



## Calls and tadpoles of the species of *Pseudis* (Anura, Hylidae, Pseudae)

Diego José Santana<sup>1,2,3</sup>, Felipe de Medeiros Magalhães<sup>1,2</sup>, Vinícius de Avelar São Pedro<sup>4</sup>, Sarah Mângia<sup>1</sup>, Talita Ferreira Amado<sup>4</sup> & Adrian Antonio Garda<sup>2</sup>

<sup>1</sup>Programa de Pós Graduação em Ciências Biológicas (Zoologia), Departamento de Sistemática e Ecologia,

Universidade Federal da Paraíba, Cidade Universitária, 58059-900, João Pessoa, Paraíba, Brazil

<sup>2</sup>Laboratório de Anfíbios e Répteis, Departamento de Botânica e Zoologia, Centro de Biociências,

Universidade Federal do Rio Grande do Norte, Campus Universitário, Lagoa Nova, 59078-900, Natal, RN, Brazil

<sup>3</sup>Laboratório de Zoologia, Centro de Ciências Biológicas e da Saúde,

Universidade Federal de Mato Grosso do Sul, Cidade Universitária, 79070-900, Campo Grande, MS, Brazil. (Current Address).

<sup>4</sup>Programa de Pós Graduação em Ecologia, Universidade Federal do Rio Grande do Norte, Campus Universitário, Lagoa Nova, 59078-900, Natal, RN, Brazil

The use of larval and bioacoustical characters has been essential to solve taxonomic problems of many anuran species. Herein, we describe the advertisement call and tadpoles of *Pseudis fusca* and *P. tocantins* and compare them with descriptions of all other Pseudae. The advertisement calls of *Pseudis* species are formed by pulsed notes. In *P. cardosoi* and *P. minuta* all pulses within a single note are concatenated, while in *P. bolbodactyla*, *P. fusca*, *P. paradoxa* and *P. tocantins* the notes are formed by sets of concatenated pulses. Moreover, the calls of *P. bolbodactyla*, *P. fusca* and *P. paradoxa* are indistinguishable. Tadpoles of *P. fusca* and *P. tocantins* resemble other *Pseudis* tadpoles described so far: their body is oval-shaped in dorsal view and triangular in lateral view and higher than wide; they possess an anteroventral oral disc with five tooth rows (two anterior and three posterior) and well developed tail musculature. The large larval size is in agreement with other species in the genus. We evaluate which characters best distinguish species within *Pseudis*.

**Key words:** bioacoustics, hydrographic basins, larvae, paradoxical frogs, taxonomy

### INTRODUCTION

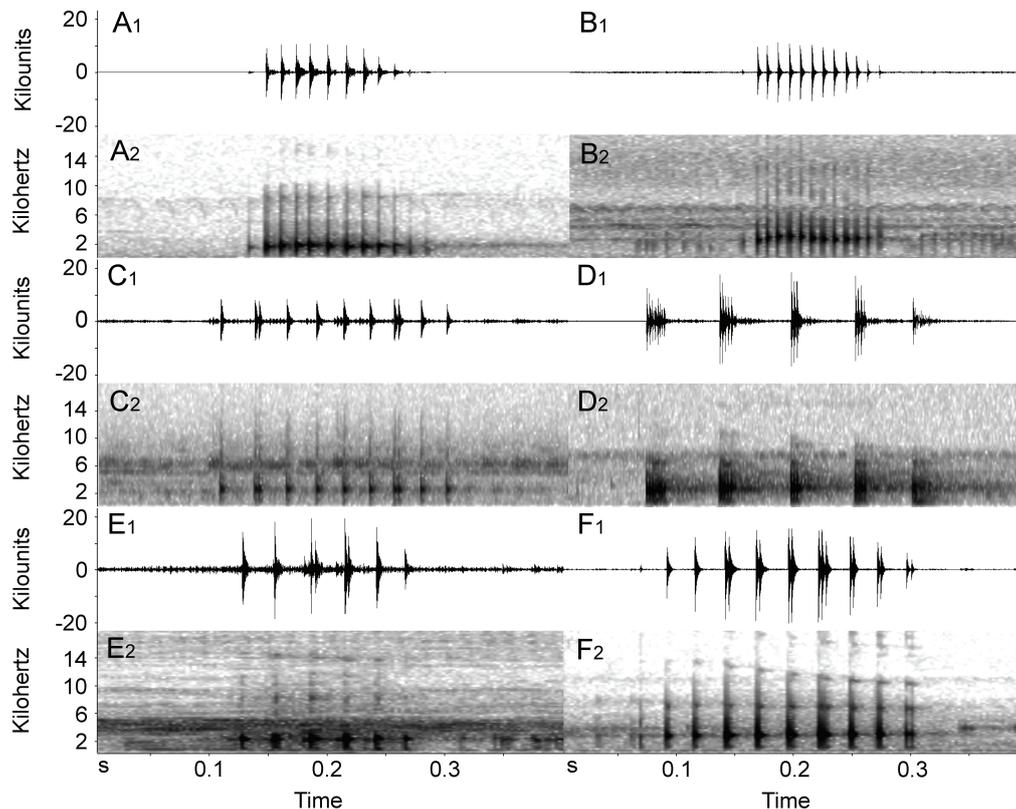
The diversity of anurans in the Neotropics is still poorly known (Bickford et al., 2007; Fouquet et al., 2007), with several morphologically similar or indistinct species (Fouquet et al., 2007). Furthermore, the lack of data on geographic variation of adults, larvae, and bioacoustic characters for most species severely hampers solid species delimitations (Gamble et al. 2008; Fouquet et al. 2014). Molecular studies are revealing significant numbers of cryptic species (Fouquet et al., 2007; Funk et al., 2011; Prado et al., 2012), but the distinct lineages found frequently lack diagnostic morphological characters (Diaz et al., 2012; Newman et al., 2012; Prado et al., 2012). Despite species descriptions based solely on non-morphological data (Haddad & Sazima, 2004; Weber et al., 2005; Leache & Fujita, 2010), descriptions of adult and larval morphology as well as of advertisement calls are fundamental for rapid field species identifications, and to evaluate the evolutionary drivers of species diversification.

