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Reproduction and population traits of the Atlantic forest tree frog *Bokermannohyla hylax*: an exploratory study in an urban forest fragment in São Paulo municipality, Brazil

FRANCINE LUENGO FERREIRA[1] & CYBELE SABINO LISBOA[2*]

[1]Mendelics Análise Genômica, 02511-000, São Paulo, SP, Brazil
[2]Zoológico de São Paulo - Reserva Paulista, Setor de Herpetofauna. 04301-905, São Paulo, SP, Brazil

[*]Corresponding author e-mail: cyb.lisboa@yahoo.com.br

ABSTRACT – *Bokermannohyla hylax* is an endemic tree frog from the Atlantic Forest and despite being a common species, very little is known about its population traits and reproduction. We undertook acoustic and visual encounter surveys, and individual marking studies of a subpopulation in an urban forest fragment, the Parque Estadual das Fontes do Ipiranga, located in the São Paulo municipality. We detected a small population that breeds in only one forest stream, and deposits eggs inside burrows along the stream's clayey border. Our findings also suggest that this subpopulation might be vulnerable due to the specific characteristics of its reproduction, which may be affected by the degree of disturbance of this fragment.

INTRODUCTION

Brazil has the greatest amphibian species richness in the world, currently comprising 1,188 species of mostly anurans (Segalla et al., 2021). About 52 % of these species occur in the Atlantic Forest domain (625 sp.; Rossa-Feres et al., 2017), one of the main biomes in Brazil considered a global Biodiversity Hotspot (Ribeiro et al., 2011). The characteristics of this biome, which has a rough topography and numerous types of humid microhabitats, favours the endemism of species and also the specialisation of reproductive modes (Silva et al., 2012; Rossa-Feres et al., 2017). As a result, the Atlantic Forest is home to the greatest diversity of anuran reproductive modes (Haddad & Prado, 2005).

The Atlantic Forest has experienced an intense historical process of deforestation and fragmentation with only approximately 12 % of its original area remaining today (Ribeiro et al., 2011). In some areas of the Atlantic Forest in south-eastern Brazil, the depletion of previously flourishing anuran communities has been recorded and this can be attributed to habitat degradation and fragmentation (Haddad & Prado, 2005; Ribeiro et al., 2011; Almeida-Gomes & Rocha, 2015). Studies on life-history are essential to better understand the species-area relationship, which is fundamental to the success of actions that preserve biodiversity (Almeida-Gomes & Rocha, 2015).

Bokermannohyla hylax Heyer, 1985, is an endemic tree frog from the Atlantic Forest with a wide geographic distribution in the south and south-east of Brazil, from the south of Rio de Janeiro to north of Santa Catarina states (Napoli, 2000). It is a medium-sized species which presents yellowish-brown tones in the dorsum with an inguinal band and the ventral surface of its thigh is a characteristic purple colour (Heyer, 1985). The genus *Bokermannohyla* comprises 30 species (Frost, 2022), of which only a few have had detailed studies of their reproductive behaviour.

Despite being a common species, almost nothing is known about the life history of *B. hylax* (Carvalho-e-Silva & Pavan, 2010). The current investigation focuses on a subpopulation that occurs in an urban forest fragment located in the São Paulo municipality and describes some reproductive behaviour of this species, including calling activity and clutch characteristics, and also makes an estimate of population size. We also evaluated the marking methods we used, verifying their effectiveness in meeting our goals.

MATERIALS & METHODS

Study Area

The study was carried out at the Parque Estadual das Fontes do Ipiranga (PEFI), in the municipality of São Paulo, São Paulo state, south-eastern Brazil (Fig. 1). It is an Atlantic Forest fragment (around 495 ha; São Paulo, 2013) surrounded by urbanisation, covered by vegetation in different successional stages with characteristics representing both dense ombrophilous and semideciduous forests (Tanus et al., 2012). For data collection, we visited the Pirarungáua Stream, which is a small tributary (around 400 m of its extension have riparian forest) of the Ipiranga River, one of the main rivers in the municipality of São Paulo (Formenton-Silva & Rancura, 2020). We sampled in this stream only because the reproductive activity of *B. hylax* occurs only at this point within the PEFI (Lisboa et al., 2021) and we didn't find any other suitable habitat there.

