



Escalated antipredator mechanisms of two neotropical marsupial treefrogs

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The sequence and intensity of antipredator mechanisms may be displayed according to the risk of predation. We tested this hypothesis using two species of marsupial treefrogs from Brazil's Atlantic Forest. We observed *Gastrotheca recava* and *G. megacephala* displaying nine antipredator mechanisms and three types of defensive calls. These behaviours were displayed in an escalated sequence from motionless (passive behaviour) to biting (the most aggressive behaviour). This diversified set of antipredator mechanisms may be related to the interaction between predator and prey at the local scale. The escalated sequence of defensive behaviours should be considered in future studies on anuran-predator interaction.

Key words: Amphibia, Atlantic forest, defensive behaviour, defensive call, *Gastrotheca*, Hemiphractidae

INTRODUCTION

Studies of predator-prey interactions continue to be one of the most fascinating and important aspects of ecological research (Mukherjee & Heithaus, 2013). Due to selective pressures from a variety of predators, anurans display a wide diversity of antipredator mechanisms (at least 31 types) that range from immobility to aposematic behaviours and defensive calls (Toledo et al., 2010, 2011; Jared et al., 2011; Haddad et al., 2013). Anuran antipredator mechanisms are diverse not only in their variety but also how and when they are displayed.

The diversity of antipredator mechanisms of amphibians may depend primarily on the characteristics of their skin secretions (Brodie, 1977, 1983), which may be noxious and toxic (Daly et al., 1987; Bevins & Zasloff, 1990; Erspamer, 1994) or adhesive (Arnold, 1982; Evans & Brodie, 1994). Skin secretions often act synergistically

with defensive behaviour and aposematic colour patterns (Johnson & Brodie, 1975; Brodie, 1977, 1983; Williams et al., 2000; Toledo et al., 2011).

Studies have shown that an individual frog may display several behaviours, which are presumably exhibited according to the degree of stress imposed by the predator (Williams et al., 2000). Some behaviours are displayed exclusively during the approach and others exclusively during handling of the potential predator (Toledo et al., 2005; Ferreira et al., 2013; Lourenço-de-Moraes et al., 2014). In addition, studies have shown that there are differences of antipredator mechanisms across species, populations, and sexes (Williams et al., 2000; Toledo et al., 2005; Heyer & Giarretta, 2009).

Predation involves several phases such as locate, identify, approach, subjugate, ingest, and digest prey (Edmunds, 1974). Therefore, one can hypothesise that the more types of antipredator mechanisms a species displays, the more likely it will escape from different

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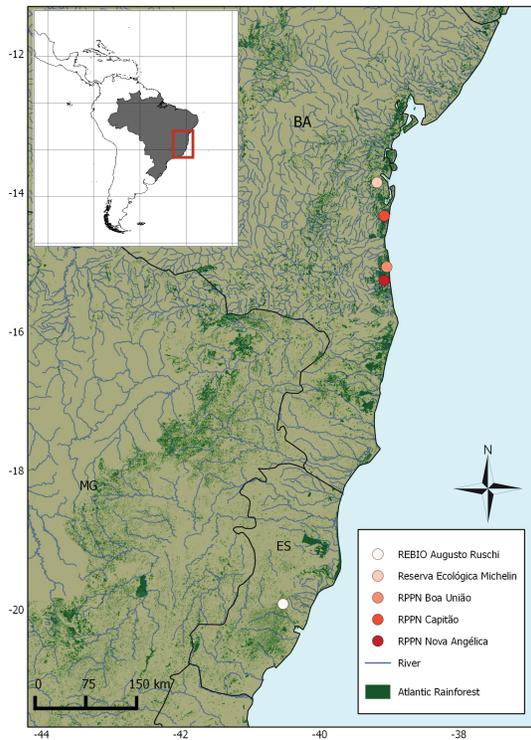


Fig. 1. Sampling sites in the Brazilian states of Bahia (BA) and Espírito Santo (ES).

predators. However the sequence of the behaviours may be more effective to defend the potential prey than only the variety of behaviours. To test this hypothesis we evaluated antipredator mechanisms of two sympatric species of Neotropical marsupial treefrogs, *Gastrotheca recava* and *G. megacephala*. In addition we described the defensive calls of both species.