Anuran advertisement calls play an important role as a pre-zygotic isolation mechanism, and are key character for taxonomic studies (Duellman & Trueb, 1986; Gerhardt,

1988). Taxonomists consider observable differences in acoustic parameters as important evidences in species descriptions, despite any evidence that such differences contribute to species isolation (Funk et al., 2008; Jovanovic et al., 2009; Toledo et al., 2010; Guerra et al., 2011). Likewise, tadpole morphology provides an important source of characters for anuran taxonomy (Cruz, 1982; Haas, 2003; Jovanovic et al., 2009). Nevertheless the calls and tadpoles of most Neotropical species are unknown (Provete et al., 2012; Santana et al., 2013).

Pseudae comprises hylid species morphologically adapted to aquatic environments and ecologically associated with river floodplains (Garda & Cannatella, 2007; Garda et al., 2010). These frogs are also known as paradoxical frogs, due to the giant tadpoles of *Pseudis*, which may reach 28 cm in length before reducing in size during metamorphosis (Bokermann, 1967; Fabrezi et al., 2009). The phylogenetic position of the group was controversial for a long time, and Pseudae have been placed in Ranidae (Günther, 1858), Leptodactylidae (Noble, 1922), Hylidae (Parker, 1935; Duellman & Trueb, 1986), and Pseudidae (Savage & de Carvalho, 1953). Currently, morphological (da Silva, 1998) and molecular

Correspondence: Adrian Antonio Garda (garda@cb.ufrn.br)



**Fig. 1.** Advertisement calls (single note) of the species of *Pseudis*. *P. cardosoi*: (A1) oscillogram and (A2) audiospectrogram, specimen from São Joaquim, RS (Air temperature 19°C); *P. minuta*: (B1) oscillogram and (B2) audiospectrogram, specimen from Taíam, RS (Air temperature not available); *P. fusca*: (C1) oscillogram and (C2) audiospectrogram, specimen from Araçuaí, MG (Air temperature 25°C); *P. tocantins*: (D1) oscillogram and (D2) audiospectrogram, specimen from Aruanã, GO (Air temperature 25°C); *P. bolbodactyla*: (E1) oscillogram and (E2) audiospectrogram, specimen from Alvorada do Norte, GO (Air temperature 23°C); *P. paradoxa*: (F1) oscillogram and (F2) audiospectrogram, specimen from Bela Vista, MS (Air temperature 25°C).

data (Darst & Cannatella, 2004; Hoegg et al., 2004; Faivovich et al., 2005; Wiens et al., 2010; Pyron & Wiens, 2011) have unequivocally placed Pseudae within Hylidae. Based on molecular evidence, *Pseudis* was seen as non-monophyletic, and Garda & Cannatella (2007) proposed the revalidation of *Podonectes* while Aguiar et al. (2007) placed *Lysapsus* as junior synonym of *Pseudis*. More recent evidence, however, recovered *Pseudis* as monophyletic in an expanded molecular phylogeny of hylids (Wiens et al., 2010). The use of external morphological characters recently supported the resurrection of *Lysapsus*, and also casted doubt on the validity of some species (Garda et al., 2010).

Herein, we describe the advertisement calls and tadpoles of *P. tocantins* and *P. fusca*, comparing the results with descriptions from the literature. Additionally, our goals were to i) reanalyse calls and tadpoles of all remaining species of *Pseudis* (sensu Garda et al., 2010) based on simultaneous and standardised comparisons, and ii) identify acoustic and larval characters that support species and genera of Pseudae.

## MATERIALS AND METHODS

We provide the first description of the calls and tadpoles of *P. fusca* and *P. tocantins* and compare them with descriptions from the literature. Throughout the paper we

**Table 1.** Summary of the advertisement call of *Pseudis* species from all localities analysed and from literature (see Online Appendix 1) (Hz=Hertz; s=seconds).

<i>Pseudis</i> species	Dominant frequency (Hz)	Note duration (s)	Number of pulses/note	Pulse rate (pulse/s)
<i>P. bolbodactyla</i>	1500–2584	0.09–0.32	5–14	30.4–114.3
<i>P. cardosoi</i>	1500–2300	0.13–0.45	10–22	71.4–82.1
<i>P. fusca</i>	2067–2584	0.10–0.24	5–16	47.2–72.4
<i>P. minuta</i>	1200–3445	0.08–0.17	7–16	90.2–128.2
<i>P. paradoxa</i>	1723–2813	0.07–0.35	8–18	34.1–183.1
<i>P. tocantins</i>	2063–2625	0.17–0.28	12–28	57.4–120.7

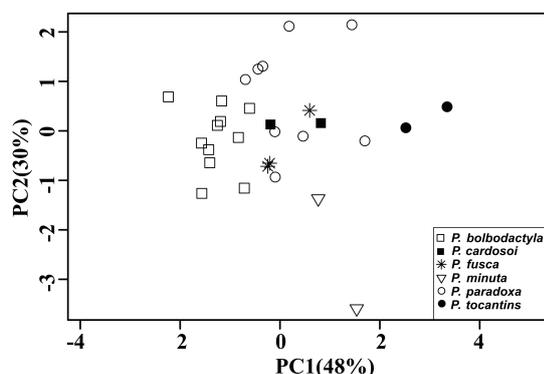
follow the nomenclature proposed by Garda et al. (2010) for *Pseudis*. The authors considered two genera valid (*Lysapsus* and *Pseudis*) and did not find sufficient evidence to support the recognition of *P. platensis* as a valid taxon.

### Vocalisations

We recorded calls of *P. fusca* in 04 March 2005, in Araçuaí municipality (-16.7796 S, -41.9358 W; approximately 300 m a.s.l.), Minas Gerais State, Brazil, using a Sony TCM-5000EV digital recorder, with an ATR55 Telemike directional microphone. The calls of *P. tocantins* were recorded on 14 March 2005, in Aruanã municipality (-14.916965 S, -51.075355 W; approximately 250 m a.s.l.), Goiás State, Brazil, using Sony TCM-5000EV digital recorder, with a ATR55 Telemike directional microphone and in February 2011, in Filadélfia municipality, Tocantins State, Brazil, using a Marantz PMD 660 digital coupled with a Sennheiser e614 directional microphone. For comparisons, we also analysed calls of the other species of the genus from several populations, from the literature, and from loans from collections (Online Appendix 1).

