

# Multimodal signaling in *Boana albopunctata* (Anura: Hylidae): reading visual and acoustic cues

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## Abstract

**Multimodal signaling in *Boana albopunctata* (Anura: Hylidae): reading visual and acoustic cues.** The acoustic mode of communication is important for anurans, but visual communication is beginning to be considered essential for some species, genera, and families. This study focuses on visual and acoustic signals in *Boana albopunctata* (Hylidae: Cophomantinae) in an attempt to increase our understanding of signaling in this nocturnal Neotropical treefrog. Visual signals were assessed to determine whether they are directed toward conspecific opponents, as has already been observed for some diurnal anurans, or associated with potential morphological asymmetries. Associations between visual and acoustic signals were explored. The results suggest that males may combine visual and acoustic signals (multimodal signaling) synergistically, thereby strengthening the efficiency of information transmission. Thus, in nocturnal anuran species, visual signals may function primarily as an alerting component and multimodal signaling may be a relevant way of communication.

**Keywords:** alerting function, territoriality, visual signaling.

## Resumo

**Sinalização multimodal em *Boana albopunctata* (Anura: Hylidae): compreendendo os sinais visuais e acústicos.** O modo de comunicação acústico é importante para os anuros, mas a comunicação visual está começando a ser considerada essencial para algumas espécies, gêneros e famílias. Ao investigar sinais visuais e acústicos, o objetivo deste estudo é a melhor compreensão da sinalização em *Boana albopunctata* (Hylidae: Cophomantinae), uma perereca noturna neotropical. Foi verificado se os sinais visuais são direcionados aos oponentes coespecíficos, como já observado para alguns anuros diurnos, ou se são relacionados a potenciais assimetrias morfológicas. Estudou-se também associações entre sinais visuais e acústicos. Esse estudo leva à compreensão de que os machos podem combinar sinergicamente sinais acústicos e visuais (sinalização multimodal), potencializando assim a eficiência da transmissão de informação. Conclui-se que para espécies noturnas de anuros os sinais visuais podem atuar como componentes de alerta e que a sinalização multimodal pode ser um meio relevante de comunicação.

**Palavras-chave:** função de alerta, sinalização visual, territorialidade.

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## Introduction

Acoustic communication is important for intraspecific interactions of anurans and has been widely studied. Calls are of central importance to social organization, such as advertisements for conspecifics and establishment of breeding territory limits (Wells 2010, Brunetti *et al.* 2014, Toledo *et al.* 2015, Chuang *et al.* 2017). Calls also play a fundamental role as prezygotic reproductive isolation mechanisms, thereby affecting the processes of speciation (Blair 1958, Doherty and Gehardt 1983, Gerhardt 1994, Grenat *et al.* 2013). Visual communication also has begun to be recognized an important and even essential component of intraspecific communication in anurans, particularly for diurnal species (Haddad and Giarretta 1999, Hödl and Amézquita 2001, de Sá *et al.* 2016, 2018). Although subject to some restrictions for message transfer (e.g., shorter range and higher light-dependence), visual signals have a lower energy cost than acoustic signals and can be as efficient as any other mode of short-range communication (Alcock 2013). Despite its high dependence on light, visual communication has been observed as a potentially relevant message transfer channel, even among nocturnal anuran species (Aho *et al.* 1988, Cummings *et al.* 2008, Gomez *et al.* 2009).

There may be a diversity of both visual and acoustic signals in some anuran families, and the social context must be considered when attempting to understand what is being communicated (Hödl and Amézquita 2001, Toledo *et al.* 2015). The selection of repertoires and signal use within them may differ according to context, such as courtship, male combat, or territorial maintenance. We know, for example, that males can modify their calls during closer interactions with conspecifics to protect their territories and ensure reproductive success, such as *Acris blanchardi* Harper, 1947 (Hylidae; Wagner Jr. 1992, Burmeister *et al.* 2002), *Bokermannohyla ibitiguara* (Cardoso, 1983) (Hylidae; Nali and Prado 2014), and *Boana*

*goiana* (Lutz, 1968) (Hylidae; Morais *et al.* 2015). Also, recent studies reveal that associations among distinct communication modes are common (e.g., associations between acoustic and visual signals, or between tactile and visual signals) and these may transfer information more accurately. These synergistic associations represent multimodal communication and have been described for different anuran species, such as *Engystomops pustulosus* (Cope, 1864) (Leptodactylidae; Rosenthal *et al.* 2004), *Crossodactylus schmidti* Gallardo, 1961 (Hylodidae; Caldart *et al.* 2014), *Hylodes japi* de Sá, Canedo, Lyra, and Haddad, 2015 (Hylodidae; de Sá *et al.* 2016), and *Hyperolius puncticulatus* (Pfeffer, 1893) (Hyperoliidae; Starnberger *et al.* 2018).

Males of the treefrog genus *Boana* (Hylidae: Cophomantinae) typically possess morphological adaptations for physical confrontations (such as a spine on Finger I and hypertrophied arm muscles; Heyer *et al.* 1990) and exhibit aggressive and territorial behaviors. Indeed, de Sá *et al.* (2014) have already reported aggressiveness and territoriality for males of the nocturnal species *Boana albopunctata* (Spix, 1824). Although advertisement and territorial calls (Heyer *et al.* 1990, De La Riva *et al.* 1997, Kwet *et al.* 2002), and the visual signals—toe trembling, finger trembling, arm lifting, leg kicking, and vocal sac display (Toledo *et al.* 2007, Ramalho 2012, de Sá *et al.* 2014)—already have been described for this species, more detailed hypotheses about variation in the use of repertoires and synergistic associations of different signaling modes have yet to be explored. Much remains to be learned about both visual signaling in nocturnal anurans and multimodal signaling in anurans in general. Intensive efforts to collect data directly in the field and new approaches to explore signaling in anurans are needed to gather previous data from the literature and to improve our current knowledge, with the goal of informing our understanding of intraspecific communication in males of *B. albopunctata* in discrete social contexts.