The banks of the stream had a low clayey edge, where burrows with water accumulated inside can be found. The stream has a slow but steady flow of running water. The bank was surrounded by vegetation and the ground covered

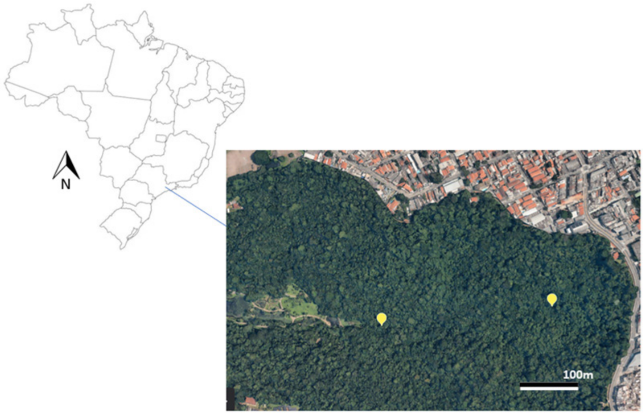

Figure 1. Location of the two sampling sites (yellow dots) in the Parque Estadual das Fontes do Ipiranga, south-eastern Brazil, showing the close proximity of this forest fragment to the urban environment

with leaf litter. We sampled two stretches of the stream (23° 38.357' S, 46° 37.117' W; 815 m a.s.l. and 23° 38.334' S, 46° 36.903' W; 781 m a.s.l.), which were between 12 to 23 m in length, 1.20 to 1.70 m in width and the depth ranged from 0.5 to 0.42 m (measures were collected during the dry season). The width, depth and intensity of the watercourse may differ throughout the seasons due to the rainfall rate. The climate of the region is characterised by a dry winter (April to September) and a rainy summer (October to March), with average temperatures varying from 18 °C in the winter to 22 °C in the summer (Santos & Funari, 2002).

Data collection

We carried out monthly field campaigns, with two to four people, lasting from one to seven days in the period from September 2013 to June 2014, totalling ten months. Observations began between 18:00 h and 19:00 h and lasted at most until 23:00 h, totalling 39 days, giving a total of 91 hours in the field. To find individuals, we conducted acoustic and visual encounter surveys (sensu Crump & Scott Jr., 1994). During behavioural calling observation, we used a red light to minimise interference or disturbance of the individuals. After the observations, we captured the individuals to obtain morphometric information, such as snout-vent length (SVL), measured with a 0.05 mm precision caliper (Digemess®), and body mass (BM), measured on a scale with a capacity of 1000 g x 0.1 g (Digital Scale®). Measurements of SVL were always taken in the same position: we held the frog by its hind limbs while it supported itself with its hands in our finger; at that time, we kept the specimen body straight to avoid arching.

In order to obtain information about the population (described below) and also about the individual's behaviour, we used two methods of marking: photo-identification and Visible Implant Elastomer (VIE). Because individuals have different colour patterns, we used photo-ID for all individuals (Fig. 2A&B), which were photographed in three standard positions: left side, right side and back. The VIE was applied to the subcutaneous tissue (Fig. 2C) of the back, one of the limbs or both limbs of the individual, using three different fluorescent colours (green, pink and orange) or a combination between them.