All calls were recorded in a sample rate of 44 kHz and 16-bit resolution. The analogical recordings were digitised also at a sampling rate of 44 kHz and 16-bit resolution in Raven Pro 1.3<sup>®</sup>. We positioned microphones approximately 1 m away from the calling frogs. We analysed calls using the software Raven Pro v. 1.3 (Cornell Lab of Ornithology) with the following parameters to build the spectrograms: Fast Fourier Transformation 512 (FFT), overlap 50. The following acoustic parameters were analysed: note duration (s); number of pulses (pulses/note); dominant frequency (Hz) and pulse rate (pulse/s). Terminology of calls follows Duellman and Trueb (1986). We searched for differences in the pattern of the modulation of frequency, number of pulses, concatenation of pulses, and pulse rate (Santana et al., 2013). The values in text are presented as “mean±standard deviation (range)”.

Advertisement call variability was explored by means of Principal Component Analysis (PCA) using the R software v. 3.1.2 (R Development Core Team, 2014) for advertisement calls recorded by us for all *Pseudis* species. We only used the acoustic variables that can be homologically compared between the species, which are listed in Table 1 (for instance, we did not include the pulse interval because there are differences in note emission and pulse composition between the species of this clade, see below).



**Fig. 2.** Distribution of *Pseudis* advertisement call variables in the two first principal components.

### Tadpoles

We collected tadpoles of *Pseudis fusca* in March 2005, at Araçuaí Municipality (-16.7796 S, -41.9358 W; approximately 300 m a.s.l.), Minas Gerais State, Brazil and *P. tocantins* tadpoles in March 2005, Britânia municipality (-15.2019 S, -51.1955 W; approximately 260 m a.s.l.), Goiás State, Brazil. All tadpoles were collected with a dip net, and anaesthetised with 5% lidocaine and fixed and preserved in 10% formalin. Although we did not raise tadpoles until metamorphosis, we collected larvae and metamorphic individuals. No syntopic species of *Pseudis* are reported to date (Garda et al., 2010), and in both localities adult males and females of their respective species were collected along with larvae. We adopted the terminology for external morphology and labial tooth row formula (LTRF) of Altig & McDiarmid (1999) and determined the development stage according to Gosner (1960). Voucher specimen lots of *P. fusca* (one tadpole at Gosner stage 35 and three at Gosner stage 40) and *P. tocantins* (nine tadpoles at Gosner stages 27–36) used for external morphology descriptions are deposited at Coleção Herpetológica da Universidade de Brasília (lots CHUNB 43491 and CHUNB 43501, respectively). Moreover, tadpole lots of *P. bolbodactyla*, *P. cardosoi*, *P. paradoxa* and *P. minuta* were used to compare with and differentiate from *P. fusca* and *P. tocantins* tadpoles (See Online Appendix 2).

We measured twenty morphometric variables in all larvae with a Mitutoyo<sup>®</sup> digital caliper (0.01 mm precision) and an ocular micrometre in a Leica-EZ4D stereomicroscope following Altig & McDiarmid (1999): total length (TL), body length (BL), tail length (TAL), maximum tail height (MTH), tail muscle height (TMH), tail muscle width (TMW), interorbital distance (IOD), internarial distance (IND). The remaining variables were measured as follows: body height (BH, maximum height), body width (BW, at widest area), maximum dorsal fin height (DFH), maximum ventral fin height (VFH), eye diameter (ED, longitudinal distance from anterior to the posterior edges of the eyes), eye-snout distance (ESD, from pupil to the tip of the snout), eye-naris distance (END, distance from pupil to naris centre), naris-snout distance (NSD, distance from naris centre to the tip of the snout), naris diameter (ND, longitudinal distance in the antero-posterior axis), spiracle length (SL, from its origins to the posterior margins), vent tube length (CTL, the portion of the vent tube that extends on to the ventral fin), oral disc width (ODW, the widest portion of the oral disc). Measurements are provided to the nearest 0.1 mm. For morphometric comparisons between tadpole species, we measured the ratio of the tail muscle height relative to the maximum tail height (TMH/MTH ratio), the ratio of body height relative to the body length (BH/BL ratio), and the ratio of P3 length relative to P2 length (when P3 length is ≤50% of P2 length we attributed a state 1; when P3 length is ≥50% of P2 length we attributed a state 2).

## RESULTS

### Vocalisations

The advertisement calls of all *Pseudis* species are formed by pulsed notes (Fig. 1). The notes are formed by groups or sets of concatenated pulses in *P. bolbodactyla*, *P.*

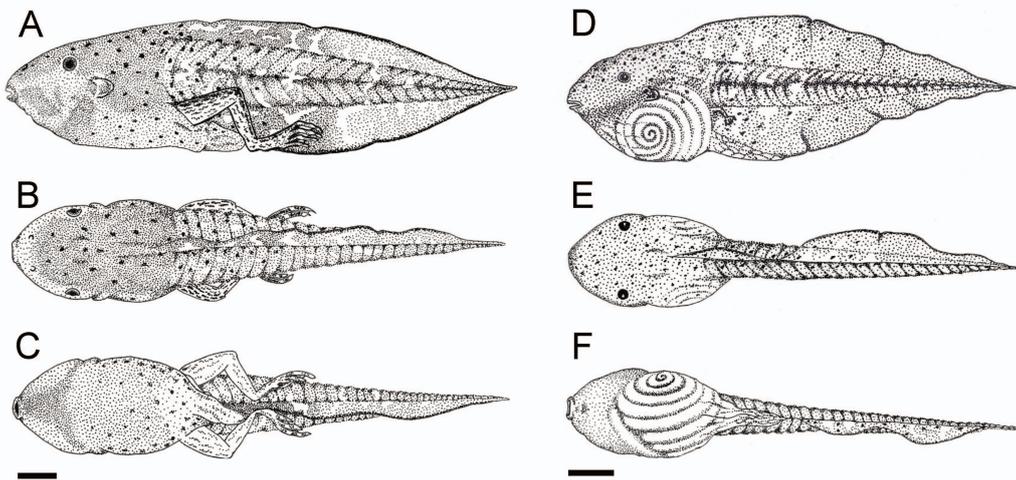
**Table 2.** Morphometric measurements (in mm) of *Pseudis* tadpoles ( $n$ =number of measured individuals). Values are mean  $\pm$ standard deviation (range).