Based on field studies, we describe the repertoires of visual and acoustic signals, along with the associated contexts of each in *Boana albopunctata*. We specifically tested whether: (1) visual signals are directed toward opponents, as previously observed for diurnal anurans, or if they are associated with potential asymmetric morphologies; and (2) visual and acoustic signals are performed together, demonstrating mutual message reinforcement and an alerting function (previously proposed for other species; Moller and Pomiankowski 1993, Hebets and Papaj 2005, Preininger *et al.* 2009, Grafe *et al.* 2012) and multimodal signaling in nocturnal anurans.

## Materials and Methods

### Study Site

We performed the study in permanent and temporary flooded portions of the Cantaclaro Ranch (22°19'36" S, 47°42'57" W; datum WGS 84), district of Itapé, municipality of Rio Claro, state of São Paulo in southeastern Brazil. The area is located in an ecotone between Cerrado (savannah vegetation) and Atlantic Forest (semi-deciduous seasonal forest). The current rural landscape contains zones of grazing and *Eucalyptus* plantations. The region has two well-established seasons—one wetter and hotter (rainy season), and the other drier and colder (dry season). The rainy season typically occurs from October–March, whereas the dry season occurs from April–September.

### Behavioral Data

All behavioral data were obtained in the field by means of in-situ natural observations during the rainy season in a total of seven expeditions between 15 October 2015 and 10 March 2016. We started to collect data immediately after the sunset (when the males began calling) and stopped when the calling activity subsided or ceased completely. We video-recorded and audio-recorded calls using a Sony Cybershot

DSC-W270 video camera (Sony Corporation) with supplementary lighting (using headlamps with red filters, which minimize disturbance to the focal males) and a Marantz PMD-660 audio recorder (Sound United, LLC) equipped with a Sennheiser ME-66 unidirectional microphone (Sennheiser electronic GmbH & Co. KG). The camera and microphone were positioned about 1.3 m from the calling male. We measured air temperature to standardize call recordings (Taigen *et al.* 1996, Guimarães and Bastos 2003, Wells 2010). We recorded and analyzed a total of 460 min of video and audio data (114 video files and 114 audio files).

### Repertoire of Visual Signals

To identify the visual signals of *Boana albopunctata*, we randomly selected and observed 30 breeding males. We filmed each individual under natural conditions for 2 min ( $N = 30$  videos) without interfering with their behavior; the context of these recordings is termed “advertisement.” Given that males *B. albopunctata* are territorial and remain at the same breeding site for months (de Sá *et al.* 2014), we chose distinct and well-spaced individuals to avoid duplication of frog recordings.

Data during “experimental territorial” contexts were gathered to understand the distinct use of signals (discussed in next section). We recorded all movements performed by resident males by watching all the video files generated and identifying visual signals that have been described in the literature (Hödl and Amézquita 2001, Hartmann *et al.* 2005). As defined in Hödl and Amézquita (2001), the following visual signaling behaviors included those that: (1) provide a visual cue during intraspecific interactions; (2) are redundant, conspicuous, and stereotyped; and (3) might provoke response by the conspecific receiver that will benefit the sender. We built the species repertoire of visual signals by concatenating the tabularized data, including the context in which each signal was performed.

### Experimental Analysis of Visual Signals

Some visual signals observed were directed to the right, to the left, or to both sides simultaneously—i.e., bilaterally. To detect whether males direct their visual signals toward an opponent, we conducted a field experiment with the same 30 males used for establishing the repertoire of visual signals. After the first 2 min of video recording under natural conditions, we inserted a conspecific male simulating an intruder in the territory of the resident male, with the “intruder male” being placed about 20 cm from resident male. As an intruder, we chose the first male randomly captured minutes before the experiment. We gently tied a nylon line to the waist of the intruder male to facilitate identification and move him wherever needed. We introduced the intruder in specific positions relative to the resident male: to his left side ( $N = 28$  videos), to his right side ( $N = 28$  videos), and in front of him ( $N = 28$  videos). To minimize interference, we employed a 2-min adaptation period from the introduction of the conspecific male to the beginning of the observations and video recordings of the resident male’s behaviors. We observed and recorded each intruder position for 2 min. We followed a random order for the positioning of the introduced conspecific male to avoid any potential bias. Experiments were classified as being in “territorial context.”

We analyzed the video files obtained from the experimental trials following the same methods used for the construction of the repertoire of visual signals, with the addition of recording where the intruder was positioned with respect to the resident male. We tested whether resident males chose sides for signals that can be performed by distinct limbs independently, according to the intruder’s position. For this we counted the number of times that each signal was performed on both sides simultaneously (frontal signal), only on the right side (right signal), and only on the left side (left signal). The frontal signal is likely detected by the intruder regardless of where the it was positioned,

whereas the right signal is likely more detectable by an intruder placed to the right side of the signaler, and the left signal is likely to be detected by placing an intruder to the left of the resident male. We excluded arm lifting, face wiping, and leg kicking in this analysis because they were so rarely recorded. The vocal sac display was excluded because it is not a bilateral signal.

For all statistical analyses here and in the next sections we used BioEstat Version 5 software (Ayres *et al.* 2007). We conducted one-way ANOVA to investigate if there were significant differences between the frequency of visual signals performed on both sides simultaneously, only on the right side, or only on the left side. When we found significant differences, we ran a post-hoc Tukey test to investigate which specific groups differed.

