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Natural history of the marsupial frog *Gastrotheca albolineata* (Anura: Hemiphractidae) in lowland Brazilian Atlantic Forest

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Abstract

Natural history of the marsupial frog *Gastrotheca albolineata* (Anura: Hemiphractidae) in lowland Brazilian Atlantic Forest. *Gastrotheca albolineata* is a marsupial frog endemic to the Atlantic Forest in southeastern Brazil. It remains poorly studied in nature and is uncommon in herpetological collections. We studied the natural history of *G. albolineata* during a four-year period (2015 to 2019), in Ubatuba, São Paulo state, Brazil, at its southernmost distribution. Our results show that *G. albolineata* is arboreal, perches from low to medium heights, and breeds during the dry season without chorus aggregation. Calling activity occurs during the day but is more intense during the first half of the night. We used dorsal body markings to identify individuals. Six individuals were recaptured during the study, indicating site fidelity during the active season. The defensive repertory of *G. albolineata* contains seven different behaviors, including a high-pitched distress call. Egg development in the female's dorsal pouch took at least 87 days, and fully formed froglets were born with a snout–vent length of 16 mm. Our data substantially add to the knowledge of the natural history of Brazilian marsupial frogs and can be helpful to delineate conservation strategies for elusive species such as *G. albolineata.*

Keywords: behavior, breeding, defense, larval development, natural marks, site fidelity.

Resumo

História natural da perereca marsupial *Gastrotheca albolineata* (Anura: Hemiphractidae) na Mata Atlântica brasileira de baixada. *Gastrotheca albolineata* é uma perereca marsupial endêmica da Mata Atlântica do sudeste do Brasil. Essa espécie permanece pouco estudada na natureza e é incomum em coleções herpetológicas. Estudamos a história natural de *G. albolineata* durante um período de quatro anos (2015 a 2019), em Ubatuba, estado de São Paulo, Brasil, em sua distribuição mais austral. Nossos resultados mostram que *G. albolineata* é uma espécie arborícola, que se empoleira em alturas baixo-médias e se reproduz durante a estação seca sem agregação de coro. A

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atividade de canto ocorre durante todo o dia, mas é mais intensa na primeira metade da noite. Utilizamos marcações dorsais naturais como identificação individual e recapturamos seis indivíduos durante o estudo, o que indicou fidelidade de sítio durante a temporada de atividade. O repertório defensivo de *G. albolineata* contém sete comportamentos diferentes, incluindo um canto de socorro estridente. O desenvolvimento do ovo na bolsa dorsal da fêmea levou pelo menos 87 dias, e os filhotes totalmente formados nasceram com 16 mm de comprimento rostro-cloacal. Nossos dados aumentam substancialmente o conhecimento da história natural das pererecas marsupiais brasileiras e podem ser úteis para delinear estratégias de conservação para espécies como *G. albolineata*.

Palavras-chave: comportamento, desenvolvimento larval, fidelidade de sítio, marcas naturais, reprodução.

Introduction

The most remarkable characteristic of frogs in the family Hemiphractidae is their capacity to brood eggs on their dorsa (Del Pino and Escobar 1981, Warne and Catenazzi 2016, Del Pino 2018). The so-called marsupial frogs have attracted scientific interest and have been studied in recent years (review in Duellman 2015). However, little scientific effort has been directed to the study of the natural history of marsupial frogs, even though many species are endangered (Duellman 2015).

Gastrotheca (Fitzinger, 1843) is the most diverse genus in Hemiphractidae, with 75 recognized species (Frost 2020). In this genus, eggs are carried by the female on the dorsum or in a dorsal pouch, with direct development into froglets (Castroviejo-Fisher *et al.* 2015), a reproductive mode classified as Mode 37 *sensu* Haddad and Prado (2005). *Gastrotheca* is well represented in the Andean highlands and in the mountain and lowland forests of Central and South America, with one species in the Amazon basin (Castroviejo-Fisher *et al.* 2015, Duellman 2015). In Brazil, species of *Gastrotheca* occur mostly in the Atlantic Forest domain, where two distinct phylogenetic groups are recognized: that of *G. fissipes* (Boulenger, 1888) and that of *G. microdiscus* (Andersson, 1910) (Blackburn and Duellman 2013, Monteiro *et al.* 2019).

