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Front cover: Black caiman *Melanosuchus niger*, see article on page 76. (© Crocodylia Brasil)

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Multilevel analysis of acoustic variation in a *Scinax fuscomarginatus* population (Anura, Hylidae) of Central Brazil

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The vocalisations of anurans are one of their principal forms of communication and are mainly used for specific recognition involving the attraction of reproductive mates and territorial defense. In this study, we analysed the advertisement calls of 101 individuals from a population of *Scinax fuscomarginatus* sampled in the type locality of *S. pusillus* (currently under the synonymy of *S. fuscomarginatus*). Specifically, we investigated acoustic variation at several levels: intraindividual, interindividual, throughout the night, and across six breeding seasons by analysing temporal and spectral parameters. We identified that all parameters of the advertisement call can be used for individual recognition, with the maximum frequency having the greatest potential. We then observed that all other acoustic parameters were influenced by the predictor variables, with the exception of maximum frequency. The air temperature negatively influenced call duration, number of pulses, dominant frequency and minimum frequency; while it positively influenced pulse rate and call rate during the breeding season. Furthermore, with the exception of call duration and pulse rate, the other acoustic parameters varied significantly across the different nocturnal periods. This study provides data on the variation in *S. fuscomarginatus* acoustic features. Besides, we also discuss the implications of individual recognition. Studies that consider different sources of variation for the same population of a given species are uncommon, but of paramount importance for understanding the behavioural dynamics of the population.

Keywords: advertisement call, behaviour, individual recognition, sexual-selection

INTRODUCTION

The vocalisations of anurans are extremely important as they represent one of their main forms of communication, especially during the breeding season (Gerhardt, 1991; Reichert, 2013; Bee et al., 2016). In anurans, vocalisations are classified based on the context in which they are emitted (Köhler et al., 2017), with the advertisement call being the most frequent (Köhler et al., 2017; Guerra et al., 2018). Calls mediate important social and sexual interactions among individuals, such as specific recognition and attraction of reproductive partners (Pettitt et al., 2013; Arini et al., 2016; Bee et al., 2016). Moreover, calls are also species-specific; therefore, can be useful in behavioural, evolutionary, and taxonomic studies (Blair, 1964; Pombal & Bastos, 2003; Glaw et al., 2010; Bee et al., 2013; Köhler et al., 2017; Tonini et al., 2020).

Several empirical studies have described the influence of environmental, morphological, and social factors on anuran vocalisations over the past few years, which have contributed to a better understanding of the communication process of these animals (see examples in Köhler et al., 2017). For example, amongst environmental factors, it is known that the acoustic parameters of calls can be greatly influenced by precipitation, temperature, relative humidity, and wind (Lemes et al., 2012; Pérez-Granados et al., 2019; Sun et al., 2019). Other factors that also influence call parameters are the characteristics of vocalising individuals, such as morphological variables (e.g. mass and SVL) and physiological conditions (Nevo & Schneider, 1976; Morais et al., 2012; Bee et al., 2013). In anurans, the allometric relationship between the size of individuals and their vocalisations is well known (Tonini et al., 2020), and an inverse relationship between body size and dominant call frequency is expected (Wagner, 1989a; 1989b; Morais et al., 2016; Köhler et al., 2017).

There is also the influence of social interactions on vocalisations (Morais et al., 2015; 2021; Dias et al., 2017). For example, the distance between conspecifics (Morais et al., 2012; Gambale et al., 2014) or the density of the chorus (Bastos et al., 2011) can influence the acoustic parameters of the vocalisations emitted by anurans. In some species it is possible to observe lek behaviour, in which several males perform courtship displays to conspecific females through their acoustic signals (Wells, 1977). In these species, the aggregation of individuals engaged in vocalisation activity is common, which can generate intense acoustic competition and consequently, the masking of some acoustic signals due to overlap (Wagner, 1989a; Gall & Wilczynski, 2016; Tanner & Bee, 2019). To minimise these effects, some anuran groups have developed strategies such as altering the dominant frequency and/or duration of their calls (Wagner, 1989b; Lucas et al., 1996; McCauley et al., 2000).

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Acoustic parameters can be classified as dynamic (variation greater than 12 %; $CV_{intra} > 12$ %) or static (variation less than 5 %; $CV_{intra} < 5$ %), suggesting that such parameters would be subjected to different types of selection (i.e. directional or stabilising) (Gerhardt, 1991). Although studies addressing acoustic variability at the intra- and interindividual levels are the most common in literature (e.g. Bee et al., 2001; Bee & Gerhardt, 2002; Gasser et al., 2009; Briggs, 2010; Bee et al., 2013; Gambale & Bastos, 2014; Guerra et al., 2017; Röhr et al., 2020). The variation in acoustic signals of anurans at distinct nocturnal periods or between distinct breeding seasons has been barely explored (Gambale et al., 2014; Dias et al., 2017; Andreani et al., 2020).

Studies addressing acoustic variation provide us with important insights into the individual recognition process in anurans, as individuals can potentially be discriminated in a reproductive aggregation based on their calls (Bee & Gerhardt, 2002; Morais et al., 2012). This is a particularly important aspect, given that females choose their reproductive partners based on the acoustic parameters of the signals emitted by them (Welch et al., 1998; Bosch et al., 2000; Schwartz et al., 2002; Byrne, 2008). Thus, changes in acoustic communication patterns in a given species, can lead to changes in reproductive rates (Warren et al., 2006; Costa & Carnaval, 2012; Klaus & Lougheed, 2013; Merrick & Koprowski, 2017). Therefore, changes in population size and structure can occur, and may render populations more susceptible to decline (Laiolo, 2010).

Scinax fuscomarginatus (Lutz, 1925) is a small hylid widely distributed in South America, including the Brazilian biomes of Amazonia, Caatinga, Cerrado, Atlantic Forest, and Pantanal (Pupin et al., 2020; Frost, 2023). The vocalisations of this species were formally described in the 1980s and since then several studies have considered its acoustic signals in different contexts (Duellman & Pyles, 1983; De la Riva et al., 1994; Pombal et al., 1995, 2011; Toledo & Haddad, 2005b; Pombal, 2010; Brusquetti et al., 2014; Jansen et al., 2016; Souza et al., 2021). The vocal repertoire of *S. fuscomarginatus* was described as being composed of four call types (Toledo & Haddad, 2005b), with the advertisement call being the most studied. In 2016, Jansen et al. investigated a character shift in the advertisement calls in allopatric and sympatric populations of two related species (*S. fuscomarginatus* and *S. madeirae*) and more recently, Souza et al. (2021) described variation in the advertisement calls emitted by males from ten populations of *S. fuscomarginatus* in Central Brazil.

Here we describe the acoustic behaviour of individuals in a population from the municipality of Rio Verde, state of Goiás, Central Brazil. The population studied was found in the type locality of *Scinax pusillus* Pombal, Bilate, Gambale, Signorelli & Bastos, 2011, currently synonymous with *S. fuscomarginatus* (Brusquetti et al., 2014). Specifically, we investigate the variation in male advertisement calls of this population considering the following levels: intraindividual, interindividual, throughout the night, and over six breeding seasons. Our expectation is to find greater variation in acoustic parameters between than within individuals, enabling acoustic discrimination of these males in a breeding aggregation (Gambale et al., 2014; Guerra et al., 2017). Additionally, we also expect that the acoustic parameters of calls that have a high potential for the discrimination of individuals will maintain this pattern at different times of the night and in different breeding seasons.

MATERIALS & METHODS

Study area

Field activities were carried out in a permanent water body located on a rural property in the municipality of Rio Verde, Goiás State, Brazil (17° 48'6.02" S, 51° 05'21 W, ~800 m a.s.l.). The water body is within the Cerrado biome and it is surrounded by different vegetational strata, such as herbs, shrubs and trees. In addition, the study site suffers from anthropogenic activities such as livestock grazing and monoculture plantations (e.g. corn and soybean). The climate in the study region is Aw (tropical wet savanna), according to the Köppen classification, having a well-defined rainy period from October to March, with average annual precipitation of 1,300 mm and average annual temperatures ranging from 20 to 25 °C (Alvares et al., 2014). Besides *S. fuscomarginatus*, several other species of anurans were recorded in the same water body: *Boana albopunctata*, *B. lundii*, *Dendropsophus cruzi*, *D. jimi*, *Scinax* aff. *fuscovarius*, *Elachistocleis cesarii*, *Leptodactylus labyrinthicus*, *Physalaemus cuvieri*, *P. nattereri*, *Pseudopaludicola* sp. and *Rhinella diptycha*.

Study species

Brusquetti et al. (2014) conducted a taxonomic revision of *S. fuscomarginatus* and related species based on acoustic, molecular, and morphological data, in which they proposed the synonymy of *S. parkeri*, *S. trilineatus*, *S. lutzorum*, and *S. pusillus* with *S. fuscomarginatus*. The population considered in this study is the one found in the type locality of *S. pusillus* (for more information see Pombal et al., 2011). Information on some aspects of the natural history of this population was obtained through a previous study (Pombal et al., 2011) and also through personal observations made in the field. The individuals of this population are active during the rainy season (October to March) and have a lek mating system, as males aggregate to perform courtship displays and consequently, attract reproductive partners. Throughout the night, males emit vocalisations in different periods (from 1800 to 0600) however; their acoustic activity peak occurs around 2100. Pombal et al. (2011) described two types of vocalisations emitted by males of this population; with the advertisement call being the most commonly observed.

Collection and Data Analysis

We recorded the calls of 101 individuals of *S. fuscomarginatus* between October to March over six breeding seasons: 2014/15 (n = 18); 2015/16 (n = 6); 2016/17 (n = 48); 2017/18 (n = 8); 2018/19 (n = 16) and 2019/2020 (n = 5). Recordings were obtained between 1900 and 0000 with Sennheiser ME-66 (Wedemark-Wennebostel, Germany) microphones coupled to Marantz PMD-660 (Kanagawa, Japan) or Tascan DR-40 (California, USA) recorders, with the following recording settings: 44.1 kHz, 16 bits, WAV format. Each recording session lasted approximately two minutes. We standardised a distance of 50 cm between the microphone and the calling males. Subsequent to the recordings, we collected the males and took the following measurements: snout-vent length (SVL), with a caliper (0.01 mm precision); and total mass, with a digital scale (0.01 g). We also recorded the air temperature following each call with a thermohygrometer (precision 0.1 °C).

Previously, Bastos et al. (2011) suggest that in the first hours of the night, vocalisations are mainly associated with defense and the establishment of vocalisation sites, while in the middle of the night they are mainly associated with the attraction of reproductive partners. This is a relevant topic, because different periods of the night are associated with different contexts and, consequently, can influence differently the acoustic parameters of the calls. To investigate this topic, we considered 13 of the 101 individuals and obtained their vocalisations at different times throughout the night (between 1930 and 2300). Specifically, we made five recordings at 30-minute intervals (e.g. sequences: A, B, C, D, and E) of each individual.