To detect whether males direct their visual signals based on their body morphology, we verified potential morphological asymmetries (Dill 1977). We visited the Célio F. B. Haddad collection (CFBH) in the Departamento de Biodiversidade, Instituto de Biociências of Universidade Estadual Paulista “Júlio de Mesquita Filho” (UNESP), Rio Claro, São Paulo state, Brazil, and measured males of *B. albopunctata* from the Cantaclaro Ranch and nearby localities ( $N = 20$ ), as follow: 9 from Cantaclaro Ranch (CFBH 07438, 04144, 06052, 06673, 06676, 22088, 22090, 39114, and 39123); 2 from Floresta Estadual Edmundo Navarro de Andrade (22°25' S, 47°33' W; datum WGS 84; 20 km NW Cantaclaro Ranch, municipality of Rio Claro, SP, Brazil) (CFBH 22084 and 38517); and 9 from Estação Ecológica de Itirapina (22°13'10" S, 47°53'54" W; datum WGS 84; 22 km SE from ranch Cantaclaro, municipality of Itirapina, SP, Brazil) (CFBH 07059–07063, 38534, 38536, 38540, and 38541). We identified adult males by the presence of sexually dimorphic traits and measured morphological features based on Heyer *et al.* (1990) and Duellman (2001): finger and toe lengths, thigh length, tibia length, tarsus length, and foot length. A Student’s t-test was

used to evaluate the presence of morphological asymmetries in male *B. albopunctata*.

### *Calls Repertoire*

We conducted audio recordings simultaneously with the video recordings described above to detect advertisement and agonistic call types of the same sample of 30 males. We analyzed the audio recordings with Raven Pro Version 1.4 software using a sampling frequency rate of 48 kHz and 16-bit resolution in the mono pattern (Center for Conservation Bioacoustics 2011). Concatenating all calls recorded, we distinguished and counted the advertisement and agonistic calls emitted by detecting notes A, B, and T (A and B as advertisement notes, and T as a territorial note; *sensu* Toledo *et al.* 2007); by associating the calls with each behavioral context (i.e., advertisement and territorial), we documented the acoustic repertoire of the species. We conducted a Friedman test to compare the numbers of A, B, and T notes performed in different contexts. All calls selected and analyzed were recorded at similar air temperatures (27–30°C).

### *Multimodal Signaling*

From all the video files analyzed, we assessed potential associations between visual and acoustic signals, and measured the intervals of transition between these two signaling modes when they were associated. Three types of association occurred, as follow: (1) visual signal performed immediately before a call; (2) visual signal performed immediately after a call; and (3) visual signal and call performed simultaneously. If the transition time between two distinct signaling modes was less than five seconds, we considered them to be associated. The vocal sac display was not included in this analysis because the studied males remained with the vocal sac inflated for long periods of time, including periods when they were not vocalizing (configuring a continuous visual

signal). Face wiping and leg kicking signals were also not included in the statistical analysis (scarce samples for comparisons).

The total numbers of visual signals and multimodal signals were recorded to assess the relative importance of each. The average time of transition between signaling modes was calculated; the values obtained when visual signals preceded acoustic signals were compared with those obtained when visual signals followed acoustic signals. In the former case, we measured the amount of time from the end of the visual signal to the beginning of the acoustic signal, and in the latter case, we measured the amount of time from the end of the acoustic signal to the beginning of the visual signal. We applied Chi-square tests to verify whether there are significant preferences for performing both signal modes (visual and acoustic) simultaneously or asynchronously. For this test, we excluded data from arm lifting, arm wiping, and leg kicking (scarce samples). We applied the Wilcoxon matched-pairs test to determine whether there was a significant difference between the average transitional times between visual and acoustic modes, and vice-versa. By testing preferences and functions in multimodal signaling, we tested the propositions of mutual message reinforcement and of alerting function (Moller and Pomiankowski 1993, Hebets and Papaj 2005).

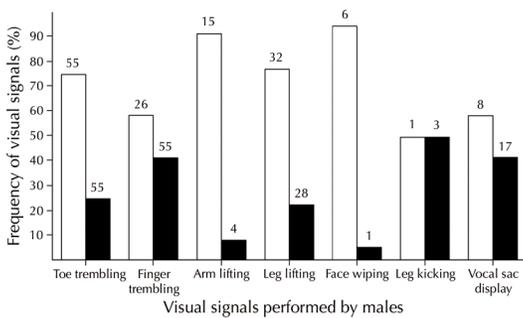
## **Results**

### *Repertoire of Visual Signals*

Male *Boana albopunctata* perform a variety of movements. We identified all the visual signals, as well as the primary occurrence of each visual signal relative to its frequency in discrete social contexts (Figure 1; Table 1). Toe trembling, finger trembling, arm lifting, leg lifting, face wiping, and vocal sac display are associated mainly in the context of advertisement, but also occur secondarily in the territorial context; leg kicking has a similar frequency in advertisement and territorial contexts. Vocal sac

**Table 1.** Repertoire of visual signals for male *Boana albopunctata* (N = 30) with a description and the respective contexts of occurrence.