Gastrotheca albolineata (Lutz and Lutz, 1939) is presently included in the *G. microdiscus* group (Monteiro *et al.* 2019), and its distribution encompasses Atlantic Forest in the states of Espírito Santo, Rio de Janeiro, and extreme northeast of São Paulo (Pontes *et al.* 2012). Like most Atlantic Forest *Gastrotheca* species, *G. albolineata* is mainly arboreal (Pontes *et al.* 2012). Some information is available in literature, including the advertisement call (Izecksohn and Carvalho-e-Silva 2008); defensive behavior (Muscat and Rotenberg 2016); breeding activity during the dry season (Pontes *et al.* 2012); and egg number and size (Caramaschi and Rodrigues 2007). Demographic data, seasonal variation in breeding activity, egg development, parturition, and newly hatched froglet size for *G. albolineata* remain unknown.

Gastrotheca albolineata is ranked as Least Concern in the IUCN Red List of Threatened species (Carvalho-e-Silva and Telles 2004), but it is poorly represented in herpetological collections and rarely seen in nature (Pontes *et al.* 2012). Although not endangered, populations are decreasing (Carvalho-e-Silva and Telles 2004). Natural history studies are essential to evaluate its local conservation status (Pontes *et al.* 2012) and to promote effective amphibian conservation (Michaels *et al.* 2014). Herein, we present new data on the natural history of a population of *G. albolineata* monitored for four

years at its southernmost distribution (Ubatuba, São Paulo state, southeastern Brazil) and provide information on temporal and habitat use, defensive behavior, and breeding biology.

Materials and Methods

Study Area and Monitoring Data

We conducted the study from August 2015 to October 2019 (~ 960 days, or 51 months) at the Projeto Dacnis private reserve in Ubatuba, São Paulo state, southeastern Brazil (23°27'46" S 45°07'58"W; WGS-84, 34 m a.s.l.). The Projeto Dacnis reserve covers 1.36 km^2 of mostly secondary lowland Atlantic Forest, with some primary forest patches. We used the average climate data, including mean, maximum and minimum temperature and rainfall, for the municipality of Ubatuba (Köppen 2020) to validate seasonal activity. The classification of Ubatuba's climate is Af, with significant rainfall throughout the year (Köppen 2020), but it is similar to Cfa in terms of hydric availability (Rolim *et al.* 2007). We defined the dry season from May to September, at which time rainfall abruptly ceases, and the rainy season from October to March.

We monitored the study area five days a week throughout the study period, with a total sampling effort of 3500 person-hours, both diurnal and nocturnal. We searched for the frogs visually and acoustically in forest strata up to 10 m in height, and we made visual and sound recordings depending on the height at which the frog perched. When we found an individual of *G. albolineata,* we recorded its location with a GPS receiver (Garmin eTrex 22×) and the height of the frog's perch from the ground with a digital laser distance meter (Bosch GLM20). When in hand, we determined the sex based on size, presence of pouch, and vocal sac. Individuals ≤ 30 mm were juveniles. In addition, we quantified the number of frogs/person-hour for each month to estimate capture rate. Results are presented as mean ± standard deviation.

Habitat Use

To understand the distribution of *G. albolineata* in the study area, we organized a Kernel Density Estimate map in QGis 3.12.1 software. This map took into account the capture density, showing the "hot spots" where we found the species more frequently. For every capture, we classified the phytocenosis type (Table 1) and calculated the observation frequency for each phytocenosis.

Non-Invasive Mark-Recapture

We photographed all individuals of *G. albolineata* with digital cameras (Canon 600D, 70D and 7D Mark II). We took pictures of the frogs' dorsa and used the pattern of whitish lines and dark spots as identification marks (e.g. Lama *et al.* 2011, Caorsi *et al.* 2012). We counted only adults in the census to avoid possible ontogenetic changes in the natural markings of juvenile frogs (Bardier *et al.* 2020). We visually identified each photographed individual, adding it to the image database and assigning it a number (for example, ID01_ date). We employed this mark-recapture technique coupled with the location to check the distance between two findings of the same individual.

We defined the breeding season as the period when we heard males vocalizing and found females carrying eggs (see Duellman 2015).