	<i>P. bolbodactyla</i> ( $n=3$ )	<i>P. cardosoi</i> ( $n=11$ )	<i>P. fusca</i> ( $n=4$ )	<i>P. minuta</i> ( $n=8$ )	<i>P. paradoxa</i> ( $n=13$ )	<i>P. tocantins</i> ( $n=9$ )
Measurements	Stages 26–31	Stages 26–40	Stages 36–40	Stages 36–39	Stages 26–40	Stages 27–36
TL	79.1 $\pm$ 30.9 (46.7–108.1)	79.2 $\pm$ 9.0 (62.7–87.2)	134.9 $\pm$ 14.3 (115.8–150.5)	72.5 $\pm$ 3.2 (66.8–78.3)	127.1 $\pm$ 23.1 (93.2–184.6)	93.7 $\pm$ 16.8 (68.5–117.6)
BL	24.3 $\pm$ 6.0 (18.5–30.5)	26.7 $\pm$ 2.2 (22.2–29.4)	43.5 $\pm$ 2.1 (40.4–45.0)	21.1 $\pm$ 0.8 (19.9–22.2)	35.8 $\pm$ 5.2 (28.0–46.7)	28.9 $\pm$ 5.8 (19.6–38.9)
BH	21.1 $\pm$ 7.4 (13.8–28.5)	19.0 $\pm$ 2.4 (16.0–22.1)	30.7 $\pm$ 2.1 (28.2–33.2)	16.9 $\pm$ 1.4 (14.4–18.5)	31.6 $\pm$ 6.9 (23.2–44.1)	20.1 $\pm$ 5.3 (11.2–27.3)
BW	14.8 $\pm$ 3.0 (11.4–16.6)	16.8 $\pm$ 1.9 (14.3–20.7)	22.3 $\pm$ 0.9 (21.0–22.9)	14.9 $\pm$ 1.2 (13.5–17.2)	24.5 $\pm$ 5.2 (19.9–35.3)	15.0 $\pm$ 2.9 (9.7–18.4)
TAL	54.8 $\pm$ 25 (28.2–77.6)	52.5 $\pm$ 7.2 (38.0–60.6)	91.4 $\pm$ 12.3 (75.4–105.5)	51.4 $\pm$ 3.0 (46.0–56.2)	91.3 $\pm$ 18.2 (64.7–138.0)	64.8 $\pm$ 12.2 (48.9–85.7)
MTH	22.3 $\pm$ 8.4 (14.6–31.3)	22.1 $\pm$ 3.3 (15.3–26.6)	29.1 $\pm$ 3.0 (25.0–32.0)	16.7 $\pm$ 1.4 (14.9–18.6)	34.6 $\pm$ 11.8 (24.0–55.5)	26.1 $\pm$ 7.7 (15.5–35.9)
DFH	8.2 $\pm$ 3.4 (4.9–11.6)	7.6 $\pm$ 1.5 (5.1–9.7)	10.9 $\pm$ 0.4 (10.3–11.3)	6.6 $\pm$ 0.6 (5.5–7.3)	13.1 $\pm$ 5.2 (6.3–22.9)	9.2 $\pm$ 2.3 (5.6–13.0)
VFH	8.4 $\pm$ 3.7 (4.9–12.3)	7.2 $\pm$ 1.6 (4.5–10.1)	9.7 $\pm$ 0.5 (9.3–10.4)	7.1 $\pm$ 0.3 (6.5–7.5)	12.7 $\pm$ 4.8 (6.9–22.4)	8.8 $\pm$ 2.3 (4.8–12.3)
TMH	8.0 $\pm$ 3.0 (5.6–11.3)	8.8 $\pm$ 2.0 (5.2–11.4)	16.0 $\pm$ 1.0 (15.0–17.1)	5.4 $\pm$ 0.8 (4.1–6.6)	13.4 $\pm$ 3.1 (7.4–16.9)	8.0 $\pm$ 2.0 (5.1–11.0)
TMW	5.7 $\pm$ 2.3 (3.9–8.3)	6.5 $\pm$ 1.2 (4.1–7.6)	15.9 $\pm$ 1.9 (13.1–17.4)	4.8 $\pm$ 0.7 (3.9–5.9)	11.6 $\pm$ 3.5 (5.9–16.4)	6.7 $\pm$ 2.0 (4.1–10.5)
ED	3.1 $\pm$ 0.4 (2.7–3.5)	3.4 $\pm$ 0.5 (2.3–3.8)	3.8 $\pm$ 0.2 (3.5–4.0)	2.5 $\pm$ 0.3 (2.1–2.9)	4.2 $\pm$ 0.7 (2.9–5.0)	3.0 $\pm$ 0.5 (2.1–4.0)
IOD	13.2 $\pm$ 3.9 (9.4–17.1)	12.3 $\pm$ 0.9 (10.8–13.7)	19.4 $\pm$ 1.0 (18.3–20.3)	11.3 $\pm$ 0.5 (10.7–12.2)	21.2 $\pm$ 4.4 (16.8–30.4)	12.4 $\pm$ 2.9 (7.5–16.9)
ESD	13.9 $\pm$ 3.9 (10.1–17.8)	11.7 $\pm$ 0.9 (10.0–12.8)	17.4 $\pm$ 1.1 (16.4–19.0)	10.2 $\pm$ 0.7 (9.0–11.1)	19.1 $\pm$ 3.4 (15.4–26.1)	12.7 $\pm$ 3.2 (8.0–18.3)
ND	1.6 $\pm$ 0.3 (1.4–2.0)	1.4 $\pm$ 0.2 (1.2–1.6)	2.0 $\pm$ 0.3 (1.7–2.2)	1.2 $\pm$ 0.2 (1.0–1.5)	1.8 $\pm$ 0.5 (0.8–2.5)	1.3 $\pm$ 0.3 (0.9–1.8)
IND	6.5 $\pm$ 2.1 (4.2–8.2)	4.9 $\pm$ 0.7 (3.5–6.0)	7.7 $\pm$ 0.8 (7.2–8.9)	4.5 $\pm$ 0.4 (3.9–5.0)	7.2 $\pm$ 1.4 (5.1–9.6)	5.7 $\pm$ 1.7 (3.4–8.2)
NSD	5.9 $\pm$ 1.7 (4.2–7.6)	5.1 $\pm$ 0.6 (3.9–6.1)	7.9 $\pm$ 1.1 (6.9–9.2)	4.2 $\pm$ 0.4 (3.8–4.7)	7.4 $\pm$ 1.0 (5.9–8.9)	4.9 $\pm$ 1.0 (3.0–6.4)
END	8.9 $\pm$ 2.2 (6.5–10.8)	6.6 $\pm$ 0.5 (5.3–7.2)	12.0 $\pm$ 1.1 (10.4–12.8)	6.0 $\pm$ 0.8 (4.3–7.1)	12.4 $\pm$ 2.4 (9.5–17.2)	8.8 $\pm$ 1.5 (6.9–11.8)
SL	3.5 $\pm$ 1.0 (2.7–4.6)	4.3 $\pm$ 0.8 (2.9–5.1)	5.2 $\pm$ 0.6 (4.8–6.0)	3.1 $\pm$ 0.2 (2.8–3.3)	4.7 $\pm$ 1.0 (3.1–6.3)	3.2 $\pm$ 0.9 (1.8–4.4)
CTL	17.0 $\pm$ 9.0 (10.0–27.2)	12.1 $\pm$ 2.2 (9.1–14.9)	17.6 $\pm$ 3.6 (12.9–20.6)	9.5 $\pm$ 1.0 (7.5–10.4)	25.1 $\pm$ 9.8 (7.1–43.4)	15.5 $\pm$ 3.5 (9.0–20.1)
ODW	4.4 $\pm$ 1.6 (2.6–5.6)	4.2 $\pm$ 0.9 (2.4–5.7)	5.9 $\pm$ 0.3 (5.5–6.1)	3.8 $\pm$ 0.4 (3.2–4.7)	6.6 $\pm$ 1.0 (5.3–8.5)	4.6 $\pm$ 0.9 (3.4–5.8)

