



Advertisement and release calls in Neotropical toads of the *Rhinella granulosa* group and evidence of natural hybridization between *R. bergi* and *R. major* (Anura: Bufonidae)

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Abstract

The *Rhinella granulosa* group currently includes 12 species distributed eastern to the Andes, from Panama to central Argentina. We studied bioacoustic features of the advertisement calls in seven of these species: *Rhinella azarai*, *R. bergi*, *R. centralis*, *R. dorbignyi*, *R. fernandezae*, *R. major*, and *R. merianae*. In addition, we analyzed the release calls of *R. azarai*, *R. bergi*, *R. dorbignyi*, and *R. fernandezae*. The advertisement calls consisted of long trills, composed by notes with a variable pulse number (2–8) that was characteristic of each species. The release calls consisted of a single note, pulsed or not. Both advertisement and release calls clearly varied between species, except for *R. dorbignyi* and *R. fernandezae*. The study of specimens sharing exosomatic characters with *R. bergi* and *R. major* from a syntopy area, which presented intermediate spectral and temporal call parameters, confirmed natural hybridization between these two species.

Key words: anuran vocalization, mating call, hybrids, South America

Introduction

Vocalization is a significant means of communication in anuran amphibians, mainly because of the relevance of the acoustic signals emitted by reproductive males to attract conspecific females (Blair 1958; Bogert 1960; Blair 1958; Wells 2007). These signals, known as advertisement calls, are usually species-specific therefore being one of the main premating isolating mechanisms. For this reason, advertisement calls have long been used in taxonomic studies on many anuran groups (Blair 1941; 1972). Another relevant acoustic signal related to anuran reproduction is the release call. This particular call is emitted by non-reproductive females or males, when attempted to mate by another male, either conspecific or not (Aronson 1944; Wells 2007). Like advertisement calls, release calls may vary between species and would aid in distinguishing between closely related taxa (Brown & Littlejohn 1972; Sullivan 1989).

Vocalizations in toads of the family Bufonidae were extensively studied mainly in the Holarctic Region (e.g. Brown & Littlejohn 1972; Martin 1972; Castellano *et al.* 2002); and to a lesser extent in the Neotropical Region (e.g. Di Tada *et al.* 2001; Alonso & Rodríguez 2003). A group of small toads scarcely studied to this respect are those included in the *Rhinella granulosa* group. Twelve taxa are currently recognized in this group, which is distributed in open habitats eastern to the Andes, from Panama to central Argentina (Narvaes & Trefaut Rodrigues 2009): *R. azarai* (Gallardo), *R. bergi* (Céspedes), *R. centralis* (Narvaes & Trefaut Rodrigues), *R. dorbignyi* (Duméril & Bibron), *R. fernandezae* (Gallardo), *R. granulosa* (Spix), *R. humboldti* (Gallardo), *R. major* (Müller &

Hellmich), *R. merianae* (Gallardo), *R. mirandaribeiroi* (Gallardo), *R. nattereri* (Bokermann), and *R. pygmaea* (Myers & Carvalho). These species have mainly allopatric distributions, but some syntopy areas have been reported (see Narvaes & Trefaut Rodrigues 2009 for review). Additionally, some authors have proposed the putative occurrence of hybridization between *R. dorbignyi* and *R. fernandezae* based on external morphology characters (Gallardo 1969).

In this work we compare the advertisement calls obtained for most of the currently recognized species, including first time descriptions for *Rhinella azarai*, *R. bergi*, *R. dorbignyi*, and *R. merianae*. The release calls described herein are those of *R. azarai*, *R. bergi*, *R. dorbignyi*, and *R. fernandezae*. Besides, we review the available information about vocalizations in the *R. granulosa* group, and study the taxonomic significance of both the advertisement and release calls of these toads. In addition, acoustic evidence of natural hybridization between two species in this group is provided for the first time.

Material and methods

Advertisement calls were recorded by the authors in the field, at seventeen localities of Argentina and Uruguay. We also studied the calls of specimens from Bolivia, Brazil, Uruguay and Panama, obtained from the Fonoteca Zoológica (Departamento de Biodiversidad y Biología Evolutiva, Museo Nacional de Ciencias Naturales de Madrid; <http://www.fonozoo.com/>; accessed 10 June 2010) and the CDs of the works of Ibáñez *et al.* (1999) and De la Riva *et al.* (2002); (see Appendix 1 and Figures 1–2). The whole sample consisted of 152 advertisement calls of 36 specimens belonging to the species *Rhinella azarai*, *R. bergi*, *R. centralis*, *R. dorbignyi*, *R. fernandezae*, *R. major*, and *R. merianae*. We recorded 1–7 advertisement calls per specimen. Besides, we recorded 13 advertisement calls from three specimens with exosomatic characters shared with both *R. bergi* and *R. major*, collected in a syntopy area at Vera, Santa Fe, Argentina. Field-work allowed the study of 366 male release calls of 22 specimens of *R. azarai*, *R. bergi*, *R. dorbignyi*, and *R. fernandezae*. We recorded 10–32 release calls per specimen. Those specimens collected for the present study were deposited at Colección Diego Baldo, housed at Museo de La Plata (MLP DB), Argentina, and Museo Nacional de Historia Natural (MNHN), Montevideo, Uruguay.

Advertisement calls obtained by the authors were recorded in the field from specimens breeding in temporary ponds. Release calls were recorded by gently pressing on the sides of males, while held between thumb and forefinger, in the field or in the laboratory until the toads stopped responding acoustically to the mechanical stimulation. Water and air temperature ($\pm 1^\circ\text{C}$) in the field near each recorded male and environmental temperature in the laboratory were measured with a digital pocket thermometer. Also, the snout-vent length (SVL) of each specimen was measured, using a Vernier caliper (to the nearest 0.1 mm).

Calls were recorded with a Sony WM-D6C recorder and Sennheiser LR 66 microphone (Argentina), and a digital Roland Edirol R-09HR recorder with an Audiotechnica AT8035 microphone (Uruguay). Recordings were analyzed employing Sound Forge 9.0a software (Sony Creative Software Inc. 2007), with a FFT of 512 points, at a sampling rate of 44.1 kHz and 16-bit precision. The following temporal acoustic variables were measured from the waveform: call duration, notes/call, notes/s (= note rate), note duration, internote duration, pulses/note, pulse duration, and interpulse duration, as defined by Heyer *et al.* (1990), Duellman and Trueb (1994), and Littlejohn (2001). Additional measures were the second pulse duration in calls of *Rhinella azarai* and *R. bergi*, the second interpulse duration and third pulse duration in *R. azarai*. Mean power spectra of those selected calls were obtained with a FFT of 512 points, overlap 93%, Hamming's sampling window and sonogram resolution of 10000 samplings. The dominant frequency was obtained from sonograms. The oscillograms and sonograms were produced with Syrinx 2.6h software (Burt 2006). All individual measurements were averaged into a unique value for each specimen and descriptive statistics of all measured acoustic variables are provided.

In the case of *Rhinella dorbignyi* and *R. fernandezae* the amount of data allowed to test for an association between acoustic variables, and the specimens SVL and environmental temperature. First, normality and homoscedasticity in the data set were evaluated following Zar (1984): for each variable we applied the ANOVA saving the residuals and the absolute residuals; then the Shapiro-Wilks test was applied to the residuals of each variable to test the normal distribution and the ANOVA was applied to the absolute residuals (Levene test) of each variable to test the homoscedasticity. Almost all advertisement call variables departed significantly from a normal distribution. Then, we tested for an association between these acoustic parameters, and the SVL of specimens and environmen-

tal temperature using the Spearman Correlation (R statistic). The dominant frequency and notes/call did not departed significantly from normality and their associations with SVL and environmental temperature were tested with the Spearman Correlation Coefficient (R^2) in Simple Linear Regression models.

