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Breeding biology and advertisement call of the horned leaf-frog, *Proceratophrys appendiculata* (Amphibia: Anura: Odontophrynidae)

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ABSTRACT. We describe the breeding biology and the advertisement call of the horned leaf-frog, *Proceratophrys appendiculata* (Günther, 1873) in the Parque Nacional da Serra dos Órgãos, municipality of Teresópolis. The reproductive period of *P. appendiculata* is short and is associated with the end of the winter and the beginning of the spring, when males call night and day under large rocks in sandy bottom rock streams. The amplexus is axillary and one female laid about 656 viscous eggs. The advertisement call of *P. appendiculata* is unique among congeners. The call lasts approximately two seconds, with about 85 pulses/call at a rate of 45 pulses/s and frequency around 0.620 kHz. It is one of the longest calls and lowest in dominant frequency. Moreover, the call of *P. appendiculata* is characterized by the greatest number of pulses so far registered, reaching 129 pulses in a single call.

KEY WORDS. Atlantic Forest; calling activity; reproductive behavior.

Breeding activity in most species of anurans begins with the emission of an advertisement call (Gerhardt 1994), which plays several roles in anuran reproductive behavior, for instance: attract females to breeding sites, defend territories against other males, and stimulate female ovulation, among others (Wells 1977).

The advertisement call is species-specific, thus presenting an important mechanism of prezygotic reproductive isolation (Ryan & Rand 1993, Funk *et al.* 2009). Because of this property, the advertisement call and all behaviors associated with it are important tools in taxonomic (Padial *et al.* 2009, Padial & De La Riva 2009, Hepp & Carvalho-e-Silva 2011) and phylogenetic studies (Cocroft & Ryan 1995, Robillard *et al.* 2006, Goicoechea *et al.* 2010).

The Neotropical *Proceratophrys* Miranda-Ribeiro, 1920 currently comprises 32 species distributed in Argentina, Brazil, and Paraguay (Ávila *et al.* 2012, Cruz *et al.* 2012, Teixeira-Jr *et al.* 2012, Dias *et al.* 2013a, Frost 2013, Godinho *et al.* 2013). These species are commonly grouped within phenetic groups or species complexes based on external morphological similarity (Prado & Pombal 2008, Dias *et al.* 2013b), despite molecular data indicating that these groups are not monophyletic (Amaro *et al.* 2009, Teixeira-Jr *et al.* 2012, Dias *et al.* 2013a).

Two main phenetic groups are recognized within *Proceratophrys* according to the presence or absence of palpebral appendages (Cruz & Napoli 2010). Among the species that possess a long and single palpebral appendage are the species complexes of *Proceratophrys appendiculata* and *P. boiei* (Izecksohn *et al.* 1998, Prado & Pombal 2008, Cruz & Napoli 2010).

Proceratophrys appendiculata (Günther, 1873) is an endemic species of the southeastern Atlantic Forest, occurring amidst the leaf-litter (Dias *et al.* 2013a). These are cryptic animals (Sazima 1978) found scattered on the forest floor. Their tadpoles develop in small streams inside the forest (Dias & Carvalho-e-Silva 2012). The breeding activity of the species is poorly studied and its advertisement call has not been described.

The great morphological similarity observed among species of *P. appendiculata* complex (Prado & Pombal 2008, Dias *et al.* 2013a,b) and the discovery of several new species (Cruz & Napoli 2010, Ávila *et al.* 2011, 2012, Martins & Giaretta 2011, Napoli *et al.* 2011, Cruz *et al.* 2012, Teixeira-Jr *et al.* 2012, Dias *et al.* 2013a, Godinho *et al.* 2013) – including cryptic species within *P. appendiculata* (see Dias *et al.* 2013a) – makes the description of the advertisement call of *P. appendiculata* very important (Mângia *et al.* 2010, Dias *et al.* 2013b). Herein we describe the advertisement call and aspects of the breeding biology of *P. appendiculata*.

MATERIAL AND METHODS

The present study was conducted in the Parque Nacional da Serra dos Órgãos (PARNASO), municipality of Teresópolis, state of Rio de Janeiro, Brazil, from September 2004 to July 2012, excepting the years of 2005 and 2007. We visited five breeding sites monthly for at least three nights and three days. During the breeding period, we stayed in the field for as long as the calling activity was taking place. Many other observations were made sporadically in different dates and sites of the study area. We considered males calling, amplexed couples, egg clutches and tadpoles in initial development stage as indicators of breeding activity (Gosner 1960, stages 25-26).