*fusca*, *P. paradoxa* and *P. tocantins* (Fig. 1C–F), whereas in *P. cardosoi* and *P. minuta* all pulses are concatenated within a single note (Fig. 1A, 1B). Males of *Pseudis fusca* and *P. tocantins* were observed calling during the night, floating on the water surface, amidst the vegetation. Less frequently, we also observed specimens calling in the twilight and some even during the day. Based on 30 calls from three different individuals, the advertisement call of *P. fusca* (Fig. 1C; Table 1) has an average duration of 0.184 $\pm$ 0.03 s (0.104–0.244 s), a dominant frequency of 2067.2 $\pm$ 45.9 Hz (2067.2–2584.0 Hz), and is composed of 11.7 $\pm$ 2.42 pulses (5–16 pulses), emitted with random intervals. Based on 23 calls from two different individuals, the advertisement call of *P. tocantins* (Fig. 1D; Table 1)

has an average duration of 0.242 $\pm$ 0.02 s (0.202–0.271 s), a dominant frequency of 2336.5 $\pm$ 237.3 Hz (2062.5–2625 Hz), and is composed of 24.6 $\pm$ 1.7 pulses (23–28 pulses), emitted with random intervals. Average parameters of the calls for each individual recorded are provided in Online Appendix 1.

The two principal components of PCA consecutively accounted for 48% and 30% of total variation (78% cumulative). The first component was loaded positively by all variables we analysed (note duration: 0.728; pulses per note: 1.567; pulse rate: 1.327; dominant frequency: 0.685), while the second component was loaded positively by note duration and pulses per note (1.379 and 0.356, respectively) and negatively by pulse rate and dominant



**Fig. 3.** Tadpole of *Pseudis fusca* at Gosner at stage 40: (A) lateral, (B) dorsal, and (C) ventral views (scale=10mm). Tadpole of *Pseudis tocantins* at Gosner stage 36: (E) lateral, (F) dorsal, and (G) ventral views (scale=10mm).

frequency (-0.752 and -0.824, respectively). Plotting variable scores on components 1 and 2 (Fig. 2) showed that there is no clear separation between the advertisement calls of *Pseudis* species, with *P. bolbodactyla*, *P. cardosoi*, *P. fusca* and *P. paradoxa* being within a single cluster (Fig. 2). *Pseudis tocantins* shows positive values in both PC1 and PC2 (e.g., high number of pulses/note) and *P. minuta* shows negative values in PC2 (e.g., high dominant frequency).

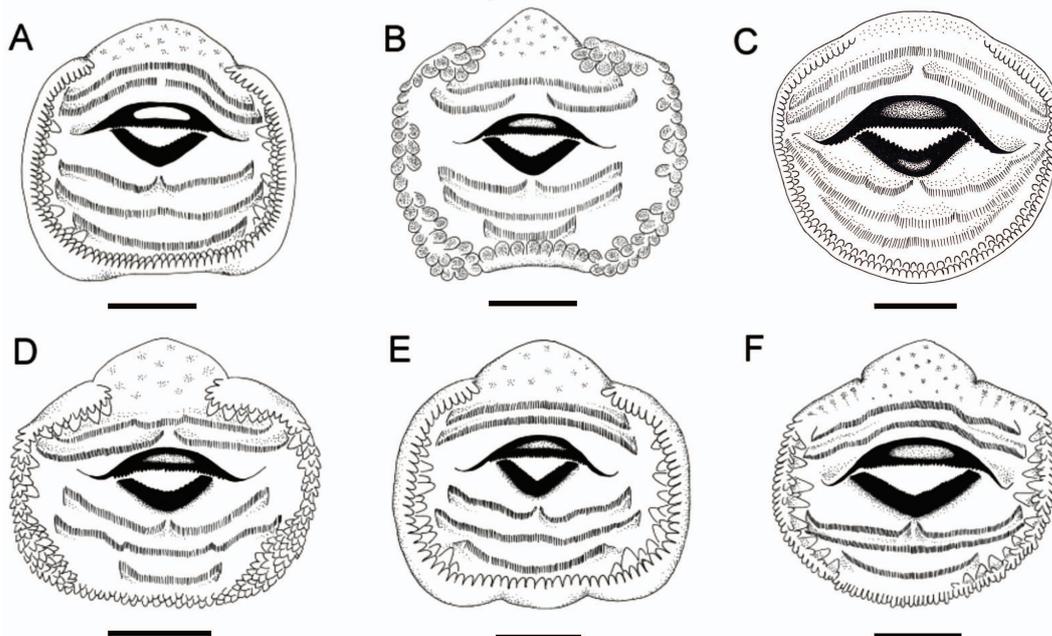
#### Tadpoles

Tadpoles of *P. fusca* and *P. tocantins* resemble other *Pseudis* tadpoles described so far (Table 3). The tadpoles of all species share an oval-shaped body in dorsal view and triangular in lateral view, a body which is higher than wide, tail musculature with strongly marked myotomes (Fig. 3), eyes arranged laterally, sinistral spiracle, ventromedial