Defensive Behavior

We induced defensive behavior in 14 individuals of *G. albolineata.* One of us grasped the frog by the hind limbs (Toledo and Haddad 2009) and, after careful restraint, applied gentle pressure on its back, taking care not to harm the frog. We filmed each behavior with a digital camera (Canon 600D). We did the test only once per frog. After the test, we released each individual at its place of capture. We followed Toledo *et al.* (2011) to classify defensive behaviors. We collected one individual as a voucher (id21) and deposited it in the UNESP-Litoral herpetological collection (HCLP-A276).

Table 1. Classification of the phytocenoses found in the study area in the municipality of Ubatuba, São Paulo state, Brazil.

	Forest	Understory density and height	Canopy	Elevation (m a.s.l.)
Type 1	Alluvial Ombrophilous Dense Forest	low density, up to 4 m	up to 25 m	20
Type 2	Submontane Ombrophilous Forest	medium density, up to 3 m	up to 25 m	35
Type 3	Forest border exposed to anthropic action	shrubs up to 3 m	absent	24
Type 4	Alluvial Ombrophilous Dense Forest	low density, up to 4 m	up to 15 m	20
Type 5	Alluvial Ombrophilous Dense Forest	low density, up to 4 m	up to 8 m	11
Type 6	Submontane Ombrophilous Forest	low density, up to 4 m	up to 25 m	93

Egg Development, Parturition, and Newly Hatched Froglet Data

We collected one egg-brooding female (Id20) and kept her in captivity for 90 days to record egg development, parturition behavior, and froglet morphology and behavior. After that, we released her and the froglets at the place of capture. We kept the female housed in a room at Projeto Dacnis in Ubatuba in a well-ventilated plastic box (80 × 40 × 50 cm) with topsoil, a variety of perches, a shelter, and a water bowl. We fed her every two days with house crickets and cockroaches. We did not control air temperature, which varied according to external air temperature, and maintained humidity by misting the box once a day. We took measurements of her eggs every five days and recorded her behavior using a web camera installed in the enclosure. We measured the embryos' snout–vent length (SVL) during their development, as in Del Pino and Escobar (1981).

Results

Monitoring Data and Habitat Use

We captured a total of 25 individuals, including 20 adult males, two egg-brooding females, and three unsexed juveniles. We caught most frogs during the dry season, especially in July and August (0.05 and 0.04 frogs/person-hour respectively). December yielded 0.0071 frogs/person-hour, October 0.0057 frogs/person-hour, January 0.0035 frogs/person-hour, and September 0.0028 frogs/person-hour. No frogs were caught from February to June, and in November (Figure 1). The number of frogs varied across years: six records in 2015, 14 in 2016, one in 2017, five in 2018, and nine in 2019. Calling activity peaked in July (N = 30) and August (N = 28) in all survey years, but we also heard a few isolated calls during the rainy season (October to March). Vocalization of *G. albolineata* was loud, heard from distances greater than 10 m, and sometimes with an interval of over two hours between calls. During the calling activity peak, we recorded a maximum of 33 calling males in one night, with no formation of aggregations or choruses. We also heard males during the day, mostly in the morning and late afternoon, but also infrequently at midday. Curiously, we observed more vocal activity during windy days in all years.

During the 51 survey months, we saw *G. albolineata* 34 times, totaling 25 individuals (Figure 2A). We observed *G. albolineata* individuals perched on vegetation at an average height of 127 ± 59 cm (N = 34; Figure 2B). Sound recordings allowed us to estimate that

individuals perched as high as 10 m. We found all juveniles of *G. albolineata* during the rainy season (2015, 2017, and 2018); two of them were perched on the same *Heliconia rostrata* Ruiz & Pav. plant (both at the same height, 200 cm). We found the third juvenile during the dry season, perched at a height of 120 cm. We observed *G. albolineata* most frequently in phytocenosis types IV (44%) and II (32%) (for a description of the phytocenoses, see Table 1). Phytocenosis type IV also yielded the most recaptures (75%). Capture rates were lower in phytocenoses types V (9%), I and III (6% each), and VI (3%) (Figure 2C).

Non-Invasive Mark-Recapture

Throughout the four years, we recaptured six individuals of *G. albolineata.* All were males, and three were recaptured twice (Figure 3; Table 2). All recaptures were in the same phytocenosis as the first capture. Almost all recaptures occurred during the same season and in close proximity to the original place of capture (< 2 m). The greatest distance between capture and recapture during the same breeding season was 20 m (Id01). We found this individual one year later only 2 m from its original place of capture. Frog Id02 was the only one found at a greater distance (100 m) from one breeding season to the next.