MATERIALS AND METHODS

Study areas

We performed the fieldwork in Atlantic forest remnants across the states of Bahia and Espírito Santo, Brazil

during two distinct periods. The first during February and November 2010 in four forest reserves in the south of Bahia state: 1) Reserva Particular do Patrimônio Natural (RPPN), RPPN Capitão, municipality of Itacaré (14°19'S, 39°04'W, 123 m a.s.l.); 2) RPPN Nova Angélica, municipality of Una (15°15'S, 39°04'W, 79 m a.s.l.); 3) RPPN Boa União, municipality of Ilhéus (15°04'S, 39°02'W, 85 m a.s.l.); and 4) Reserva Ecológica Michelin (REM), municipality of Igrapiúna (13°50'S, 39°10'W, 95 m a.s.l.). The second sampling period was conducted during December 2012, and June and July 2013 in a rocky outcrop of a private property (19°54'S, 40°31'W, 878 m a.s.l.) inserted in the buffer zone of a biological reserve (REBIO - Reserva Biológica Augusto Ruschi), municipality of Santa Teresa, state of Espírito Santo (Fig. 1).

Sampling

We conducted the sampling at night with two to four observers through both active and calling surveys. We conducted focal animal sampling to evaluate their antipredator behaviours in the field (Altmann, 1974). In some cases ($n=10$), treefrogs were brought to the laboratory for tests of predator attack simulation (below). Frog sex was determined in the laboratory based on the presence or absence of dorsal pouch, exclusively present in females (Duellman, 1992). Voucher specimens were deposited at three zoological collections: Universidade Estadual de Santa Cruz, Ilhéus, state of Bahia, Brazil (*G. recava*: MZUESC 9291), Museu de Zoologia "prof. Adão José Cardoso", Universidade Estadual de Campinas, Campinas, state of São Paulo, Brazil (*G. recava*: ZUEC 16611-12, 16643-44, and *G. megacephala*: ZUEC 16650), and Museu de Biologia Professor Mello Leitão, Santa Teresa, state of Espírito Santo, Brazil (*G. megacephala*: MBML 7590-92, 7633).

Simulation of predator attack

We adopted two methods for simulating the predator attack (details in Table 1). In the first method (finger-only stimuli) the researcher's fingers were used to stimulate the frogs, considering humans as possible predators (e.g., Cooper et al., 2008). For the second method (multiple stimuli) we used forceps, human fingers,

Table 1. Description of stimuli methods to elicit antipredator mechanisms.

	Stimulus	Degree of stress	Description
Finger-only stimuli	1	Low	Approaching the frog without touching it
	2	Intermediate	Touching a finger on frog's dorsal, chin, and lateral regions
	3	High	Holding the frog and lightly squeezing it with fingerprints
	4	High	Releasing the frog and tapping it with the fingertips
Multiple stimuli	1	Low	Approaching the frog without touching it
	2	High	Holding the frog and lightly squeezing it with fingerprints
	3	Low	Moving a plastic-tarantula spider toward the frog without touching
	4	Low	Moving an alive snake toward the frog without touching it
	5	Intermediate	Touching frog's dorsal, chin, and lateral regions with metal tweezers
	6	Intermediate	Tapping frog's dorsal, chin, and lateral regions with metal tweezers
	7	High	Pinching the anterior and posterior frog's limbs with metal tweezers

Table 2. Antipredator mechanisms displayed by *Gastrotheca recava* (six males and four females) and *G. megacephala* (five males).