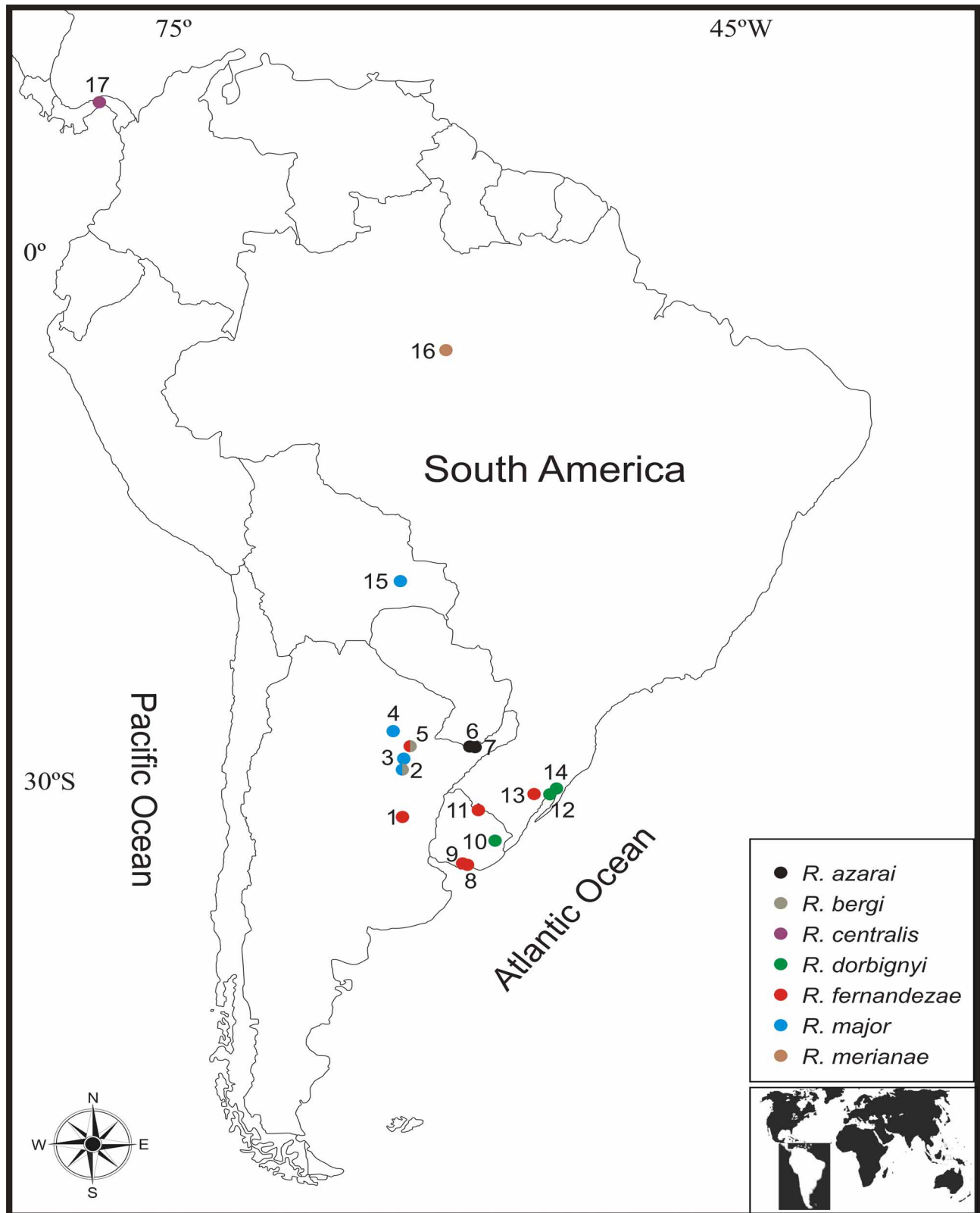


FIGURE 1. Geographic distribution of the populations of the *Rhinella granulosa* group studied in this work. The numbers indicate the localities sampled in Argentina and Uruguay. For details of the localities see Appendix 1.

Advertisement call variability was explored by means of Principal Component Analysis (PCA) in the whole data set of the standardized variables with both the corresponding environmental or water temperature, and the SVL of the specimens, also standardized. We used the water temperature when the environmental temperature was not available. When the SVL was not available we used the mean value of the corresponding species following Narvaes and Trefaut Rodrigues (2009). *Rhinella centralis* and some specimens of *R. major* were excluded from the PCA because the temperature data of the corresponding recordings were not available. We performed a PCA with a varimax rotation and extracted those factors with an eigenvalue greater than 1.

All routines were done using the Statistica 6.0 and Infostat/E software.

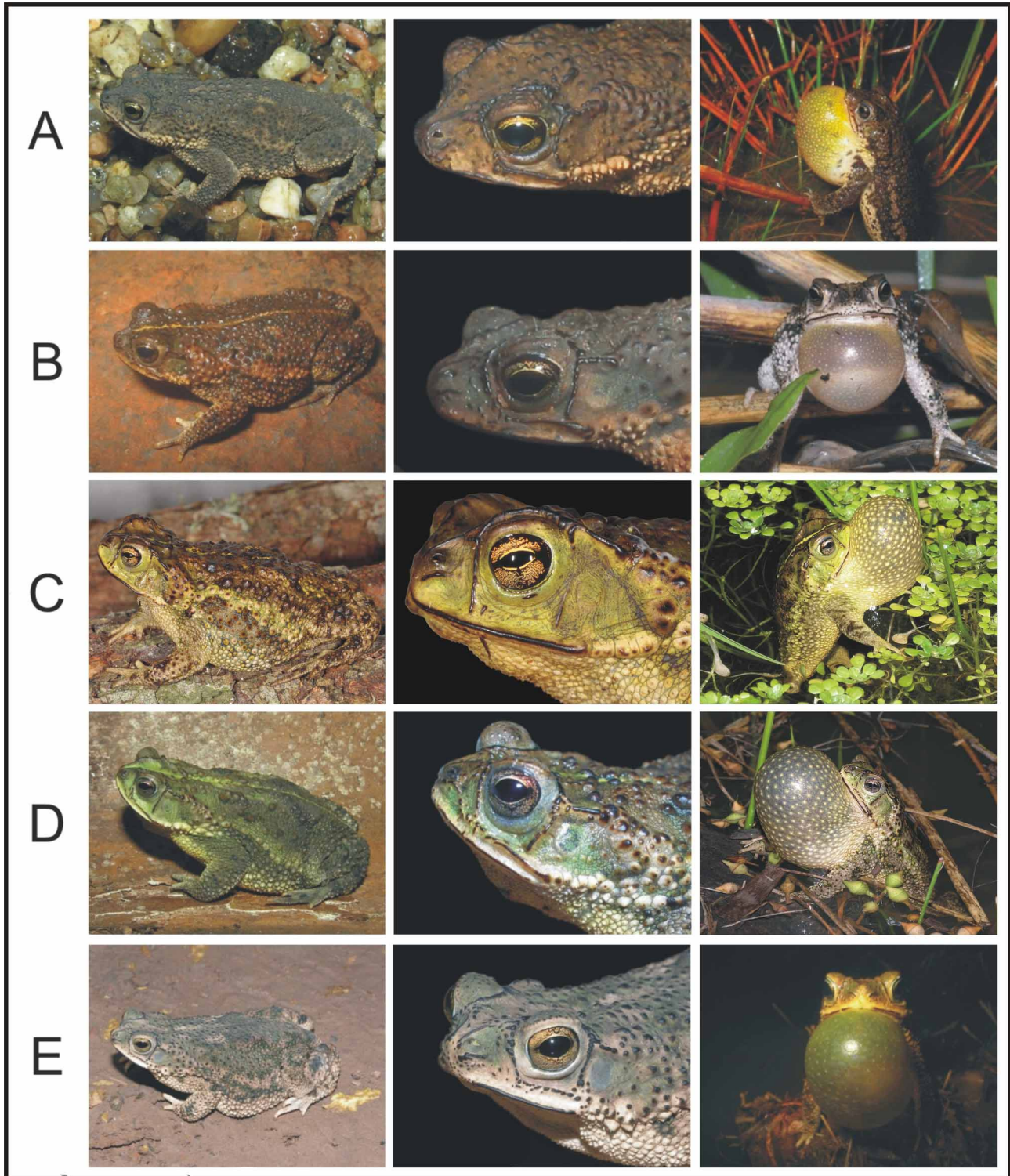


FIGURE 2. Adult specimens of (A) *Rhinella azarai*, (B) *R. bergi*, (C) *R. dorbignyi*, (D) *R. fernandezae*, and (E) *R. major*.

Results

Advertisement calls in the *Rhinella granulosa* group (Table 1, and Figures 3–5) consist of regular series of pulsed notes (trills) that last a few seconds, usually between 5 s and 20 s (2–36). The number of notes/call greatly varied within and between species, for instance we recorded 42–326 in *R. bergi* and 118–499 in *R. fernandezae*. The variation in dominant frequency was narrower between species, and ranged from 1800 to 4000 Hz in the whole sample. Shorter advertisement calls of about 5 s were observed for *Rhinella major*, *R. centralis*, and *R. merianae*, intermediate calls of approximately 8 s were those of *R. fernandezae* and *R. dorbignyi*, and *R. bergi* and *R. azarai* were the species with relatively longer calls of approximately 14 s and 19 s respectively. As expected, the dominant frequency was higher in *R. bergi* (3643–3966 Hz), the smallest species in the sample; it almost not overlapped with that of the rest of the species, in which usually fluctuated between 1800 and 3000 Hz. The mean number of notes/call varied from 85.4 ± 22.6 in *R. major* to 318.3 ± 66.1 in *R. fernandezae*. Note duration was around 20 ms in *R. merianae*, *R. fernandezae*, *R. centralis*, and *R. dorbignyi*, but relatively longer in *R. major* (45 ms), *R. azarai* (53 ms), and *R. bergi* (62 ms).

