtship calls, and performed fast vibratory movements of the body without lifting any limb from the ground. These vibratory movements began as rapid shakes of the abdominal region towards the right and left that flowed to the posterior part of the body before reaching the cloaca (about 2 s). The male, hitherto vocalising without moving, jumped towards the side of the female, exhibited the same vibratory movement, and continued emitting courtship calls. The two individuals maintained the interactions for about 30 min, before another male positioned itself a few centimetres from the pair and remained motionless throughout the observation. The mating pair continued to interact without amplexus.

At the Brazilian site, we only rarely observed reproductive interactions before ( $n=1$ ) and after ( $n=2$ ) *L. bufonius* pairs entered the chamber, and no vibratory movements were observed. After being approached by a female, males entered the chamber followed by the female. Amplexus and spawning occurred inside the chamber. As the female was releasing eggs, the male produced a foam nest by churning the spawn with alternated leg movements. Foam nest production lasted 30 min in the first observation and 2 h in the second. Neither of the two chambers was closed after the spawning process. We did not observe males



**Table 2.** Linear regression results for snout-vent length (SVL) vs. head width (HW), head length (HL), tibia length (TL), ratio head width/SVL (RHW), ratio head length/SVL (RHL) and ratio tibia length/SVL (RTL) in male and female *Leptodactylus bufonius* frogs sampled in the municipality of Porto Murtinho, State of Mato Grosso do Sul, central Brazil.

Sex	Linear regression equation	p-value	r <sup>2</sup>	n
Male	HL=2.050 + 0.350 SVL	<0.01	0.46	25
	HW=8.650 + 0.180 SVL	<0.01	0.45	25
	TL=9.640 + 0.190 SVL	<0.01	0.38	25
	RHL=not significant	0.62	-	25
	RHW=0.530 – 0.004 SVL	<0.01	0.39	25
	RTL=0.600 – 0.004 SVL	<0.01	0.38	25
Female	HL=5.320 + 0.250 SVL	<0.01	0.53	31
	HW=5.750 + 0.240 SVL	<0.01	0.62	31
	TL=6.470 + 0.280 SVL	<0.01	0.67	31
	RHL=0.480 – 0.002 SVL	0.01	0.20	31
	RHW=0.490 – 0.003 SVL	<0.01	0.33	31
	RTL=0.560 – 0.003 SVL	<0.01	0.32	31

constructing chambers or contact between the male and female before amplexus.

We found eight open and empty reproductive chambers indiscernible in shape and size from other chambers containing clutches. Chambers resemble the shape of volcanoes, and the mean diameter of the external cone was  $6.9 \pm 1.4$  cm (range=4.7–9 cm,  $n=8$ ). The opening on top of the cone was oval, with a larger diameter of  $2.0 \pm 0.4$  cm (range=1.1–2.5 cm,  $n=8$ ) and a smaller diameter of  $1.4 \pm 0.4$  cm (range=1–2 cm,  $n=8$ ). The opening was  $1.9 \pm 0.5$  cm (range=1–2.7 cm,  $n=8$ ) above ground, and the subterranean spawning compartment had a depth of  $8.0 \pm 1.7$  cm (range=6.0–10.0 cm,  $n=8$ ). Thus, the average height of chambers was  $9.9 \pm 1.5$  cm (range=8.3–11.9 cm,  $n=8$ ). Four closed and one open chamber contained spawn. The mean temperature inside the sealed chambers at 1300 was  $30.2^\circ\text{C} \pm 1.3^\circ\text{C}$  (range= $29.1^\circ\text{C}$ – $32.1^\circ\text{C}$ ,  $n=4$ ), whereas the average external temperature was  $34.3^\circ\text{C} \pm 1.6^\circ\text{C}$  (range= $32.9^\circ\text{C}$ – $36.0^\circ\text{C}$ ,  $n=4$ ; paired  $t=5.74$ ,  $p=0.01$ ,  $df=3$ ). For the open chamber, the difference between the internal and external temperatures ( $35.6^\circ\text{C}$  and  $35.7^\circ\text{C}$ , respectively) was  $0.1^\circ\text{C}$ .