Defensive Behavior

All 14 frogs we tested displayed at least one defensive strategy. Five of the seven defensive behaviors displayed had not been previously recorded for *G. albolineata:* cloacal discharge, active escape, mouth gaping, distress call, and hiding (Figure 4). In order of frequency, the tactics we recorded were: cloacal discharge (N = 14, or 100% of frogs); inflating the body (N = 13, 93%); active escape (N = 6, 42.8%); thanatosis (N = 3, 21.4%); mouth gaping (N = 3, 21.4%); distress call (N = 3, 21.4%); and hiding (N = 1, 7.14%).

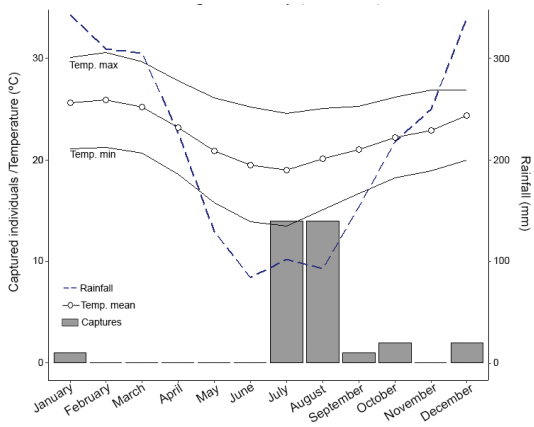

Figure 1. Captures and temporal activity (2015–2019) of *Gastrotheca albolineata* vs. climate data recorded in the municipality of Ubatuba, São Paulo state, Brazil.

Egg Development, Parturition, and Newly Hatched Froglet Data

We found two egg-brooding females during the study. The first was found on 23 July 2016 (Id06) carrying 17 eggs in her dorsal pouch (Figure 5A). We caught the second female on 29 July 2019, with 18 eggs in the pouch. It took 87 days from capture until the last froglet was expelled from the female's pouch (detailed embryo development in Appendix I). In the first 74 days, egg diameter doubled (from 6 to 12 mm) and the proportion of yolk decreased from 80% to none as the embryos grew. On 19 October 2019, at 06:30 h, the first froglet was released from the dorsal pouch after the mother performed a series of jumps. On the next day, at 22:50 h, she began using her foot to push froglets out of the pouch. After a few minutes, she inflated her body and expelled two live froglets (Figure 5B) and seven dead ones. On 21 October 2019, at 02:00 h, two froglets left the mother's pouch by themselves. On 23 October 2019, the internal pouch membrane completely everted with six other froglets and/or embryos still attached to it. Again, the female jumped, inflated her body, and used her foot to