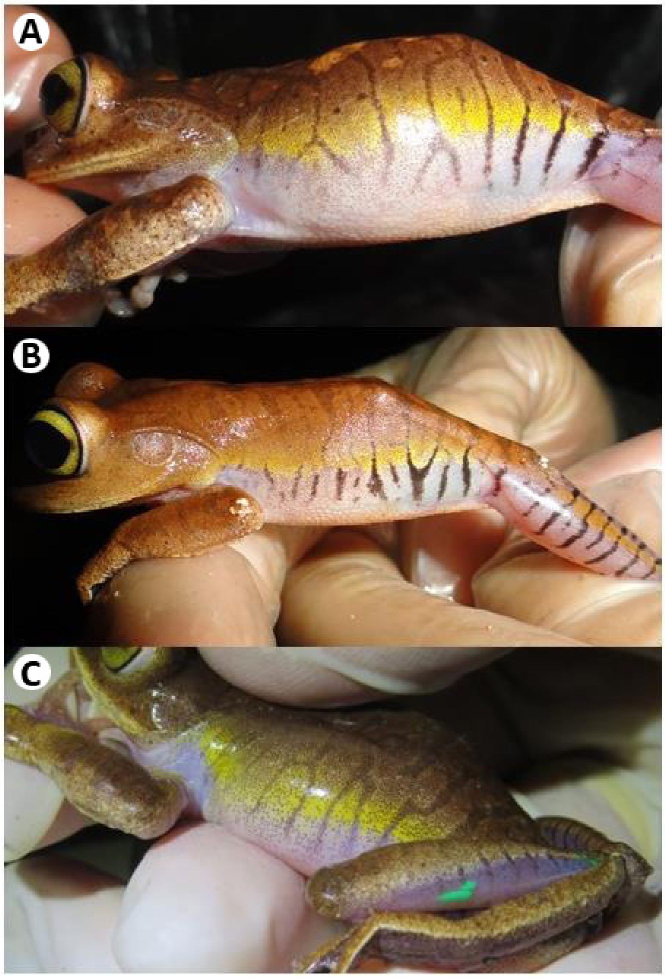

Figure 2. Identifying individual *Bokermannohyla hylax* - **A.** & **B.** Natural colour patterns used for photo-identification, and **C.** Green visible implant elastomer applied subcutaneously into the left leg

We determined the sex of males through their calling activity or by the presence of vocal sacs and well-developed pre-pollical spines (Heyer, 1985). Females were determined by the presence of eggs in the abdominal region and also by the absence of vocal sacs. For individuals in which we could not detect these characteristics, we considered them to be 'undetermined sex'.

Population data analyses

For population size estimation we used a model for closed populations (i.e. no significative births, deaths, immigration, or emigration are assumed), due to the characteristics of our population and length of study (ten months): we studied an adult population (not enough time for births) and isolated from others (no immigration and emigration were considered). We performed analyses using the Schnabel-Schumacher method in the Ecological Methodology Version 7.4 software (Krebs, 2019), with 95 % of confidence limits (lower and upper).

Bioacoustic analyses

We recorded male's vocalisations with a SONY-PCM-D50 sound recorder, at a sample frequency of 44.1 kHz and a sample size of 16 bits, which was positioned at a distance of 0.5 to 1.5 m from the calling male. We analysed 194 calls from nine males in the Raven Pro 1.4 software (K. Lisa Yang Center for Conservation Bioacoustics, 2011), using the following spectrogram parameters: window type Hann and size of 256 samples, which resulted in a 3 dB filter bandwidth of 248 Hz, overlap at 50 % (locked; hop size = 128 samples) for time grid, and DFT of 256 samples (grid spacing = 172 Hz) for frequency grid. We used the configurations of 52–64 % brightness, 60–70 % contrast, and a Fast Fourier Transform length (FFT) of 256 points. All other settings followed the software default values. We measured spectral traits using the spectrogram and temporal traits using the oscillogram.

To describe acoustic call traits, we followed Köhler et al. (2017). For spectral parameters, we obtained the dominant frequency with the Peak Frequency function and the minimum and maximum frequencies with the Frequency 5 % and Frequency 95 % functions, respectively. We also used the two last functions for estimating the bandwidth that concentrates 90 % (BW 90 %) of the energy of the call.

For temporal parameters, such as note duration, intervals between pulse groups, notes and calls, we used the Delta Time function. We measured call rate by calculating the ratio of the number of calls and the duration in which these calls were emitted. We measured pulses per note manually using the oscillogram and pulse rate by calculating both the ratio of the number of pulses and the duration of the note. We excluded notes that were masked by background noise from the pulse count. For the definition of note, we followed McLister et al. (1995), where 'note' refers to the unit of sound produced during a single airflow cycle (lung to vocal sac), and we determined the number of notes per call by observing the movement of the vocal sacs.

We used linear regression based on Pearson's correlation coefficient (r) to assess whether dominant frequency, call rate and call duration were correlated with air temperature and SVL. The analyses were performed using the Past 2.17 software (Hammer et al., 2001) with significance set at the level of 0.05. All recordings were deposited in Fonoteca Neotropical Jaques Veilliard (FNJV 50714–50732).

RESULTS

Population characteristics

In total, we recorded 48 individuals of *B. hylax*, of which 35 were males (72.9 %), one was female (2.1 %), and 12 were of undetermined sex (25 %). Males had SVL ranging from 45.1 to 53.3 mm (mean ± sd - 49.3 ± 2.3 mm, N = 35) and BM from 5.4 to 8.7 g (6.84 ± 0.87 g, N = 32). The only female found measured 55.4 mm and presented 8.98 g of BM. Individuals whose sexes were not determined had SVL ranging from 39 to 57 mm (51.5 ± 5.3 mm, N = 9) and BM from 3.1 to 9.7 g (7.57 ± 2.05 g, N = 9).