Vocalizations were recorded with a Marantz PMD-670 and Tascam DR-07 digital recorders, at sample rate of 44.100 kHz and sample size of 16 bits and microphone Sennheiser ME-67. The recordings were made in October, 2011 and September, 2006 both with temperature of about 17.5°C. Sound analyses and graphs were made with Raven Pro ver. 1.3 from the Cornell Laboratory of Ornithology (Bioacoustics Research Program). The following parameters were measured: call duration; number of pulses per call; pulse rate; pulse periods (measured from the beginning of one pulse to the beginning of the following one, thus encompassing the pulse duration and interpulse interval); dominant frequency; and fundamental frequency. Numerical call parameters are given as a range, followed by the Mean ± SD, Mode and N within parenthesis.

The temporal parameters were measured directly from the waveform and frequency parameters were measured directly from the audiospectrogram (with window function Hann, amplitude logarithmic, window size 512 samples and overlap 99%). We also counted the number of harmonically related frequencies observed in the audiospectrogram and power spectrum (see Hepp *et al.* 2012). Technical terms and definitions adopted follow Littlejohn (2001) and Hepp & Carvalho-e-Silva (2011).

Individuals recorded were collected, fixed in 10% formalin, and preserved in 70% ethyl alcohol. They are housed in the Amphibian collection of the Laboratório de Biossistemática de Anfíbios of the Universidade Federal do Estado do Rio de Janeiro (UNIRIO 2542-2543, 4471-4473) and in the Amphibian collection of the Departamento de Zoologia of the Universidade Federal do Rio de Janeiro (ZUFRJ 8970-8971). The permit for collecting zoological material provided by Instituto Chico Mendes (ICMBio) and Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais e Renováveis (IBAMA) receives the number12164-2.

Comparative acoustic data for other species were obtained from the following references: *Proceratophrys avelinoi* Mercadal de Barrio & Barrio, 1993 – Kwet & Baldo (2003) and Lima (2007); *P. bigibbosa* (Peters, 1872) and *P. brauni* Kwet & Faivovich, 2001 – Kwet & Faivovich (2001); *P. boiei* (Wied-Neuwied, 1824) – Heyer *et al.* (1990); *P. carranca* Godinho, Moura, Lacerda & Feio, 2013 – Godinho *et al.* (2013); *P. cururu* Eterovick & Sazima, 1998 – Eterovick & Sazima (1998); *P. concavitympanum* Giaretta, Bernarde and Kokubum, 2000 – Santana *et al.* (2010); *P. cristiceps* (Müller, 1883) – Nunes & Juncá (2006); *P. huntingtoni* Ávila, Pansonato & Strüssmann, 2012 – Ávila *et al.* (2012); *P. melanopogon* (Miranda-Ribeiro, 1926) – Mângia *et al.*(2010); *P. moehringi* Weygoldt & Peixoto, 1985 – Weygoldt & Peixoto (1985); *P. moratoi* (Jim & Caramaschi, 1980) – Brasileiro *et al.* (2008) and Martins & Giaretta (2012); *P. palustris* Giaretta & Sazima, 1993 – Martins & Giaretta (2012); *P. paviotii* Cruz, Prado & Izecksohn, 2005 – Cruz *et al.* (2005); *P. renalis* (Miranda-Ribeiro, 1920) – Santana *et al.* (2011); *P. sanctaritae* Cruz & Napoli, 2010 – Cruz & Napoli (2010); *P. vielliardi* Martins & Giaretta, 2011 – Martins & Giaretta (2011).

RESULTS

Breeding Biology

The reproductive period of *P. appendiculata* was short and associated with the end of winter and spring, from July to November, although the males called only one or two months per year (Table I).

Table I. Month of calling activity of *Proceratophrys appendiculata* per year in the period of 2004 to 2012. Breeding site not visited in the years of 2005 and 2007.

Year	Month
2004	October
2006	September
2008	August
2009	September
2010	July and November
2011	October

During the breeding season, males called continuously at night and during the day in large choruses inside the forest, along shallow rocky streams with sandy bottoms (Fig. 1). Calling activity began a few days after the first heavy rainfall within the period above mentioned. Males of *P. appendiculata* were only observed calling under large rocks or in small burrows formed by vegetation and tree branches, always with their bodies in contact with the water.