We used the Raven Pro 1.5 software (Bioacoustics Research Program, 2011) to analyze the acoustic parameters of *S. fuscomarginatus* calls. For each recording session, we randomly selected five advertisement calls and measured the following acoustic parameters: call duration (s), number of pulses (pulses/call), dominant frequency (Hz), maximum frequency (Hz), and minimum frequency (Hz). We also measured the pulse repetition rate (pulses/s), which is estimated by dividing the number of pulses per call by the total duration of the call. Finally, we calculated the call repetition rate (calls/min), which is done by dividing the number of calls by the total duration of the recording. The acoustic terminology is according to Köhler et al. (2017) and the figures (spectrogram and oscillogram) were prepared in the R software (R Core Team, 2020) using the Seewave 1.6.4 package (Sueur et al., 2008), with the following settings: window name (Fourier transform window) = Hanning; window length = 256 samples; overlap = 90 %. The collected specimens were deposited in the Coleção de Vertebrados Alípio de Miranda-Ribeiro (CVAMR-An 316-329; 338; 384-390; 400) and the audio recordings were deposited in the Collection of Sound Archives of Neotropical Amphibians (CASAN 025-030; 032-040; 047-049; 147-148; 151-156; 196-198; 200-204; 209-215; 236-302; 360-365; 399-401; 427-443; 479-533) both located in the Rio Verde Campus, Instituto Federal Goiano (IF Goiano), Brazil.

Intra- and interindividual variation

The influence of temperature on the acoustic parameters of anuran calls is well established in the literature (Köhler et al., 2017). Prior to the analyses of intraindividual and interindividual variation, we controlled the effect of temperature on the acoustic parameters using the equation described by Kaefer & Lima (2012): where *Y* is the raw value, Y_{adj} is the adjusted value of the parameter, *b* is the regression coefficient, T_{local} is the temperature at the time of recording, and $T_{average}$ is the average temperature of all recordings.

To describe the intraindividual variation of each acoustic parameter, we obtained its coefficient of variation (CV_{intra}), based on a recording session for each individual, where we calculated the CV_{intra} through the individual's standard deviation divided by the mean of the parameter analysed. For the interindividual coefficient of variation (CV_{inter}), we used the same formula, but considering the standard deviation and the mean of a given parameter of all individuals analysed, regardless of reproductive season.

We classified the acoustic parameters according to their intraindividual coefficients of variation, following Gerhardt (1991). Thus, the parameters were classified as static (CV_{intra} < 5%); intermediate (CV_{intra} 5 % to 12 %), and dynamic (CV_{intra} > 12 %). To determine whether a given acoustic parameter has a greater variation at the interindividual level than its counterpart at the intraindividual level we calculated the CV_{inter}/CV_{intra} ratio. Acoustic parameters with CV_{inter}/CV_{intra} values above 1.0 determine that the variation between individuals is greater than the variation within individuals, indicating that this acoustic parameter can potentially be used for individual species recognition (Bee et al., 2001).

Finally, in order to identify statistical differences in the acoustic parameters between individuals, we performed a simple Analysis of Variance (ANOVA). However, when the data did not meet the assumptions (i.e. homogeneity of variances and normal distribution) the non-parametric Kruskal-Wallis test was used. We used subjects as predictor variables and acoustic parameters as response variables for these analyses.

Variation throughout the night

As described above, we calculated the coefficients of variation (CV_{inter}/CV_{intra}) and the CV_{inter}/CV_{intra} ratio for the different periods throughout the night (i.e. A, B, C, D, and E). In addition, we performed a repeated-measures Analysis of Variance (ANOVA) to investigate the differences in acoustic parameters between the different periods throughout the night, using data corrected for ambient temperature. We used each period of the night as predictor variables, the individual as a repeated measures factor, and the response variables were the acoustic parameters of the individuals. Finally, we applied the Tukey test on the results, which makes pairwise comparisons with the nocturnal periods, making it possible to identify which periods of night differed from each other. The analyses throughout the night were performed using the Jamovi software version 1.8. We adopted a significance level of 0.05 for these analyses and the ones mentioned in the previous topic (Zar, 1996).

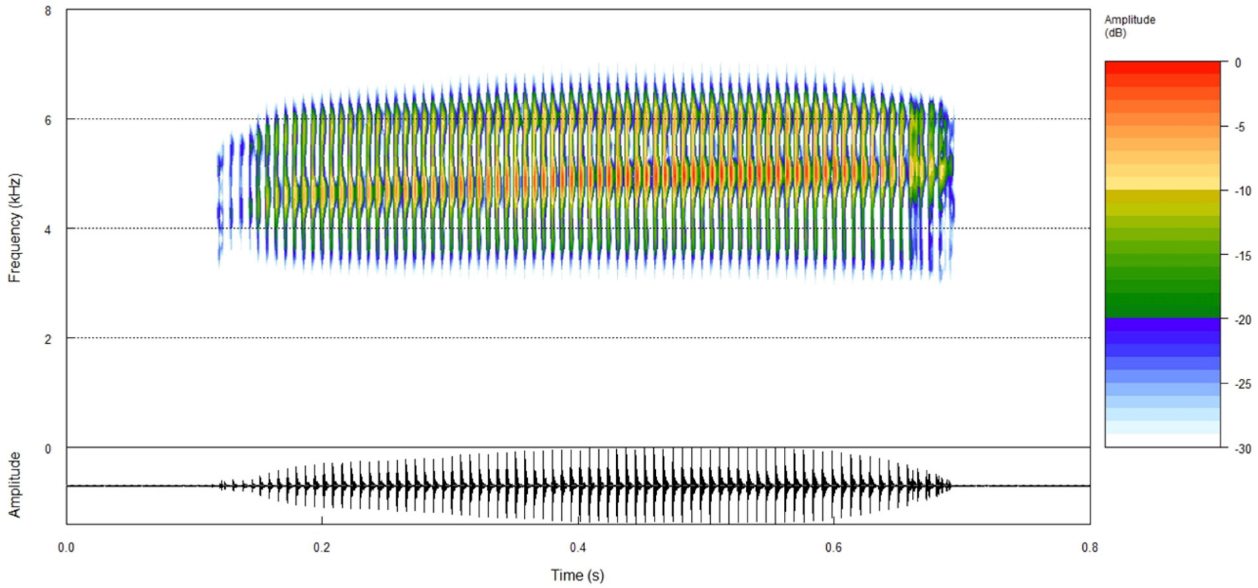

Figure 1. Audiospectrogram (above) and oscillogram (below) of the advertisement call of *Scinax fuscomarginatus* from Central Brazil. Air temperature = 21.7 °C; air humidity = 94 %; SVL = 16.53 mm; mass = 0.3 g; CASAN_0479).

Variation over breeding seasons

To evaluate the variation in the acoustic parameters of advertisement calls across breeding seasons, we followed the approach described by Andreani et al. (2020). First, in order to eliminate the correlation between the SVL and mass body variables, we created a single value by multiplying these variables and named them body condition (Gambale et al., 2014). Subsequently, we also transformed the reproductive season variable into a multistate discrete variable, where the value 0 was assigned to the first reproductive season in our sampling (i.e. our starting point of study observation) and the value of 1 was added to subsequent years. Thus, we have the following values: 2014/2015 = 0; 2015/2016 = 1; 2016/2017 = 2; 2017/2018 = 3; 2018/2019 = 4 and 2019/2020 = 5.

Finally, we applied a Multiple Linear Regression for each acoustic parameter separately to understand how years, environmental temperature and body condition influence acoustic parameters, using the acoustic parameters as response variables and the years,

Table 1. Values of acoustic parameters of *S. fuscomarginatus* males including mean, standard deviation, amplitude, intraindividual coefficient of variation (%) (mean, minimum and maximum), interindividual coefficient of variation (%), and CV_{inter}/CV_{intra} ratio

	Mean ± SD (Range)	Within-male mean CV %	Within-male range CV %	Between-male mean CV %	Ratio CV_{inter}/CV_{intra}
Call duration (s)	0.54 ± 0.07 (0.25 – 0.82)	5.21	1.01 – 31.1	9.76	1.87
Number of pulse (pulse/call)	69.33 ± 7.80 (30 – 97)	5.56	0.80 – 29.2	9.75	1.76
Dominant frequency (Hz)	5019.74 ± 670.49 (656.5 – 6563.5)	2.32	0.00 – 12.6	10.17	4.39
Maximum frequency (Hz)	6189.67 ± 295.14 (5062.50 – 7125)	1.03	0.00 – 5.59	4.66	4.52
Minimum frequency (Hz)	3805.33 ± 1072.22 (93.80 – 4875)	7.6	0.00 – 121	17.7	2.33
Pulse repetition rate (pulses/s)	129.34 ± 12.30 (105.1 – 202.15)	2.72	0.413 – 1.4	7.17	2.63

Table 2. Coefficient of variability of advertisement call (CV_{inter}/CV_{intra}) for *S. fuscomarginatus* males in each reproductive season sampled. We also present the ANOVA or Kruskal-Wallis results of the interindividual comparisons of the acoustic parameters.

	Acoustic parameters					
Breeding seasons	**Call duration**	**Dominant frequency**	**Maximum frequency**	**Minimum frequency**	**Number of pulses**	**Pulse repetition rate**
2014/2015	2.51 H = 67.32 p = 0.0000	2.23 H = 68.59 p = 0.0000	2.84 H = 71.51 p = 0.0000	2.73 H = 73.79 p = 0.0000	1.93 H = 65.28 p = 0.0000	2.81 H = 68.02 p = 0.0000
2015/2016	1.79 H = 15.91 p = 0.0031	1.03 H = 14.37 p = 0.0062	10.22 H = 23.32 p = 0.0001	0.97 H = 18.47 p = 0.0010	2.55 F = 31.29 p = 0.0000	1.60 H = 14.62 p = 0.0056
2016/2017	1.55 H = 170.88 p = 0.0000	5.14 H = 154.51 p = 0.0000	3.55 H = 202.74 p = 0.000	3.46 H = 193.02 p = 0.000	1.35 H = 155.99 p = 0.0000	1.74 H = 166.02 p = 0.0000
2017/2018	1.23 F = 6.99 p = 0.0097	1.24 F = 6.23 p = 0.0140	1.92 H = 9.76 p = 0.0076	1.77 H = 8.17 p = 0.0168	1.45 F = 8.48 P = 0.0050	0.72 F = 2.53 P = 0.1212
2018/2019	1.61 H = 62.57 p = 0.0000	3.30 H = 73.04 p = 0.0000	4.67 H = 70.14 p = 0.0000	2.01 H = 73.68 p = 0.0000	1.45 H = 59.71 p = 0.0000	3.51 H = 60.93 p = 0.0000
2019/2020	3.48 F = 44.81 p = 0.0000	3.30 H = 19.69 p = 0.0006	16.13 H = 23.08 p = 0.0001	2.05 H = 21.51 p = 0.0003	4.87 F = 103.89 p = 0.0000	4.98 F = 112.92 p = 0.0000
Total	1.87 F = 11.6 p = 0.001	4.39 F = 54.0 p = 0.001	4.52 F = 55.7 p = 0.001	2.33 F = 15.7 p = 0.001	1.75 F = 10.9 p = 0.001	2.63 F = 23.1 p = 0.001

temperature and body condition as predictor variables. We used the multi-model inference framework based on Akaike's selection criteria (AICc) to select the best combinations of variables in each model (Burnham et al., 2011), considering a 0.8 threshold of importance to determine the permanence of the predictor variable in a given model (Calcagno & Mazancourt, 2010). We used the R software (R Core Team, 2020) for all analyses with the Glmuti package for multi-model analyses (Calcagno & Mazancourt, 2010).