Thirty-seven individuals received VIE marking and all of them photo-ID. During the entire study period, the recaptured animals marked with VIE remained with the marking, however, in some of them there was migration of the polymer to the ends of the limbs. The natural marks present on the lateral region of the thighs and on the back presented a unique pattern for each individual, thus allowing for the easy recognition of individuals through photo-ID.

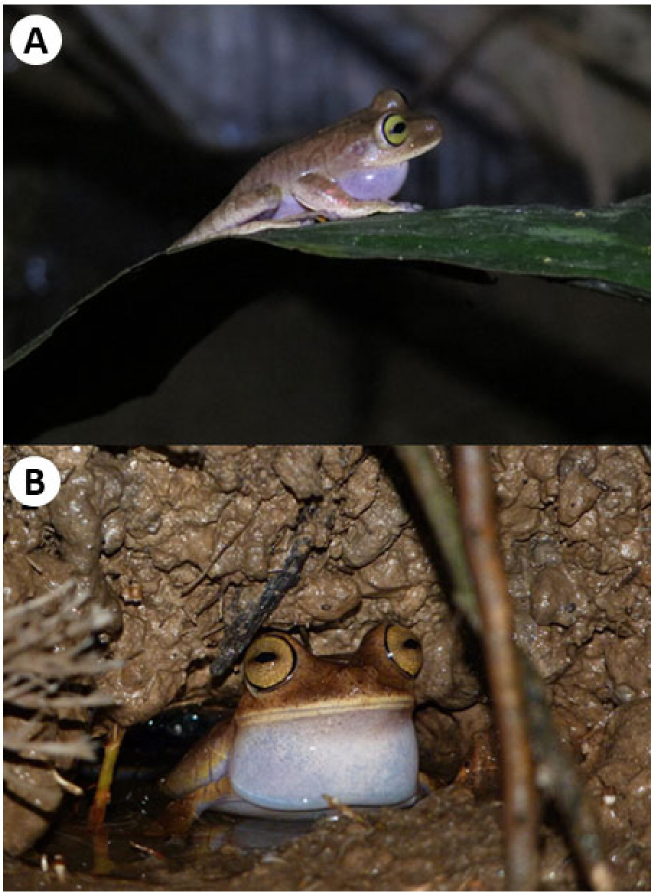

Figure 3. Male *Bokermannohyla hylax* calling - **A.** Exposed, and **B.** In a burrow on the banks of the stream

We found the 48 individuals 122 times, of which 74 were recaptures referring to 28 individuals (58.3 % of the study population was recaptured). The Schnabel-Schumacher method estimated a total of 45.9 individuals (confidence limits: 37.1–60) and of 52.2 individuals (44.6–62.8), respectively. Twenty individuals were captured only once, but one individual was captured nine times. Regarding the time span between the first time the individual was found and the last time, most individuals were recaptured in the same month or in the month following capture, however, two individuals were recaptured after six months and one individual after seven months.

Calling activity and bioacoustic traits

Male calling activity was recorded throughout the study period (September to June), but the months with lower intensity were March and April. They began to call around 19:00 h, and the chorus became more intense around 21:00 h. Males' calling site varied, but it was always associated with the margins of the Pirarungáua Stream. We registered 61 times the marked males in calling activity. We found them mostly calling exposed (Fig. 3A), perched on branches, stems, or broad leaves (77 %, N = 47 times), but also hidden, on the floor in litter-covered stream puddles (19.7 %, N = 12 times) or in holes on the banks (3.3 %, N = 2 times; Fig. 3B).

Table 1. Advertisement call variables of *Bokermannohyla hylax*, a total of 194 calls were recorded from nine males at 21.08 ± 1.15 °C (19.2–22.7) air temperature. Data are presented as mean ± standard deviation (range).