Males called at an approximately equal distance from one another, which varied from one to two meters. A wave like chorus was observed along the streams, where one male called followed by the most adjacent male and so on down and up the river. No satellite males were observed, and it seems that calling activity was also related to territorial defense.

In a plot of 5 x 5 m, about eight to twelve males were observed calling actively. This number decreased each day, and about three to four days after the first record of calling activity, only two or three males were still calling. The frequency of call emission decreased drastically and males called in duets closer than previously, only about 40 cm apart. After this period, the calling activity ceased completely. The end of the calling period was not associated with any abiotic parameter, such as temperature, rainfall or any other.

Three days after the calling activity had stopped, we observed hatched capsules strongly adhered to submerged rocks and aquatic vegetation. We also saw several newly hatched tadpoles (Gosner stage 25 or less) close to these capsules.

In October 2004, one single male was observed calling in a very small stream. It was in contact with the running water. Adhered to its body and to the sandy bottom and to rocks were several eggs (Fig. 2). They were collected and raised in the laboratory (Fig. 3). Four days later, tadpoles at Gosner stage 23 hatched.

In September 2006, during calling activity, an amplexed coupled was found in contact with shallow water after removing a large rock. The male was strongly clasped to female's armpits (Fig. 4). The couple was collected and later the female spontaneously oviposited in a plastic box.

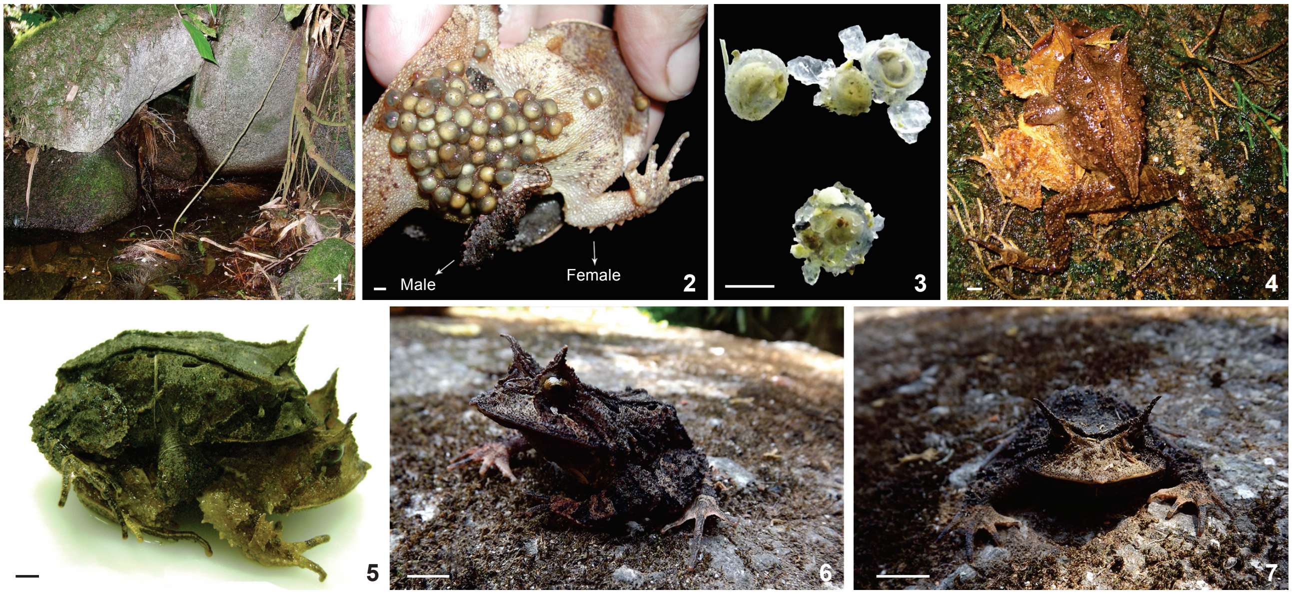
This clutch contained 656 viscous eggs of brownish color; some of these eggs remained adhered to the female's abdomen (Fig. 3). Each egg was 3.4 ± 0.4 (2.7-4.3 n = 25) millimeters in diameter.

In August 2008, a solitary male was heard calling from under a large rock. About five minutes later, a female was intercepted moving through the water in the direction of the male'. Both were captured and maintained in a plastic bag with a small amount of water and vegetation. In the laboratory, they spontaneously engaged in amplexus (Fig. 5), although no eggs were laid.