vent tube, an anteroventral oral disc with five tooth rows (two anterior and three posterior, Fig. 4). All species exhibit giant tadpoles in comparison with the adult size. The tadpoles can be distinguished mainly by oral disc characteristics, relative height of the tail musculature in comparison to the tail height, relative height of the body in comparison to the body length, spiracle position, tail shape and some colouration aspects (Table 3). Below we describe the tadpoles of *P. fusca* and *P. tocantins* in detail.

#### *Pseudis fusca*

External morphology (Fig. 3A–C): At stage 40, the body is overall oval in dorsal and ventral views, triangular in lateral view, laterally compressed, with a small constriction on its middle third and represents about 31% of total length (Table 2). Snout is rounded in dorsal and ventral views, and slightly truncated in lateral view. Eyes are small



**Fig. 4.** Tadpole oral discs of *Pseudis* species. (A) *P. bolbodactyla* from Flores de Goiás, GO; (B) *P. cardosoi* from Bom Jesus, RS; (C) *P. fusca* from Araçuaí, MG; (D) *P. minuta* from Sapucaia do Sul, RS; (E) *P. paradoxa* from Ribeirão Preto, SP; (F) *P. tocantins* from Britânia Goiás, GO (scale=2mm).

**Table 3.** External morphological characteristics of *Pseudis* tadpoles described in the literature and in the present paper. TL: total length (in millimeters); LTRF: labial tooth row formula; ODF: oral disc folds; MP: marginal papillae; PR: P3/P2 ratio; BBR: BH/BL ratio; TMR: TMH/MTH ratio; BBR: BH/BL ratio; DC: dorsal crest; SP: spiracle position

Taxa	Stage	TL	LTRF	ODF	MP	PR	TMR	BBR	Viscera	DC	SP
<i>P. bolbodactyla</i> <sup>a</sup>	39	129.6	2(2)/3(1)	absent	simple	2	–	82%	not visible*	present	below body's middle line
<i>P. bolbodactyla</i> <sup>a</sup>	26–31	46.7–108.1	2(2)/3(1)	absent	simple or double	2	37%	86%	visible	present	below body's middle line
<i>P. bolbodactyla</i> <sup>a</sup>	26	82.6	2(2)/3(1)	absent	simple and laterally irregular	2	33%	88%	visible	present	below body's middle line
<i>P. cardosoi</i> <sup>f</sup>	26–40	62.7–87.2	2(2)/3(1)	ventral or absent	simple or double	1 or 2	40%	71%	visible	absent	at body's middle line
<i>P. cardosoi</i> <sup>f</sup>	34–37	79–87	2(2)/3(1)	absent*	multiple	1*	–	–	visible	absent	at body's middle line
<i>P. fusca</i> <sup>g</sup>	36–40	115.8–151.5	2(1,2)/3(1) or 2(2)/3(1)	absent	simple anteriorly and double posteriorly	2	55%	70%	not visible	absent	at body's middle line
<i>P. minuta</i> <sup>g</sup>	36–39	66.8–78.3	2(2)/3(1), 2(1,2)/3(1), 2(2)/3 or 2/3(1)	ventral or absent	multiple (two to four)	1	32%	80%	visible	absent	below body's middle line
<i>P. minuta</i> <sup>c</sup>	–	55–58	2(2)/3(1)	absent	multiple (two to four)	2*	–	–	–	absent	below body's middle line*
<i>P. minuta</i> <sup>o</sup>	36	–	2(2)/3	absent*	double	1	–	–	not visible*	absent	at body's middle line*
<i>P. minuta</i> <sup>e</sup>	31–35	35.7–45.5	2(2)/3(1)	absent	multiple (two to four)	2*	35%	–	visible	absent	below body's middle line
<i>P. paradoxa</i> <sup>a</sup>	26–31	93.2–125.4	2/3(1)	absent	simple	2	23%	97%	visible	present	at body's middle line
<i>P. paradoxa</i> <sup>a</sup>	36	120.7	2(2)/3(1)	ventral	simple	2	30%	87%	not visible	present	at body's middle line
<i>P. paradoxa</i> <sup>a</sup>	40	153.3–184.6	2(2)/3(1)	ventral	simple	2	31%	95%	not visible	present	below body's middle line
<i>P. paradoxa</i> <sup>a</sup>	39–40	114.8–142.9	2/3(1), 2(2)/3(1) or 2/3(1,2)	absent	simple	2	49%	83%	visible	present	below body's middle line
<i>P. paradoxa</i> <sup>f</sup>	37	169.1–208.2	2(2)/3(1)	ventral	double	2	–	–	not visible	present	at body's middle line
<i>P. paradoxa</i> <sup>s</sup>	21–38	45.7–135	2/3 or 2(2)/3*	absent*	simple	2*	–	–	visible*	present	at body's middle line*
<i>P. paradoxa</i> <sup>o</sup>	36	–	2/3(1)	absent*	double*	2*	–	–	not visible*	present	at body's middle line*
<i>P. paradoxa</i> <sup>h</sup>	36	168	2/3(1)	absent	double	–	–	–	not visible*	present	–
<i>P. paradoxa</i> <sup>i</sup>	39	270	2/3(1)	absent*	simple	2	–	87%	not visible	present	at body's middle line*
<i>P. tocantins</i> <sup>g</sup>	27–36	68.5–117.6	2/3(1), 2(2)/3(1) or 2(1,2)/3(1)	absent	simple and double laterally	2	31%	70%	visible	absent	at body's middle line

<sup>a</sup>Caramaschi & Cruz (1998); <sup>b</sup>Kwet (2000); <sup>c</sup>Fernández (1921); <sup>d</sup>Cei (1980); <sup>e</sup>de Sá (1997); <sup>f</sup>Rossa-Feres & Nomura (2006); <sup>g</sup>Dixon (1995); <sup>h</sup>Vizotto (1967); <sup>i</sup>Bokermann (1967); <sup>o</sup>present work. \*Characters based on tadpole illustrations or figure provided on the description.