Call traits		**Note A**	**Note B**	**Note C**
Note duration (ms)		512.5 ± 127.7 (282–1014)	434 ± 64.4 (306–554)	61.8 ± 12.7 (47–86)
Dominant frequency (Hz)		1991.1 ± 253.9 (1378.1–2584)	2044.7 ± 163.1 (1722.7–2411.7)	1615 ± 321.6 (1033.6–2067.2)
Maximum frequency (Hz)		2456.1 ± 176.5 (2239.5–2928.5)	2599 ± 263.5 (2239.5–3273)	2261 ± 250.2 (1894.9–2756.2)
Minimum frequency (Hz)		1117.1 ± 199.5 (689.1–2067.2)	1078 ± 216.8 (689.1–1550.4)	818.3 ± 142.8 (689.1–1033.6)
Frequency bandwidth (Hz)		1339 ± 166.7 (516.8–1722.7)	1520.4 ± 207.3 (1205.9–2067.2)	1422.7 ± 209.9 (1205.9–1894.9)
Internote interval (ms)		–	–	156.9 ± 68.5 (12–221)
Pulses/note		53.9 ± 19.6 (25–156)	104.3 ± 28 (65–152)	23 ± 6.5 (14–30)
Pulse rate (pulses/second)		106.5 ± 34.7 (61.6–257.4)	239.3 ± 54.5 (137.5–342.1)	372 ± 64.9 (297.9–500)
Interval between pulse groups (ms)		40.5 ± 17.4 (4–1)	43.2 ± 16.3 (19–74)	–
Call rate (calls/min)	26.6 ± 11.6 (9.7 – 49.4)			
Intercall interval (ms)	26.6 ± 11.6 (9.7 – 49.4)			

We recognised only one call type in our records and field observations, the advertisement call (quantitative traits are summarised in Table 1). This call presents a multipulsed structure, released at a rate of 26.6 ± 11.6 calls/minute (range 9.7–49.4; N = 194). The intercall interval ranged from 169–9493 ms (2440.3 ± 1954.3; N = 183). Advertisement calls (N = 194 calls from 9 males) are composed mostly by one single note (95.9 %) or eventually by two (4.1 %). In the single note call, we recognised two types of notes, herein referred to as Note A and Note B. In the two note calls, we also identified a third type, Note C, which is always emitted before Note A or Note B (Fig. 4).

Note A is the typical advertisement call and was the most frequent in our records (84 %). This note presents a duration of 512.5 ± 127.7 ms (282–1014; N = 163) and a dominant frequency of 1991.1 ± 253.9 Hz (1378.1–2584). Notes have 53.9 ± 19.6 pulses (25–156; N = 106 notes), which are emitted at rates of 106.5 ± 34.7 pulses/second (61.6–257.4). Pulses are usually arranged in well-defined pulse groups and, eventually, silent intervals between pulses can occur (up to four per note) which last 40.5 ± 17.4 ms (4–91; N = 146 intervals).

Note B is released randomly during calling activity and appeared 11.8 % of the time in our records. This note presents a duration of 434 ± 64.4 ms (306–554; N = 23) and a dominant frequency of 2044.7 ± 163.1 Hz (1722.7–2411.7). Notes have 104.3 ± 28 pulses (65–152; N = 20 notes), which are emitted at rates of 239.3 ± 54.5 pulses/second (137.5–342.1). Pulses are usually arranged in well-defined pulse groups in the first third and juxtaposed in the final portion of the note, which sounds like an off-key call. Eventually, silent intervals between pulses of the first half of the note can occur (up to two per note) and they last 43.2 ± 16.3 ms (19–74; N = 16 intervals).

Note C is also released randomly during calling activity, but less frequently (4.1 %), and it sounds like a hiccup. This note presents a mean ± sd duration of 61.8 ± 12.7 ms (range 47–86; N = 8) and a dominant frequency of 1615 ± 321.6 Hz (1033.6–2067.2). Notes have 23 ± 6.5 pulses (14–30; N = 7 notes), which are emitted at rates of 372 ± 64.9 pulses/second (297.9–500). Pulses are not arranged in pulse groups and there is no silent interval between pulses. The interval between Note C and Note A or B is 156.9 ± 68.5 ms (12–221; N = 8 intervals).

Neither dominant frequency (r = -0.12; p = 0.76), call rate (r = -0.12; p = 0.76) or call duration (r = 0.25; p = 0.52) were correlated with air temperature. Call rate (r = 0.18; p = 0.65) and call duration (r = 0.44; p = 0.24) were also not correlated with SVL, but dominant frequency was inversely correlated with SVL (r = -0.77; p = 0.01).