In 2010, two breeding periods were recorded, one in July and another in November, although not in all breeding sites. The July activity took place only at one of the sites, and the November one in all other sites except the first one.

Advertisement Call

Eighty-two calls from three (UNIRIO 4471-73) specimens were analyzed (Figs 6 and 7). The advertisement call was composed of a sequence of 51 to 129 pulses ($\bar{x}$ = 85.5 ± 18.7, Mo = 70.0, n = 79) repeated quasi-periodically. All pulses have a shorter attack than decay, probably as a consequence of the natural reverberant environment of the calling sites (rock caves). The amplitude of the call usually increases and decreases abruptly, although, on occasion, this rise and fall were gradual. The middle or the last quarter of the call is the part with most amplitude (Fig. 8). The difference between the amplitude of the pulses throughout the call is variable. Some calls (63.4%)



Figures 1-7. (1) One of the breeding sites utilized by *Proceratophrys appendiculata* in Parque Nacional da Serra dos Órgãos. Sandy bottom rocky stream (22°27'05.8"S, 43°00'02.0"W) about 1,165 meters above sea level. (2) Eggs of *Proceratophrys appendiculata* collected in field in 2004 with sediments adhered to then – scale bar = 2.5 mm; (3) egg clutch not fertilized laid in laboratory – scale bar = 2.5 mm; (4) couple collected in field in September 2006 – male (ZUFRJ 8970, SVL 58.0 mm) and female (ZUFRJ 8971, SVL 62.0 mm) – scale bar = 3.5 mm; (5) couple whose got spontaneously amplexed in laboratory in August 2008 – male (UNIRIO 2542, SVL 56.2 mm) and female (UNIRIO 2543, SVL 66.7 mm) – scale bar = 3.5 mm. (6) Voucher specimens recorded. UNIRIO 4471, SVL 58.0 mm – scale bar = 5.0 mm; and (7) UNIRIO 4473, SVL 57.2 mm – scale bar = 5.0 mm.

have pulses with approximately similar amplitudes while others (36.6%) have middle pulses with clearly more amplitude, resulting in a prominent middle amplitude peak (Fig. 9). The calls without a prominent peak vary from a low pulse rate with few pulses to a high pulse rate with high number of pulses (Fig. 13). The calls with a prominent peak have a high pulse rate with high number of pulses (Figs 9 and 11). Call duration ranges from 1.322 s to 2.416 s ($\bar{x}$ = 1.907 ± 0.223, n = 79). The call has a harmonic structure with up to four visible harmonics, though only the first two harmonics are highlighted. The dominant frequency corresponds to the fundamental one and ranges from 0.562 to 0.656 kHz ($\bar{x}$ = 0.621 ± 0.046, Mo = 0.656, n = 79) (Fig. 8). The pulse period varies from 0.012 to 0.119 s ($\bar{x}$ = 0.021 ± 0.008, Mo = 0.015, n = 944). The pulse periods decrease slightly and gradually until to the middle of the call where they begin to increase slightly and gradually until the end in calls with prominent amplitude peak. In calls without a prominent peak, they only increase slightly and gradually all the way until the end (Fig. 11). Eventually (19.5%), the first or second period is the longest one, mainly (93%) in calls with a prominent peak (Fig. 8). The pulse rate ranges from 30.0 to 65.4 pulses per second ($\bar{x}$ = 45.3 ± 10.4, n = 79) and varies inversely to the pulse periods throughout the call, being higher in the middle or beginning of the call (Fig. 11).

In September 2006, sixty sections with calls partially overlapped, emitted by two or three specimens in a chorus, were recorded (Fig. 12). In this social context, an isolated pulse (introductory pulse) precedes the overlapping calls and the final pulse periods are long and their pulses have high amplitudes. Moreover, on many occasions, a long pulse with modulated and low amplitude was emitted immediately after the end of the overlapping calls (Figs 12 and 13).

