Reproduction of *Agalychnis granulosa* Cruz, 1989 (Anura: Hylidae)

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We provide a detailed description of the reproduction of the tree frog *Agalychnis granulosa*, including its advertisement call. *Agalychnis granulosa* breeds in ponds and the backwaters of streams within primary forests. The breeding season started at the beginning of the wet season and ended two months before the beginning of the dry season. Males called at night in the vegetation around the ponds at a maximum distance of 5 m from the water. The call was composed of a single note with three to seven pulses and a dominant frequency ranging from 1490 to 2101 Hz. Oviposition sites included tree trunks, attached leaves, fallen leaves and bromeliad roots, and ranged in height from 0 to 300 cm above the water. Egg clutches were composed of approximately 45 eggs encapsulated by a transparent gelatinous matrix. Empty egg capsules (4–7 per clutch) were recorded in 36% of the clutches. Great plasticity was observed in the reproductive strategies of *A. granulosa*, probably reflecting the varying competition and predation at the different life stages of the species.

Keywords: Phyllomedusinae; reproductive ecology; advertisement call; Atlantic Forest

Introduction

The Phyllomedusinae clade includes the characteristic hylid tree frogs of the Neotropical region. A recent molecular phylogenetic study of this group indicated that the genus *Hylomantis* was paraphyletic and it was suggested that *Hylomantis* and *Pachymedusa* should be considered as junior synonyms of *Agalychnis* (Faivovich et al. 2010). Consequently, the genus *Agalychnis* now comprises 14 species divided into three groups, all of which inhabit the tropical forests of Central America, Amazonia and the east coast of South America (Frost 2013).

The *Agalychnis aspera* group (*sensu* Faivovich et al. 2005) includes two unique species from the Brazilian Atlantic Forest (BAF): *A. aspera* (Peters, 1873), which occurs in southern and central Bahia State (Pimenta et al. 2007); and *Agalychnis granulosa* (Cruz, 1989), which occurs in the northern states of Alagoas and Pernambuco (Carnaval et al. 2003; Nascimento and Skuk 2007). Both species have

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restricted ranges and inhabit one of the most threatened and unprotected BAF regions (Ribeiro et al. 2009).

Although *A. granulosa* is classified as Least Concern (LC) by the International Union for Conservation of Nature (IUCN) red list (Carnaval and Peixoto 2004), it is considered Critically Endangered (CR) by the Brazilian red list of threatened species (MMA 2003). Nevertheless, basic ecological data of *A. granulosa* are still missing, a fact that delays conservation actions towards the protection of this species. The only published information currently available is on adult and larval external morphology and a few distribution occurrence records (Cruz 1989; Carnaval et al. 2003; Nascimento and Skuk 2007). In this context, the objective of this study is to provide data on the reproductive ecology (including advertisement call) of this enigmatic species. Such data could also clarify our understanding of the evolutionary relationships of the species and how its traits have evolved.

Materials and methods

Study area

This study was conducted between January 2010 and December 2011 on three BAF fragments in the state of Alagoas, northeastern Brazil. Most of the survey effort was concentrated on an open ombrophilous forest fragment located in the municipality of Maceió, known as Mata do Catolé (9°35'00"S, 35°49'00" W, 140 m elevation; here and throughout DATUM WG84) – an area of 492 ha encompassed by the Environmental Protection Area (EPA) of Catolé and Fernão Velho. This region is characterized by two main climatic seasons: (1) a dry period (October to March), with high temperatures (mean monthly temperatures of between 21.2 and 29.5°C) and relatively low rainfall ($\bar{X} = 413 \pm 34$ mm); and (2) a wet period (April to September), with lower temperatures (19.8–28.4°C) and higher rainfall ($\bar{X} = 1388 \pm 73$ mm) (climatic data obtained from Worldclim v.1.4 [<http://www.worldclim.org>]).

Data were also collected from two additional forest fragments: Serra da Saudinha (09°22'00" S, 35°45'00" W, 150 m elevation), also located in the municipality of Maceió; and the Ecological Station of Murici (09°12'00" S, 35°52'00" W, 500 m elevation), in the municipality of Murici. Both regions follow the climatic regime described in the previous paragraph.

Reproductive ecology

Habitat preference was assessed by searching for the presence of *A. granulosa* in all potentially suitable aquatic environments that could support nektonic tadpoles. The following measurements were taken for each water body: area; maximum water depth; distance to forest edge; pH; conductivity; salinity; and dissolved oxygen. The final four parameters were measured using the Hanna multi parameter meter model HI 929828-1.1.