RESULTS

Call description and intra- and interindividual variation

During field activities, air temperature ranged from 18.6 to 24.4 °C (x = 21.1 ± 1.6 °C), while the SVL and mass of individuals ranged from 16.11 to 20.73 mm (x = 17.9 ± 1.11 mm) and from 0.17 to 0.49 g (x = 0.3 ± 0.06 g), respectively. We analysed 505 advertisement calls emitted by 101 *S. fuscomarginatus* males of the same population (see an example in Figure 1). The mean, minimum, maximum, and standard deviation values for each acoustic parameter analysed are shown in Table 1.

Overall, none of the acoustic parameters were classified as dynamic (Table 1). Call duration (CV_{intra} = 5.21 %), number of pulses (CV_{intra} = 5.55 %), and minimum frequency (CV_{intra} = 7.60 %) were classified as having intermediate acoustic properties, whereas pulse rate (CV_{intra} = 2.72 %), dominant frequency (CV_{intra} = 2.32 %), and maximum frequency (CV_{intra} = 1.03 %) were classified as static (Table 1). Additionally, all acoustic parameters of the advertisement calls had $CV_{inter}/CV_{intra} > 1$ (i.e. they varied more between than within individuals). Overall, the spectral parameters of the advertisement call had the highest values, with the maximum frequency (CV_{inter}/CV_{intra} = 4.52) exhibiting the greatest potential for individual recognition (Tables 1 & 2). However, only call duration, dominant frequency, maximum frequency and number of pulses are the parameters that had $CV_{inter}/CV_{intra} > 1$ across all six breeding seasons (Tables 1 & 2).

Variation throughout the night

Except for call duration and pulse rate, all other acoustic parameters varied significantly throughout the night ($p < 0.05$ for all cases). We especially noticed the following differences: pulse number (period A ≠ B; $p_{Tukey} < 0.05$); dominant frequency (period B ≠ D; $p_{Tukey} < 0.05$), maximum frequency (period A ≠ C and period A ≠ D; $p_{Tukey} < 0.05$), minimum frequency (period A ≠ E and period B ≠ E; $p_{Tukey} < 0.05$), and call rate (period A ≠ B and period D ≠ E; $p_{Tukey} < 0.05$). Despite the observed differences, we noticed that intraindividual variation remained constant throughout the period analysed. That is, with the exception of minimum frequency, all acoustic parameters were classified as static ($CV_{intra} < 5$ %) or intermediate (5 % < CV_{intra} < 12 %) throughout the night (Table 3).

Influence of temperature, body condition (allometry) and breeding seasons

Acoustic parameters of the advertisement call varied according to the body condition of individuals, the air temperature, and also throughout the breeding seasons. However, each parameter showed distinct patterns of variation and influence. All acoustic parameters were influenced by ambient temperature, except for maximum frequency (Table 4). We observed an inverse relationship between air temperature and the following acoustic parameters: call duration [Importance = 1.0000; Partial regression coefficient (PRC) -0.0297], number of pulses (Importance = 0.9999; PRC -1.2545), dominant frequency (Importance = 0.9522; PRC -49.1484), and minimum frequency (Importance = 0.9999; PRC -149.7444). On the other hand, we observed a positive relationship between air temperature and pulse rate (Importance = 1.0000; PRC 4.7617), and call rate (Importance = 0.9999; PRC 4.7617) (Table 4).

It was evident that temporal parameters [i.e. pulse rate (Importance = 0.8074; PRC 0.6640) and call rate (Importance = 0.8774; PRC 0.6894)] increased over the years, while spectral parameters [i.e. maximum frequency (Importance = 0.9999; PRC -64.0891) and dominant frequency (Importance = 0.9040; PRC -47.0812)] decreased as the breeding seasons passed. When we took body condition into consideration, call duration (Importance =0.9999; PRC 0.0112) and number of pulses (Importance = 0.9999; PRC 1.8645) were positively related, while maximum frequency (Importance = 0.9998; PRC -55.8453) and minimum frequency (Importance = 0.9954; PRC -115.8327) were negatively influenced by body condition.

Table 3. Means of the intraindividual coefficient of variability (%) of the advertisement call of all *S. fuscomarginatus* males throughout the same night by periods (A, B, C, D, E).

	Acoustic parameters					
Different periods throughout the night	**Call duration**	**Number of pulses**	**Dominant frequency**	**Maximum frequency**	**Minimum frequency**	**Pulse repetition rate**
A	4.03	4.16	2.38	0.79	6.33	1.89
B	3.42	3.39	2.67	0.69	3.29	1.91
C	4.08	4.30	2.33	0.65	2.12	1.99
D	3.38	3.40	3.43	0.56	2.40	1.83
E	3.81	4.65	2.59	0.50	17.43	2.52

Table 4. Results of multiple linear regressions between *S. fuscomarginatus* males and multi-model inference using the threshold of 0.8.

Acoustic parameters	**Variables**	**Importance**	**Partial regression coefficient**	**Unconditional variance**	**Confidence interval**
Call duration	Body size	0.9999	0.0112	6.2257	0.0049
	Reproductive season	0.3760	-0.0008	2.0456	0.0028
	Temperature	1.0000	-0.0297	3.7400	0.0038
Number of pulses	Body size	0.9999	1.8645	0.1102	0.6526
	Reproductive season	0.3382	0.0790	0.0256	0.3148
	Temperature	0.9999	-1.2545	0.0662	0.5059
Pulse repetition rate	Body size	0.7166	0.6249	0.2919	1.0621
	Reproductive season	0.8074	0.6640	0.2061	0.8924
	Temperature	1.0000	4.7617	0.1285	0.7046
Call repetition rate	Body size	0.3448	-0.1151	0.0518	0.4475
	Reproductive season	0.8774	0.6894	0.1436	0.7449
	Temperature	0.9999	4.7617	0.0808	0.5588
Dominant frequency	Body size	0.4706	15.2666	494.5974	43.7204
	Reproductive season	0.9040	-47.0812	551.9694	46.1866
	Temperature	0.9522	-49.1484	412.1078	39.9084
Maximum frequency	Body size	0.9998	-55.8453	155.0935	24.4825
	Reproductive season	0.9999	-64.0891	102.6456	19.9173
	Temperature	0.6426	-10.7501	120.3722	21.5686
Minimum frequency	Body size	0.9954	-115.8327	1075.1604	64.4607
	Reproductive season	0.2723	-2.1464	83.8771	17.9734
	Temperature	0.9999	-149.7444	634.9085	49.5352

DISCUSSION

Several descriptions of the advertisement call of *S. fuscomarginatus* based on different sample sizes collected from different populations throughout the species' geographic range have been conducted in recent decades (Duellman & Pyles, 1983; De la Riva et al., 1994; Pombal et al., 1995; 2011; Toledo & Haddad, 2005b; Pombal, 2010; Brusquetti et al., 2014; Jansen et al., 2016; Souza et al., 2021). Considering that the advertisement calls of anuran species are important taxonomic tools, bioacoustic studies based on a large sampling effort are important contributors to improving our understanding about the taxonomy of our target species. In this sense, it is important to highlight that the present study characterised the advertisement call of *S. fuscomarginatus* based on an expressive number of individuals that belong to a population that is taxonomically relevant, as it was sampled in the type locality of *S. pusillus* (currently under the synonymy of *S. fuscomarginatus*).

Our results expand the previously known variation of the advertisement call's acoustic parameters for individuals of this population (Pombal et al., 2011). We also observed that some acoustic parameters (e.g. dominant frequency and pulse rate) of the call differ from what was previously described for other populations of *S. fuscomarginatus*, as was reported by Brusquetti et al. (2014). On average, the advertisement calls of individuals considered here have a higher dominant frequency and lower pulse rate than calls recorded for individuals from other localities (Duellman & Pyles, 1983; De la Riva et al., 1994; Pombal et al., 1995; Toledo & Haddad, 2005b; Silva et al., 2008; Brusquetti et al., 2014; Jansen et al., 2016; Souza et al., 2021). Considering that divergences in acoustic parameters between individuals from distinct populations have revealed the existence of a species complex in anurans (Lopes et al., 2020), we suggest that future studies evaluate whether the acoustic differences reported here impact the specific recognition process between individuals from different populations (e.g. through experimental playbacks). This is particularly important considering the recent history of taxonomic change experienced by individuals from the target population of this study (Brusquetti et al., 2014).

Overall, we observed low intraindividual variation in the advertisement calls, as none of the acoustic parameters were classified as having dynamic properties (CV_{intra} < 12 % for all cases). These results are similar to those described by Souza et al. (2021), as these authors also found that none of the parameters of the *S. fuscomarginatus* calls could be classified as dynamic when analysing acoustic variation among populations from Central Brazil. On the other hand, Jansen et al. (2016) reported high intraindividual variability in some acoustic parameters of the species' call. *Scinax fuscomarginatus* is a widely distributed species and consequently, individuals from distinct populations experience different environmental and/or social conditions that may affect their vocalisations in distinct ways. Therefore, this might explain the discrepancies found between different studies on acoustic variation in *S. fuscomarginatus*.