Comparison with other speciesThe advertisement call of *P. appendiculata* is unique when compared with those of its congeners. The numerical call parameters given in this section in parenthesis are the range of *P. appendiculata* followed by the combined range of the species compared. The call of this species is longer than those of *P. avelinoi*, *P. boiei*, *P. brauni*, *P. concavitympanum*, *P. cristiceps*, *P. cururu*, *P. huntingtoni*, *P. melanopogon*, *P. moratoi*, *P. palustris*, *P.paviotii*, *P. renalis*, *P. sanctaritae* (1322-2416 ms *vs*. 146-1184 ms), and shorter than that of *P. moehringi* (1.3-2.4 s *vs*. 3.5-4.0 s). The pulse rate is lower than those of *P. carranca*, *P. concavitympanum*, *P. cristiceps*, *P. melanopogon*, *P. sanctaritae*, *P. vielliardi* (30.0-65.4 pulses/s *vs*. 68.0-142.4 pulses/s) and higher than that of *P. bigibbosa* (30.0-65.4 pulses/s *vs*. 23.0-27.0 pulses/s). Its number of pulses is higher than those of *P. bigibbosa*, *P.boiei*, *P. brauni*, *P. melanopogon*, *P. moratoi, P. palustris*, *P. paviotii*, *P. renalis* (51-129 pulses/call *vs*. 12-45 pulses/call). The dominant frequency is lower than those *P. avelinoi*, *P. bigibbosa*, *P. brauni*, *P. carranca*, *P. concavitympanum*, *P. cristiceps*, *P. huntingtoni*, *P. melanopogon*, *P. moratoi*, *P. palustris*, *P. paviotii*, *P. renalis*, *P. sanctaritae*, *P. vielliardi* (0.562-0.652 kHz vs. 0.660-2.300 kHz). Additionally, the call of *P. appendiculata* differs from that of *P. carranca* and *P. vielliardi* by consisting of a single pulsed note (*vs*. call consisting by a sequence of pulsed notes, with a longer last note in *P. vielliardi* and in most calls of *P. carranca*); and from *P. sanctaritae* by absence of trill of notes (*vs*. presence of sequence, or trill, of notes in *P. sanctaritae*).

DISCUSSION

Breeding behavior

Few studies have presented detailed information about the reproductive behavior of *Proceratophrys* species, and the information available generally corresponded to natural history notes in species descriptions (e.g., Weygoldt & Peixoto 1985, Cruz & Napoli 2010, Martins & Giaretta 2011) with few exceptions (e.g., Boquimpani-Freitas *et al.* 2002).

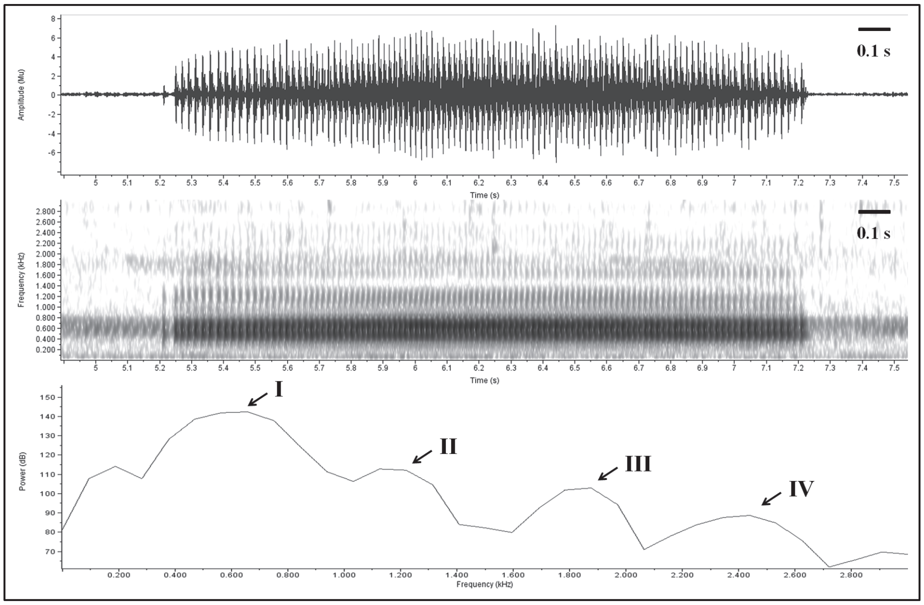
The utilization of sandy bottom streams as a reproductive site seems to be the norm for *Proceratophrys* species. Several species like *P. bigibbosa*, *P. brauni*, *P. concavitympanum*, *P. cristiceps*, *P. cururu*, *P. melanopogon*, *P. moheringi*, *P. paviotii*, *P. tupinamba* Prado & Pombal, 2008, *P. sanctaritae*, and *P. vielliardi* adopt this kind of environment as breeding sites (Weygoldt & Peixoto, 1985, Eterovick & Sazima 1998, Kwet & Faivovich 2001, Boquimpani-Freitas *et al.* 2002, Cruz *et al.* 2005, Nunes & Juncá 2006, Cruz & Napoli 2010, Mângia *et al.* 2010, Santana *et al.* 2010, Barros *et al.* 2011, Martins & Giaretta 2011).