| Visual signal                 | Description  | Context   |
|-------------------------------|--|---|
| Toe trembling<br>(N = 110)    | Fast lifting or trembling of one or more toes without a pattern. May be combined with finger trembling (N = 49).   | Mainly advertisement, with secondary occurrence in territorial context. |
| Finger trembling<br>(N = 81)  | Fast lifting or trembling of one or more fingers without a pattern. May be combined with toe trembling (N = 49).   | Mainly advertisement, with secondary occurrence in territorial context. |
| Arm lifting<br>(N = 19)       | Fast and short lifting of an arm, with it not passing above the head. May be combined with leg lifting (N = 6).  | Mainly advertisement, with secondary occurrence in territorial context. |
| Leg lifting<br>(N = 60)       | Fast and short lifting of a leg. It can be completely vertical or laterally oriented (N = 3). May be combined with arm lifting (N = 6) or face wiping (N = 5). | Mainly advertisement, with secondary occurrence in territorial context. |
| Face wiping<br>(N = 7)        | Lifting of one arm and setting upon the head. Similar to a head-whipping movement. May precede leg lifting (N = 5).  | Mainly advertisement, with secondary occurrence in territorial context. |
| Leg kicking<br>(N = 4)        | Fast and long movement of one leg. It is a fast stretching with the return of the leg to the initial position.   | Territorial and advertisement.  |
| Vocal sac display<br>(N = 25) | Vocal sac remains inflated between two calls (N = 24) or rarely when the male was not calling (N = 1; Figure 2).   | Mainly advertisement, with secondary occurrence in territorial context. |



**Figure 1.** Frequency of visual signals performed by male *Boana albopunctata* in the field without the introduction of a conspecific male (advertisement context; white bars) and with the conspecific male (territorial context; black bars). The total number of records for each signal is indicated at the top of the bars, but frequencies (%) from territorial contexts are divided by three to correct the three times longer recording in that context, and allowing comparison with frequencies from advertisement context.

display (Figure 2) is not associated with any other visual signal. To estimate the main contexts in which each signal occurs, the frequencies of each signal performed in the territorial context were divided by three. Because recordings in the territorial context were three times longer than those in the advertisement context, we divided the territorial frequencies by three to bring the values into parity with one another.

#### Direction of Visual Signals

On 13 out of 30 occasions, the resident male *Boana albopunctata* became immobile and quiet during the entire period that the conspecific intruder was inside its territory. However, in the other 17 observations, we occasionally observed the resident frog to shift the orientation of his body when the intruder was present. Sometimes



**Figure 2.** Male *Boana albopunctata* performing the vocal sac display.

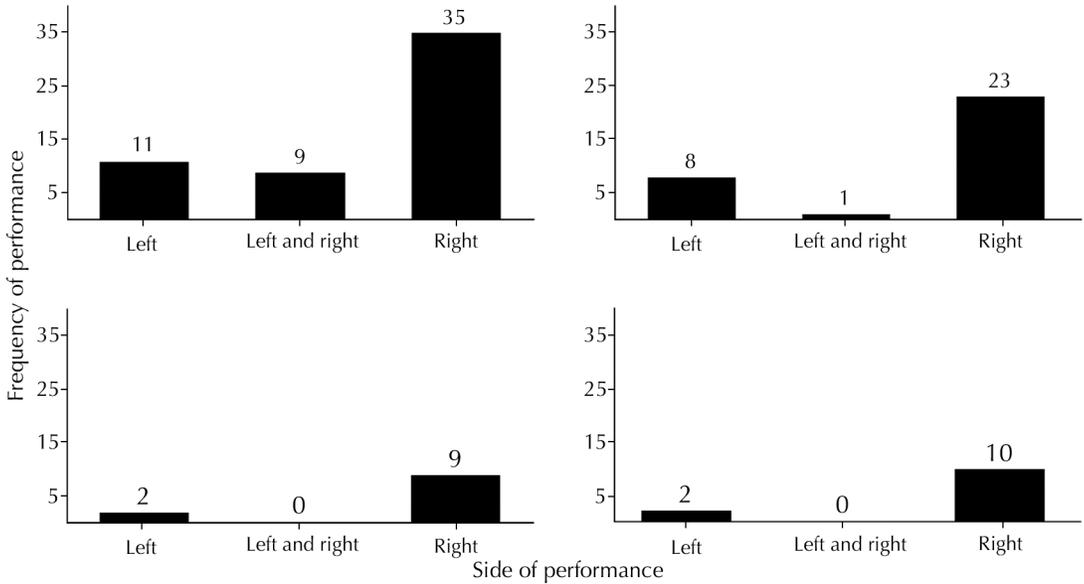
the resident male turned its entire body to the side where the intruder was located ( $N = 4$ , recorded in 2 males), whereas other times the resident male only turned its head toward the intruder ( $N = 5$ , recorded in 3 males). On two occasions, the resident male moved ahead and attacked the intruder. These three movements indicated some agonistic and territorial defense levels by the resident males. Moreover, twice the resident male was observed to jump and attack the intruder, which was obviously aggressive behavior associated with territorial defense. These responses indicate that resident males recognized the presence of the conspecific intruder and were bothered by the invasion of their territories.

We selected three visual signals to analyze intentional direction of visual signals by resident males—viz., toe trembling, finger trembling, and leg lifting. Toe trembling and finger trembling were performed more often with only the right foot or right hand, respectively, regardless of the location of the intruder (Figures 3, 4). Leg lifting was performed more often with only the left leg when the conspecific intruder was absent, but more often with the right leg when the intruder was present; these signals also were independent of the location of the intruder (Figure 5). According to a one-way ANOVA, the side of the