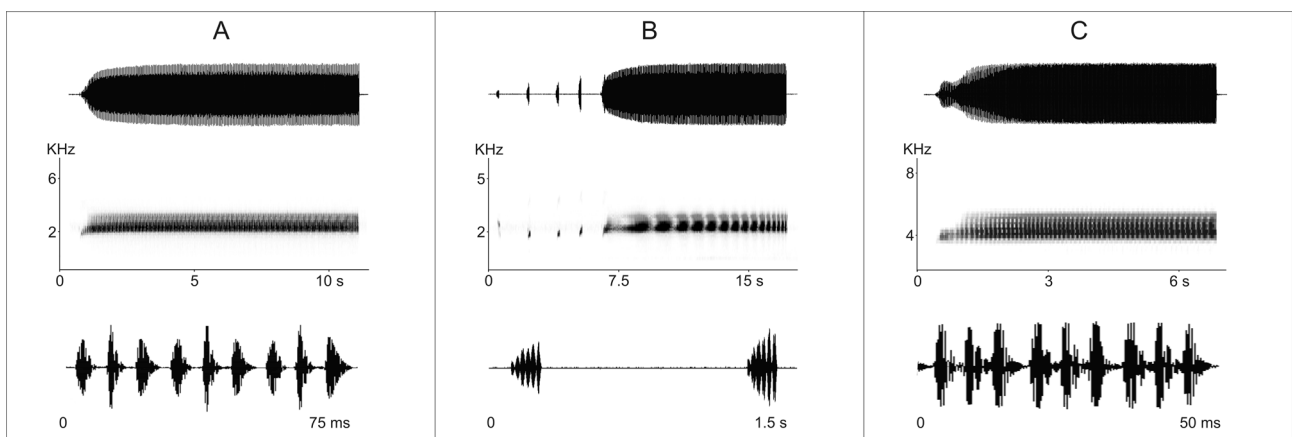


FIGURE 3. Advertisement calls of *Rhinella dorbignyi* (A and B), and *R. fernandezae* (C). Oscillogram (top) and sonogram (center) of a single call, and oscillogram with some few notes (bottom of A and C), and short trills (bottom of B).

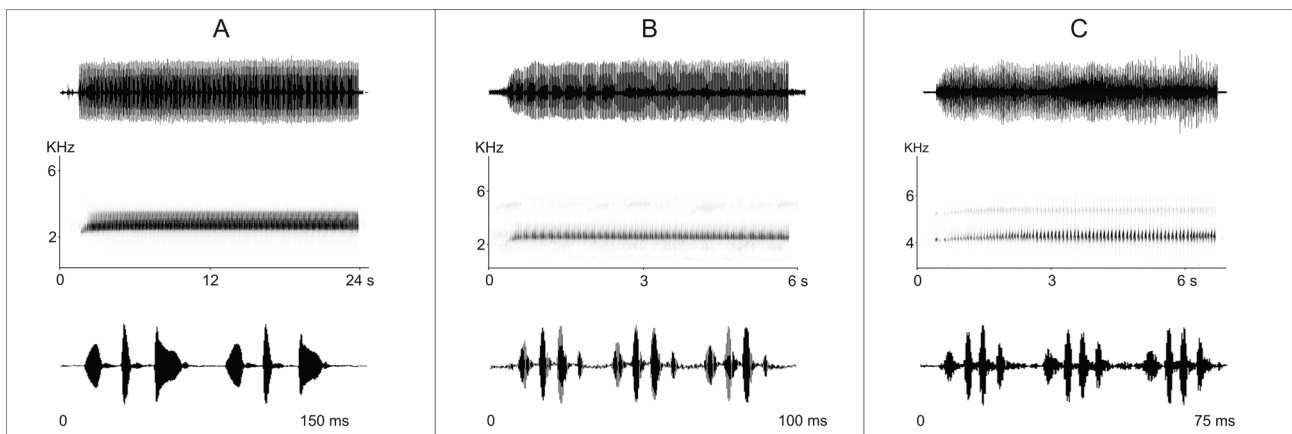


FIGURE 4. Advertisement calls of *Rhinella azarai* (A), *R. centralis* (B), and *R. merianae*. Oscillogram (top) and sonogram (center) of a single call, and oscillogram with some few notes (bottom).

The number of pulses/note noticeably differed among taxa: 2 in *Rhinella bergi*, 3 in *R. azarai*, *R. dorbignyi*, and *R. fernandezae*, 4 in *R. merianae* and *R. centralis*, and 6–8 in *R. major*. One specimen of *R. azarai* presented 4 pulses in the first notes of the call, but 3 in the rest. In most specimens of *R. fernandezae* (9/12), the first notes exhibited 2 pulses and 3 in the rest. Pulse duration in *R. merianae*, *R. centralis*, *R. major*, *R. dorbignyi*, and *R. fernandezae* range between 3–5 ms, somewhat shorter than in *R. azarai* and *R. bergi*, 12–15 ms. Pulse duration also varied within species: the second pulse in *R. bergi* is longer than the first; the third pulse of the advertisement call

in *R. azarai* (15 ± 3 ms; 7–22 ms) is longer than the others; and the fourth pulse in *R. centralis* is shorter than the others. The first interpulse interval duration of the advertisement call in *R. azarai* is shorter than the second (10 ± 3 ms; 6–17 ms). Curiously, 8% of the advertisement calls emitted by specimens of *R. dorbignyi* were preceded by a series of 2–6 short trills (Figure 3B) that have 74–726 ms of duration and a dominant frequency (1496–2357 Hz) usually lower than the corresponding advertisement call. The relevance and function of these short trills are unknown.

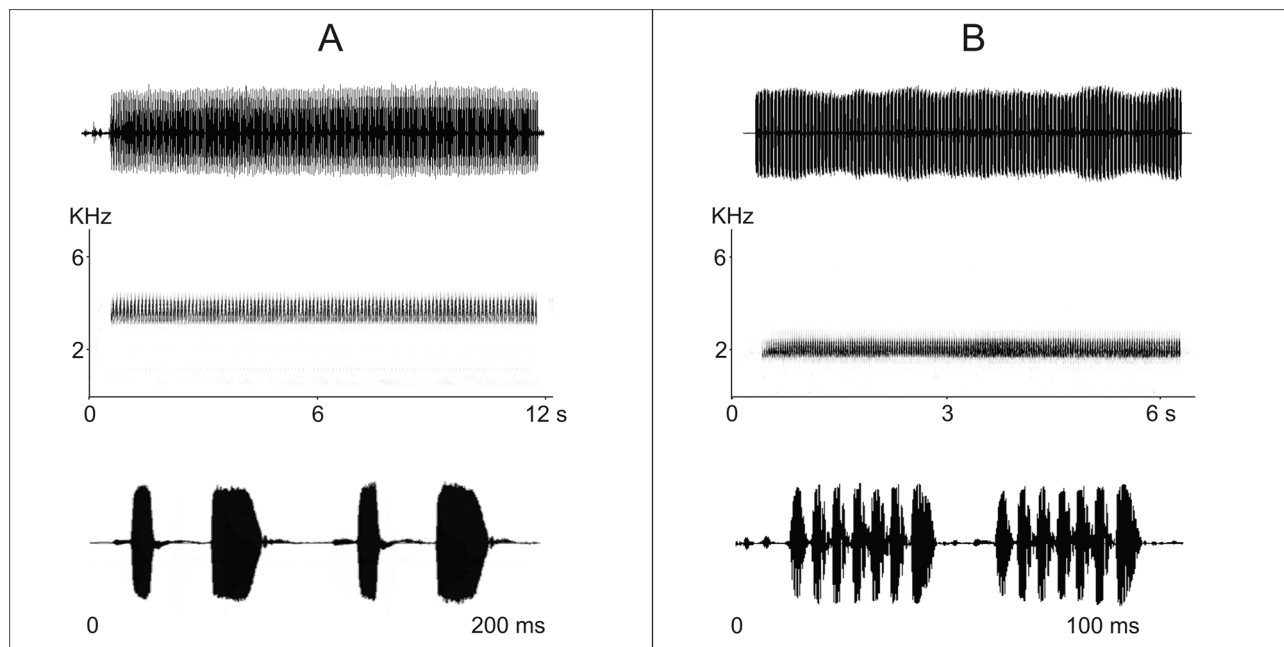


FIGURE 5. Advertisement calls of *Rhinella bergi* (A), and *R. major* (B). Oscillogram (top) and sonogram (center) of a single call, and oscillogram of two notes (bottom).