and positioned laterally. Nares opening are small, oval, directed anteriorly and located closer to the snout than to the eyes and without projections on the internal rim. A short and sinisterly spiracle is located at about midway of the body length, at the body's middle line with the internal wall fused to the body; spiracle opening is rounded and posteriorly oriented. Vent tube is long and medial with anterior walls attached to the ventral fin; its opening is rounded and directed posteriorly. Dorsal fin is slightly arched and emerges at the body terminus in a low slope; ventral fin originates at the posterior ventral terminus of the body and gradually curves toward the tail tip; dorsal and ventral fins of about equal heights and are lower than the body and tail musculature heights. Tail tip end is triangular and acuminate. Tail musculature with strongly marked myotomes and its width corresponds to 71% of the body width. Lateral line system is not visible. Oral disc (Fig. 4C) is anteroventral, not emarginated and represents 30% of the maximum width of the body. Marginal papillae arranged in a single row laterally and anteriorly, and in two rows posteriorly, with a wide rostral gap. Papillae are conical with rounded tips. Submarginal papillae are present on each side of the disc commissure. LTRF: 2(2)/3(1); anterior tooth rows are similar in length, whereas P3 is slightly shorter than P1 and P2. Jaw sheaths are pigmented and have a serrated edge; upper jaw sheath is arc-shaped and lower jaw sheath is U-shaped.

Morphological variation: The LTRF of one tadpole at stage 35 is 2(1,2)/3(1). Two tadpoles at stage 40 exhibited marginal papillae arranged in a single row around the entire oral disc.

Colouration: In 10% formalin, the body varies from a light brown to a pale grey colouration on the dorsal region and is light grey on the ventral portion. Black dots are scattered along the dorsal and lateral region of the body, on the posterior third of body ventral surface, and on the first third of the tail musculature. The tail musculature follows the same pattern as the dorsal surface of the body. Viscera are not visible. Fins are slightly translucent and pigmented with light grey colouration. Spiracle margin is not pigmented.

#### ***Pseudis tocantins***

External morphology (Fig. 3D–F): At stage 36, the body is overall oval in dorsal and ventral views, slightly triangular in lateral view, laterally compressed, with a small constriction on its middle third and represents about 32% of the total length (Table 2). Snout is rounded in dorsal and ventral views, and slightly truncated in lateral view. Eyes are relatively small and positioned laterally. Nare openings are small, oval, directed anteriorly and located closer to snout than to the eyes and without projections on the internal rim. A short and sinistrally spiracle is located about midway of the body length, at body's middle line with the internal wall fused to body; spiracle opening is rounded and posteriorly oriented. Vent tube is long and medial with anterior walls attached to the ventral fin; its opening is rounded and directed posteriorly. Dorsal fin is convex and emerges at the body terminus in a high slope; ventral fin originates at the posterior ventral terminus of the body and gradually curves towards the tail tip;

dorsal and ventral fins of about equal heights and lower than the body. Tail tip end is triangular and acuminate. Tail musculature with strongly marked myotomes and its width corresponds to 45% of the body width. Lateral line system is not visible. Oral disc (Fig. 4F) is anteroventral, not emarginated and represents 31% of the maximum width of the body. Marginal papillae arranged in a single row anteriorly and posteriorly, and in two rows laterally; papillae are conical with rounded tips. Submarginal papillae are present laterally on each side of the disc commissure; their tips are triangular and some are larger than the marginal papillae. LTRF: 2/3(1); anterior tooth rows are similar in length, whereas P3 is slightly shorter than P1 and P2. Jaw sheaths are pigmented and have a serrated edge; upper jaw sheath arc-shaped and lower jaw sheath V-shaped.

Morphological variation: One tadpole at stage 36 exhibited the LTRF 2(1,2)/3(1) and two tadpoles at stages 27 and 34 exhibited the LTRF 2(2)/3(1). In two tadpoles at stage 35, the marginal papillae are arranged in three rows on the disc commissure.

Colouration: In 10% formalin, the dorsal and ventral body surfaces are mostly translucent with a pale grey pigmentation. Dark dots are homogeneously scattered along the body, tail musculature and fins, except for the ventral region of the body. Viscera are visible. Both fins are light grey pigmented and slightly translucent. Spiracle margin is not pigmented.

## DISCUSSION

#### **Vocalisations**

Advertisement calls are similar among the three lineages of *Pseudis*. A general structure containing pulsed notes with or without concatenated pulses is seen in the sister genus *Lysapsus* (Santana et al., 2013), in both clades of *Pseudis* (*P. minuta* + *P. cardosoi*, and the remainder of *Pseudis* species). The similarity is most striking among *P. bolbodactyla*, *P. fusca* and *P. paradoxa*, which are indistinguishable based on acoustic parameters (see Table 1 and Fig. 2). Because these three species are morphologically different and do not form a monophyletic clade (Garda & Cannatella, 2007; Garda et al., 2010), the similarity of their calls is unexpected. Mate recognition signal divergence is complex, and genetic drift, indirect selection or direct selection via sexual or environmental selection may contribute to the evolution of advertisement calls (Ryan et al., 1990). Conversely, acoustic signal evolution can be constrained by physical features of the habitat, community composition, ambient noise, phylogenetic history and sender and receiver morphology and neurophysiology (Wilkins et al., 2013). *Pseudis* species show a remarkable similarity in habitat and microhabitat used when calling: all occur in ponds associated with medium to large river floodplains east of the Andes (Garda et al., 2010) and call with most of the body underwater (Brandão et al., 2003). Furthermore, most hydrographic basins harbour only one species of the genus (Gallardo, 1961), precluding secondary contacts that would reinforce the effects of acoustic divergence. The most divergent of the advertisement calls in all

*Pseudis* species is that of *P. tocantins*, which shares the Tocantins river with *P. bolbodactyla* (Garda et al., 2010).