Reproductive data were collected from three temporary ponds with visits being conducted once every two weeks between April and December 2011. Visits generally started at 15:00 and finished at 01:00, though on some occasions observations were recorded beyond this specific time frame. During each visit, we recorded the presence of males in breeding activity, amplexant pairs, tadpoles, egg clutches (including their

horizontal and vertical distance to the water and the substrate used), territorial disputes and the presence of satellite males. A red cellophane filter was mounted on the flashlights for observations to minimize the disturbances that white light could cause to the species. The presence of other frog species and predators in the breeding sites was also recorded.

Several measurements of eggs and clutches were also collected: maximum diameter of the egg clutches; number of visible eggs per clutch; and maximum diameter of the eggs (without the jelly capsule). The presence and the maximum diameter of eggless capsules were also recorded. All measurements were collected by photographing the egg clutches next to a ruler and then processing the images using Image Tool (v.3.0). In addition, we collected three egg clutches so that we could obtain the total number of egg and eggless capsules.

In April and June 2011, the advertisement calls of two males were recorded in Mata do Catolé, where one of the two individuals was collected (MUFAL 8798). Calls were recorded with a Sony PCM-D50 digital recorder and a Sennheiser ME66 external directional microphone, using a sample frequency of 44 kHz and 16 bit resolution. The files were recorded in wav format. Calls were analysed using the programs Avisof T-lite SASLab for Windows (v.1.5.16; Avisoft Bioacoustics, Berlin, Germany) based on the following parameters: Fast Fourier Transformation (256 points); frame (100%), window (Flat Top); and overlap (75%). The dominant frequency was obtained through the program Cool Edit Pro (v.2.1) using Fast Fourier Transformation 1024. Terminology describing call characteristics is according to Duellman and Trueb (1994). Voucher specimens were placed in the Herpetological Collection of the Natural History Museum of the Federal University of Alagoas.

Most of the measures are presented as mean ($\bar{X}$) followed by standard deviation ($\pm$); in some cases the median and the range are presented as they summarize the data better.

Results

Agalychnis granulosa breeds around temporary ponds and backwaters of streams within primary forests. The species was not observed in ponds with total area $<6 \text{ m}^2$ and a depth of $\leq 5 \text{ cm}$. The distance of the reproductive environments to the edge of the forest did not influence the presence of *A. granulosa*, with breeding environments found both close to the forest edge ($\cong 25 \text{ m}$) and in the centre of the fragment ($\cong 480 \text{ m}$).

In all surveyed breeding sites we registered calling males and tadpoles of *A. granulosa*. The following species were also recorded calling simultaneously: *Dendropsophus haddadi*; *D. minutus*; *Hypsiboas albomarginatus*; *Pristimantis* gr. *ramagii*; *Leptodactylus mystaceus*; *L. natalensis*; *L. vastus*; *Physalaemus cuvieri*; *Scinax skuki*; and *S. x-signatus*. Tadpoles co-occurred with *Chiasmocleis alagoana*, *D. haddadi*, *D. minutus*, *H. albomarginatus*, *L. mystaceus*, *L. natalensis*, *L. vastus*, *Macrogenioglottis alipioi*, *P. cuvieri*, and *S. skuki* e *S. x-signatus*. Breeding sites also contained fish, Odonata larvae and other insects that are known to prey on tadpoles.

Breeding activity coincided with the start of the wet season; calling males and egg clutches were recorded from April to July 2011, and tadpoles from April to October 2011. Based on the last record of egg clutches, it is possible to estimate that tadpoles take approximately two months to complete metamorphosis.

The daily vocalization period began around 17:30 (post-sunset), with activity reaching its peak between 19:00 and 21:00. Some individuals were calling until 04:00 (sunrise). We did not observe breeding events during nights of heavy rain (>50 mm). Calling males were located above the water to a horizontal maximum distance of 5 m from the edge of the water (median = 1 m, $n = 23$) and the vertical position of individuals over the water ranged from 15 cm to 5 m (median = 1.6 m, $n = 23$). The substrate used for calling varied among green leaves (30%), including bromeliads, dry leaves (4%), tree trunks (9%) and tree branches (57%), all suspended above the ground.

The two recorded males were calling from the marginal vegetation of a temporary 37 m², 40 cm deep pond. One of the males was 2.5 m above the water while the other was at 1.5 m, with both being at the edge of the pond. The average air temperature during the recording was between 20.9 and 30.9°C, and relative humidity was between 88 and 94%. A total of 30 separate calls were recorded (20 of the individual not collected, 10 of the collected individual). The advertisement call of *A. granulosa* ($n = 2$; Figure 1) is composed of one single note formed by 3–7 closely packed pulses ($\bar{X} = 5.4 \pm 0.7$) with a duration of 19–49 milliseconds ($\bar{X} = 30 \pm 0.9$ ms) and an ascending modulation amplitude. The call emission rate was 7.62 (individual not collected) and 1.07 (MUFAL 8798) calls per minute. The interval time between calls ranged from 1.74 to 139.3 s ($\bar{X} = 27.09 \pm 38.35$ s). Every pulse took between 0.77 and 20 ms ($\bar{X} = 42 \pm 0.99$ ms), generally the first three or four pulses with lower intensity than the others (Figure 1D); the pulse rate ranged from 151.21 to 364.09 pulses per