There are several advertisement call variables that were affected by SVL in the available data of *Rhinella dorbignyi*: internote duration ($R = -0.6$; $p < 0.000$), interpulse duration ($R = 0.4$; $p = 0.004$); *R. fernandezae*: call duration ($R = -0.3$; $p = 0.02$), notes/s ($R = 0.3$; $p = 0.03$), note duration ($R = -0.4$; $p = 0.002$), pulse duration ($R = -0.6$; $p < 0.000$); and *R. dorbignyi* and *R. fernandezae*: dominant frequency ($R^2 = -0.6$; $p < 0.000$). Also, there are several advertisement call variables that were affected by environmental temperature in *R. dorbignyi*: notes/s ($R = 0.4$; $p = 0.002$), note duration ($R = -0.3$; $p = 0.02$), internote duration ($R = -0.6$; $p < 0.000$); and in *R. fernandezae*: notes/s ($R = 0.3$; $p = 0.03$), pulse duration ($R = -0.3$; $p = 0.02$). However, we considered that all this variation did not hamper species comparison because variables with main interspecific differences seem not to be greatly affected within our SVL and temperature ranges.

On February 3, 2004 we found several males of *Rhinella bergi* and *R. major* calling together in syntopy at Vera, Santa Fe, Argentina. Moreover, we identified irregular advertisement calls emitted by some specimens that were diagnosed as putative hybrids between these species (Figure 6). The study of exosomatic characters of these specimens (MLP DB 2674, 2726, 2734) revealed the presence of the following features that are shared with *R. bergi*: (1) small size (mean SVL = 40.48 mm); (2) cephalic crests predominantly continuous; (3) lateroposterior expansion of infraorbital crest visible in dorsal view; (4) longitudinal dorsal stripe; (5) parietal crest present. In addition, the following characters are shared with *R. major*: (1) infraorbital crest short; (2) supratympanic crest short; (3) belly not pigmented (see diagnostic characters of both *R. bergi* and *R. major* in Narvaes & Trefaut Rodrigues 2009).

The advertisement calls of these specimens were more irregular than those recorded for specimens unequivocally diagnosed as belonging to *Rhinella bergi* and *R. major* (see Table 2, Figure 7). The acoustic parameters were in general intermediate between those of the parental species, except for the number of pulses/note (between 4 and 8) that more closely resembles the call of *R. major* and dominant frequency that differ from parental taxa.

TABLE 1. Advertisement call parameters in species of the *Rhinella granulosa* group obtained for the present work and from literature: means±SD (range). CD = call duration; DF = dominant frequency; ND = note duration; IND = internotes duration; PD = pulse duration; IPD = interpulse duration; PD2 = second pulse duration; IPD2 = second interpulse duration; PD3 = third pulse duration. ^A from Zweifel (1965); ^B from Salas *et al.* (1998); ^C from Zimmerman (1983); ^D from Köhler *et al.* (1997); dashes indicate no data available.

Species (SVL) °C, males, calls	CD (sec)	DF (Hz)	Notes / Call	Notes / s	ND (ms)	IND (ms)	Pulses / Note	PD (ms)	IPD (ms)	PD2 (ms)	IPD2 (ms)	PD3 (ms)
<i>R. azarai</i> (41.6 mm) 16–21°C, 2, 9	19.3±1.1 (18.6–20.1)	2499.3±36.9 (2473.2–2525.4)	264.4±51.4 (228–300.7)	13.7±3.4 (11.3–16.2)	51±15 (40–62)	24±3.7 (21–26)	3	11±3.7 (9–14)	7±2.6 (5–9)	8±1.9 (7–9)	9±2.8 (7–11)	15±4.5 (12–18)
<i>R. bergi</i> (35.2–36.3 mm) 21–23°C, 3, 12	15±5.6 (9.9–21)	3828.8±151.9 (3653.5–3923)	144.2±47 (101–194.2)	9.7±0.5 (9.2–10.2)	62±2.4 (61–65)	40±3.4 (36–43)	2	13±2.9 (11–17)	27±7.5 (18–33)	23±2 (21–25)	–	–
<i>R. centralis</i> (–) 24°C, 2, 17 ^A	4.3 (3.5–5.3)	(2200–3300)	–	32.7	–	–	–	–	–	–	–	–
(–) –, 1, 2	5.2±0.3 (4.9–5.5)	2541.4±44.5 (2500–2587)	171.5±11.2 (160–182)	33±0.2 (32.9–33.2)	21±0.7 (20–23)	9±0.8 (7–10)	4	4±0.7 (2–5)	2±0.5 (1–3)	–	–	–
<i>R. dorbignyi</i> (44.6–55 mm) 16–20.9°C, 13, 56	8.3±1.5 (6–10.8)	2128.1±104.9 (1952.8–2349)	291.7±58.1 (219.2–396.6)	35±2.9 (29.1–37.8)	23±1.4 (20–25)	5±2.2 (3–10)	3	5±0.4 (5–6)	3±0.6 (3–4)	–	–	–
<i>R. fernandezae</i> (–) 19–28°C, 2, 31 ^B	3.9±0.5	2400 (2100–3200)	–	–	–	–	–	–	–	–	–	–
(–) 16–23.5°C, 12, 49	8.1±2.8 (5.6–12.8)	2043.5±122.2 (1840.8–2338.5)	313.4±60.6 (183.7–386.4)	40.6±8.1 (29.5–56.5)	21±4.2 (15–27)	5±2.2 (2–8)	3	5±0.8 (4–6)	3±1.2 (2–5)	–	–	–
<i>R. humboldti</i> (25.4 mm) 22–25°C, 1, – ^C	–	2100	135	–	–	–	–	–	–	–	–	–
<i>R. major</i> (34.5 mm) 24.5°C, –, – ^D	5.3 (4.1–7.5)	2960 (2500–3900)	75–133	18.3	–	–	6	–	–	–	–	–
(49.2–55 mm) 20°C, 4, 21	5.1±1.6 (3.6–7.2)	2725.8±178.9 (2500.4–2936.7)	84.3±18.5 (61.4–106)	16.8±1.8 (14.3–18.6)	45±4.7 (42–52)	15±2.2 (13–18)	6.6±0.6 (6–7.3)	4±0.5 (4–5)	3±0.7 (2–3)	–	–	–
<i>R. merianae</i> (–) 25°C, 1, 3	5.4±1.5 (4–7.1)	2315±112 (2220–2436)	213±64.8 (149–283)	39.1±1.1 (37.2–39.9)	18±1.5 (16–22)	7±1.3 (4–9)	4	3±0.9 (2–6)	1±0.4 (1–2)	–	–	–

TABLE 2. Advertisement call parameters of the hybrid specimens between *Rhinella bergi* and *R. major*: mean±SD (range). CD = call duration; DF = dominant frequency; ND = note duration; IND = internotes duration; PD = pulse duration; IPD = interpulse duration.

Specimen (SVL; °C; calls)	CD (sec)	DF (Hz)	Notes / Call	Notes / s	ND (ms)
MLP DB 2674 (42.6 mm; 21°C; 5)	8.9±2.6 (4.8–12.2)	3001±28 (2953–3018)	105.6±32.1 (57–146)	11.8±0.2 (11.4–12)	49±2 (45–54)
MLP DB 2736 (38.5 mm; 21°C; 4)	5.4±2.1 (2.9–8)	3260±105 (3125–3362)	83.2±37.4 (50–136)	15.3±2.1 (13–17.1)	44±3 (40–51)
MLP DB 2734 (40.3 mm; 24.5°C; 4)	7.9±0.5 (7.3–8.4)	3239±21 (3212–3255)	139.5±9.7 (126–149)	17.7±0.3 (17.2–18)	38±1 (36–40)

continued.