Behaviour	<i>Gastrotheca recava</i>	<i>Gastrotheca megacephala</i>
Biting	♂ 5 ♀ 1	♂ 3
Body-tilting	♂ 1	♂ 2
Cloacal discharge	—	♂ 2
Contracting	♂ 2 ♀ 1	—
Flee away	♂ 5 ♀ 2	♂ 4
Motionless	♂ 6 ♀ 4	♂ 5
Mouth-gapping	♂ 3 ♀ 2	♂ 2
Puffing-up the body	♂ 6 ♀ 4	♂ 5
Thanatosis	—	♂ 1
Alarm call	—	♂ 1
Distress call	♂ 3 ♀ 1	♂ 4
Warning call	♂ 3 ♀ 1	♂ 4

plastic spider, and live snakes (*Xenodon neuwiedii*) (modified from Williams et al., 2000). Finger-only stimulus was applied on both species (10 individuals of *G. recava* and one individual of *G. megacephala*) and the multiple stimuli were applied only to four individuals of *G. megacephala*.

Call recording and analysis

We recorded the defensive calls with a Marantz® PMD 660 digital audio recorder with one unidirectional Sennheiser® ME45 microphone and a K6 power module positioned 50 cm from the calling individual. Recordings were analysed at a resolution of 16 bits at a sampling rate of 48 kHz. Waveform and spectrogram were analysed using the Software Raven Pro v. 1.4 with a Fast Fourier Transform (FFT) of 256 points, 50% overlap for an entire call and Window Hamming format. For all other configurations the default settings of Raven were used. Call terminology follow Duellman & Trueb (1994). Defensive call terminology follows Toledo et al. (2015) that defined three defensive call types: warning, distress and alarm calls. The intensity of call was classified as follows: type 1 (emitted after stimulus 2 of finger-only method); type 2 (emitted after stimulus 3 of finger-only method); and type 3 (emitted after stimuli 3 and 4 of finger-only method and stimulus 2, 5, 6 and 7 of the multiple stimuli method).

RESULTS

Fifteen individuals of *Gastrotheca* (10 *G. recava* and 5 *G. megacephala*) displayed a total of nine antipredator behaviours (Table 2; Figs. 2 and 3) and three defensive calls (Table 2). The behaviours motionless, thanatosis, body-tilting, cloacal discharge and skin secretions were the first recorded for the subfamily Hemiphraetinae (Table 3). Most antipredator behaviours were displayed in synergy with other types (except for motionless). Sequentially after motionless, frogs either displayed contracting or thanatosis (Fig. 4). Both species sequentially displayed motionless, puffing-up the body,

and mouth-gapping, followed by defensive calls and biting.

Gastrotheca megacephala displayed eight antipredator behaviours and three defensive calls (distress, warning and alarm calls). Cloacal discharge, thanatosis and alarm call were only displayed by this species. *Gastrotheca megacephala* showed similar

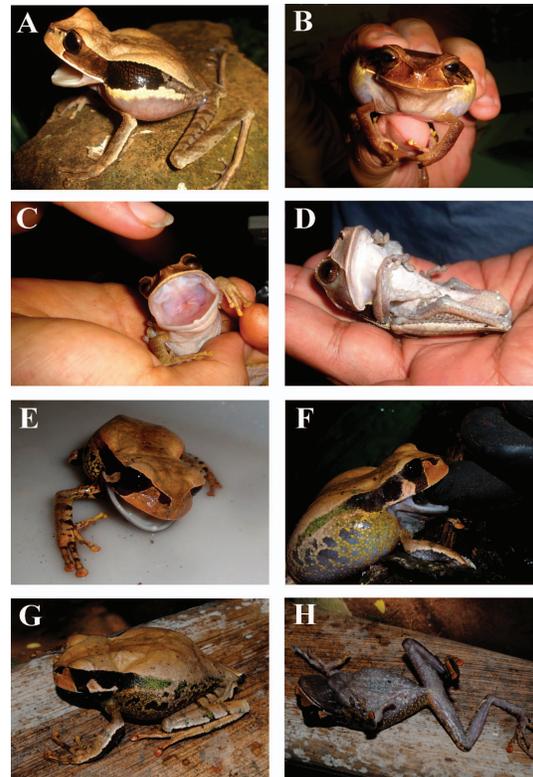


Fig. 2. *Gastrotheca recava* displaying: puffing-up the body and mouth-gapping in synergy (A); puffing-up the body (B); puffing-up the body and mouth-gapping (C); contracting (D); *Gastrotheca megacephala* displaying body-tilting (E); biting (F); puffing-up the body (G); and thanatosis (H).