On the other hand, the peculiar behavior of calling from under large rocks is less diffused in the genus, and seems to be shared only with *Proceratophrys moheringi* (Weygoldt & Peixoto 1985). As Gerhardt (1994) pointed out, natural selection usually favors morphological characteristics and behaviors that increase the range of effective communication in the context of mate attraction. In the burrowing frogs *Eupsophus* Fitzinger, 1843, acoustic properties of calling sites may affect signal emission and reception (Penna & Solís 1999), contributing to the antiphonal and chorus activity. The ongoing antiphonal calling would in turn direct the females toward the breeding areas (Penna 2004). The rocks may play a similar role in the social interactions of *P. appendiculata*, working as natural sound amplifiers.

Several behavioral traits may be indicative of mate choice by the female, such as 1) males calling from a fixed site; 2) absence of physical interactions between males; and 3) females moving toward the male (Wells 1977). The breeding behavior recorded for *P. appendiculata* match these characteristics.

In addition, the absence of egg clutches and amplexed pairs during peak calling activity and the constant decline in the number of males in the chorus per day may indicate that females are choosing the males who remain in the chorus for longer. Despite the fact that our data support this hypothesis, it still must be tested.

The short reproductive period recorded for *P. appendiculata* may be a consequence of the calling activity pattern, acting as a



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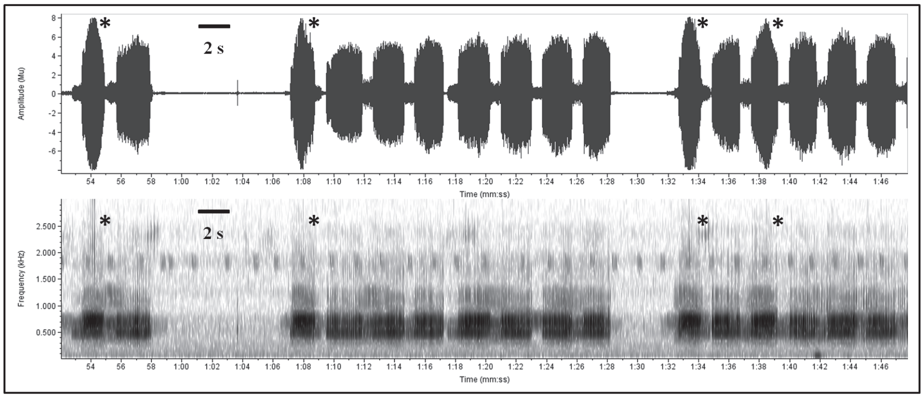

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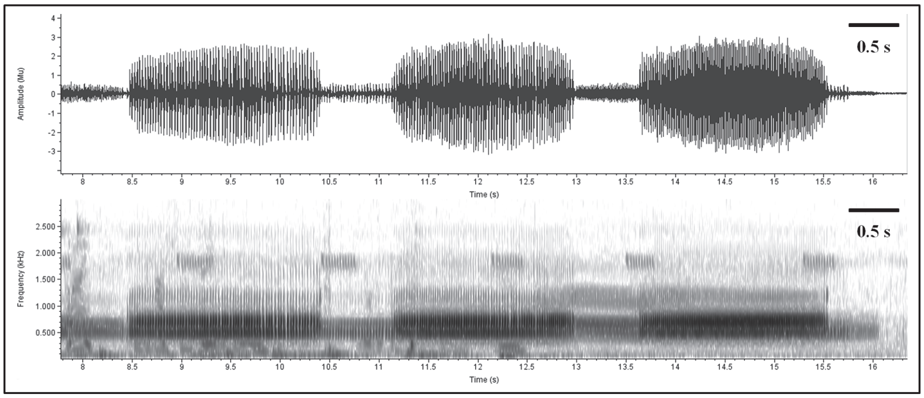