Specimen (SVL; °C; calls)	IND (ms)	Pulses / Note	PD (ms)	IPD (ms)
MLP DB 2674 (42.6 mm; 21°C; 5)	36±6 (28–58)	5.9±0.8 (4–8)	5±4 (2–23)	2±3 (0–10)
MLP DB 2736 (38.5 mm; 21°C; 4)	33±50 (10–350)	4.6±0.6 (4–6)	7±4 (2–17)	2±2 (0–8)
MLP DB 2734 (40.3 mm; 24.5°C; 4)	18±1 (15–21)	4.9±1 (4–8)	6±3 (2–14)	2±2 (0–6)

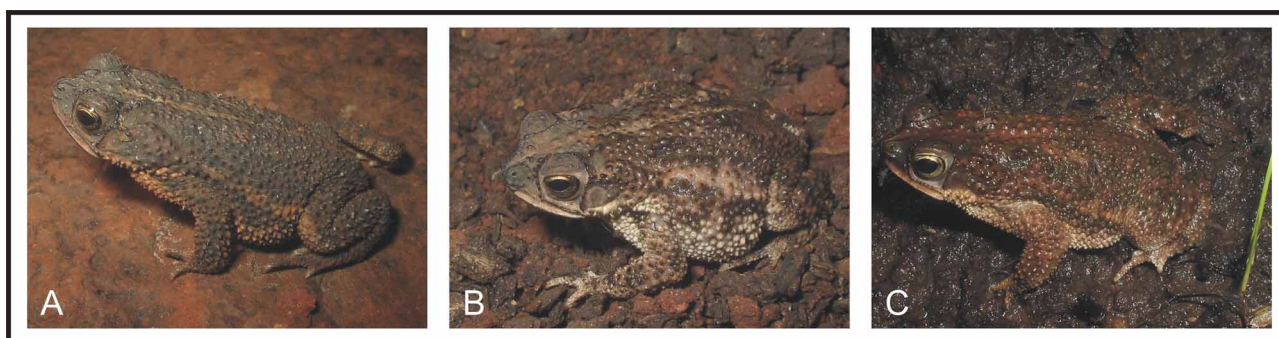


FIGURE 6. Adult specimens of (A) *Rhinella bergi*, MLP DB 2845, (B) hybrid of *R. bergi* x *R. major*, MLP DB 2736, and (C) *R. major*, unvouchered specimen, from Vera, Santa Fe, Argentina.

Three factors were extracted from the PCA that consecutively explained 56.27%, 20.30%, and 9.67% of total variation (86.24% cumulative). The corresponding factor loadings are presented in Table 3. The first factor was temperature-independent and was loaded heavily and positively by note duration, internote duration, dominant frequency, pulse duration, and interpulse duration; and negatively by note rate and SVL; the second one was temperature and SVL-independent and was loaded positively by pulses/note; at last, the third factor represented the temperature effect on call structure. Plotting variable scores on factors one and two (Figure 8) showed that there is a considerable separation between the advertisement calls of the different species in the *Rhinella granulosa* group, which were segregated in at least three non-overlapping clusters. One of them is on positive values of the factor one and negative values of the factor two (i.e. long notes and low number of pulses/note), composed by the advertisement calls of *R. azarai* and *R. bergi*, with some overlapping. The second cluster is on positive values of the factors one and two (i.e. long notes and high number of pulses/note) grouping the calls of *R. major* and the specimens of intermediate morphological characters, with no overlapping between them. The third cluster is approximately on negative values of the factors one and two (i.e. short notes and low number of pulses/note), represented by the advertisement calls of *R. dorbignyi*, *R. fernandezae* and *R. meriane* and with considerable overlapping among the first two species. The advertisement call of *R. centralis* (not included in the PCA) seems to be more related with this last cluster according to their spectral and temporal parameters.

TABLE 3. Factor loadings of variables included in the Principal Component Analysis (varimax rotation) of advertisement calls of the *Rhinella granulosa* group.

Variables	Factor 1	Factor 2	Factor 3
Call duration	0.632939	-0.542406	0.291393
Notes/call	-0.504333	-0.673576	0.348732
Note rate	-0.921452	-0.291757	-0.016570
Note duration	0.965644	0.164657	-0.038713
Internote duration	0.916864	0.126169	-0.194262
Pulses/note	0.081725	0.935584	0.026803
Pulse duration	0.836368	-0.381298	0.035551
Interpulse duration	0.727666	-0.439661	-0.245345
Dominant frequency	0.866997	0.086959	-0.401688
Temperature	0.134540	0.067537	-0.919482
SVL	-0.809962	0.101302	0.346983

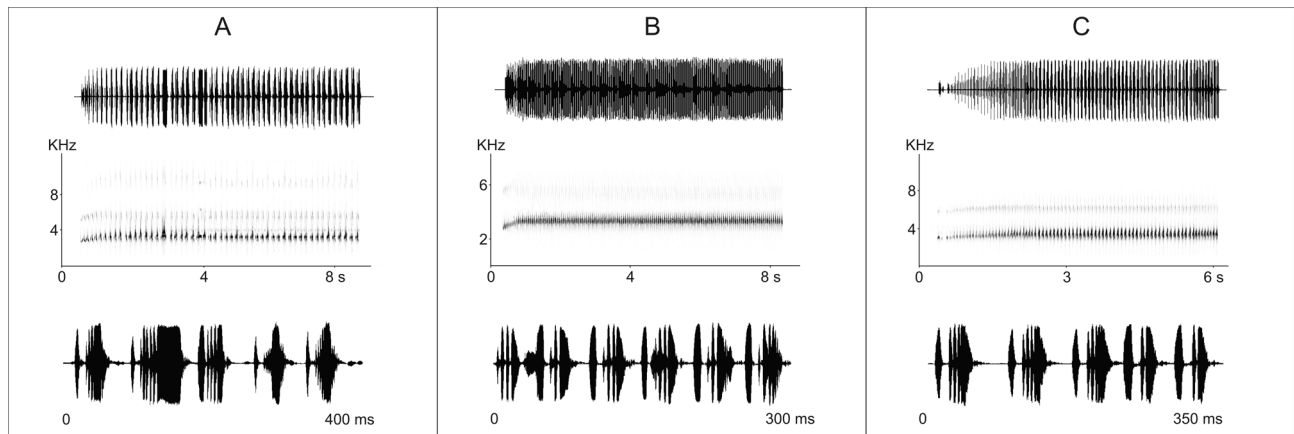


FIGURE 7. Advertisement calls of three hybrid specimens of *Rhinella bergi* x *R. major*, MLP DB 2674 (A), MLP DB 2736 (B), and MLP DB 2734 (C). Oscillogram (top) and sonogram (center) of a single call, and oscillogram with some few notes (bottom).

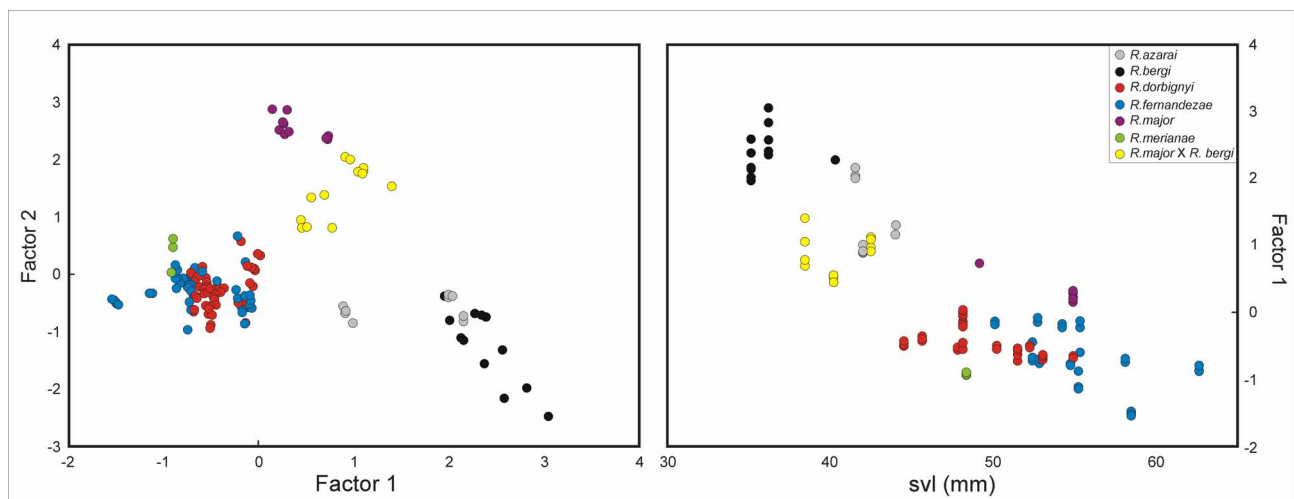


FIGURE 8. Ordination of advertisement calls in species of the *Rhinella granulosa* group as resulted from the Principal Component Analysis: temperature-independent Factor 1 vs. temperature dependent Factor 2 (A), and Factor 1 vs. SVL of specimens (B).

Release calls in the *Rhinella granulosa* group (Table 4, and Figure 9) are shorter than the advertisement calls, and somewhat variable regarding duration and pulse number. The dominant frequency is lower than those of advertisement calls of the same species, except in *R. dorbignyi* in which both dominant frequencies are similar. Two types of release calls were observed: 1) a non-pulsed release call was exhibited by *R. bergi*, and also observed in some *R. azarai* and *R. fernandezae*; 2) a pulsed call with a variable number of pulses, either between 2 and 14 as observed in *R. azarai*, *R. dorbignyi*, and *R. fernandezae* or with more than 14 pulses, which was only recorded in *R. dorbignyi*.