Table 3. Antipredator mechanisms reported for marsupial treefrogs of the subfamily Hemiphractinae.

Species	Motionless	Mouth-gapping	Puffing-up the body	Biting	Contracting	Defensive call	Flee away	Thanatosis	Body-tilting	Skin secretion	Cloacal discharge	References
<i>Gastrotheca fissipes</i>					X							Haddad et al., 2008
<i>G. helenae</i>		X				X						Duellmann & Trueb, 1994
<i>G. megacephala</i>	X	X	X	X		X	X	X	X	X	X	Present study
<i>G. microdiscus</i>					X							Haddad et al., 2008
<i>G. recava</i>	X	X	X	X	X	X	X		X	X		Present study
<i>Hemiphractus fasciatus</i>		X		X		X						Meyrs, 1996
<i>H. johnsoni</i>				X		X						Toledo et al., 2011
<i>H. scutatus</i>						X						Toledo et al., 2011
<i>Stefania woodleyi</i>		X	X	X		X	X					Kok et al., 2007

behavioural responses to both stimuli methods. Puffing-up the body and motionless were most frequently displayed by this species. Thanatosis was the least displayed. *Gastrotheca megacephala* emitted warning ($n=4$ individuals), distress ($n=4$ individuals), and alarm calls ($n=1$ individual).

Gastrotheca recava displayed seven antipredator behaviours and emitted distress calls ($n=4$ individuals) and warning calls ($n=4$ individuals). Contracting was only exhibited by this species. Most behaviours were observed in both males and females. Puffing-up the body and motionless were the most displayed behaviours. Puffing-up the body and mouth-gapping were also associated with active release of glandular secretions from the lateral surface (Fig. 3A, B). Body-tilting was the least displayed behaviour. Mouth-gapping was displayed for up to 30 seconds after the individuals were stimulated by the stimulus 2 and 3 of fingers-only method. *Gastrotheca recava* emitted warning ($n=4$ individuals) and distress calls ($n=4$ individuals).

Three types of warning calls were emitted by both species. Only type 1 could be analysed for *G. megacephala*, and only type 2 and 3 could be analysed for *G. recava*. The warning call type 1 consists of a note with eight pulses. The duration of the call was 28 ms. Pulses were analysed separately due to their different size and duration. The first and second pulses with 2.5 ms (± 0.7 ms), the third with 20 ms, and the fourth with 3 ms. The peak of dominant frequency was at 0.75 kHz, maximum frequency was 1.75 kHz, and minimum frequency was 0.29 kHz (Fig. 5A).

The warning call type 2 was recorded for a total of 11 defensive calls, consisting of a pulsed note with five to seven pulses (Fig. 5B). The duration of the call was 140 ± 4 ms (130–150 ms). Pulses were analysed separately because they had different duration. Thus, the duration of the first pulses was 15 ± 2 ms (9–119 ms) and the last pulse was 72 ± 6 ms (60–84 ms). Peak of dominant frequency was 1.49 ± 0.04 kHz (1.34–1.53 kHz), minimum frequency was 0.34 ± 0.12 kHz (1.70–