Clutch and oviposition site

During the entire study period we found only one clutch of eggs, on 9 June 2014. The clutch contained approximately 120 eggs, wrapped in a gelatinous mass, deposited in a burrow formed in the stream's clayey border. We found

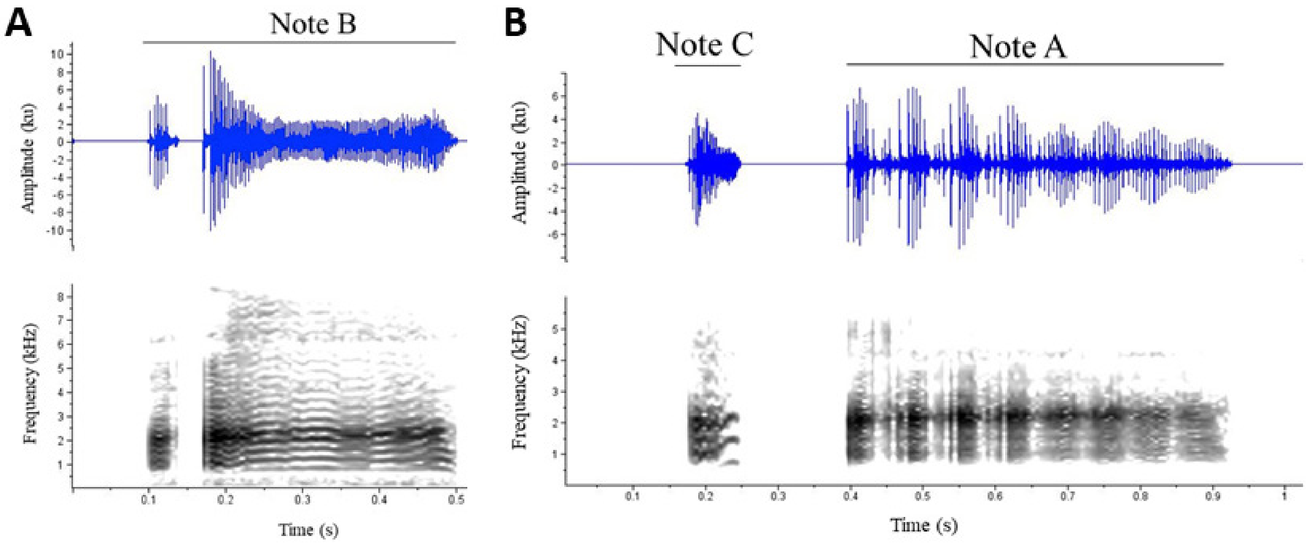

Figure 4. Audio spectrograms (below) and corresponding oscillograms (above) of the advertisement calls of *Bokermannohyla hylax* at the Parque Estadual das Fontes do Ipiranga - **A.** The call can be composed by one single note, or **B.** Composed of two notes. Three different notes were identified (Note A, B, and C). Air temperature during record was 22.7 °C.

this burrow due to the presence of a male calling at the entrance, where three eggs were observed (Fig. 5A), which were possibly dragged there by this male. The burrow was about 15 cm above the water surface. The entrance to the burrow measured 4 cm in diameter with a corridor with 17 cm in length reaching a 9 x 6 cm chamber with a thin layer of water, where most of the eggs were concentrated (Fig. 5B). The eggs were not completely submerged, just in contact with water. Another burrow found with the presence of a male vocalising, but without eggs, was measured and had the same entrance diameter, a corridor with 29 cm in total length and was located at the height of the water surface.

We collected 28 eggs and took them to the laboratory to monitor their development, from which 19 tadpoles emerged after seven days. On the following day after hatching, the tadpoles had a total length that ranged from 12.9 to 15.6 mm (14.2 ± 0.8, N = 19) and body length that ranged from 3.2 at 4.5 mm (4.1 ± 0.3, N = 19). On some occasions in the field, we recorded tadpoles of the species in the stream, close to the edge and in front of the burrows in the stream bank, where we often found males calling.