TABLE 4. Release call characteristics in four species of the *Rhinella granulosa* species group, obtained for the present work and from literature: mean±SD (range). CD = call duration; DF = dominant frequency; ICD = intercalls duration; PD = pulse duration; IPD = interpulse duration.

Species (males, calls)	Call type	CD (ms)	DF (Hz)	ICD (ms)
<i>R. azarai</i> (16, 102)	Unpulsed	29±34 (2–260)	2097±174 (1736–2431)	406±1008 (84–9024)
<i>R. azarai</i> (13, 192)	Pulsed	38±19 (9–91)	2143±156 (1910–2431)	247±466 (51–5462)
<i>R. bergi</i> (1, 25)	Unpulsed	15±3 (11–23)	2712±93 (2587–3061)	479±637 (120–2900)
<i>R. dorbignyi</i> (3, 19)	Pulsed	471±200 (195–775)	2109±82 (2005–2252)	452±482 (168–1876)
<i>R. fernandezae</i> (1, 21)	Unpulsed	41±17 (13–88)	1557±67 (1466–1703)	402±278 (94–968)
<i>R. fernandezae</i> (1, 7)	Pulsed	206±65 (155–321)	1937±26 (1897–1961)	238±40 (167–292)

continued.

Species (males, calls)	Pulses / s	Pulses / Call	PD (ms)	IPD (ms)
<i>R. azarai</i> (16, 102)	75.7±86.3 (3.8–500)	–	–	–
<i>R. azarai</i> (13, 192)	137±44.7 (23.5–222.2)	4.8±2.4 (2–14)	8±8 (1–58)	5±3 (1–25)
<i>R. bergi</i> (1, 25)	69.5±12.6 (43.5–90.9)	–	–	–
<i>R. dorbignyi</i> (3, 19)	47.5±15.1 (19.9–68.5)	23.6±14.7 (7–49)	5±5 (1–28)	19±13 (2–87)
<i>R. fernandezae</i> (1, 21)	30±16.5 (11.4–76.9)	–	–	–
<i>R. fernandezae</i> (1, 7)	54.5±10.7 (36.9–64.5)	10.7±1.5 (10–14)	11±4 (6–26)	9±11 (1–48)

Discussion

The trilled advertisement calls in toads of the *Rhinella granulosa* group studied herein are similar to those of closely related species groups of *Rhinella*, like the *R. crucifer*, *R. margaritifera*, and *R. marina* groups (see interspecific relationships in Pramuk 2006 and Pramuk *et al.* 2008). All these species groups present advertisement calls that consist of periodic trains of these complex pulses (type II of Martin 1972), except for *R. margaritifera* (*R. margaritifera* group) and *R. icterica* (*R. marina* group) that also present unpulsed calls (type I of Martin 1972; see Appendix 2 and references therein). However, there are some noticeable differences among these species groups. For instance, the advertisement calls in species of the *R. marina* group and *R. crucifer* (Wied-Neuwied, *R. crucifer*

group) present relatively lower dominant frequencies, which is coherent with their larger size. In addition, the call in species of the *R. marina* group is usually shorter —*R. poeppigii* (Tschudi), *R. schneideri* (Werner)— and the number of notes per call is lower —*R. marina* (Linnaeus), *R. rubescens* (Lutz), *R. poeppigii*, *R. schneideri*—. The advertisement calls in species of the *R. margaritifera* group present a lower number of notes per call and their dominant frequencies are usually lower than in the *R. granulosa* group. Even when some differences could be associated with the size of the species, the higher dominant frequency allow distinguishing clearly the calls of the *R. granulosa* group from those of the other groups of *Rhinella* that emit advertisement calls (*R. marina* and *R. crucifer*).

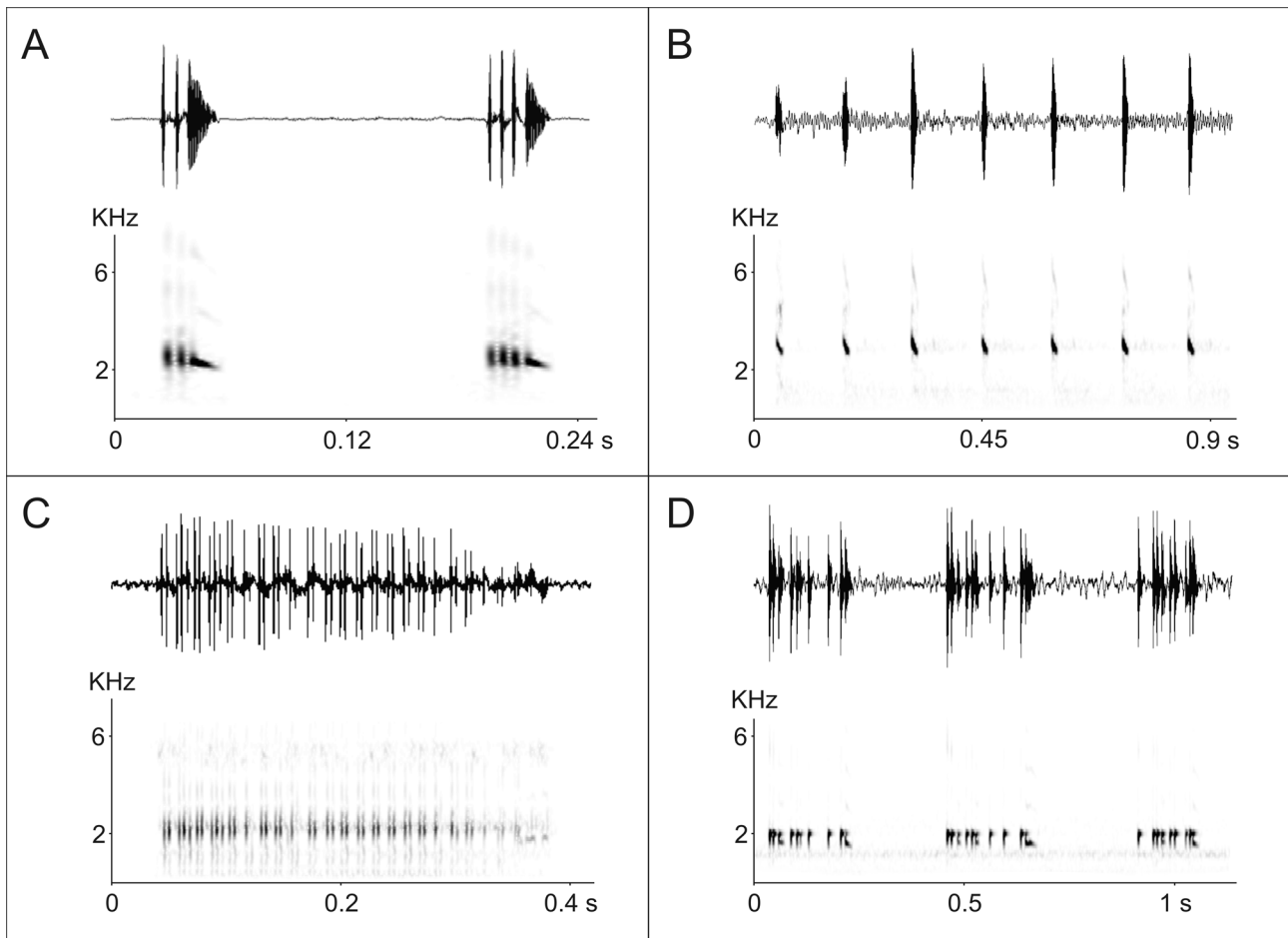


FIGURE 9. Release calls of *Rhinella azarai* (A), *R. dorbignyi* (B), *R. fernandezae* (C), and *R. major* (D). Oscillogram (top) and sonogram (bottom).