We also observed that all acoustic parameters of *S. fuscomarginatus* calls had CV_{inter}/CV_{intra} ratios above 1. This implies that all parameters analysed have the potential to discriminate individuals in a breeding aggregation. These results are similar to those previously reported for *S. fuscomarginatus* (Souza et al., 2021) as well as for other Neotropical hylids (*Aplastodiscus albosignatus* - Moser et al., 2022; *Dendropsophus minutus* - Morais et al., 2012; and *Boana goiana* - Signorelli et al., 2016). As discussed by Bee & Gerhardt (2001) and Gasser et al. (2009), the pattern observed in the present study reinforces that the individual recognition process in anurans takes place through a set of different acoustic parameters. This is an important issue particularly in regions with high species richness (e.g. Neotropics), because dozens or hundreds of individuals of different species may coexist in the same site and therefore, generate some level of acoustic interference between them (Sugai et al., 2021).

Few studies have considered Neotropical anuran species to assess whether vocalisations vary at different times throughout the night. Two examples are Bastos et al. (2011) and Toledo & Haddad (2005b), who compared the emission rates of different call types of *Scinax centralis* and *S. fuscomarginatus* males throughout the night, respectively. Recently, Dias et al. (2017) included other acoustic parameters (e.g. dominant frequency, note duration, number of pulses, inter-note interval, and repetition rate) in their analysis and found that only the rate of call emission of the advertisement call of *B. goiana* differed over successive night periods. Unlike previous reports by Dias et al. (2017), our results demonstrate that distinct acoustic parameters (e.g. dominant frequency, maximum frequency, minimum frequency, pulse number, and call rate) of the advertisement call of *S. fuscomarginatus* differed throughout the night. Considering that vocal activity is energetically costly (Gerhardt, 1994; Prestwich, 1994), it is expected that individuals will exhibit distinct vocalisation behaviours throughout the night as a way to minimise body mass losses during the course of the breeding season (Robertson, 1986; Mac Nally, 1981). We hypothesise that vocalisation behaviours adopted by *S. fuscomarginatus* males throughout the night can be attempts to optimise female attraction by balancing their energy expenditure. However, future studies are necessary to better evaluate the pattern described here.

There are few studies that address how the acoustic parameters of advertisement calls vary across breeding seasons. In contrast to the lack of variation reported for some species (e.g. *Dryophytes versicolor* – Gerhardt et al., 1996; *Dendrobates pumilio* – Pröhl, 2003; *Scinax constrictus* – Gambale et al., 2014), the temporal (i.e. pulse rate and song rate) and spectral (e.g. maximum and dominant frequency) acoustic parameters of *S. fuscomarginatus* decreased across the breeding seasons. The change in temporal parameters of anuran vocalisations over reproductive seasons has also been reported by other authors (Howard & Young, 1998; Smith & Hunter, 2005; Andreani et al., 2020) and is correlated to the decrease in body mass of *Boana goiana* males over time (Andreani et al., 2020). On the other hand, Smith & Hunter (2005) reported that the variation in spectral parameters in the calls of males of *Litoria booroolongensis* across breeding seasons is caused by changes in body size.

In addition to expanding the known variation of this population, our results indicate that all parameters analysed are potentially important in the process of individual recognition, which may have implications for aggressive interactions and also in the preference of females for reproductive partners.

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What can studying anacondas tell us about *Titanoboa cerrejonensis*? Exploring the life of an extinct giant snake using an extant pretty big snake

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The maximum size that snakes can reach has been a matter of long held debate until the discovery of *Titanoboa cerrejonensis* (Boidae). It was an aquatic predator that lived 60–58 million years ago in what is currently eastern Colombia, occupying tropical swamps. It was calculated to measure 12.82 metres and it was speculated that it grew so much due to a warmer planetary weather. Its life history and ecology are speculated to be very similar to that of current day anacondas. Using data from a long-term study involving hundreds of green anacondas *Eunectes murinus*, this study makes inferences about aspects of the natural history of *T. cerrejonensis* that perhaps will not be easily available by studying the fossil record. Drawing parallels with anaconda's biology, I estimate that a non-breeding female *T. cerrejonensis* weighed approximately 1,232 kg, and 1,465 kg when pregnant. It would have started breeding at 480 cm SVL, weighing 95 kg. New-born *Titanoboa* were between 181 and 215 cm. Its average meal was estimated to be 505 kg, with a potential maximum of 1,799 kg. I estimate that *Titanoboa* had a growth rate of 0.046 mm/day compared with 0.036 in anacondas; which does not support the notion that it grew more due to a warmer planet. Although the results are largely speculative, they help give a better idea of what the life of an extinct snake was like.

Keywords: Gigantism, prey size, reproductive biology, life history, palaeothermometer, giant snake, allometry, palaeoecology

INTRODUCTION

The study of palaeontology has given us great insights into the lives of extinct organism but it is limited by the availability of fossils and how incomplete the fossil record is (Dzik, 2005; Wang & Marshall, 2016; Darwin, 1859). The field has advanced in leaps and bounds lately due to sophisticated technologies that allow more details analysis of the fossil remains we have (Mallison et al., 2009). However, recruiting the assistance of other disciplines can help shed light on the life of extinct organism that analysis of fossils might not. It is possible to use our knowledge of the ecology of extant species that used similar habitat, or that are phylogenetically related to our target extinct species, to make educated guesses about their lives. This approach has been used successfully studying body size of crocodilians (Godoy & Turner, 2020), the evolution of wing morphology (Burch, 2014) and tails in birds (Pittman et al., 2013), as well as gliding among vertebrates (Socha et al., 2014) even among fairly distantly related organisms.

The heart of this approach is based on the assumption that different organisms will respond in similar ways when exposed to a particular set of evolutionary and ecological pressures, especially if they share recent common ancestors and share a given blueprint that sets them in similar tendencies and constraints. This is the reason that adaptations of natural history traits among vertebrates have shown remarkably similar trends across taxa and variety of habitats (Meiri et al., 2021). Case in point, in birds as distantly related as penguins (Sphenisciformes) and puffins (Charadriiformes) that co-opt their wings in a very similar manner when they evolved diving, despite having split more than 70 m.y.a (Kimball et al., 2019). Simply put, the body plan of a bird will predispose the bearer to swimming in a similar manner even if Charadriiformes are nowhere closely related to penguins. Similarly, data from great white shark *Carcharodon carcharias* has been used to reconstruct the biology of the extinct giant relative megalodon *Carcharocles megalodon* (Pimiento & Balk, 2015). Thus, approaches to use extant species to understand the lives of extinct ones are not limited to very closely related individuals; and still allows us to gain insights on the lives of extinct species that we would not be able to gain using only palaeontological evidence.

One such extinct species which biology is very unknown is *Titanoboa cerrejonensis*, a giant snake that lived 60–58 m.y.a in what is now eastern Colombia (Head et al., 2009). The maximum size a snake can reach has been a long held debate among herpetologists, and among those interested in megafauna. There are numerous reports about the maximum size snakes can attain. Some of them are more reliable than others (Murphy & Henderson, 1997). There might be natural limits on the maximum size set by the "blueprint" of a snake (Rivas, 2020a). The finding of *T. cerrejonensis* gave the scientific community undisputable evidence that a snake can reach at least 13 metres in total length (Head et al., 2009).

Titanoboa lived in tropical swamps, surrounded by rainforest, that was likely part of an extensive wetlands that scattered over western South America resulting from the damming of the Palaeo-Amazonas river, by the rise of the Andes. This process would have started since the late Cretaceous, and lasting until the Neogene creating an extensive flooded system of estuaries and marshlands in most of northern South America. This swamp would have fluctuated from freshwater to marine-like conditions on occasions when local weather resulted in higher evaporation rates, in a system that may have been intermittently disconnected from the ocean (Rivas, 2020b). *Titanoboa* belonged to the group of Neotropical Constrictores (Georgalis & Smith, 2020). Vertebrae and skull morphology places *Titanoboa* firmly within the family Boidae, with an association with Madagascar boas (Head et al., 2013) and *Boa constrictor* (Head et al., 2012); however, no formal phylogenetic analyses have been done on the species (Smith & Georgalis, 2022). The taxonomy within the family is problematic because only the use of cranial material can provide the most reliable phylogenetic information (Georgalis et al., 2021) and the only skull known is incomplete (Head et al., 2013); Boinae genera are not diagnosable by vertebrae morphology alone (Head et al., 2006). However, the vertebral evidence is strong enough to consider *Titanoboa* within the family Boinae (Head et al., 2013; 2009). *Titanoboa* may have lived in excess of 60 years based on Lines of Arrested Growth (LAGs) in the vertebras, but the authors express uncertainty on this estimate based on the lack of complete breaks in tissue deposition between some of the LAGs (Werning et al., 2014). *Titanoboa* was hypothesised to have fed on a variety of fish, crocodiles, and turtles that inhabited the swamps where it lived (Bloch et al., 2005; Head et al., 2009; Cadena & Jaramillo, 2015; Hastings et al., 2010). Its size was estimated to be 1,201 cm long in snout-vent length (henceforth SVL) and 1,282 cm in total length using morphological data from extant boas. Head et al. (2009) argued that it grew so large because of higher global temperatures that promoted faster growth, but this notion has been challenged based on biases of the sample used that fail to predict other Squamata reptiles (Sniderman, 2009), conflict with other estimations of other species (Shackleton & Boersma, 1981), and the fact that it ignores the behavioural abilities of animals to regulate their own temperature (Huber, 2009; Denny et al., 2009; Sniderman, 2009).

Although we do not know exactly how closely related they were, they were related enough that the ecological similarities and phylogenetic proximity between anacondas and *Titanoboa* offers the possibility to use anacondas as a proxy to understand parts of the ecology of *Titanoboa* that perhaps are very difficult to learn from palaeontological methods. To use data from anaconda biology as a model to understand the biology of *Titanoboa* is well justified because of the following reasons. First, they are closely related phylogenetically, belonging to the same family. It is reasonable to expect that their shared ancestry results in similar responses to environmental variables. Second, they share a similar ecological niche and their ecological interactions with other members of their community would have produced similar selection pressures. Both are top predators living in tropical aquatic environments, mostly swamps. There are some similarities and differences in the diet of adult female anacondas and what *Titanoboa* was hypothesised to feed on. They both were aquatic predators, that preyed in crocodyliforms and other riverine vertebrate fauna (Head et al., 2009; Rivas, 2020a); however, there were also difference in that *Titanoboa* likely included fish in their diet, as evidenced by abundant palatine teeth, (Head et al., 2013) while fish are conspicuously absent in anacondas diet (Rivas, 2020a; Miranda et al., 2016). So, it is reasonable to expect that they faced comparable ecological and evolutionary pressures. Third, anacondas are, by far, the best studied genus of the family Boidae (Rivas, 2015; 2020a). In fact, the authors that described *Titanoboa* used an anaconda dataset to estimate its mass. So, using knowledge on anacondas to predict other aspects of *Titanoboa* biology is consistent with existing patterns in the literature. Thus, this study used data on anaconda biology to make predictions about the life of *Titanoboa*. Specifically, this anaconda dataset facilitates data-based speculation about its size and morphometric relationships, reproductive biology, size at first reproduction, and aspects of its natural history such as neonate size, prey size, growth rate, and demography. I analyse this information in light of the ecological and evolutionary landscape of the time.