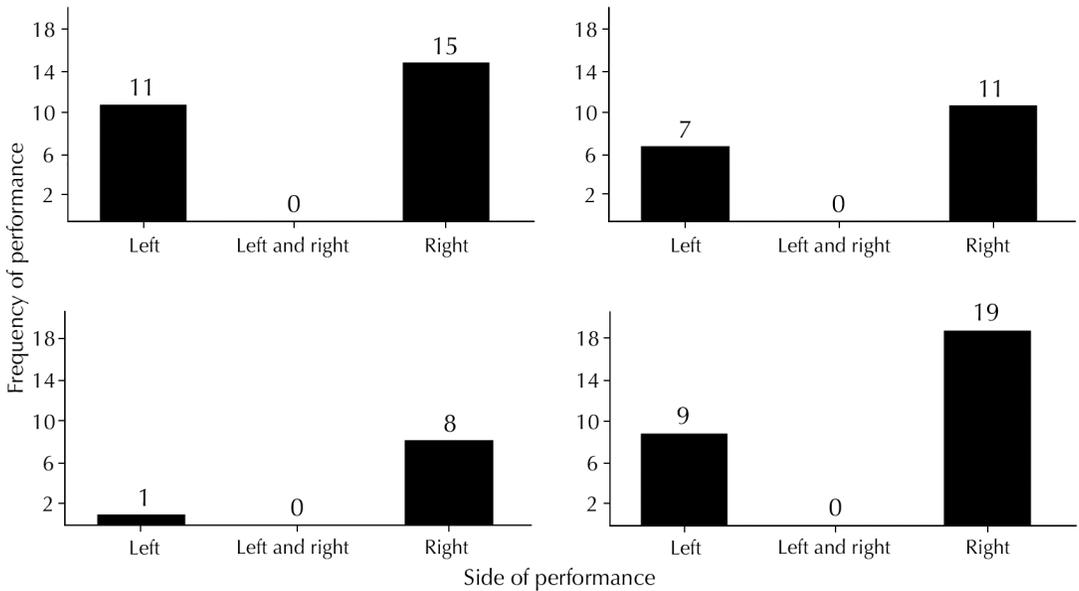
frog's body used for the toe- and finger-trembling signals are significantly different {Toe-trembling:  $[F(2, 9) = 4.979, p = 0.035]$  and finger trembling  $[F(2, 9) = 12.679, p = 0.028]$ }, whereas there is no significant difference for leg lifting  $[F(2, 9) = 1.653, p = 0.244]$ . A post-hoc Tukey test revealed significant differences between signals performed with the right side (most frequent) and with both sides simultaneously (least frequent) for toe trembling ( $p < 0.05$ ) and for finger trembling ( $p < 0.01$ ). The similarity of the sizes of all the right and the left fingers, toes, and forelimbs is statistically significant (Table 2).

### *Calls Repertoire*

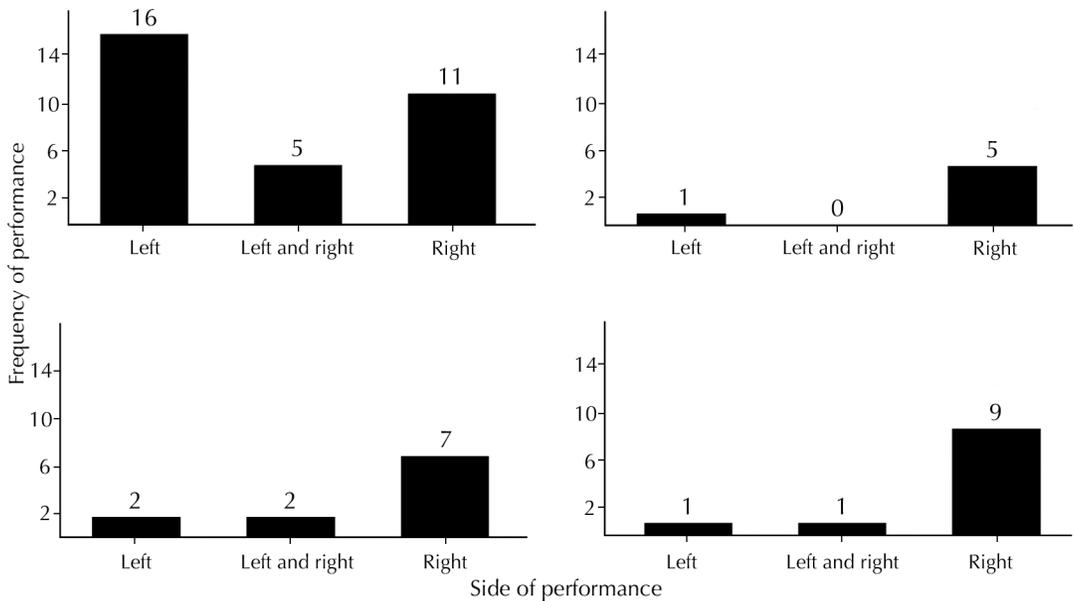
The calls of male *Boana albopunctata* are simple, having only one note per call (notes A, B, or T), and composite calls with two or more notes per call. We recorded the advertisement notes A and B, and the territorial note T (*sensu* Toledo *et al.* 2007) more frequently in the composite calls than in the simple calls. Some common note combinations for the composite calls were AABT, ABB, ABT, and BBT. When used, note A was always the first note emitted and always emitted in composite calls, whereas note T was always the last. Both notes A and B have similar dominant frequencies, with A having  $2.28 \text{ kHz} \pm 0.1$  ( $N = 56$ ; 2.06–2.43 kHz) and B having  $2.35 \text{ kHz} \pm 0.1$  ( $N = 33$ ; 2.06–2.43 kHz). Nonetheless, the notes can be distinguished by their durations, with A lasting  $0.37 \text{ s} \pm 0.04$  ( $N = 56$ ; 0.26–0.45 s) and B lasting  $0.56 \text{ s} \pm 0.12$  ( $N = 33$ ; 0.46–0.94 s). Note that T is clearly differentiated audibly from notes A and B (note T:  $N = 14$ ; dominant frequency of  $1.31 \text{ kHz} \pm 0.6$ ; 0.19–2.25). Also, T has a different duration from note A (note T:  $0.53 \text{ s} \pm 0.08$ ; 0.43–0.69 s;  $N = 14$ ). The number of notes A, B, and T emitted during distinct contexts (advertisement and territorial) is statistically similar [Note A  $\chi^2(1) = 0.833$ ;  $p = 0.361$ ; Note B  $\chi^2(1) = 0.533$ ;  $p = 0.465$ ; Note T  $\chi^2(1) = 0.133$ ;  $p = 0.715$ ]. Our results here confirmed the acoustic repertoire



**Figure 3.** Frequency of performance of the toe-trembling signal according to side by male *Boana albopunctata* in discrete contexts, (A) without and with conspecific intruder male (B) in front, (C) on the left, and (D) on the right side of the resident male. Signals were performed by the resident male using the left side, the left and right sides simultaneously, or the right side. The number of records for each direction is at the top of the bars.