DISCUSSION

Population traits and marking effectiveness

Our findings suggest that the adult population of *B. hylax* in the PEFI might be small, as most encounters were related to recaptures and estimated population sizes were similar to our study population, with a maximum of 62 individuals. However, we must take into account that our samples were composed of males, as these are easier to detect, which may have contributed to an underestimation of the real population size (Pham et al., 2007). Moreover, imperfect detection is not a trivial fact in any field study (Schmidt & Pellet, 2010). Even without comparative population data for this species in larger forest areas, it is expected that isolated

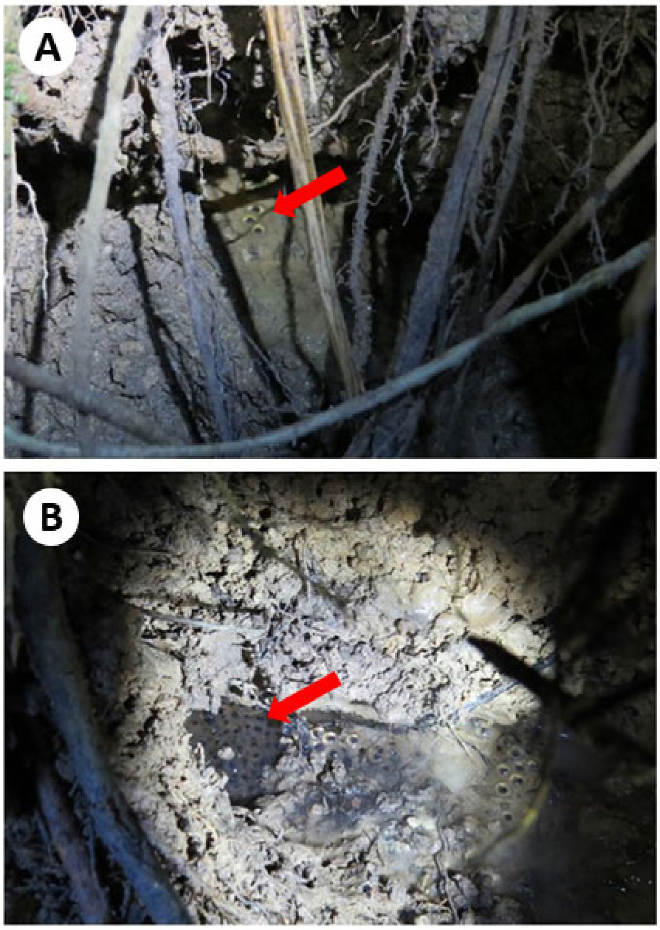

Figure 5. Egg clutch of *Bokermannohyla hylax* found in a clay burrow at stream border - **A.** Entrance of the burrow with some eggs, **B.** Chamber inside the burrow, distance of 17 cm from the entrance, containing approximately 120 eggs. Red arrows are pointing to the eggs. Note that the chamber was opened manually.

areas such as the PEFI present unbalanced situations, such as very abundant or extremely reduced populations (Becker et al., 2007). The isolation may also have affected the body size of the individuals from our study site as males were smaller (45–53 mm) than those from the type locality in Boracéia municipality, State of São Paulo, Brazil (55–62 mm; Heyer, 1985). However, despite standardising our technique for collecting measurements to avoid biases, it remains possible that some of these differences are attributable to different methodological approaches. In this context, it would be interesting to carry out a more current study and measure the individuals of the type locality with the same methodology that we used or vice versa.

The reliability of our population data, obtained from capture and recapture, was supported by the marking methods, VIE and photo-ID, which proved to be efficient for this study. The association of both methods allowed us to recognise all the recaptured individuals, as it reduced the percentage of error or difficulties that each one can generate (Campbell et al., 2009; Ferner, 2010; McHarry et al., 2018) and, for *B. hylax*, these are good alternatives to replace the toe clipping method (Campbell et al., 2009). The VIE was visible due to the translucency of the skin at the application site and was helpful for identification in the field, sometimes without the need to capture the animal. However, the similarity of some colours (orange and pink were very similar in the skin) and elastomer migration (Campbell et al., 2009), created uncertainty on some occasions. The photo-ID method was essential to confirm the individual after comparison with registered photos, reinforcing the accuracy of this method for some species of anurans (Ferner, 2010). Although long-term studies with VIE are scarce, causing uncertainties about future problems in individuals (Antwis et al., 2014), the technique has already been shown to be efficient for salamanders, with records of individuals recaptured five years after marking (Lunghi & Bruni, 2018). In this study, we did not observe damage in the frogs caused by marking with VIE, as it was possible to find individuals up to six or seven months after application.