MATERIALS & METHODS

Morphometry

Since 1992, I have conducted a long-term study on the life history of anacondas in the Venezuelan Llanos, addressing aspects of its ecology, such as its mating system (Rivas et al., 2007a; Rivas & Burghardt, 2001), reproductive biology (Rivas, 2015), diet, mobility and habitat use (Rivas et al., 2007b; 2016; Rivas, 2020a). As part of this study, I have caught in excess of 800 animals from which I have gathered representative morphometric information (Table 1, and supplementary material). I measured the animals by stretching a non-elastic string over the middle line of their body and later measuring the string on a measuring tape (Rivas et al., 2008) after muzzling them for ease of handling (Rivas et al., 1995). Data were analysed using histograms and linear regression models. I log-transformed the data of SVL and mass to better model their relationship (Huxley,

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Table 1. Summary of the measures of 823 anacondas caught between 1992 and 2014 years. N = sample size, SVL = snout-vent length, NP = non-pregnant adult females, P = pregnant adult females. All the measures of length are given in cm and all the masses are given in grams.

Measure	Mean	STD	Max	Min	N	Regression from SVL	R^2
Total length	263.6	83.1	522	64.5	823	y = 1.1068x (SVL) + 10.571	0.99
SVL	228.0	75.0	477	54.7	813	n/a	
Mass	11437.1	13316.2	97000	107	794	Log(y) =2.9813x (LogSVL) - 3.1141	0.94
Tail length	35.9	10.3	64	10.8	231	y = 0.115x SVL + 91479	0.86
Perimeter	28.42	12.09	85	10	91	Log(y) = 1.0345x Log(SVL) - 1.0081	0.79
Head	86.4	20.49	150	45.6	82	y = 0.3149x + 9.1166	0.92
Females							
Total length	304.1	101.2	522	64.6	458	y = 1.1065x SVL+8.388	0.996
SVL	267.3	91.2	477	54.7	458	n/a	
Mass	19407.3	17518.6	97000	107	458	Log(y) = 2.9629x Log(SVL) - 3.0336	0.95
Tail length	36.4	12.7	64	10.8	114	y = 0.1203 SVL + 5.1685	0.96
Perimeter	36.15	14.39	85	14.5	39	Log(y) = 1.0562x (log(SVL) - 1.0268	0.73
Mass (NP)	22496.4	14699.4	54000	4700	104	Log(y) = 2.9386x Log(SVL)-3.1103	0.99
Mass (P)	26413	18046	97000	7250	235	Log(y) = 2.9843x Log(SVL)-3.0245	0.93
Males							
Total length	238.6	51.2	339.0	68.8	460	y = 1.1432x (SVL) + 5.0762	0.99
SVL	204.3	44.6	293.7	58	460	n/a	
Mass	5678.6	2906.9	17000	180	460	Log(y) = 2.6471x (LogSVL)+2.3927	0.92
Tail length	35.3	7.3	46	11.3	117	y = 0.1601x SVL + 2.4007	0.88
Perimeter	22.63	4.86	34.5	10	52	Log(y) = 0.8014x (log(svl))-0.4991	0.82

1932). No fossil specimens were measured in this study. All data from fossil snakes were obtained from the literature (Head et al., 2009).

For the predictions in this study I used a specific dataset that best applied to the category I was trying to predict. For instance, for maximum mass, I used a dataset that included only females with complete tails, as they may suffer injuries that shorten their tails. Additionally, the decision to only use females in the regression is justified because, among boas, larger females are the rule (Shine, 1994), so it is reasonable to assume that the largest *Titanoboa* was female. Males have longer tails and slimmer bodies, so using an only-female dataset is more appropriate to predict the dimensions of the largest, likely female, *Titanoboa*.

General natural history

For most of my calculations I assume than the largest reported size of *Titanoboa*, of 1,201 cm in SVL, is the maximum size it reached (but see below). Data from current snakes show that snake maximum size is between 1.5 to 2.5 the length at which they start breeding (Pritchard, 1994). My data on reproduction of anacondas confirm that females grow up to 2.27 of the size at which they start reproducing (Rivas & Burghardt, 2001; Rivas et al., 2007a; Rivas, 2015; 2020a; 2020b). I thus use the Pritchard's 2.5 ratio to make predictions about *Titanoboa* in terms of its size and first reproduction, clutch size, and neonates size.

In my calculations I use two approaches. First, I scale up what I know about anaconda biology to the size that *Titanoboa* was reported to be and speculate about clutch size and neonate size. Second, and more realistically, I assume that *Titanoboa* was not merely a scaled up anaconda but rather the ecological equivalent of current anacondas (the largest aquatic snake of its time). Likely, a young female *Titanoboa* ready to breed was faced with the same decisions as our current anacondas. She had to be able to produce offspring that were ready to survive in terms of both avoiding predation and finding food, as well as having some energy for her own metabolic needs and growth. Natural selection would have shaped the natural history of *Titanoboa*, like that of anacondas, so they would breed as soon as they could produce a viable clutch, both to increase their reproductive output and to secure some offspring in case she was preyed upon before the next breeding opportunity (Rivas, 2020a). Therefore, I assume the investment per offspring for *Titanoboa* was the same as for anacondas at different parts of their ontogenetic developments.

Growth rate

Having the estimated size of neonates, the maximum reported size, and the estimated life span of 60 years, I calculated the growth rate. While the authors that estimated its lifespan to more than 60 years express doubts about the accuracy of their estimate based on LAGs, no better estimate of age has been attempted. In this contribution I use their estimation of 60+ years as the potential life span of *Titanoboa*, since LAGs are a well established method to estimate age in fossils (Castanet et al., 1993)

Maximum size

Last, because *Titanoboa* lived in tropical swamps that regularly have acidic water (Rivas, 2020b) where fossil formation is unlikely, I assume that the individuals found are not necessarily the largest they reached but a representation of the most common size of the population (the average) of *Titanoboa*. Thus, I use the size distribution of existing anacondas to speculate about the maximum size *Titanoboa* could reach. Given their close phylogenetic association and similar ecological roles, these assumptions are well granted.

RESULTS

Body size and body mass estimations

Using an only-female dataset, I calculate that an average *Titanoboa* would have weighed 1,232 kg and measured 168 cm in circumference at its widest point. A non-pregnant female, just after giving birth, would have weighed 870 kg because of a strong investment in reproduction. Conversely, a pregnant female would have weighed an amazing 1,465 kg. Using data from the general population (males and females), I calculate that its head (from the end of the quadrate to the tip of the nasal scale) would have been 38 cm long (Table 1).

At first glance *Titanoboa* may come across as having a very short tail to the trained eye. Current day anacondas of 500 cm SVL have tails in excess of 50 cm. So their tail length is 0.16 % of the total body size. However, in *T. cerrojonensis* it is 0.06 %. At the size *Titanoboa* reached, one would expect it to have a much larger tail if the proportion of tail to body size were the same in both species (Fig. 1A). However, looking at the allometric relationship of tail and body length it is possible to see that as anacondas grow larger, the relative tail length decreases. If a current day anaconda were to grow to the size of *Titanoboa*, it would have a comparably shorter tail (Fig. 1B).

Reproductive biology

Using the 2.5 factor proposed by Pritchard (1994), if *Titanoboa* grew to a maximum of 1,201 cm SVL, it must have started to breed at 480 cm SVL. At this size it would have weighed 95 kg (Table 1). This is about the maximum size reached by current anacondas in the hyper-seasonal savannah of the Venezuelan Llanos (Rivas, 2020a).

If a current day anaconda were to grow to the size of *Titanoboa*, she would produce 1,321 neonates (clutch size = 0.0009 x mass + 2.5327, R^2 = 0.8421) weighing 513 g each. She would invest 55 % of her body mass in babies amounting to 678 kg (Rivas, 2020b; Rivas et al., 2016). However, this figure assumes that *Titanoboa* was just a scaled up anaconda, which is likely not the case. For a more responsible calculation, I assume that the neonates of *Titanoboa* were under the same selection pressure as current day neonate anacondas, so its neonates had to be the same proportional size as the neonates of current anacondas. If a *Titanoboa* new mother had babies 4 % of her non-pregnant mass, as anacondas do (Rivas, 2020a; 2015), her babies will weigh 4.0 kg at birth and measure 1.81 metres SVL (Log (SVL) = 0.3141x + 1.1265, R^2 = 0.94). Using a similar reasoning it is possible to estimate neonate size from a 12.82 m long *Titanoboa*. The relative investment per offspring declines as the animals gets larger. In anacondas it drops to 0.56 % of the female's mass (Rivas, 2020a; 2015). At 1,232 kg of mass, a *Titanoboa* would have had neonates weighing 6.9 kg and measuring 2.15 m in SVL at birth.

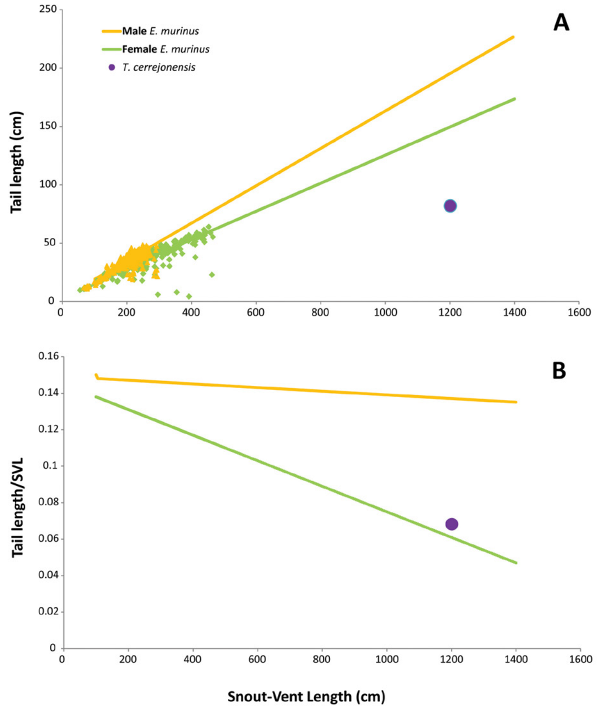

Figure 1. **(A)** regression of tail length vs snout-vent length in male (R^2=0.88) and female (R^2=0.96) green anacondas. The scatter plot in the lower values shows data from actual green anacondas (yellow triangles males; green squares females). The regression lines offer predictions for males (green) and females (yellow) on the larger sizes. The purple dot represent the tail of *Titanoboa*. **(B)** regressions showing the allometric relationship between relative tail length (tail length/SVL) and SVL in male and female anacondas. In the size range of current day anacondas it is not obvious to see the shortening of the tails as they get larger (females relative tail length=-0.0001x SVL+ 0.1664, R^2 0.53). The purple dot shows where *Titanoboa* fell in this relationship. This chart shows that *Titanoboa*'s tail/SVL proportions were just on par with those of current day anacondas.