**Figure 4.** Frequency of performance of the finger-trembling signal according to side by male *Boana albopunctata* in distinct contexts, (A) without and with conspecific intruder male (B) in front, (C) on the left, and (D) on the right side of the resident male. Signals were performed by the resident male using the left side, the left and right sides simultaneously, or the right side. The number of records for each direction is at the top of the bars.



**Figure 5.** Frequency of performance of the leg lifting signal according to side by male *Boana albopunctata* in distinct contexts, (A) without and with conspecific intruder male (B) in front, (C) on the left, and (D) on the right side of the resident male. Signals were performed by the resident male using the left side, the left and right side simultaneously, or the right side. The number of records for each direction is at the top of the bars.

described for the species (Haddad *et al.* 1988, Heyer *et al.* 1990, De la Riva *et al.* 1997, Kwet *et al.* 2002, Bastos *et al.* 2003, Toledo *et al.* 2007).

### Multimodal Signaling

There is an association between visual and acoustic signaling modes (visual and acoustic), thereby documenting multimodal signaling. All visual signals that were considered part of the multimodal signaling were performed by resident males immediately prior to calling, immediately after calling, or while in the process of calling. The vocal sac display, as it is defined, was performed when the male was not calling as a pure visual signal (Figure 2); however, by necessity, it was also inflated during call emission, presenting a multimodal signal. The other visual signals recorded ( $N = 281$ ; Table 1) often were performed in association with

acoustic signals ( $N = 268$ ). Therefore, around 95% of visual signals recorded were observed in association with acoustic signals, indicating a high degree of association between these two signaling modes (Figure 6).

There are statistically significant preferences for performing visual signals such as toe trembling, finger trembling, and leg lifting before or after calls based on Chi-square tests [ $\chi^2$  (2,  $N = 240$ ) = 9.836;  $p = 0.007$ ]. The interval between both modes together with the number of transitions (visual to acoustic and vice-versa) corroborated the revealed preferences (Table 3). The numbers of visual signals preceding and following calls are about the same (Table 3). For all visual signals that were statistically analyzed, the transition intervals between signaling modes with visual signals preceding calls are significantly shorter than the intervals with visual signals being performed after calls (Figure 7; Table 4).

**Table 2.** Measurements of fingers, toes, and forelimbs, and Student-t values from morphological asymmetry tests for male *Boana albopunctata* (N = 20). Vouchers are from Cantaclaro Ranch and nearby localities and are housed at Célio F. B. Haddad collection (CFBH) in the Departamento de Biodiversidade, Instituto de Biociências of Universidade Estadual Paulista (UNESP), Rio Claro, São Paulo state, Brazil. Separating traits bilaterally, average (mm) SD values for morphological measurements are shown with t- and p-values from statistical comparisons. All morphological traits measured are statistically symmetric ( $p > 0.05$ ).

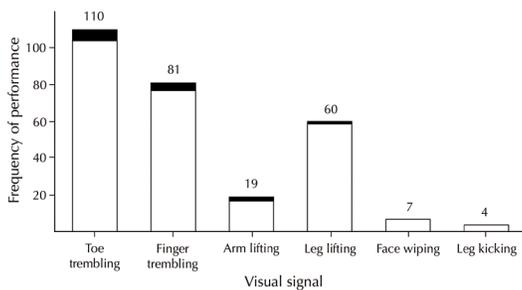
| Trait      | Left      | Right     | t     | p     |
|------------|-----------|-----------|-------|-------|
| Finger I   | 5.65 0.6  | 5.46 0.6  | 0.966 | 0.34  |
| Finger II  | 6.34 0.6  | 6.01 0.6  | 1.587 | 0.121 |
| Finger III | 8.1 0.87  | 7.83 0.71 | 1.043 | 0.304 |
| Finger IV  | 6.78 0.8  | 6.22 0.8  | 1.943 | 0.06  |
| Toe I      | 5.08 0.7  | 5.38 0.8  | 1.245 | 0.221 |
| Toe II     | 6.25 0.7  | 6.44 0.9  | 0.716 | 0.479 |
| Toe III    | 9 1.2     | 9 1.2     | 0.164 | 0.871 |
| Toe VI     | 12.8 1.4  | 12.5 1.6  | 0.466 | 0.644 |
| Toe V      | 8.3 0.8   | 8.5 1     | 0.504 | 0.618 |
| Thigh      | 26.22 2.1 | 25.92     | 0.427 | 0.672 |
| Tibia      | 27.28 2.1 | 27.25     | 0.052 | 0.959 |
| Tarsus     | 16.25 1.2 | 16.5 1.4  | 0.535 | 0.596 |
| Foot       | 20.3 1.8  | 19.9 1.8  | 0.664 | 0.511 |

**Table 3.** The number of transitions of visual signals performed before or after acoustic signals, and the number of visual signals performed simultaneously with acoustic calls. We also show average transition intervals between visual signals and acoustic signals performed by male *Boana albopunctata* (SD; N = 30 males).