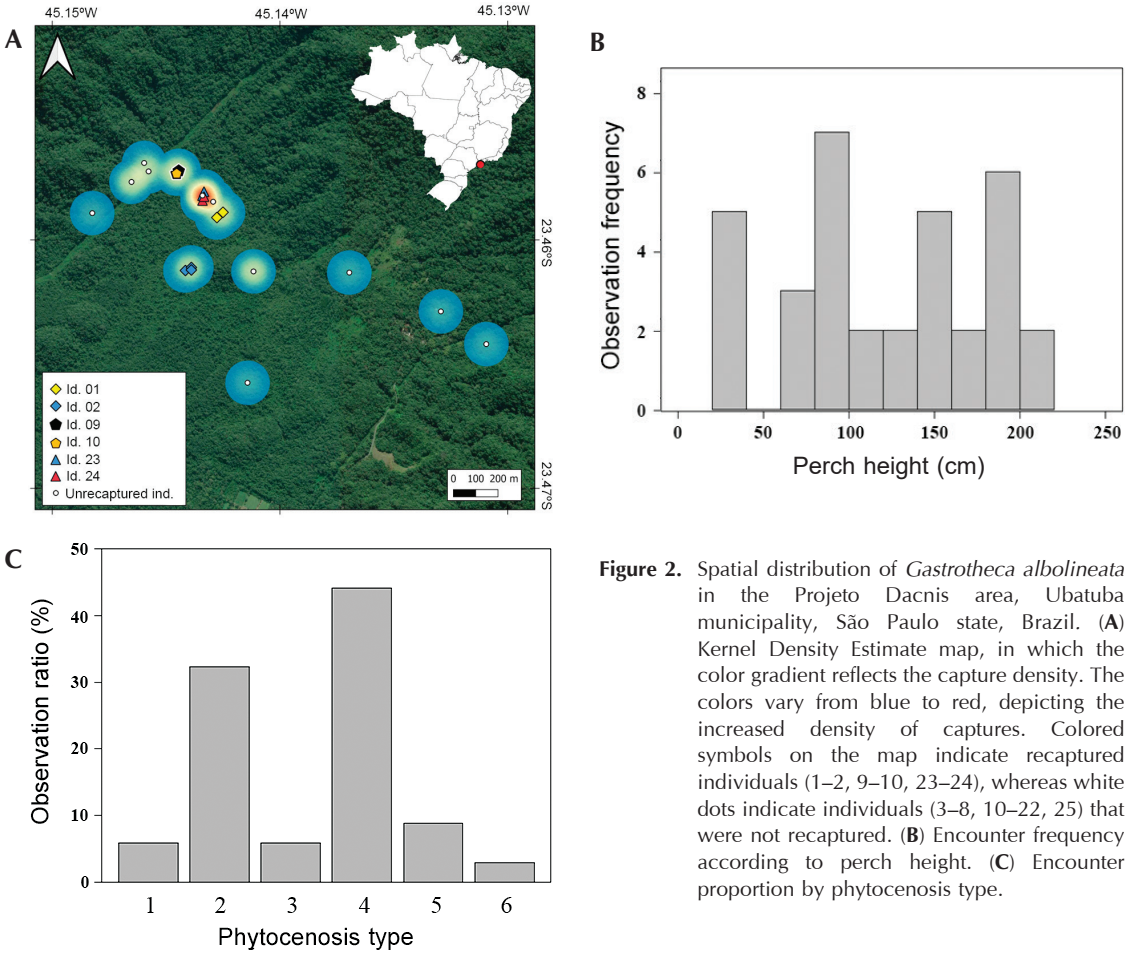

Figure 2. Spatial distribution of *Gastrotheca albolineata* in the Projeto Dacnis area, Ubatuba municipality, São Paulo state, Brazil. (**A**) Kernel Density Estimate map, in which the color gradient reflects the capture density. The colors vary from blue to red, depicting the increased density of captures. Colored symbols on the map indicate recaptured individuals (1–2, 9–10, 23–24), whereas white dots indicate individuals (3–8, 10–22, 25) that were not recaptured. (**B**) Encounter frequency according to perch height. (**C**) Encounter proportion by phytocenosis type.

Table 2. Recaptures of *Gastrotheca albolineata* in the study area in the municipality of Ubatuba, São Paulo state, Brazil. Distance calculated as a straight line between consecutive captures.

ID	Capture	1st Recapture	Distance (m)	2nd Recapture	Distance (m)
01	30/07/2015	06/10/2015	20	29/08/2016	< 2
02	23/08/2015	30/09/2015	< 2	29/08/2016	100
09	23/07/2016	03/08/2016	< 2	30/08/2016	< 2
10	31/07/2016	30/08/2016	< 2	-	-
23	02/08/2019	13/08/2019	< 2	-	-
24	08/08/2019	13/08/2019	< 2	-	-