Prey size

Average prey size of a neonate is about 32 % of their body weight (Rivas et al., 2016). So, neonatal prey items must have ranged between 1.3 and 2.2 kg. The average prey size for adult females is 41 % (Rivas, 2020a; 2015), so the average *Titanoboa* meal would have been in average 505 kg. Furthermore, data on the diet of anacondas suggests that anacondas can go for prey as large as 146 % of its body weight (Rivas, 1998). If a large *Titanoboa*, at 1,232 kg, had been able to attack prey of this size, this prey would had weighed 1,799 kg.

Last, using the current size distribution of anacondas, it is possible to speculate about the size distribution of *Titanoboa* under the assumption that the animals recovered from the fossil record were representative of the average *Titanoboa*. Figure 2 shows the current distribution size of female anacondas (Fig. 2A) and the hypothetical distribution size of *Titanoboa* (Fig. 2B).

Growth rate

Bone histology suggests that *Titanoboa* had a lifespan in excess of 60 years (Werning et al., 2014). If neonate *Titanoboa* was born between 181 cm and 215 cm SVL, it would have experienced a growth rate between 0.046 and 0.047 mm per day to reach 1,201 cm in 60 years.

Maximum size

Figure 2A shows the distribution of female anacondas from my study, suggesting a typical normal distribution. If *Titanoboa* had a similar size distribution, it is not impossible that the largest snake that ever lived was much larger than the 13 m long *Titanoboa* found. If the *Titanoboa* specimens that have been found were average in size, the theoretical maximum *Titanoboa* could have reached in excess of 20 m in total length (Fig. 2B). At this size it would have had a mass of 5,091 kg.

DISCUSSION

This study offers educated guesses on the life of *Titanoboa* that would be difficult to obtain using other methods. Studying the life of extinct animals is fraught with limitations from the nature of the evidence that can be found in the fossil record (Darwin, 1859). However, using an extant organism that is closely related phylogenetically, and that plays the same ecological role as the extinct one, may offer unprecedented opportunities to make educated guesses on the biology of the extinct one, thereby aiding our capacity to understand better the life and ecology of the extinct organism (Kimball et al., 2019; Pimiento & Balk, 2015).

The original paper describing *Titanoboa* calculated its mass to be 1,135 kg using available regressions from pythons and anaconda sizes (Head et al., 2009). However, the anaconda dataset used in that study included males, which are thinner than females (Rivas, 2015; Rivas et al., 2007a; Rivas & Burghardt, 2001). It also included terrestrial pythons, that are slimmer than aquatic snakes. An aquatic lifestyle releases the organism from constraints of gravity and allows for the development of a larger body mass (Denny, 1990; Andersson, 1994). Taken together, I believe that a dataset only including female anacondas is more appropriate to predict *Titanoboa*'s mass and body proportions.

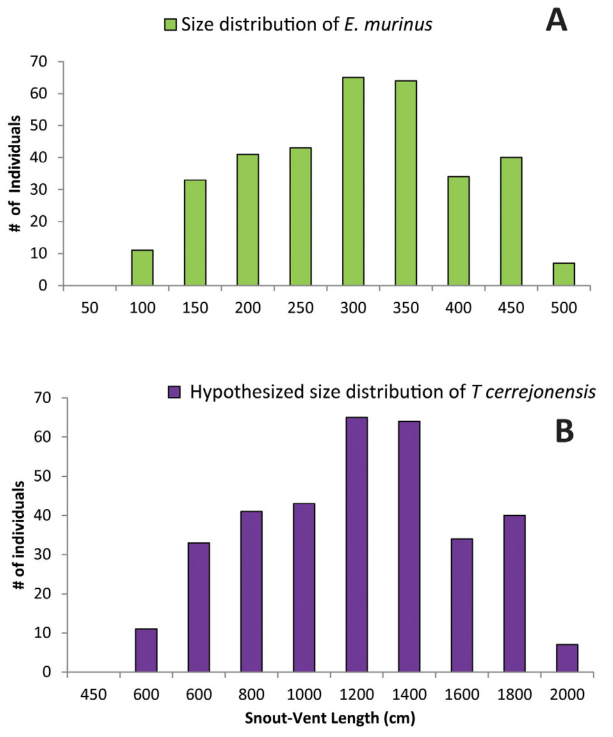

Figure 2. **(A)** Size distribution of female anacondas in the Venezuelan Llanos. **(B)** Hypothetical size distribution of *Titanoboa* assuming their demographic distribution was similar.

I assume that the ecology of *Titanoboa* was comparable to that of anacondas and thus used what we know of anacondas to speculate about the life of *Titanoboa*. *Titanoboa*'s world was likely filled by large predators, several species of crocodiles and predatory fish that abounded in the waters (Bloch et al., 2005; Hastings et al., 2010; Hastings & Bloch, 2007). There would have been a size after which a juvenile *Titanoboa* would have outgrown its predators. But until then, predation was expected to be high if the biology of anacondas and other large reptiles is any indication (Rivas et al., 2016). During *Titanoboa* times, a neonate 350 g in mass and 90 cm SVL (the largest anaconda neonate) would have faced a large amount of very large predators for a long time. This probably would have prevented a large proportion of them from reaching adulthood and reproducing. Furthermore, the assemblage of prey we have in current times provides a variety of smaller prey, including many birds, that neonate anacondas feed on (Rivas et al., 2016). Likely, smaller prey items were not that common in *Titanoboa*'s time, as *Titanoboa* predated the radiation and diversification of Neornithes birds (Claramunt & Cracraft, 2015). Therefore, a neonate *Titanoboa* the size of a neonate current-day anaconda, scaled up as it may be, would really have faced very hard odds, both for finding small food items they could prey on and also by facing strong predation during their long time of vulnerability. Because of this, I believe that the first calculation of clutch size of 1,321 neonates is unrealistic. Rather, assuming conservatism in the proportions of female mass to neonate mass yields sounder estimates (0.56–4 % of female's mass). Furthermore, the relationships of clutch size and reproductive investment is similar in *Boa constrictor* (Cardozo & Chiaraviglio, 2011), suggesting that these natural history traits are conserved within the family.

My data suggest that the average prey size of a 1,282 cm SVL *Titanoboa* was approximately 505 kg. Clearly an animal as large as *Titanoboa* had to consume very large prey. There were a whole assortment of crocodiles, turtles, and fish at the time that likely were common in its diet (Cadena et al., 2010; Bloch et al., 2005; Hastings et al., 2010; Hastings & Bloch, 2007). Furthermore, many snakes that feed on very large prey are able to shut down their metabolism when they are not foraging. Allowing its digestive system atrophy saves energy when digestive tissue is not being used (Secor & Diamond, 1997; 2000).Moving from feast to famine is common among many snakes, and certainly among Boidae. This may be the reason that anacondas drop smaller prey from their diet as they grow larger (Rivas, 2020a; 2015). It does not pay for a very large snake to restart its large metabolic machinery for a prey that is not big enough to warrant the investment. If *Titanoboa* shared this physiological adaptation, that seems to be common among basal snakes (Secor & Diamond, 2000), it would have been also advantageous for it to drop smaller prey items from it diet. So, an average human being weighing 70 kg would weigh only 5.7 % of *Titanoboa* mass. Anacondas hardly ever take a prey this small (Rivas, 2020a; 2015). So, a full size *Titanoboa* might not have been interested in eating a person!

Studies from wild anacondas show that wild neonates may have a growth rate of 0.071 mm per day (Rivas et al., 2016). Captive bred neonates may grow as fast as 0.14 to 2.15 mm per day (Rivas et al., 2016; Lamonica et al., 2007). However, these figures are not comparable because they are from neonates fed *ad libitum* and the calculated growth rate of *Titanoboa* is over its 60-year lifespan; neonates are supposed to experience a higher growth rate than adults. A more reasonable comparison would be comparing growth rate of adult anacondas in the wild with that of *Titanoboa*, since neonatal growth rate is supposed to last a relatively short period. Female adult anacondas have an average growth rate of 0.036 mm/day (Rivas, 2020a) which seems very similar to the growth rate found in *Titanoboa* along its lifespan.

The original paper that described *Titanoboa* (Head et al., 2009) speculated that its large size was associated with faster growth due to the higher temperature of the planet at the time. My data suggest that the contribution of higher temperature to *Titanoboa*'s size might have been marginal, at best. While 0.047 mm per day seems higher than 0.036 mm per day, the seemingly higher growth rate in *Titanoboa* could be the combination of faster juvenile growth and slower adult growth. If *Titanoboa* was born at, for example, 200 cm SVL and grew at the same growth rate as neonate anacondas (0.071 mm per day) until it reached adulthood (at 480 cm SVL), it would take 10.8 years to reach that size. If then it grew at the same growth rate of adult anaconda (0.036 mm per day) it will take her another 54.9 years to reach 1,201 cm, needing a total of 65.9 years to reach that size. This calculation suggests that *Titanoboa* experienced a comparable growth rate as current day anacondas. Its large size can be explained by its long lifespan alone, without invoking higher global temperatures.

Last, I will speculate about the maximum size *Titanoboa* could have reached. Palaeontologists know all too well how difficult it is to find fossils. The odds that an organism would fossilise are low to begin with. Furthermore, the fossil then needs to be found by someone who can identify it properly. Considering that *Titanoboa* lived in tropical swamps that likely had acidic black water (Rivas, 2020b) that dissolves the calcium phosphate from the bones, we are lucky to have any fossils at all. All of the knowledge the scientific community accepts, regarding the size of the largest snake that ever lived, is the calculated size based on the fossils found. But what are the odds that the largest *Titanoboa* is the one found? It is unlikely that the *Titanoboas* found represent really the largest of its kind. The largest individual is only one, and the top largest are only few. In fact, in the Cerrejón mines where *Titanoboa* was found, they have found several individuals of about the same size. So it is quite possible that the 13 m long *Titanoboas* found might have been just your run-of-the-mill, regular-size, impress-nobody individuals. Supporting this notion is the later finding of a partial skull whose owner was estimated to be 14.3 m in total length (Head et al., 2013). If the size distribution of *Titanoboa* was similar to that of anacondas and the animals found are average size individuals, the maximum possible size of *Titanoboa* would have been in excess of 20 m. We may never find a fossil of a snake quite that large. However, this calculation shows at least the theoretical maximum that a snake could reach.