Figure 1. Advertisement call of *Agalychnis granulosa* (MUFAL 8798). Air temperature: 20.9°C, relative humidity 94%. (A) Oscillogram, (B) sonogram, (C) frequency spectrum and (D) detail of the advertisement call showing the first four pulses of low intensity and spaced from each other.

second ($\bar{X} = 248.01 \pm 56.54$ p/s). The dominant frequency ranged from 1490 to 2101 Hz ($\bar{X} = 1832 \pm 65.31$ Hz) with a slight ascending modulation throughout the note.

Only four amplexant pairs were observed (Figure 2A and B), all of them between 19:00 and 21:30. All couples were moving slowly and were located on tree trunks ($n = 2$), tree branches ($n = 1$) and on a bromeliad's leaf ($n = 1$). Paired males were approximately 20% smaller than the females (as an approximate measure made by photographs so as not to disturb the amplexant pair). In general, one to two hours elapsed between pair detections until the beginning of the spawning. During this time, the pairs were apparently searching for an appropriate oviposition site. The spawning sites chosen included holes in tree trunks ($n = 1$), leaves ($n = 2$) and bromeliad's roots ($n = 1$), all at a maximum horizontal distance of 20 cm from the water. Each egg took 4–6 minutes to be laid. Based on the number of eggs per clutch, the spawning process is estimated to last 2–4 hours. Two of the pairs entered the water around 30 and 50 minutes before spawning.

Other egg clutches ($n = 16$; Figure 2C, D) were located at a maximum horizontal distance of 1 m from the water's edge ($\bar{X} = 29 \pm 33$ cm), and from the ground level to a height of 3 m ($\bar{X} = 73 \pm 91$ cm). Eggs were recorded on the following substrates: tree trunks, leaves (including bromeliads), fallen leaves and bromeliad roots. Some oviposition sites were used repeatedly throughout the breeding season.

Figure 2. Amplexant pairs of *Agalychnis granulosa* in spawning process (A and B); and egg clutches of *A. granulosa*, in epiphyte roots (C) and in a bromeliad's leaf (D).

Egg clutches were composed of unpigmented eggs surrounded by a jelly capsule with one clutch appearing to have greenish capsules. The maximum diameter of the clutches ranged from 28.35 to 56.98 mm ($\bar{X} = 39.18 \pm 10.09$ mm; $n = 12$). The maximum diameter of the eggs ranged from 2.68 to 5.48 mm ($\bar{X} = 4.08 \pm 0.49$ mm; $n = 12$). The number of visible eggs per clutch ranged from 22 to 64 ($\bar{X} = 42 \pm 14$; $n = 12$). Visible empty egg capsules were present in 41% of the clutches (five of 12 clutches), but only 4–7 per clutch ($\bar{X} = 5.0 \pm 1.0$), representing on average 15% ($\pm 9.5\%$) of the total number of eggs. The empty egg capsules had a maximum diameter that ranged from 1.18 to 4.66 mm ($\bar{X} = 2.7 \pm 0.83$). The three collected egg clutches for the total count of eggs presented 63, 60 and 45 eggs and 6, 5 and 13 eggless capsules respectively (MUFAL 11153, 11154 and 11159). A clutch took approximately 8–10 days to hatch. However, some clutches did not hatch; three clutches were desiccated (1 m distant from the water) and two clutches were submerged when the water level increased.

Two conflicts were observed between males. One male was observed trying to overthrow another male from a tree branch. In the second case, one of the males held the other and attempted to stay on the top of its back. Non-calling males were also observed in close proximity to calling males, but it was not possible to verify whether these males were behaving as satellites.

Discussion

The reproductive period of *A. granulosa* seems to begin at the start of the wet season and finishes two months before the end of the wet season, allowing the complete development of the tadpoles before the aquatic environment dries out. The species breeds in lentic as well as in lotic environments. Reproduction in streams is found in all genera of the group, except for *Cruziohyla*. In *Agalychnis*, only *Agalychnis lemur* (Jungfer and Weygoldt 1994) and now *A. granulosa* are reported to reproduce both in ponds or streams, while all other species have been observed reproducing only in ponds.