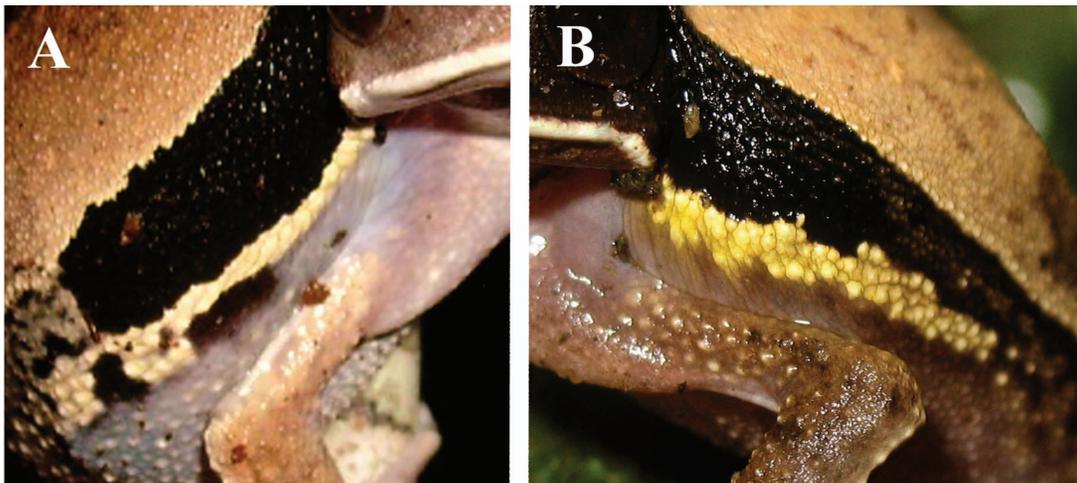


Fig. 3. *Gastrotheca recava* releasing skin secretions: (A) without secretions; (B) releasing yellow secretions.