While the results of this study are speculative, they are based on well justified assumptions and a solid dataset. Using the ecology of extant anacondas allows us to speculate about the biology of *Titanoboa* and create sound predictions on their biology and life history. There is no doubt that fossil studies will continue to yield sound information about extinct species but this study shows that cross-pollination with other disciplines can help better understand the lives of extinct organisms.

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Incidence and characteristics of crocodilian incidents on humans in Brazil in the period 2000–2022

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Brazil has the largest diversity of crocodilians in the world, with six species present in the country. Considered as opportunistic generalist predators, these animals occupy the top of the food chain in river ecosystems. Anthropic actions result in an impact both on habitats and on the behaviour of the crocodilians, in addition to facilitating the encounter between humans and crocodilian species. In this study, we evaluated the characteristics of alligator incidents on humans in Brazil during the years 2000–2022. We used online platforms for scientific articles and news, collecting information about the victims, the species involved, and the locality of occurrence. We found 86 records of incidents, of which 18 resulted in the victim's death. The Amazon biome encompass the highest number of incidents, and the black caiman *Melanosuchus niger* was the species involved most. Most incidents (n = 35) occurred with people who were fishing or on boats. Considering the size of the country's population, alligator incidents on humans in Brazil can be considered rare, but they should not be overlooked. The advancement of activities that degrade the environment, causing imbalances, can cause an increase in the likelihood of encounters and, consequently, incidents, which usually generates critical medical problems and negative consequences for the populations of these animals.

Keywords: Alligator, attack, bite, Caiman, *Melanosuchus*

INTRODUCTION

Crocodilian attacks on humans are considered rare, and when they do occur, they are mainly caused by the improper approach or non-visualisation of the animal (Caldicott et al., 2005; Bury et al., 2012). The incidents caused by these animals can be categorised into territory defense, nest and/or nest defense, self-defense, hunting and mistaken identity (Caldicott et al., 2005). Worldwide, the vast majority of reported cases are related to incidents caused by crocodilians considered to be large (Caldicott et al., 2005), such as the Nile crocodile *Crocodylus niloticus* Laurenti, 1768, saltwater crocodile *Crocodylus porosus* (Schneider, 1801), and american alligator *Alligator mississippiensis* (Daudin, 1802). In Brazil, the species most involved in this type of incident are the black caiman *Melanosuchus niger* (Spix, 1825). In non-fatal cases, the bite of these animals can lead to morbidity and necrosis (Da Silva et al., 2010). Another concern is the potential for infection caused by micro-organisms present in the mouths of these animals (Flandry et al., 1989; Burgess et al., 1997).

Crocodilians, in general, are opportunistic predators (Somaweera et al., 2020) and are considered to be at the top of the food chain in river ecosystems in Brazil (Bataus et al., 2013). These animals have an ontogenetic variation in their diet, which consists of invertebrates in the juvenile phase, and of vertebrates, such as fish and mammals, in the adult phase (Tucker et al., 1996; Da Silveira & Magnusson, 1999; Melo, 2002; Borteiro et al., 2009). Young or smaller individuals of crocodilians can be easily seen in urbanised environments (Beal & Rosenblatt, 2020; Mascarenhas Jr. et al., 2021).

Six species of crocodilians live in Brazil, all belonging to the Alligatoridae family (Costa & Bérnils, 2018). The largest species, *M. niger*, known locally as 'jacaré-açu' (black caiman), can exceed five metres in length (Thorbjarnarson, 2010), and is distributed mainly the Amazon region (Marioni et al., 2013). The species *Paleosuchus palpebrosus* (Cuvier, 1807), or 'jacaré-paguá' (dwarf caiman) and *Paleosuchus trigonatus* (Schneider, 1801), or 'jacaré-coroa' (crown alligator), rarely exceed two metres in length and inhabit much of the Brazilian territory (Costa & Bérnils, 2018). *Caiman latirostris* (Daudin, 1801), or 'jacaré-do-papo-amarelo' (broad-snouted caiman), has a wide distribution in Brazil.*Caiman yacare* Daudin, 1802, or 'jacaré-do-Pantanal' (yacare caiman), occurs in the Pantanal region (Medem, 1983). Finally, the *Caiman crocodilus* species (Linnaeus, 1758), or 'jacaretinga' (common caiman), occurs in all states in the north and central west regions of Brazil. These last three species are considered small to medium-sized, reaching up to three metres in length (Brazaitis, 1973).

Currently, no Brazilian species of crocodilians are considered threatened (ICMBIO, 2018). This can be explained by the implementation of environmental laws and conservation programs (Da Silveira & Thorbjanarson, 1999), which successfully reversed the results of overhunting that occurred in the 1970s (Rebêlo & Magnusson, 1983). However, the conservation of these animals remains affected by poaching and the loss of their natural habitats (Mittermeier et al., 2004; Marques et al., 2016). The advancement of urbanisation has caused negative effects, such as the ingestion of anthropic residues by animals (Dutra-Araújo et al., unpublished data), and the reduction in the size of crocodilian populations (Abercrombie & Verdade, 2002; Filogonio et al., 2010). In addition, deforestation, pollution, and the consequent decrease in the number of natural prey species are factors that directly and indirectly increase the likelihood of these animals encountering humans (Campos et al., 1995; Da Silveira & Thorbjanarson, 1999; Campos & Mourão, 2006; Magnusson & Campos, 2010; Dutra-Araújo et al., unpublished data).

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In several regions of Brazil, crocodilians have a bad image. For fishermen, they are often considered as a competitor, which may even cause damage to fishing nets (Barboza et al., 2013). For others, these animals are considered a great threat because they are large and predatory, or even because of the lack of knowledge about them, causing incidents with animals or humans (Pooley et al., 2021). These negative relationships between crocodilians and humans are relatively well documented all over the world (Caldicott et al., 2005; Dunham et al., 2010; Bergamasco et al., 2018; Das & Jana, 2018; Grajales & Silva, 2018; Woodward et al., 2019; Sideleau et al., 2021). However, conservation strategies for crocodilian species involve measures of peaceful coexistence with humans, depending on the quantitative documentation, spatialisation, and understanding of the characteristics of these interactions.

In this study, we compiled all the incidents caused by crocodilians on humans, which occurred in Brazil, analysing the general characteristics of these negative interactions.

MATERIALS & METHODS

We performed searches of online databases (Google Scholar, Lilac, and Pubmed), using the words: attack, alligator, crocodilians, and Brazil. We consulted the Worldwide Crocodilian Attack Database (Crocbite), which is associated with Australia's Charles Darwin University, filtering the records that have occurred in Brazil. We also conducted searches of online news sites on Google, with the sentences: alligator attack, alligator bite, and alligator accident, in addition to their Portuguese versions, accompanied by the names of all Brazilian states. We performed all searches from January 2020 to December 2022. We only considered records that reported the presence of witnesses, in addition to prioritising records present in more than one media outlet, or that were found in reputable news programs. For the purpose of comparison with the number of fatalities caused by other animals and the number of homicides, we consulted data from the information system for notifiable diseases (Sinan), the International Shark Stack File (ISAF) and the Brazilian Public Security Forum (FBSP).

The term 'incident' in this paper includes actions from the animal attacking or defending itself, many of which are not easily distinguishable from each other. In this way, we considered 'attacks', as sudden actions or bites from crocodilians involving human beings without any apparent provocation or threat beforehand. We considered unintentional incidents, when the animal reacted to a possible threat from the human being, which can be considered self-defense. We did not use data from attacks intentionally provoked by human beings, such as an attempt to capture, handle or harm the animal. We also disregarded attacks that have been carried out by animals in captivity. We compiled the results highlighting information such as the age of the person affected by the incident, date of the incident, location, and identification of the crocodilian. We searched for the most accurate location co-ordinates, using the information available in the news and articles, and for the elaboration of the map, we used the software QGIS version 2.18.9 'Las Palmas.'

We categorised the activities or situations in which the victims or animals were at the time of the incidents, such as: fishing or in boats, near the water; swimming in or in the water; situations in which the animal felt threatened; and when cleaning fish. To calculate the average number of incidents per year, we used the number of records between the period from 2000 to December 2022, and to compare with the deaths caused by other wild animals, we used data from 2010 from though 2020. The victims' age groups were divided into 10-year intervals. The analyses on the months in which the attacks occurred were carried out only for the Amazon biome due to the low number of attacks that occurred in the other biomes.

We obtained information about the seasonal distribution of the number of attacks by crocodilians in the Amazon biome using circular statistical analysis (Zar, 2010), using ORIANA 4:02 program (Kovach, 2009). The months were converted into angles (30° intervals), and the number of attacks observed in each month was considered as their frequency in each angle (month) (Prudente et al., 2014). From this method, the following values were estimated: a) average vector (μ); b) length of the average vector (r); c) circular standard deviation (SD) in relation to μ; and d) Reyleigh's Uniformity Test. The Rayleigh uniformity test (Zar, 2010) calculates the probability of the data being uniformly distributed (P > 0,05) (null hypothesis). A significant result of the Rayleigh Uniformity Test (P < 0,05) indicates that the data is not evenly distributed.

RESULTS

We recorded a total of 86 cases of crocodilian incidents on humans in Brazil during 2000–2022, 76 of which were unprovoked attacks, ten unintentional attacks, of which only three records came from scientific articles (Supplementary material).