Calling activity

Apparently, *B. hylax* is active throughout the year in the PEFI, with a lower rate of reproductive activity between months March and April, corroborating the studies by Peres (2010) and Bertoluci et al. (2021). The calling site of males, perched in low vegetation or inside holes on the banks of the stream, also corroborates other studies (Pombal Jr. & Gordo, 2004; Silva et al., 2011; Carvalho et al., 2012; Bertoluci, 2021). Although our data showed a low rate of males calling in holes, these data are underestimated, as we only counted them if we could identify the individuals concerned, and often capturing them in holes was not possible. The choice of calling site can be related to the proximity to the females, because the location can influence the call intensity and sound propagation quality (Wells, 2007). Thus, it is possible that perched males of *B. hylax* were calling to attract more distant females, while buried males would likely attract females that were closer by.

In our recordings, it was only possible to identify the advertisement call, as the individuals observed did not exhibit any differentiated behaviour that could allow us to characterise other types of calls, such as courtship for example (Wells, 2007; Köhler et al., 2017). We identified three types of notes emitted during the advertisement call, whose spectral and temporal parameters were very similar to those described by Carvalho et al. (2012). Comparing the description and structure of the notes, we assume that Note A of our study is the same 'Note A' of the study by Carvalho et al. (2012), however our Note B is probably related to the 'Note C' of these authors and our Note C is more similar to their 'Note D'. We did not identify in our recordings the note identified by them as 'Note B'.

Temporal parameters of the anuran calls are usually influenced by air temperature (Köhler et al., 2017). In our study, temporal features showed no correlation with the air temperature, probably due to the small range of air temperature when we made the recordings. Regarding the intrinsic factors of the calling male, the dominant frequency showed a negative correlation with SVL, i.e. the larger the individual's size, the lower the frequency emitted. This type of correlation is considered the most common among anurans (Wells, 2007) and can be explained by the fact that larger frogs have longer vocal cords. This affects the female's choice for mating, which recognises the suitable male through the call frequency (Köhler et al., 2017).

Clutch characteristics and reproductive mode

Based on our observations of the egg clutch we found inside a burrow and the encounter of tadpoles in the water body close to the edge that contains these burrows, the best fit for *B. hylax* is reproductive mode #4 (Haddad & Prado, 2005) described as "eggs and early larval stages are found in natural or constructed basins; subsequent to flooding, exotrophic tadpoles in ponds or streams". Although we were unable to observe the complete reproductive behaviour (courtship, amplexus and oviposition), we believe that our data, together with that of other studies (Bertoluci et al., 2003; Pombal Jr. & Gordo, 2004; Silva et al., 2011), provide evidence to support this reproductive mode. We also suggest that males can construct nests, as the burrow containing a clutch that we observed, and another one with no eggs that we opened, seem to have been built, as the entrance was very narrow and opened into a concave chamber. Silva et al. (2011) observed a male building a concave nest, pushing the earth to the edge with his hind limbs and leaving the backwater. We recorded several other burrows with calling males, most with the external characteristics similar to this one: made of clay and with the entrance disguised by roots or fallen dry leaves.

The clutch we found in this study had 120 eggs, but Bicudo et al. (2013) recorded about 400 eggs per clutch. There are several possible reasons for this difference including the size or age of females, the number of clutches produced by a female in one season, the existence of communal masses (Wells, 2007), or even to the variation among populations that is usually attributed to differences in temperature, altitude, or latitude (Morrison & Hero, 2003). We found the clutch in June, and Bicudo et al. (2013) found the clutches between the months of April and September, indicating that the greater oviposition activity of *B. hylax* is in the winter.

This can be advantageous for the species, as stream tadpoles tend to develop more slowly, metamorphosis will complete during the rainy season, when environmental conditions are more suitable for the froglets (Eterovick et al., 2010).

Considering the characteristics of the reproduction of *B. hylax*, the findings of our study suggests that subpopulations of this species that occur in disturbed fragments may be endangered by that (Becker et al., 2007). Reproductive modes that present aquatic larvae, especially stream-breeders, are particularly vulnerable, since they depend on forest humidity (Haddad & Prado, 2005; Costa et al., 2012). These factors, associated with the small size of this subpopulation that reproduces in only one forest stream, serve as a warning to the risks for the species' maintenance in the PEFI. This fragment is heavily impacted by fragmentation and urbanisation and the disappearance of some anuran species within an approximate interval of ca. 50 years has already been detected (Lisboa et al., 2021).

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