| Visual signal    | Visual signal performed before call (N) | Visual signal and call performed simultaneously (N) | Visual signal performed after call (N) | Time from visual signal to call (s) | Time from call to visual signal (s) |
|------------------|---|---|--|-------------------------------------|-------------------------------------|
| Toe trembling    | 39%                                     | 25%   | 36%                                    | 1.69 1.3                            | 2.33 1.2                            |
| Finger trembling | 41%                                     | 21%   | 38%                                    | 1.6 1.3                             | 2.65 1.2                            |
| Arm lifting      | 46%                                     | 8%  | 46%                                    | 1.02 0.7                            | 2.36 1.3                            |
| Leg lifting      | 45%                                     | 12%   | 43%                                    | 1.42 1                              | 1.83 1.1                            |
| Face wiping      | 46%                                     | 0   | 54%                                    | 1.77 1.1                            | 1.78 0.1                            |
| Leg kicking      | 57%                                     | 0   | 43%                                    | 1.66 1.2                            | 2.15 0.8                            |

**Table 4.** Wilcoxon test (two tailed), matching pairs of transitions (s) from visual to acoustic signals and from acoustic to visual signals for male *Boana albopunctata* ( $N = 30$  males). All results were statistically significant ( $p < 0.05$ ), indicating that those time intervals are different.

| Visual signal    | Z      | P     | N (pairs) |
|------------------|--------|-------|-----------|
| Toe trembling    | -2.912 | 0.004 | 57        |
| Finger trembling | -1.716 | 0.001 | 37        |
| Arm lifting      | -2.49  | 0.013 | 11        |
| Leg lifting      | -2.12  | 0.034 | 37        |



**Figure 6.** Frequency of visual signals recorded in association with calls for male *Boana albopunctata* ( $N = 30$  males). White areas of the bars indicate the incidence of visual signals performed associated with calls and black areas indicate the incidence of visual signals not associated with calls. Numbers at the top of the bars indicate total frequency of performances for each visual signal.

## Discussion

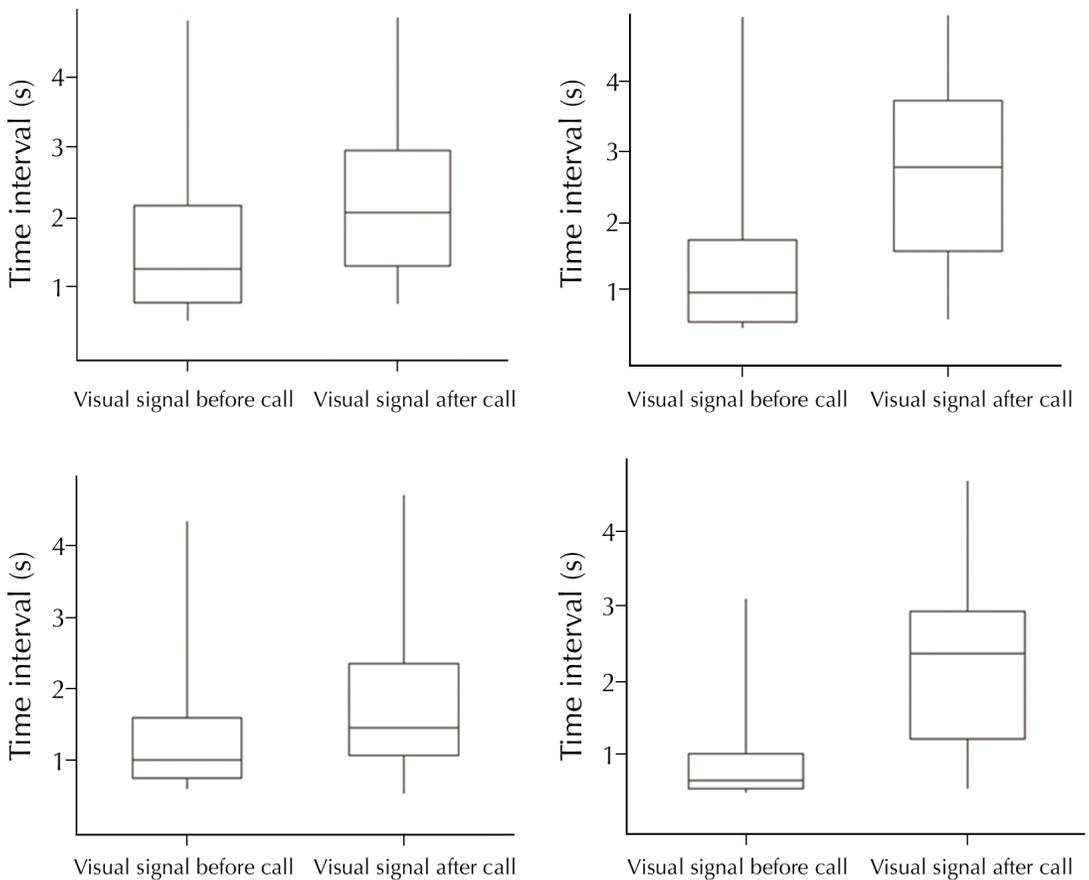
### Visual Signaling for *Boana albopunctata*

Our total of seven visual signals constitutes the most complete repertoire of visual signals for male *Boana albopunctata* reported thus far. These include all signals previously recorded for this nocturnal treefrog (Toledo *et al.* 2007, Ramalho 2012, de Sá *et al.* 2014), with the addition of two newly recognized signals (leg

lifting and face wiping), thereby improving the understanding of visual signaling by the species. We document that visual signaling by male *B. albopunctata* is more closely associated with advertisement than territorial contexts. Also, the observation that resident male frogs perform visual signals frequently in the absence of a conspecific intruder male further confirms that these stereotyped movements are true visual signals, not only a byproduct of some social excitement from visual detection.