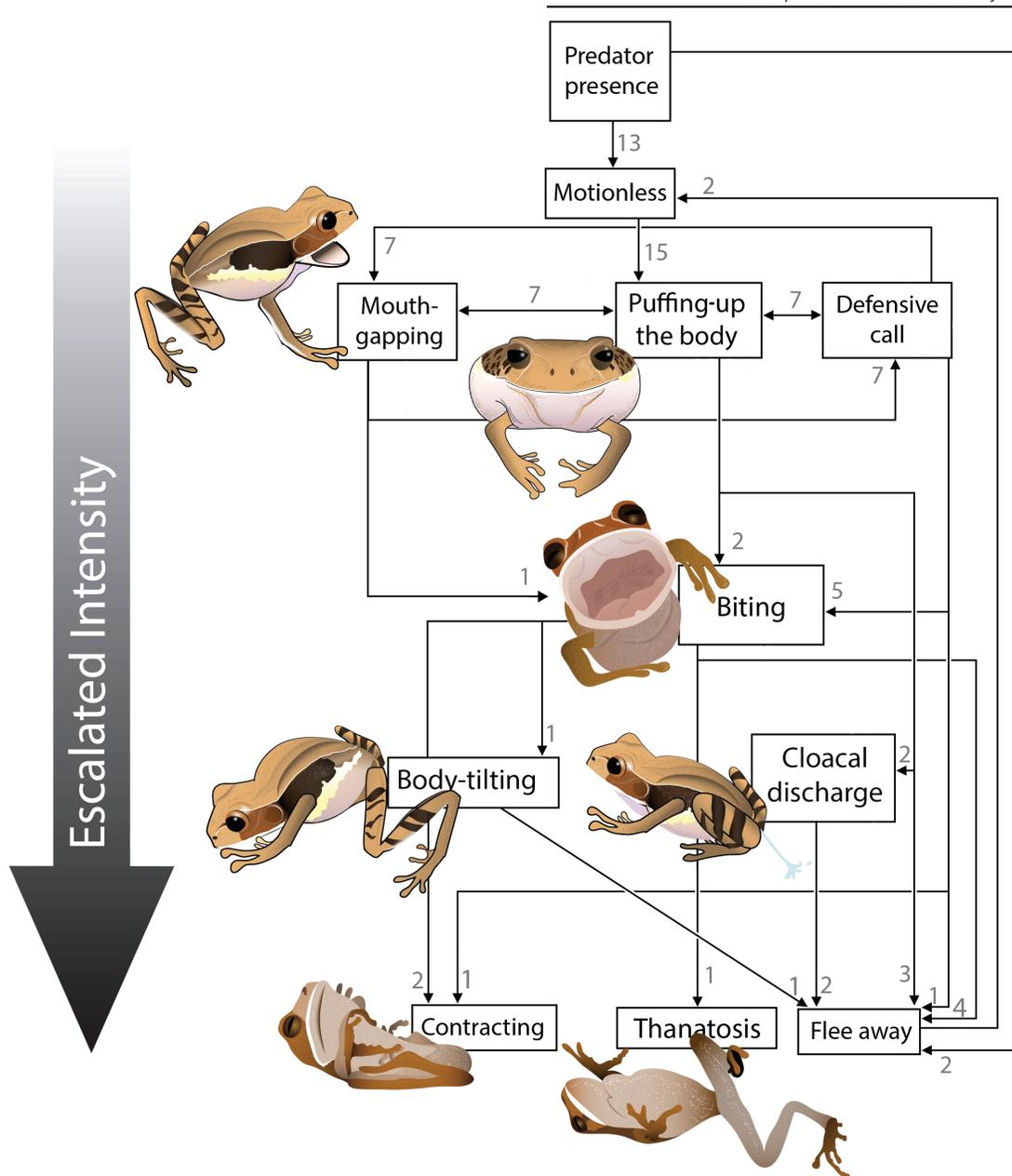


Fig. 4. Ethogram of the sequence of antipredator behaviours displayed by *Gastrotheca megacephala* and *G. recava*. Numbers by arrows indicate the number of individuals.

5.71 kHz), and maximum frequency was 10.89 ± 3.01 kHz (8.00–16.57 kHz). This type of call has harmonics, starting at 6 kHz.

The warning call type 3 (Fig. 5C) was recorded from 6 defensive calls, which consisted of one multipulsionate note and may have modulations at the beginning and end of the note. The pulses are irregular, and were not analysed. These calls had duration of 1940 ± 0.5 ms (1270–2640 ms). The peak of dominant frequency was 5.58 ± 0.37 kHz (5.11–6.05 kHz), minimum frequency was 1.64 ± 0.34 kHz (1.12–2.04 kHz), and maximum frequency 13.25 ± 0.02 kHz (10.54–15.74 kHz).

DISCUSSION

Our results show that the two species of marsupial treefrogs display an escalated sequence of antipredator mechanisms according to the stress level imposed by predator stimuli. The two species of *Gastrotheca* seem to flee away as a last resource of defense maybe because mobility represents a high-energy cost (Wells, 2007) or because postures are ineffective when the predator has already located the prey. In this study, two individuals fled away as a first antipredator strategy. Possibly because the individuals were already walking on tree branches.

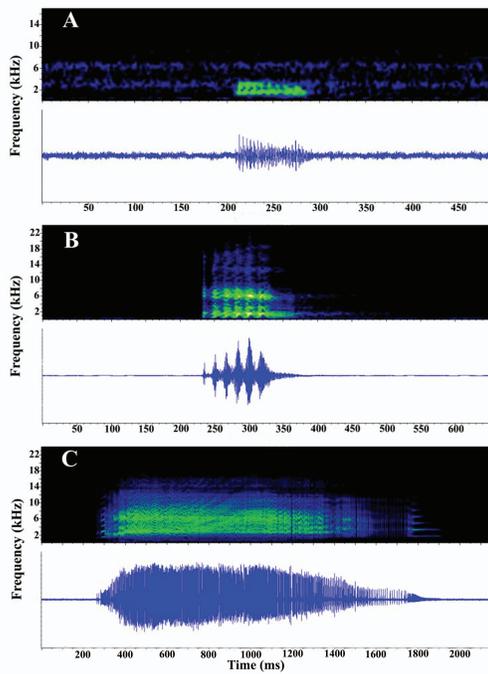


Fig. 5. Waveform (above) and spectrogram (below) of three warning calls from two species of *Gastrotheca*. Warning call type 1 of *G. megacephala* (A); warning call type 2 of *G. recava* (B); and warning call type 3 of *G. recava* (C).

The robustness and large size of *G. recava* and *G. megacephala* may be related to the evolution of aggressive behaviours such as puffing-up the body, defensive calls, mouth-gapping and biting. They initially avoid active behaviours when facing a potential predator. Only after additional stimuli do they make a posture such as contracting or thanatosis. Two individuals used cloacal discharge after puffing-up the body in synergy with flee away. Cloacal discharge may be used to deceive the predator and/or to enhance escape by reducing body weight (Toledo et al., 2011). The individuals that displayed cloacal discharge were captured walking on tree branches.