Within the *Rhinella granulosa* group, the advertisement calls present some clear interspecific differences of relevant taxonomic value. A simple combination of acoustic characters like call and note duration, number of pulses per note and dominant frequency allows distinguishing between *R. azarai*, *R. bergi*, *R. centralis*, *R. major*, *R. meriane*, and *R. dorbignyi* + *R. fernandezae*. For instance, the broadly sympatric species *R. bergi* and *R. major* (Narvaes & Trefaut Rodrigues 2009) can be unequivocally distinguished by a relatively long call with two pulses per note in the first, and a shorter call with 6–8 pulses per note in the former. The only two species whose advertisement calls are indistinguishable are *R. dorbignyi* and *R. fernandezae*, the southernmost taxa of the *R. granulosa* group. Their distributions overlap in Argentina, Brazil, and Uruguay (Gallardo 1969; Narvaes & Trefaut Rodrigues 2009). These two species are distinguished by some minor differences in the development and extension of cephalic crests (Gallardo 1957; Narvaes & Trefaut Rodrigues 2009). However, resemblance between the larval and adult forms of both species has been indicated (e.g. Borteiro *et al.* 2006). Moreover, some authors reported difficulty to find constant diagnostic characters in their external morphology (e.g. Klappenbach & Langone 1992; Prigioni & Achaval 1992). The results presented herein for both taxa (also the similitude in their release calls) suggest that their specific status must be reassessed as they probably are conspecific.

Herein, we describe for the first time release calls of species in the *Rhinella granulosa* group. Nevertheless, the preventive vibration associated to the release calls was studied in *R. dorbignyi* (Rengel 1948; 1949) and *R. fernandezae* (Ceí 1964). The release calls in the *R. granulosa* group, although irregular, clearly differ between species in three out of seven acoustic parameters; intercall duration, pulse rate, and pulse duration (Table 4). The pulsed and non-pulsed calls of *R. azarai* and *R. fernandezae* are respectively quite similar, and only differ in pulse number. The pulsed calls of *R. fernandezae* and the corresponding one of *R. dorbignyi* were also very similar. Like we observed in *R. azarai* and *R. fernandezae*, pulsed and non-pulsed release calls were reported for *R. arenarum* (Hensel, *R. marina* group), *R. dapsilis* (Myers & Carvalho, *R. margaritifera* group), and *R. achalensis* (Ceí), *R. limensis* (Werner), and *R. spinulosa* (Weigmann, *R. spinulosa* group), see Appendix 3 and references therein. As expected, the dominant frequencies in the relatively smaller species of the *R. granulosa* group are usually higher.

The natural hybridization between closely related bufonid species is a relatively common phenomenon in the Holarctic Region (e.g. Blair 1972; Masta *et al.* 2002; Vogel & Johnson 2008). In contrast, the occurrence of natural hybridization was only occasionally confirmed amongst the large diversity of Neotropical bufonids; e. g. *Rhinella arenarum* x *R. spinulosa* and *R. icterica* (Spix) x *R. crucifer* (Brown & Guttman 1970; Haddad *et al.* 1990). This bias is likely due to the large amount of work that has been done on the holarctic anuran fauna. Our observations on putative hybrids between *R. bergi* and *R. major* from Argentina confirmed the occurrence of natural hybridization in the *R. granulosa* group for the first time. The calls of these hybrid specimens were quite irregular but their acoustic parameters resulted intermediate between those of the presumed parental species, which is coincident with other previous works (Volpe 1952; Cory & Manion 1955; Blair 1956). The dominant frequency, note rate and inter-note intervals could be distinguished from those of both parental species, similarly as reported by Zweifel (1968) for hybrids between the toads *Anaxyrus* (as *Bufo*) *americanus* (Holbrook) and *A. woodhousii* (Girard). The occurrence of natural hybridization between bufonid species whose advertisement calls clearly differ like in our study case (Blair 1958), reinforce the idea that call variation do not warrant species isolation in this group. As reported for many other toads (Wells 1977; Zimmerman & Bogart 1984; Wells 2007), species in the *R. granulosa* group present explosive reproduction (*sensu* Wells 1977). They commonly form dense breeding aggregations for brief periods (Narvaes & Trefaut Rodrigues 2009). We observed that males of the different species usually attempt to mate with both males and females present at the breeding site, even belonging to different species. This reproductive trait exhibited by the *R. granulosa* group increases the potential for interspecific hybridization (Blair 1958), given the several zones of sympatry between some of their species. The evolutionary implications of the hybrid zone between *R. bergi* and *R. major* and the possibility of introgression should be investigated using molecular markers.

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APPENDIX 1

List of studied specimens, with locality (numbers correspond to sites indicated in Figure 1), recording date, and associated air/water temperature data. Abbreviations: AC, advertisement call; RC, release call.

Rhinella azarai.—ARGENTINA: PROVINCIA DE MISIONES: Departamento Capital: 6) Posadas, western access (27° 23' 00"S; 55° 57' 43"W), MLP DB 5775–76 (RC: 18/9/2007, 24°C/–); MLP DB 5775 (AC: 18/9/2007, 16°C/19°C); Departamento Candelaria: 7) Ñu Pyahú, Ruta Provincial N° 3, 3.5 Km from Ruta Nacional N° 12 (27° 29' 25"S; 55° 40' 06"W), MLP DB 5270–71 (RC: 1/11/2006, 26°C/–); MLP DB 5928 (RC: 11/10/2007, 23°C/–); 7) Ruta Nacional N° 12 and Ruta Provincial N° 3 (27° 27' 46"S; 55° 40' 55"W), MLP DB 5914–22, 5929, unvouchered specimen (RC: 11/10/2007, 23°C/–); 6) Villa Lanús (27° 26' 13"S; 55° 53' 39"W), unvouchered specimen (AC: 27/09/2004, 21°C/–).

Rhinella bergi.—ARGENTINA: PROVINCIA DE SANTA FE: Departamento Vera: 2) Vera (29° 29' 00"S; 60° 13' 00"W), MLP DB 2739 (23°C/–), MLP DB 2741 (21°C/–) (AC: 3/2/2004), unvouchered (AC: 4/2/2004, 21°C/–); PROVINCIA DE CHACO: Departamento San Fernando: 5) Ruta Nacional N° 11, near Arroyo Palmira (27° 54' 00"S; 59° 57' 00"W), MLP DB 3455 (RC: 3/11/2004, 28°C/–).

Rhinella centralis.—PANAMA: 17) Monumento Natural Barro Colorado (09° 09' 00"N; 79° 51' 00"W), acronym number not given.

Rhinella dorbignyi.—URUGUAY: Departamento de Treinta y Tres: 10) Ruta N° 8, near Treinta y Tres (33° 14' 00"S; 54° 22' 00"W), MNHN 9413–14, 9420, unvouchered (–/20°C), MNHN 9415–16 (AC-RC: 3/11/2009, –/20°C), MNHN 9417–18, unvouchered (AC: 3/11/2009, –/20.9°C), MNHN 9419 (AC-RC: 3/11/2009, –/20.9°C). BRAZIL: Estado de Rio Grande do Sul: 12) Municipio de Porto Alegre, Ipanema (30° 01' 00"S; 51° 15' 00"W), acronym number not given - Fonoteca Zoológica recorder numbers 5711 (AC: 5/10/1997, 16°C/–), 5964 (AC: 5/10/1997, 16°C/–); 14) Municipio de Viamão (30° 05' 00"S; 51° 00' 00"W), acronym number not given - Fonoteca Zoológica record number 5828 (AC: 28/9/1996, 17°C/–).

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Rhinella fernandezae.—ARGENTINA: PROVINCIA DE ENTRE RÍOS: Departamento Paraná: 1) Balneario Thompson (31° 43' 00''S; 60° 31' 00''W), MLP DB 2112 (AC: 19/12/2002; 23°C/–); PROVINCIA DE CHACO: Departamento San Fernando: 5) Ruta Nacional N° 11, near Arroyo Palmira (27° 54' 00''S; 59° 57' 00''W), MLP DB 3454 (RC: 3/11/2004, 28°C/–). URUGUAY: Departamento de San José: 9) Delta del Tigre (34° 46' 00''S; 56° 21' 00''W), MNHN 9404, 9406–07 (AC: 5/10/2009, –/17°C), MNHN 9405 (AC-RC: 5/10/2009, –/17°C); Departamento de Montevideo: 8) Paso de la Arena (34° 49' 00''S; 56° 15' 00''W), MNHN 9408–10 (AC: 26/11/2009, 16/22.5°C), MNHN 9411–12 (AC: 26/11/2009, 18/22°C); Departamento de Rivera: 11) near Tranqueras (31° 11' 00''S; 55° 45' 00''W), acronym number not given - Fonoteca Zoológica record number 6256 (AC: 20/10/2003, 19.5°C/–); BRAZIL: Estado de Rio Grande do Sul: 13) Municipio de São Jerônimo (29° 59' 00''S; 51° 43' 00''W), acronym number not given - Fonoteca Zoológica record number 6045 (AC: 13/12/1998, 23.5°C/–).