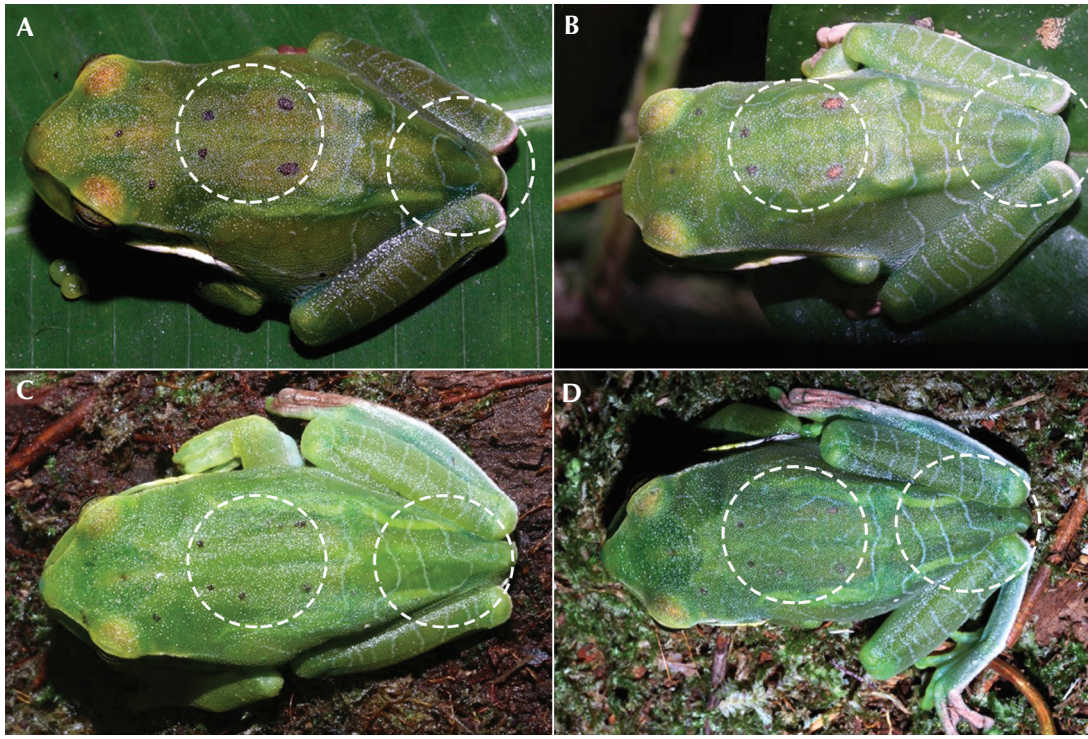

Figure 3. Examples that validate the recapture photo identification of individuals of *Gastrotheca albolineata* in the Projeto Dacnis area, Ubatuba municipality, São Paulo state, Brazil. (**A, B**) Individual 1 recaptured for the first time on 06 October 2015 and again on 29 August 2016; (**C, D**) Individual 2 recaptured on 30 September 2015 and recaptured again on 29 August 2016. Dashed circles indicate natural marks (dark spots and light lines).

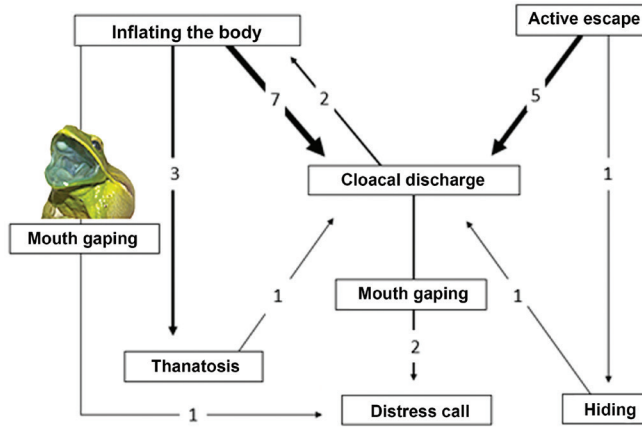

Figure 4. Ethogram of defensive behaviors displayed by *Gastrotheca albolineata*. The arrows and the numbers indicate the frequency of observation and the sequence in which each defensive behavior was displayed. The thicker arrows point out the most common defensive behaviors.