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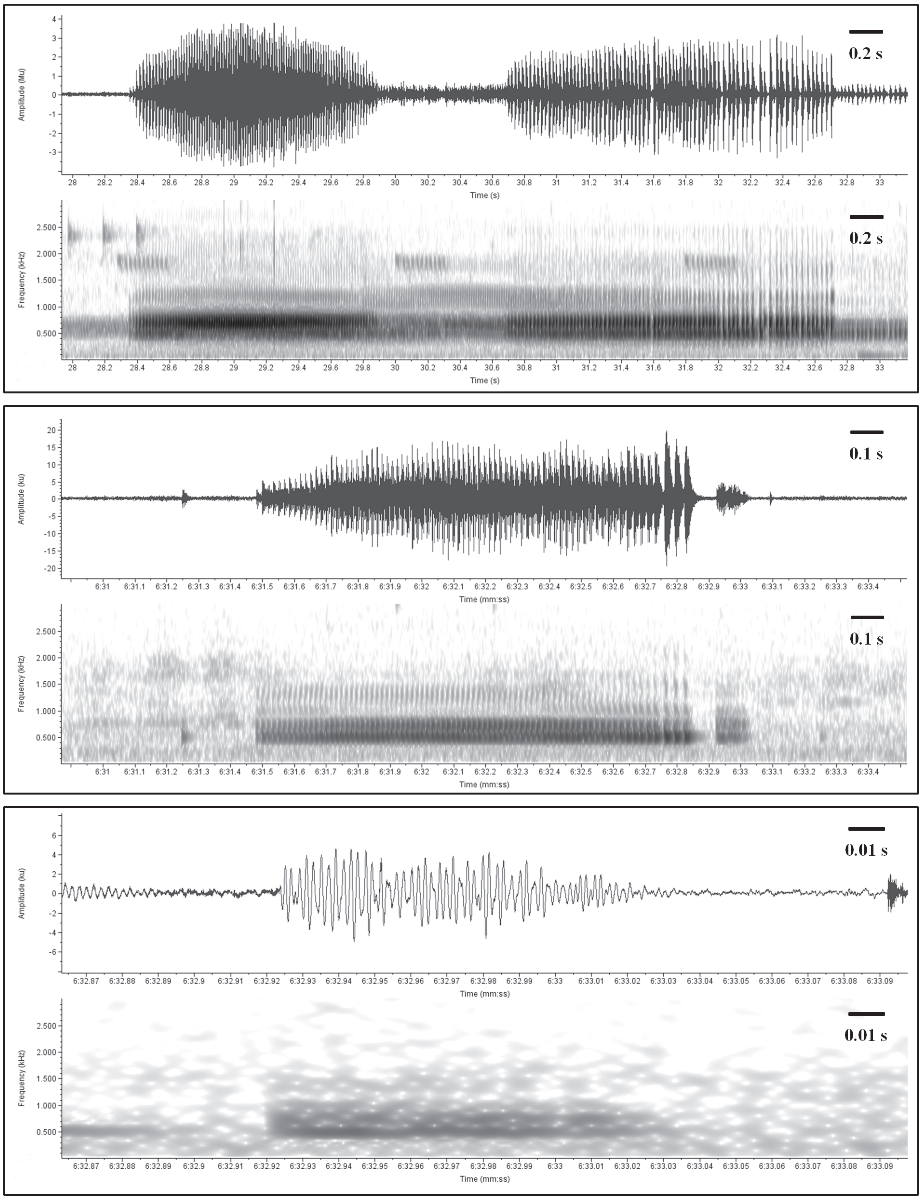
Figures 8-10. Advertisement call of *Proceratophrys appendiculata*, spectrogram and power spectrum with window function Hann, amplitude logarithmic, window size 512 and 256 samples respectively, overlap 99%. (8) Oscillogram (top), spectrogram (middle) and power spectrum (bottom) of one call. Note the first longest period and the first two harmonics with more energy (harmonics indicated by Roman-numbered arrows in power spectrum). (9) Oscillogram (top) and spectrogram (bottom) of sixteen calls with and without prominent amplitude peak (see text). The first ones are flagged with asterisk and show higher pulse rates. (10) Oscillogram (top) and spectrogram (bottom) of three calls without prominent amplitude peak. Note the pulse rate variation, increasing from the first to the last call.