The studied *Gastrotheca* species tended to counter-attack at first (mouth-gapping, puffing-up the body and biting), and tended to flee away or display more passive (motionless) behaviours later, such as thanatosis or contracting. In fact, these latter two behaviours are used by several treefrogs as a secondary antipredator strategy (Toledo et al., 2011; Table 3). Haddad et al. (2008) recorded contracting (at that time misinterpreted as thanatosis: see Toledo et al., 2010) for *G. fissipes* and *G. microdiscus*. Contracting is often reported for Phyllomedusids and species of the genus *Rhinella*, highly toxic organisms that could induce the predator to regurgitate (Toledo et al., 2011). *Gastrotheca* spp. likely also produces skin toxins with similar effects on predators.

A visually oriented predator may avoid attacking frogs displaying puffing-up the body, because it increases the frog's size (Stebbins & Cohen, 1995; Williams et

al., 2000; Toledo et al., 2011; Haddad et al., 2013). Puffing-up the body may either intimidate a potential predator or avoid subjugation after being captured. Predators may also avoid attacking frogs that display mouth-gapping, because the frog exposes contrasting colours in their mouth, which could be interpreted as an aposomatic signal (Toledo et al., 2011). Perhaps biting can be effective against small sized and sensitive skinned predators (Wells, 2007). Biting is poorly documented for treefrogs; it is more common among heavy and large frogs such as *Ptychocheilus adpersus*, *Calyptocephalella gayi* and some species of *Ceratophrys*; these species can inflate lungs, raise body, open mouth, emit defensive calls, and can jump up and attack the potential predator (Veloso, 1977; Duellman & Trueb, 1994; Toledo & Haddad, 2009; Toledo et al., 2011). Mouth-gapping and biting seem to be related to a diet consisting of small vertebrates (Toledo et al., 2011). However, Teixeira et al. (2012) analysed stomach contents of *G. recava* and found only invertebrates; orthopterans were the most common prey. Besides diet, biting was suggested to be related to parental care (Toledo et al., 2011), as, while displaying mouth-gapping and puffing-up the body, females of *G. recava* were found with eggs in the dorsal pouch or with newly born young in the bromeliad axils (present study). These observations suggest that mouth-gapping and biting may be related to parental care. These aggressive behaviours may increase the survivorship of the young by intimidating the potential predator.

The three types of warning calls displayed by both species seem to be related to the intensity of stress stimuli. Individuals of both species under intermediate degree of stress displayed warning call type 1, in synergy with puffing-up the body. Individuals of *G. recava* under intermediate and high degree of stress displayed warning call type 2 in synergy with puffing-up the body and mouth-gapping. Warning call type 3 was emitted under the highest degree of stress and was in synergy with biting, mouth-gapping, puffing-up the body and flee away for *G. recava*; and with cloacal discharge while fleeing away for *G. megacephala*. Defensive call is presumably induced by apprehension as observed for both *Gastrotheca* species in our study and the same individual may use distress, alarm or warning calls. Defensive vocalisation may intimidate sound oriented predators such as birds and mammals (Toledo & Haddad, 2009).

During a predation attempt, an alarm call may be used for intraspecific communication (Toledo et al., 2015). We observed *G. megacephala* emitting alarm calls in the field, which presumably alerted other conspecifics because they stopped calling for about 15 minutes. Alarm calls have also been reported for other treefrogs, as *Hypsiboas bischoffi* (Toledo et al., 2015).

We suggest some defensive behaviours may be used as phylogenetic characters. Mouth-gapping, puffing-up the body, and biting were displayed by our studied *Gastrotheca* and also recorded for other species of Hemiphractinae such as *G. helenae*, *Hemiphractus fasciatus*, *H. johnsoni* and *Stefania woodleyi* which may

suggest a homology for marsupial treefrogs and could also constitute a synapomorphy for different clades. The diverse antipredator repertoire observed for both *Gastrotheca* species suggest that it may have resulted from an evolutionary adaptation to different predators. Furthermore, defensive behaviour displayed in an escalated sequence may be related to the distribution of species.

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