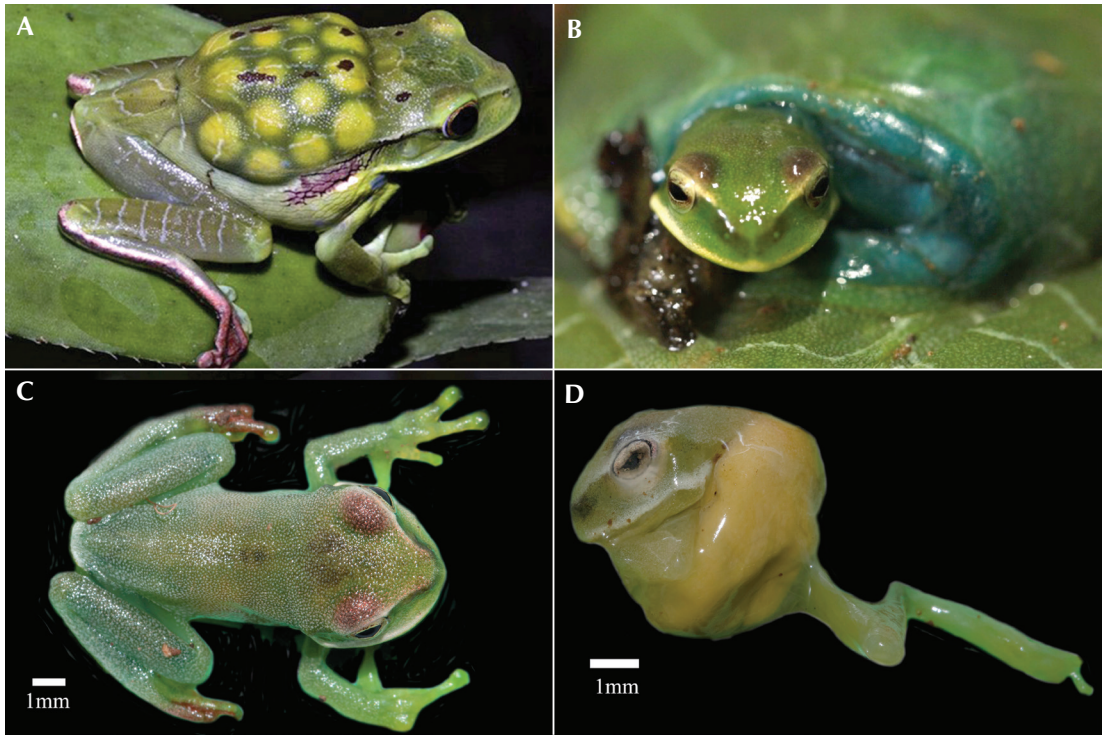

Figure 5. (**A**) *Gastrotheca albolineata* female (Id20) with eggs in her dorsal pouch. (**B**) A froglet emerges from the pouch (note bluish color of internal pouch membrane). (**C**) A newly expelled froglet. (**D**) A dead embryo with incomplete development.

push the rest of the froglets out of the pouch. This last effort resulted in two live froglets (Figure 5C) and four dead embryos (Figure 5D) expelled from the pouch. Live newly released froglets averaged 16 ± 1.68 mm SVL and 0.34 ± 0.05 g, while dead froglets averaged 12 ± 3.22 SVL mm and 0.25 ± 0.08 g.

Discussion

Monitoring Data and Habitat Use

Our data indicate that *G. albolineata* reproduces mainly during the dry season (mostly in July and August, austral winter), which agrees with Pontes *et al.* (2012), who found males calling on trees during the dry season. We recorded no breeding activity outside of the dry season. During our study, several *G. albolineata* males vocalized during cold, windy nights, which matches observations in the escarpments of the municipality of Nova Friburgo, state of Rio de Janeiro, Brazil (Renato Bernils, pers. comm.). We recorded calling activity of *G. albolineata* during the day, but mostly during the first half of the night, the same as observed in other Atlantic Forest species of *Gastrotheca* (Monteiro *et al.* 2019). We often found *G. albolineata* in low to medium understory up to 3–4 m high, in areas with a deep leaf litter layer and abundant bromeliads and lianas, which agrees with a similar habitat description for *G. microdiscus* (Monteiro *et al.* 2019). Several species of *Gastrotheca* are arboreal and seem to

prefer perches 1–4 m high (Catenazzi and Von May 2011, Teixeira Jr. *et al.* 2012, Monteiro *et al.* 2019), which again agrees with our findings.

Non-Invasive Mark-Recapture

Using natural body markings and photography as an individual marking technique is non-invasive, but restricted to species with distinct and identifiable color patterns (e.g., Lama *et al.* 2011, Caorsi *et al.* 2012). Further studies may use photographic identification of *G. albolineata* because each adult individual has a unique combination of lines and dorsal spots that are easily recognizable. Using this technique, we were able to conclude that *G. albolineata* displays site fidelity at least during the breeding season, and that males remain close to the same calling places for long periods. A similar pattern is reported for *Gastrotheca marsupiata* (Duméril and Bibron, 1841), which uses the same places for at least four consecutive weeks (Sinsch and Joermann 1989).