Our comparisons of the advertisement calls of all species endorse the existence of three lineages within Pseudae previously identified through sperm ultrastructure (Garda et al., 2004), cytogenetics (Suarez et al., 2013), morphology (Garda et al., 2010) and DNA (Aguiar et al., 2007; Garda & Cannatella, 2007). The advertisement calls of *P. cardosoi* and *P. minuta* are unique by having all pulses concatenated (Fig. 1), while all other species have groups of concatenated pulses in the same note. *Lysapsus*, conversely, shows a second type of advertisement call (Call B) unique to the genus (Santana et al., 2013), and the remaining *Pseudis* (*P. fusca*+*P. tocantins*, *P. bolbodactyla*+*P. paradoxa*) contain only the basic set of a call composed of pulses or sets of concatenated pulses also found in Call A of *Lysapsus*.

Some species of *Pseudis* can be readily distinguished by their advertisement calls. *Pseudis tocantins* has the highest number of pulses per call (23–28, Fig. 1. See other species in Table 1), in four or five groups of several concatenated pulses. The call of *P. minuta* has the highest dominant frequency in the genus, reaching 3445 Hz (Table 1). However, there is a large overlap in this parameter among most species, likely because of the intraspecific variation in size at maturity (Garda et al., 2010) resulting from the lack of postmetamorphic growth in the genus (Downie et al., 2009). Despite the low number of individuals, the PCA analyses corroborate these observations suggesting a segregation of *P. tocantins* (high number of pulses/note) and *P. minuta* (high dominant frequency) from the cluster formed by *P. bolbodactyla*, *P. cardosoi*, *P. fusca* and *P. paradoxa* (Fig. 2).

The large overlap among calling parameters in *P. bolbodactyla* and *P. paradoxa* coincides with a wide distributions of both species, the fact at least one of them has had subspecies recognised in the past (*P. paradoxa*, Gallardo, 1961), and the fact that they are morphologically difficult to separate (Garda et al., 2010). We also compared the advertisement calls of several populations of *P. paradoxa* and *P. bolbodactyla* (Online Appendix 1), and found no clear differences in call parameters. However, genetic data (D.J. Santana and A.A. Garda, unpublished) and the similarity of the calls of *P. bolbodactyla*, *P. fusca* and *P. paradoxa* suggest that these taxa might correspond to more than one species.

### Tadpoles

Except for the clade formed by *P. minuta* and *P. cardosoi*, whose adult males are readily distinguished by paired vocal sacs (Garda et al., 2010), no other adult morphological trait supports the phylogenetic groups of *Pseudis* recovered in Aguiar et al. (2007) and Garda & Cannatella (2007). Conversely, tadpole morphology provides relevant information that supports such groupings. For instance, tadpoles of *P. minuta* and *P. cardosoi* share some exclusive features: P3 is usually reduced and its length is smaller or equal to 50% of P2 length (on other species tadpoles P3 is smaller but its length represents more than 50% of P2 length) and the body is entirely black pigmented

in living specimens (other species do not exhibit such dark colouration, instead they exhibit a greyish or dark brown pigmentation). Such characteristics corroborate the molecular relationships of *P. cardosoi* and *P. minuta* as sister taxa (Aguiar et al., 2007; Garda & Cannatella, 2007). Although they share these characteristics, the body and tail shape distinguishes the tadpole of *P. cardosoi* from that of *P. minuta*. Kwet (2000) further mentions that *P. cardosoi* exhibits a darker uniform colouration without light stripes or blotches along the body and tail (light stripes present on *P. minuta*), and characterises the marginal papillae of *P. cardosoi* as arranged in “multiple rows”. However, we did not observe multiple rows in any tadpole analysed and therefore consider that the row of marginal papillae is a good diagnostic characteristic that distinguishes these closely related species.

The larvae of *P. paradoxa* and *P. bolbodactyla* also share characteristics that support their sister taxa relationship: both exhibit dorsal crests parallel to dorsal fins, and in general the body is almost as high as wide (values of BH/BL ratio are higher than 80%, Table 3). We did not observe characteristics that unequivocally separate the tadpoles of *P. bolbodactyla* from *P. paradoxa*, as most of their external morphological features overlap (Table 3). Caramaschi & Cruz (1998) mentioned that *P. bolbodactyla* tadpoles are smaller in size in comparison to *P. paradoxa* tadpoles. Tadpoles of *Pseudis* can vary in size due to ecological conditions even in the same developmental stage (Downie et al., 2009; Garda et al., 2010). Hence, we believe that size should not be used as a diagnostic characteristic to distinguish these species tadpoles.

Despite the close relation of *P. fusca* and *P. tocantins* as sister taxa (Garda & Cannatella, 2007; Aguiar et al., 2007), their tadpoles share no exclusive characteristics, but each present unique features that differentiate from each other and from all other tadpoles of *Pseudis* species. For instance, the tadpole of *P. tocantins* differs from tadpoles of other species by the following combination of characters: BH/BL ratio around 70% (BH/BL ratio higher than 80% in *P. paradoxa* and *P. bolbodactyla*), marginal papillae conical, taller than wide, arranged at least in two rows and P3/P2 ratio always more than 50% (marginal papillae rounded and wider than tall, and P3/P2 ratio equal or less than 50% in *P. cardosoi*; numerous marginal papillae and always arranged in more than two rows and P3/P2 ratio equal or less than 50% in *P. minuta*), meanwhile a higher TMH/MTH ratio (over 50%) with reduced dorsal fin easily distinguishes *P. fusca* tadpoles from all other tadpoles of *Pseudis* species. Other comparisons between *Pseudis* tadpoles are available in Table 3.

Even though larval characters provide relevant information for taxonomic researches in several anuran groups (Candioti, 2007; Cruz, 1982; Rossa-Feres & Nomura, 2006), the oral formula and the marginal papillae arrangement of most *Pseudis* species overlap and exhibit high inter- and intra-specific variation (Table 3), except for the multiple row arrangement of the marginal papillae in *P. minuta*. A similar variation was also observed in tadpoles of *Lysapsus*, for which the documented variation in the oral formula might be caused by injuries in the oral apparatus (Santana et al., 2013). We accounted for different oral

formulas in specimens with the same or similar stage of development (for example, *P. tocantins* and *P. minuta* presented from three to four different oral formulas, Table 3), but adequate research on the ontogenetic changes of the oral apparatus during the larval development of *Pseudis* species is still needed to confirm our observations. Moreover, such variation and overlap on the oral apparatus characteristics of *Pseudis* larvae hampers the use these features for taxonomic comparisons.

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