Figures 11-13. Advertisement call of *Proceratophrys appendiculata*, oscillogram(top) and spectrogram (below). Spectrogram with window function Hann, amplitude logarithmic, window size 512 samples, overlap 99%. (11) Two calls with and without prominent amplitude peak respectively. Note that the first call shows the highest pulse rate section in the middle while the last one shows the same section in the in the beginning, decreasing until the end. (12) Overlapping calls of three males. Note the introductory pulse and the territorial final pulse after the loud pulses at the end of the main emission. (13) Territorial pulse in detail.

trade-off mechanism where males call intensively throughout the day but for only a few days. The emission of the advertisement call has a high energy cost (Woolbright 1983) and, as noted by Boquimpani-Freitas *et al.* (2002), probably constrains the calling period of some species of *Proceratophrys*.

This pattern is recurrent for several species. Males of *Proceratophrys moheringi* also call at night and during the day, and the reproductive activity of this species was recorded for about three months – August to October (Weygoldt & Peixoto 1985). The same was observed for *P. tupinamba* in Ilha Grande, where the species breeds from one to three months per year (Boquimpani-Freitas *et al.* 2002).

This pattern was also registered for species of the *Proceratophrys bigibbosa* group. Caldart *et al.* (2010) reported that *P. avelinoi* reproduces from September to November, and males can be heard both at night and during the day. The same period was cited by Kwet & Faivovich (2001) for *P. avelinoi*. These authors also mentioned that *P. brauni* calls throughout the day from November to February and *P. bigibbosa*, which calls during the night and sometimes during the day, breeds from September to November.

Female fecundity of *P. appendiculata* is very similar to that of *P. tupinamba* (729 to 946 eggs) and *P. melanopogon* (664 eggs) (Boquimpani-Freitas *et al.* 2002, Almeida-Gomes *et al.* 2007). There are no other records for *Proceratophrys* species, and more data are necessary to generalize this pattern. The hatching time for *P. appendiculata* is about three to four days after the eggs are laid. This fact was observed in the field and in the laboratory.

There are many forms of parental care in anurans, of which egg attendance the most common (Kluge 1981). Martins *et al.* (1998) mentioned the facultative parental care in the Hylid frog *Hypsiboas faber* (Wied-Neuwied, 1821), in which males keep calling near fertilized clutches, thus protecting their territory and, consequently, the eggs from other males. The single male captured in 2004 was calling near laid fertilized eggs and no other male or female was caught in the area. This behavior may be associated with facultative parental care, presenting the first record for the genus, although more observations are necessary.

Advertisement Call

Possibly, the function of the introductory pulse in the chorus (Fig. 12) is to advertise to neighboring males that the main call is about to begin. This pulse could be important to stimulate the partial overlap of the calls. The long pulse recorded after the overlapping calls may be related to a territorial or agonistic context, since it is frequently emitted after intense overlap and/or after high amplitude pulses in the chorus (Fig. 12).

The advertisement call of *P. appendiculata* is quite different from the calls of fourteen congeners. Within the *P. appendiculata* complex, the call differs from those of *P. melanopogon* and *P. paviotii* in all the numerical aspects that were compared [call duration (ms): 1322-2416 in *P. appendiculata*; 170-480 in *P. melanopogon*; 347-427 in *P. paviotii* – number of pulses (pulses/call): 51-129 in *P. appendiculata*; 12-41 in *P. melanopogon*; 26-32 in *P. paviotii* – dominant frequency (kHz): 0.56-0.65 in *P. appendiculata*; 0.99-1.27 in *P. melanopogon*; 0.66-1.28in *P. paviotii* – pulse rate (pulses/s): 30.0-65.4 in *P. appendiculata*; 68-96 in *P. melanopogon*; unknown in *P. paviotii*], and from that of *P. moehringi* in call duration (1.3-2.4 s in *P. appendiculata*; 3.5-4 s in *P. moehringi*). On the other hand, it is similar to the call of *P. moehringi* and *P. boiei* (*P. boiei* complex) in dominant frequency at around 0.600 kHz [dominant frequency (kHz): 0.621 in *P. appendiculata*; 0.600 in *P. boiei*; 0.200-0.700 in *P. moehringi*]. The greater similarity with these species than with *P. melanopogon* in unexpected, since the phylogenetic proximity between *P. appendiculata* and *P. melanopogon* found by Amaro *et al.* (2009). However this similarity may be a consequence of the relatively larger snout-vent length of those three species within the genus [adult males (in mm; Prado & Pombal 2008): 40.4-59.9 in *P appendiculata*; 39.8-61.9 in *P. boiei*; 59.2-62.6 in *P. moehringi*] (see Márquez 1995, to more details about the relationship between body size and call frequency).

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