Of the 14 species that currently belong to the genus *Agalychnis*, nine have had their vocalization described: *A. annae* (Duellman 1970); *A. aspera* (Pimenta et al. 2007); *A. dacnicolor* (Duellman 1970); *A. lemur* (Cannatella 1980); *A. medinae* (Hertz and Lotzkat 2010); *A. moreletii* (Briggs 2010); *A. psilopygion* (Cannatella 1980); *A. saltator* (Duellman 1970); and *A. spurrelli* (Duellman 1970). The advertisement calls of species that belong to *Agalychnis* are composed of a single note (an exception is *A. saltator* which uses two notes and *A. medinae* which uses five notes) and have a dominant frequency around 1800 Hz (with the exception of *A. spurrelli* which has a mean of 568 Hz). *Agalychnis granulosa* is characterized by the highest pulse rate per second among species with an already described call (mean of 248.01 per second versus 42 in *A. annae*, 159 in *A. danicolor*, 40 in *A. lemur*, 108 in *A. saltator* and 75 in *A. spurrelli*). *Agalychnis granulosa* and *A. aspera* have similar spectral and temporal parameters, including an ascending modulation amplitude and a short call duration compared with congeneric species (mean of 30 ms against 280 ms in the other species; the exception is *A. psilopygion* with 40–50 ms). Nevertheless, the average number of pulses in *A. granulosa* was slightly higher than *A. aspera* (5.4 against 3 or 4 in *A. aspera*; Table 1).

The fact that two amplexant couples visited the water before they began the spawning process could indicate the presence of bladder-filling behaviour; however,

Table 1. Comparisons of some call parameters among *Agalynchnis* species.

Species	Call duration (ms)	Number of notes per call	Number of pulses per note	Pulse rate (p/s)	Dominant frequency range (Hz)	Source
<i>Agalynchnis annae</i>	160–440	1	≅13	38–50	1044–1295	Duellman (1970)
<i>A. aspera</i>	14–50	1	3–4	–	1679.6–2110	Pimenta et al. (2007)
<i>A. dactylor</i>	160–360	1	–	120–190	1120–2240	Duellman (1970)
<i>A. granulosa</i>	19–49	1	3–7	151–364	1490–2101	This study
<i>A. lemur</i>	20–29	1	–	97–118	2272–2520	Cannatella (1980)
<i>A. medinae</i>	670–720	5	–	–	2092.7–2506.6	Hertz and Lotzkat (2010)
<i>A. moreletii</i>	21.5–87.8	1	1–26	–	1046–1396	Briggs (2010)
<i>A. psilopygion</i>	40–50	1	–	–	≅1900	Cannatella (1980)
<i>A. saltator</i>	80–120	1 (sometimes 2)	≅11	105–110	1844–1890	Duellman (1970)
<i>A. spurrelli</i>	340–400	1	≅28	60–90	435–700	Duellman (1970)

further investigation is necessary to verify this statement. This behaviour, which is found in some species of *Agalychnis* (e.g. Pyburn 1980; Roberts 1994) and in *Cruziohyla* (Savage 2002), is supposed to facilitate water acquisition in order to hydrate the eggs before the spawning process begins. The origin of this character in the phylogeny is still uncertain (Faivovich et al. 2010).

Agalychnis granulosa displays one of the highest plasticities known for phyllomedusines in its choice of egg-laying substrate. In the present study three substrates were recorded (tree trunks, leaves and epiphytic roots). Other species showing high plasticity in egg-laying substrate are *Cruziohyla calcarifer* (Marquis et al. 1986; Caldwell 1994) and *A. saltator* (Roberts 1994).

Some of the egg clutches ($n = 4$) were laid directly on leaves on the ground (the possibility that the clutches had fallen accidentally was rejected as all the clutches had no signs of fall and the places where the leaves were found seemed to be carefully chosen). This suggests that tadpoles of *A. granulosa* can move actively towards the water source, or more probably that the tadpoles may simply swim off once the nest is flooded. However, more investigation related to the subsequent development of the clutch is needed to clarify this subject.

Agalychnis granulosa is the only species of the genus for which empty egg capsules have been reported, although for *A. aspera* only one clutch was examined from a collected amplexant couple that spawned in a plastic bag (Pimenta et al. 2007). Having empty capsules is considered a synapomorphy of Phyllomedusinae that was subsequently lost in *Agalychnis* (Faivovich et al. 2010). For some species, the ecological function is thought to be avoidance of premature desiccation of egg clutches. Empty egg capsules also seem to reduce the concentration of toxic substances resulting from metabolic processes and to act as glue in the leaf folding behaviour of the genera *Phyllomedusa* and *Phasmahyla* (Pyburn 1980). *Agalychnis granulosa* has retained this characteristic (in a lower frequency per clutch and fewer empty capsules); however, the role of eggless capsules in *Cruziohyla*, *Phrynomedusa*, and now *A. granulosa* still remains unknown.

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