Male *Boana albopunctata* do not direct their visual signals toward their conspecific receivers in territorial contexts. Overall, we found that the male frogs prefer to use their right sides to perform visual signals, regardless of the social context and the location of the intruder male. Our statistical analyses reveal that these preferences of sides are not associated with morphological asymmetries, in contrast from observations of *Pseudacris regilla* (Baird and Girard, 1852) (Hylidae; Dill 1977). As detected in the present study for *B. albopunctata*, preferential use of the right side has also been documented in other nocturnal frog species, such as *Bufo bufo* (Linnaeus, 1758) and *Rhinella marina* (Linnaeus, 1758) (Bufonidae; Bisazza *et al.* 1997, Robins *et al.* 1998). These reports coupled with the present study provide some insights into the evolution of brain lateralization among anurans, indicating that it might have a long evolutionary history, dating back to early tetrapods (Bisazza *et al.* 1997; Robins *et al.* 1998).

Behavioral studies of diurnal anuran species of the genera *Hylodes* and *Micrixalus* have demonstrated that individuals direct their visual signals toward their conspecific receivers (Preininger *et al.* 2013, de Sá *et al.* 2016). Male *Hylodes japi*, for example, perform visual signals by using their double vocal sacs independently, inflating either the right or left vocal sac depending on the location of the target conspecific (de Sá *et al.* 2016, Elias-Costa *et al.* 2017). Similarly, male *Micrixalus saxicola* (Jerdon, 1853) coordinate the use of their



**Figure 7.** Time interval of transition from visual signals to calls and from calls to visual signals from the visual signals analyzed for resident male *Boana albopunctata* ( $N = 30$  males): **(A)** toe trembling ( $N = 57$ ), **(B)** finger trembling ( $N = 37$ ), **(C)** leg lifting ( $N = 37$ ), and **(D)** arm lifting ( $N = 11$ ). Box plots show the median, mean (line in the middle of the box), maximum, and minimum (vertical lines above and below) time interval.

forelimbs toward the position of a conspecific (Preininger *et al.* 2013). Although the directionality of visual signals is unknown in nocturnal species of hylids, these species commonly have relatively rich repertoires of visual signals (as shown here for *B. albopunctata*), suggesting some level of importance for these stereotyped movements during intraspecific communication (Hartmann *et al.* 2005, Toledo *et al.* 2007, Gomez *et al.* 2009). Moreover, visual

signals in nocturnal hylids seem to function in attracting females, as reported for *Aplastodiscus eugenioi* (Carvalho-e-Silva and Carvalho-e-Silva, 2005) (Hartmann *et al.* 2004), *Boana curupi* (Garcia, Faivovich, and Haddad, 2007) (Lipinski *et al.* 2012), and *Scinax cardosoi* (Carvalho-e-Silva and Peixoto, 1991; = *Oloolygon cardosoi*) (Moroti *et al.* 2017). In addition, movements that produce visual signals also produce seismic signals (Narins 1990), especially

when on high perches, with less stable substrates, such as breeding sites. For example, in the nocturnal frog *Hyperolius puncticulatus*, tapping signals produce vibrations on the substrates, acting in close-range signaling (Starnberger *et al.* 2018). Because daylight is believed to have facilitated the evolution of visual signaling among anurans (Hödl and Amézquita 2001), it is possible that diurnal species may have more accurate and highly developed visual signaling than nocturnal species; however, it does not exclude the possibility that visual communication has evolved in nocturnal anurans. Nocturnal habits do not provide a significant barrier to signal perception among anurans because they can see well in the dark (Kelber *et al.* 2017, Mohun and Davies 2019).

#### *Multimodal Signaling for a Nocturnal Anuran Species*

Our results show that associations between visual and acoustic signals are surprisingly frequent in male *Boana albopunctata*. We noted that males tend to perform visual signals immediately before or after emitting calls, rather than performing them while calling. There is less time between visual signals performed before the start of a call than between the end of the call and the start of visual signals performed following it. Frequently, male *B. albopunctata* combine visual and acoustic signals to form multicomponent signals, with visual signals functioning as alerts for the calls. According to Hebets and Papaj (2005), a first signaling mode gets the attention of the receiver for the subsequent and associated signaling mode, with the latter carrying a more important message. This synergistic combination of distinct signaling modes may reinforce the message sent by an emitter to a conspecific (Moller and Pomiankowski 1993).

Previous information on multimodal signaling (visual and acoustic) has been reported for *B. albopunctata* (Toledo *et al.* 2007, Ramalho 2012) and for other hylids with nocturnal habits,

such as *Aplastodiscus perviridis* Lutz, 1950 and *B. bischoffi* (Boulenger, 1887) (Toledo *et al.* 2007), *A. eugenioi*, *B. albomarginata*, and *Scinax eurydice* (Bokermann, 1968) (Hartmann *et al.* 2005), and *Hyla arborea* (Linnaeus, 1758) (Gomez *et al.* 2009). Our findings open a new window of knowledge, better suggesting how visual signals might work for a nocturnal anuran. Visual signals in nocturnal anurans (likely more connected with advertisement contexts) may have a more subtle role, alerting and actually improving communication performed via calls. This possibility reinforces that in nocturnal anurans calls convey the main message during intraspecific communication, but it does not exclude an additional important communication role for visual signaling. As far as we know, we provide the first evidence that visual signals may have an alerting function among anurans (Moller and Pomiankowski 1993, Hebets and Papaj 2005). Similar tests as those conducted here have reported calls as the alerting message for subsequent visual signals among diurnal species of the genus *Staurois* (Grafe and Wanger 2007, Preininger *et al.* 2009, Grafe *et al.* 2012). Therefore, visual and acoustic signals may have distinct roles among diurnal and nocturnal anuran species, and the synergistic combination of visual and acoustic signals might have evolved differently, most likely affected by the presence or absence of daylight (Hödl and Amézquita 2001).

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