Defensive Behaviors

Inflating the body was the second most frequent defensive behavior observed, and *G. recava* Teixeira, Vechio, Recoder, Carnaval, Strangas, Damasceno, Sena, and Rodrigues, 2012 and *G. megacephala* Izecksohn, Carvalho-e-Silva, and Peixoto, 2009 employ this strategy frequently (Lourenço-de-Moraes *et al.* 2016). Thanatosis is reported only for *G. albolineata* (Muscat and Rotenberg 2016) and *G. megacephala* (Lourenço-de-Moraes *et al.* 2016). We also observed synergistic behaviors in several individuals. The combination of different behaviors can increase the defensive effectiveness against a variety of predators in different contexts (Toledo *et al.* 2011).

Egg Development, Parturition, and Newly Hatched Froglet Data

The direct-development reproductive mode, as observed in *Gastrotheca albolineata,* is linked to a series of physiological modifications (Duellman 2015, Warne and Catenazzi 2016, Del Pino 2018). One of its limitations is how many eggs the female can brood. The number of eggs carried by the two egg-brooding females in our study is close to the 16 eggs previously reported (Caramaschi and Rodrigues 2007) and is similar to those of other direct-development *Gastrotheca* species such as *G. fissipes, G. megacephala,* and *G. recava* (Caramaschi and Rodrigues 2007, Izecksohn *et al.* 2009, Teixeira *et al.* 2012). The number of eggs produced by species of *Gastrotheca* with direct egg development is smaller than in species that rely on indirect egg development. For example, Del Pino and Escobar (1981) report 128 eggs for the indirect egg development *G. riobambae* (Fowler, 1913). The egg diameter is larger than in indirect-development species of *Gastrotheca:* indirect cycle species, 2.3–6.3 mm; direct cycle species, 4.0–13.2 mm (review in Duellman 2015). The eggs of *G. albolineata* have among the largest diameters reported for any species of *Gastrotheca* (Duellman and Köhler 2005, Duellman and Chavés 2010, Gagliardo *et al.* 2010, Del Pino 2018). The behavior of the female pushing the embryos out of the dorsal pouch with her feet has been reported both for species with direct egg development that release their froglets in the water (Del Pino and Escobar 1981) and for species with indirect egg development (Del Pino and Escobar 1981, Duellman and Köhler 2005). A female *G. recava* kept in captivity for a month produced 20 live froglets weighing 0.8 g each (Teixeira *et al.* 2012), which is twice the weight of *G. albolineata* froglets. Duellman and Chavés (2010) induced expulsion of froglets in *G. testudinea* (Jiménez de la Espada, 1870); the froglets had SVLs of 9.4–11.7 mm, but only those over 11 mm were fully developed. Size of newly expelled froglets of *G. albolineata* in our study follows the same pattern as that reported by Duellman and Chavés (2010). Although most frogs are strictly lecithotrophic (including marsupial species with indirect egg development),

matrotrophy was reported in the direct-development species *G. excubitor* Duellman and Fritts, 1972 (Warne and Catenazzi 2016), so it is possible that female brood investment in *G. albolineata* exceeded pre-fecundation yolk production, and that the nutritional condition in captivity affected egg development and expulsion of live froglets.

As a final note, our four-year study substantially adds to the knowledge of natural history of *G. albolineata,* an elusive treefrog endemic to the Atlantic Forest of southeastern Brazil. Natural history oriented studies are fundamental to conservation projects, both of a target species or the biome in which the target species dwells (Michaels *et al.* 2014).

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Appendix I. *Detailed embryo development of* Gastrotheca albolineata *recorded from a captive female in the municipality of Ubatuba, São Paulo state, Brazil.*

Date	Egg diameter (mm)	Embryo morphology
29 July 2019	6	Eggs yellowish-green with no clear embryonic structure
9 August 2019	6	Increase in the number of vessels irrigating the egg. The embryo eyes were visible as two symmetrical dark spots
18 August 2019	7	A thin, dark, median line appeared in the middle of the egg
2 September 2019	7	Yolks decreased in volume, taking up about 80% of the eggs. Head contours visible in some of the embryos
27 September 2019	8	First embryo movements inside the pouch. The yolk took up about 50% of the egg
01 October 2019	10	The yolk occupied barely 20% of the egg, and the embryos were constantly moving
10 October 2019	12	Most of the yolks had disappeared
19 October 2019	-	First froglet ejected from the dorsal pouch
20 October 2019	-	The female expelled two live froglets and seven dead
21 October 2019	-	Two live froglets left the mother's pouch by themselves
23 October 2019	-	The internal pouch membrane completely everted. Two live froglets and four dead embryos were expelled from the pouch