## SSD

All traits were considered normally distributed after statistical and visual examination of the data. All analysed females had mature oocytes in their ovaries. On average, females with and without oocytes were heavier than males ( $t=3.89$ ,  $p<0.01$ ,  $df=54$ ;  $t=2.61$ ,  $p=0.01$ ,  $df=54$ , respectively). Females also attained larger SVL ( $t=1.99$ ,  $p=0.05$ ,  $df=54$ ; Table 1), despite a overlap between the sexes. Females had larger TL ( $t=4.29$ ,  $p<0.001$ ,  $df=54$ ) and RTL ( $t=1.97$ ,  $p=0.05$ ,  $df=54$ ), whereas HL ( $t=-2.19$ ,  $p=0.03$ ,  $df=54$ ), RHL ( $t=5.05$ ,  $p<0.01$ ,  $df=54$ ) and RHW ( $t=2.32$ ,  $p=0.02$ ,  $df=54$ ) of males were larger than those of females (Fig. 2). HW did not differ between the sexes ( $t=0.59$ ,  $p>0.05$ ,  $df=54$ ; Table 1). For both males and females, HL, HW and TL were positively correlated with SVL (Table 2). With the exception of males' RHL, all trait

ratios were negatively correlated with SVL in both sexes (Table 2, Fig. 2).

## DISCUSSION

### Reproductive activity

The reproductive activity of anurans from tropical seasonal habitats is strongly influenced by rainfall (Wells, 2007; Nali & Prado, 2012). Although the construction of terrestrial chambers for incubating foam nests represents a partial independence from water, species in the *L. fuscus* group still rely on rainfall to build the chambers in moist soil and for the development of aquatic tadpoles. However, as the first developmental stages take place inside the chambers, they are protected against aquatic predators (Heyer, 1969; Arzabe & Almeida, 1997; Reading & Jofré, 2003; Lucas et al., 2008). We observed male *L. bufonius* vocalising between 1830 and 0100 during the months of December 2009 and January 2010 when rainfall was high (170 mm and 220 mm, respectively; INMET, 2010). In Argentina, *L. bufonius* also reproduced during the rainiest months, with calling activity observed between 2000 and 0130 (Philibosian, 1974; Reading & Jofré, 2003). Because we did not have access to the study site before December 2009, it was not possible to determine when *L. bufonius* males started to vocalise. However, we did not record any calling activity in April 2010, which may be explained by the low precipitation at the end of the rainy season (75 mm and 30 mm in March and April, respectively; INMET, 2010).

Male *L. bufonius* from Argentina have been found calling near or inside subterranean chambers (Crump, 1995; Reading & Jofré, 2003). In the present study, males were found calling from approximately 1 m away or from pre-existing cavities (probably crab burrows) where no amplexed pairs or clutches were observed. Digging behaviour has already been observed for other species such as *L. fuscus* (Arzabe & Almeida, 1997; Kokubum et al., 2009). The ancestral species of *L. fuscus*

was probably semi-fossorial, which may be the origin of the behaviour observed in extant species (Heyer, 1978). At present, male *L. bufonius* may use burrows as calling sites (Kokubum et al., 2009; present study) or for protection against fire (Miranda et al., 1993; Arzabe & Almeida, 1997) and predators (Arzabe & Almeida, 1997; Arzabe & Prado, 2006).

#### Courtship, spawning and subterranean chambers

The courtship behaviour of *L. bufonius* was similar to that described for other species in the genus (e.g., *L. furnarius*: Giaretta & Kokubum, 2003; *L. fuscus*: Lucas et al., 2008), except for the emission of reciprocal courtship calls by the female and the performance of rapid vibratory movements of the body by both sexes which is described here for the first time. Reciprocal calls by females have previously been observed in anurans from Africa, North America, Europe and Asia (Roy et al., 1995; Tobias et al., 1998; Bosch & Márquez, 2001; Bosch, 2002; Krishna & Krishna, 2005; Shen et al., 2008) as well as in *Leptodactylus* species (Schlaepfer & Figueroa-Sandí, 1998; Davis et al., 2000; Bernal & Ron, 2004; Silva et al., 2008; Kokubum et al., 2009). The function of female vocalisation varies among species (Emerson & Boyd, 1999; Toledo et al., 2014), and is usually related to mate location, synchronisation of complex courtship and nesting, or female receptiveness (Schlaepfer & Figueroa-Sandí, 1998; Tobias et al., 1998; Emerson & Boyd, 1999; Gibson & Buley, 2004; Shen et al., 2008; Kokubum et al., 2009; Toledo et al., 2014). Female *L. bufonius* may vocalise to signal receptiveness or to coordinate the courtship while moving to the chamber, as suggested for *Aplastodiscus* hylids (Haddad & Sawaya, 2000). Females of the sister species *L. troglodytes* also emit reciprocal calls before amplexus (Kokubum et al., 2009; de Sá et al., 2014).