Rhinella major.—ARGENTINA: PROVINCIA DE CHACO: Departamento 9 de Julio: 4) Las Breñas (27° 06' 00''S; 61° 06' 00''W), MLP DB 2733 (AC: 8/2/2004, 20°C/18°C); PROVINCIA DE SANTA FE: Departamento Vera: 3) Ruta Provincial N° 7, near Toba (29° 11' 18.3''S; 60° 09' 26.6''W), MLP DB 4570 (AC: 24/11/2005, 19.4°C/–); BOLIVIA: 15) Locality and acronym number not given - Fonoteca Zoológica recorder number 5582 (AC); DEPARTAMENTO DEL BENI: 15) Estación Biológica del Beni, acronym number not given (AC).

Rhinella merianae.—BRAZIL: Estado de Amazonas: 16) Aldeia dos Lagos, Silves (02° 50' 00''S; 58° 12' 00''W), acronym number not given - Fonoteca Zoológica record number 6240 (AC: 7/7/2003, 25°C/–).

Rhinella bergi x *R. major*.—ARGENTINA: PROVINCIA DE SANTA FE: Departamento Vera: 2) Vera (29° 29' 00''S; 60° 13' 00''W), MLP DB 2674, 2736 (21°C/–), MLP DB 2734 (24.5°C/–), (AC: 3/2/2004).

APPENDIX 2. Advertisement call parameters in the *Rhinella crucifer*, *R. margaritifera*, and *R. marina* groups, obtained from the literature as species of genus *Bufo*, except for Maciel *et al.* (2007) and Garda *et al.* (2010): mean±SD (range). CD = call duration; DF = dominant frequency; ND = dominant frequency; ND = note duration.

Species	CD (sec)	DF (Hz)	Notes / Call	Notes / s	ND (ms)	Pulses / Note	SVL, °C, males, calls	Source
<i>R. crucifer</i> group								
<i>R. crucifer</i>	(4–7)	(960–1750)	(60–150)	(11.5–15.5)	(30–60)	(4–7)	63–77 mm	Heyer <i>et al.</i> (1990)
<i>R. margaritifera</i> group								
<i>R. dapsilis</i>	0.12±0.01 (0.08–0.14)	2415 (1560–3360)	–	–	–	–	22–25°C, 1, 20	Zimmerman & Bogart (1988)
<i>R. margaritifera</i>	–	1500	(3–5)	–	(150–200)	–	62 mm	Duellman (1978; as <i>Bufo typhonius</i>)
<i>R. margaritifera</i>	–	(800–2200)	–	–	–	–	–	Schlüter (1981)
<i>R. margaritifera</i>	0.238±0.023 (0.197–0.292)	1332.3±107 (1211.5–1544.7)	6.3±0.6 (5–7)	26.6±2.1 (22.4–30.1)	–	2±0.5 (1–4)	23.5°C, 3, 32	De la Riva <i>et al.</i> (1996)
<i>R. margaritifera</i>	0.316 (0.291–0.339)	1140 (750–1450)	(7–8)	24.9	–	(1–4)	24.7°C	Köhler <i>et al.</i> (1997)
<i>R. margaritifera</i>	0.296	(240–1670)	7	–	–	–	78 mm, 24–28°C	Lescure & Marty (2001)
<i>R. marina</i> group								
<i>R. arenarum</i>	4.019±1.062	1250 (950–1500)	–	–	–	–	19–28°C, 3, 17	Salas <i>et al.</i> (1998)
<i>R. cerradensis</i>	(1.9–12.4)	839±8.9	19–129	9.8±0.6	–	3	117.7 mm, 17.1°C	Maciel <i>et al.</i> (2007)
<i>R. ictérica</i>	(4–20)	646±0.2 (320–850)	(40–180)	(8.5–9)	(40–60)	(1–3)	119–140 mm	Heyer <i>et al.</i> (1990)
<i>R. jimi</i>	6.29±2.29 (3.83–13.97)	600.59±51.71 (516.8–689.1)	95.16±34.13 (52–201)	–	–	(2–3)	27.1–28.6°C, 4	Garda <i>et al.</i> (2010)
<i>R. marina</i>	1.4	(300–1000)	20	–	–	–	–	Schüttler (1981)
<i>R. marina</i>	2	1000	>20	–	–	–	–	Easteal (1986)
<i>R. marina</i>	0.241±0.077 (0.121–0.368)	460	(24–26)	–	–	–	98.7 mm, 5–10, 3 by male	Bernal <i>et al.</i> (2004)
<i>R. marina</i>	14.3	(180–1010)	–	–	–	–	140 mm, 24–28°C	Lescure & Marty (2001)
<i>R. marina</i>	2.94	521	51	–	–	3	–	Ibáñez <i>et al.</i> (1999)
<i>R. poeppigii</i>	(2.717–3.312)	1033.2±71.6 (907–1141.3)	29.9±10.1 (10–45)	17±0.6 (15.8–17.8)	–	3.3±0.6 (3–5)	25.4°C, 1, 19	De la Riva <i>et al.</i> (1996)
<i>R. rubescens</i>	1.760±0.598 (0.631–2.681)	1015.6±41.1	5–25	–	–	2	115.5 mm	Maciel <i>et al.</i> (2007)
<i>R. schneideri</i>	2.38 (1.75–2.91)	700 (350–900)	24–40	14.11	–	3	155 mm, 25.2°C	Köhler <i>et al.</i> (1997; as <i>Bufo paracnemis</i>)

APPENDIX 3. Release call parameters in the *Rhinella margaritifera*, *R. marina*, and *R. spinulosa* groups obtained from the literature as species of genus *Bufo*, except for Garda *et al.* (2010): mean±SD (range). CD = call duration; DF = dominant frequency; ICD = intercalls duration; PD = pulse duration; IPD = interpulse duration.

Species (males, calls)	Call type	CD (sec)	DF (Hz)	ICD (sec)	Pulses / s	Pulses / Call	PD (sec)	IPD (sec)
<i>R. margaritifera</i> group								
<i>R. dapsilis</i>	Pulsed	0.08±0.01 (0.07–0.11)	4810±350 (4220–5250)	0.13±0.1 (0.06–0.36)	55.42±9.56 (44.78–71.27)	(1–4)	0.003±0.001	–
Zimmerman & Bogart (1988)								
<i>R. marina</i> group								
<i>R. arenarum</i>	Unpulsed	0.036 (0.005–0.196)	804 (653–1256)	–	–	1	–	–
Di Tada <i>et al.</i> (2001)								
<i>R. arenarum</i>	Pulsed	0.034 (0.007–0.228)	979 (703–1206)	–	187.5 (97.6–375)	6 (2–23)	0.003 (0.002–0.007)	0.002 (0.001–0.006)
Di Tada <i>et al.</i> (2001)								
<i>R. arenarum</i>	Pulsed	0.352 (0.072–0.792)	854 (703–1256)	–	39.2 (30.8–92.8)	16 (3–28)	0.013 (0.006–0.025)	0.014 (0.008–0.017)
Di Tada <i>et al.</i> (2001)								
<i>R. arenarum</i>	–	–	–	–	45.3	–	–	–
Brown & Guttman (1970)								
<i>R. jimi</i>	–	–	600.59±51.71 (516.8–689.1)	–	(2–5)	–	–	–
Garda <i>et al.</i> (2010)								
<i>R. marina</i>	–	–	–	–	6.9 (5–9)	–	–	–
Blair (1947)								
<i>R. spinulosa</i> group								
<i>R. spinulosa</i>	Unpulsed	0.078 (0.009–0.187)	1055 (704–1381)	–	–	–	–	–
Di Tada <i>et al.</i> (2001)								
<i>R. spinulosa</i>	Pulsed	0.578 (0.223–0.290)	1080 (954–1306)	–	46.5 (33.1–48.7)	28 (9–46)	0.012 (0.009–0.018)	0.01 (0.002–0.013)
Di Tada <i>et al.</i> (2001)								
<i>R. spinulosa</i>	–	–	–	–	71.4	–	–	–
Brown & Guttman (1970)								
<i>R. achalensis</i>	Unpulsed	0.048 (0.011–0.187)	954 (603–1231)	–	–	–	–	–
Di Tada <i>et al.</i> (2001)								
<i>R. achalensis</i>	Pulsed	0.455 (0.058–0.878)	929 (829–1080)	–	61.8 (48.8–86.2)	28 (4–56)	0.015 (0.005–0.019)	0 (0–0.01)
Di Tada <i>et al.</i> (2001)								
<i>R. limensis</i>	Unpulsed	0.047 (0.013–0.184)	728 (603–979)	–	–	–	–	–
Di Tada <i>et al.</i> (2001)								
<i>R. limensis</i>	Pulsed	0.052 (0.017–0.114)	854 (653–954)	–	164.7 (115.4–233.3)	9 (2–20)	0.004 (0.002–0.006)	0.003 (0.002–0.003)
Di Tada <i>et al.</i> (2001)								