The rapid vibratory movements displayed by both sexes appear similar to the abdominal movements observed in male *L. fragilis* (Bernal & Ron, 2004). Another visual communication displayed by frogs where neither the front nor hind limbs are lifted is body jerking (Hödl & Amézquita, 2001). However, body jerking is characterised by forward and backwards or up and down movements of the body performed by males as part of the territorial display (Hödl & Amézquita, 2001; Wogel et al., 2004; de Sá et al., 2016; see also Weygoldt & Carvalho-e-Silva, 1992), or by males and females during courtship (de Sá et al., 2016). Because the body movements we observed were laterally orientated undulations performed by males and females close to each other, we argue that the vibration of the body during the courtship of *L. bufonius* is different from body jerking, and may be part of visual or chemical communication between males and females.

*Leptodactylus bufonius* from Brazil exhibited the reproductive mode previously reported elsewhere for this species (Crump, 1995; Reading & Jofré, 2003) and other congeners in the *L. fuscus* group (Heyer, 1969; Prado et al., 2002). The observation of a satellite male next to a pair exhibiting courtship behaviour, along with reports of more than one male with a single female in a

subterranean chamber (Reading & Jofré, 2003; Faggioni et al., 2011), reinforce the possibility of polyandry in *L. bufonius* (see also Prado & Haddad, 2003).

The dimensions of the chambers were similar to those previously reported (Philibosian et al., 1974; Reading & Jofré, 2003; for slightly larger chambers see Crump, 1995), although males from our study site were smaller than those from Argentina (Reading & Jofré, 2003). We also observed chambers that had not yet been used for breeding (see also Philibosian et al., 1974; Reading & Jofré, 2003). Four out of five of the subterranean chambers containing eggs were closed, confirming Philibosian et al. (1974) and Crump (1995) that reported females closing the nests. Closed chambers were also observed for *L. fuscus* (Lucas et al., 2008), and may represent an adaptation to seasonal environments; however, such behaviour has not been registered for *L. troglodytes* from arid areas in northeastern Brazil (Kokubum et al., 2009). Our results showed that closure of the opening maintains internal temperatures below external temperature, likely contributing to reduce tadpole desiccation and confirming Reading & Jofré (2003).

#### SSD and chamber construction

Although females were on average larger and heavier than males (as previously reported: Heyer, 1978; Reading & Jofré, 2003), our results showed a large overlap in size between the sexes. In anurans, females are larger than males in about 90% of species, which can be explained by the fecundity advantage hypothesis (Shine, 1979; Nali et al., 2014). Although head width was not sexually dimorphic, males had larger head lengths (confirming Heyer, 1978; Reading & Jofré, 2003). In *L. fuscus*, males have longer heads due to a chisel-shaped snout used for digging (Heyer, 1978; Ponssa, 2008). Contrary to Heyer (1978) and Reading & Jofré (2003), *L. bufonius* males in our study had shorter tibia than females. Tibia length may also be related to digging, as shorter legs may confer additional power during chamber excavation (Heyer, 1978). The fact that our study was conducted at the periphery of *L. bufonius* range could explain the observed population differences (for other population differences in *Leptodactylus* see Lucas et al., 2008; Ponssa et al., 2011). The new information reported here highlights the importance of natural history studies on anurans from the Neotropics, a region harbouring a large diversity of amphibians (Duellman, 1988; Haddad & Prado, 2005).

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