We highlight a record of a fatal attack in 1886 in Iguape, state of São Paulo, where a boy died from serious injuries

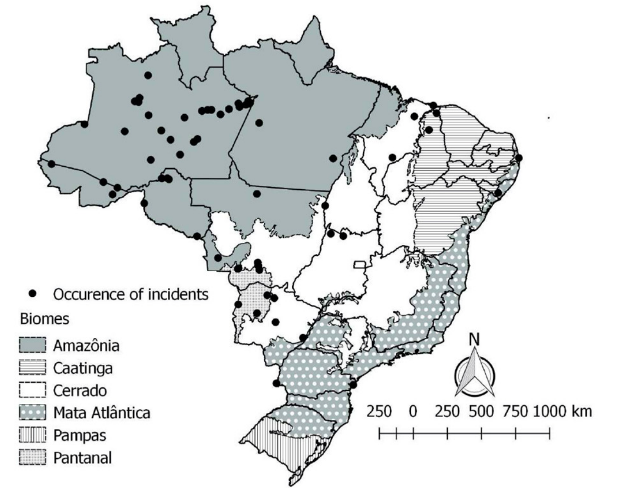

Figure 1. Spatial distribution of records of human crocodile incidents from 2000 to 2022 in Brazil

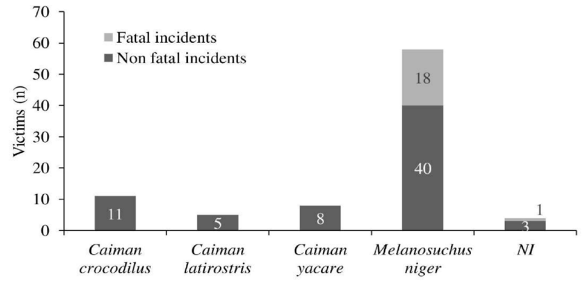

Figure 2. Identification of the species of crocodilians involved in incidents on humans in Brazil, from 2000 to 2022. NI: Not identified

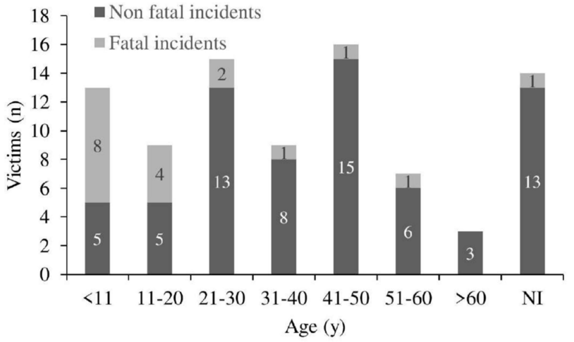

Figure 3. Age classification of victims of incidents by crocodilians in Brazil. NI: Not identified

caused by *C. latirostris*. Eight records where individuals of *M. niger* were found with human parts in their stomach, or pieces of clothing from the victims in their jaws, however, the cause of death cannot be confirmed; the animal may have fed on bodies from drowning or other causes, considered as an occasional event. Both data were not included in the analyses.

The Amazon is the Brazilian biome that concentrates the largest number of records, with 70 % (n = 61) of incidents and 94 % (n = 17) of deaths. The Cerrado biome had the second highest number of records, being a fatal incident, while in Pampas, in the south of Brazil, it is the only Brazilian biome with no records of incidents by crocodilians (Fig. 1 and Table 1).

Table 1. Records of accidents by crocodilians in Brazil from 2000 to 2022

Biome	Fatal accidents	Non fatal accidents	Total accidents per biome
Amazônia	17	44	61
Caatinga	0	3	3
Cerrado	1	10	11
Mata Atlântica	0	4	4
Pantanal	0	7	7
Pampa	0	0	0
Total	18	68	86

Of the six species of crocodilians that occur in Brazil, four were involved in incidents on humans: *C. crocodilus*, *C. latirostris*, *C. yacare* and *M. niger*. In four of the cases, the species could not be identified, being registered as "alligator". The *M. niger* species was the main species involved in incidents, with 67 % of the records (n = 58) being responsible for all the deaths caused by incidents by crocodilians in Brazil, during 2000–2022 (Fig. 2).

Most incidents (n = 16; 18 %) occurred in people aged between 41–50 y, one being fatal. The under 11-y age group had the highest number of fatal incidents (n = 8; 39 %). The age group over 60-y was the only one that did not present any records of fatal incidents. Of all records, 14 did not report the victim´s age (Fig. 3).

In most incidents the victims were fishing or on boats (n = 35; 40 %). In other cases, with people swimming or being present in the water (n = 21; 23 %); were close to the water (n = 18; 22 %); animal felt threatened (n = 9; 11 %); or cleaning fish (n =3; 4 %) (Table 2).

There was a statistically significant difference between the number of records and seasons (p = 0.002). In the Amazon biome, most incidents occurred in the rainy season, between the months of September and December (n = 35; 57 %) (Fig. 4). During the drought period, between April and June, there was a lower number of recorded incidents (n = 6; 8 %).

In the period from 2000 to December 2022, there were a total of 86 crocodilian incidents in Brazil, with an average of 3.7 ± 3.2 incidents per year, of which 22 % resulted in deaths (n = 19; $\overline{x}$ = 0.8 ± 0.7). Comparing with the records of deaths caused by other wild animals in Brazil, during the period 2010–2020, we found that the number of people killed by crocodilians was lower when compared to deaths by snakes, scorpions, bees, and spiders, surpassing only incidents by sharks (Fig. 5). On the other hand, the number of deaths caused by all these animals added together is not close to the number of intentional homicides that occurred in Brazil, only in 2019 (Fig. 5).

DISCUSSION

The reported cases of crocodilian incidents in Brazil may be underestimated due to the absence of a formal record system, especially when it does not result in serious injury or the death of the victim. In addition, many incidents take place in areas far from large urban centres and areas difficult to access. A platform with official and detailed records could provide full conditions for more detailed studies, such as those in the United States (Woodward et al., 2019). Even so, Brazil is the country with the most crocodilian bite records in South America (Pooley et al., 2021).

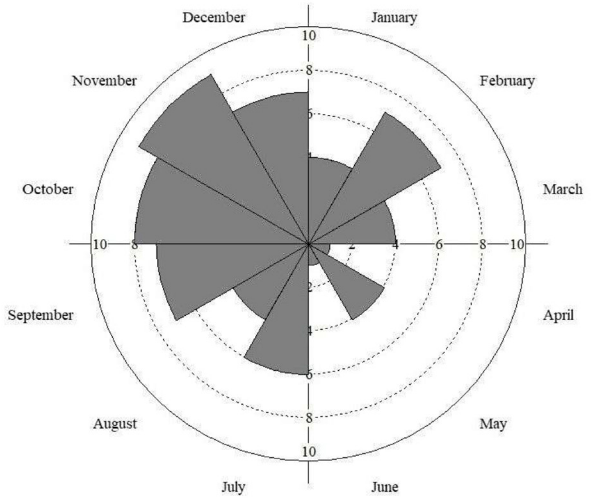

Figure 4. Rose diagram of the circular analysis showing the seasonal distribution of the number of incidents by crocodilians in the Amazon biome

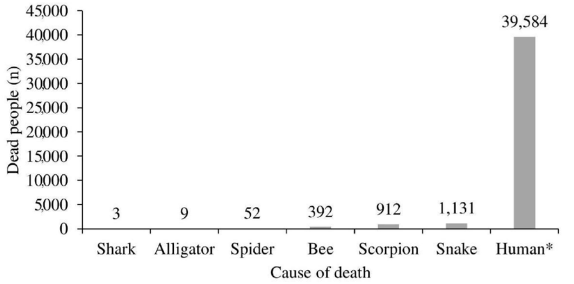

Figure 5. Number of deaths in the period 2010–2020 in Brazil. Number of homicides only in the year 2019. Sources: Ministério da Saúde - Sistema de Informação de Agravos de Notificação - Sinan Net, and Forum Brasileiro de Segurança Pública

Table 2. Activities or situations in which the victim was engaged in at the time of the crocodilian incident

Activities	Incidents (n)	Incidents (%)
Fishing and/or by boat	35	40 %
Swimming or in water	21	23 %
Near the water	18	22 %
Animal felt threatened	9	11 %
Cleaning fish	3	4 %

During the period 2000–2022, the average number of incidents observed in this study (3.7 / year) was small when compared to other regions, such as Indonesia or India with *C. porosus* (Das & Jana, 2018; Sideleau et al., 2021), or Mozambique, caused by *C. nyloticos* (Dunham et al., 2010). Our results were close to those obtained in Florida (United States) caused by the *A. mississippiensis* (Woodward et al., 2019), and were only higher than those obtained in Australia (Caldicott et al., 2005), by *C. porosus* and by *C. acutus*, in the coastal region of Oaxaca in Mexico (Grajales & Silva, 2018).

Melanosuchus niger was involved in more than 67 % of incidents, and responsible for all fatal incidents that occurred in Brazil. Studies points out that larger adult individuals remain in their habitat, being indifferent to human presence (Medem, 1981). The increase in the natural habitat degradation increases the possibility of human contact with wild animals, both due to competition for space as well as attacks on domestic animals and people (Dunham et al., 2010). Another relevant factor is the population density of *M. niger* which, in some isolated regions in the Amazon, has had a very expressive increase in recent years (Da Silveira, unpublished data, 2001), which may have caused an ecological imbalance in relation to the number of natural preys, as also seen in Florida and Australia with *A. mississippiensis* and *C. porosus*, respectively (Caldicott et al., 2005; Woodward et al., 2019).

Because it is a large species, deaths caused by *M. niger* involving children aged 0-10 y can be considered as predation. This can be reinforced by the fact that *M. niger*, like other predators, prefers to attack young or elderly prey (Cott, 1961; Caldicott et al., 2005). Because they are smaller and more fragile, children tend to die when compared with adults, which is why this age group has such a high proportion of fatalities, which was also observed in Mexico (Grajales & Silva, 2018). The 41–50 y age group has the highest number of incidents and corresponds to the age group with the largest number of fishermen in the Amazon (Inomata & De Carvalho, 2018), the main region of crocodilian incidents.

Most of the victims were fishing or were on boats. Such an observation, added to the victims who were cleaning fish, has also been noted in other studies in Malaysia and Australia (Steubing, 1983; Caldicott et al., 2005). The smell or movement of fish, the main component of the diet of adult crocodilians (Magnusson et al.,1987), can attract these animals as they are opportunistic predators (Somaweera et al., 2020). Their diet varies according to their size, in addition to changing their feeding behaviour according to the availability and abundance of prey (Horna et al., 2003). Thus, attacks on people who were swimming, or were in the water, can be considered an attempt at predation. This information can be reinforced by the high number of fatal attacks on children, as seen above. It is common, in the Amazon and in other parts of Brazil, for children to use rivers as places of play or recreation, especially during the rainy season (Brandão et al., 2020), staying longer in the rivers and, consequently, more time exposed to these predators, causing attacks. Crocodilians are extremely territorial, especially when in the presence of nests (Caldicott et al., 2005), which may explain attacks on people close to the shore. The other situations can be characterised as incidents related to the animal's defense reflex.

We observed that, in the Amazon, the number of incidents accompanies the rainy season, with more records occurring during the rainy season, which runs from November to March (Fisch et al., 1998). This may be related to the fact that, during the rainy season, there is a natural expansion of the dispersion of these animals due to flooded rivers (Rebêlo & Lugli, 2001), increasing, therefore, the likelihood of encounters between these animals and riverside residents. In addition to the increase in river levels, the nesting period for Amazonian crocodile species occurs at the end of the drought period, starting in September (Herron et al., 1990), making crocodilians more aggressive (Pooley, 2015). These results were similar to those obtained for *Crocodilus acutus* (Grajales & Silva, 2018).

Comparing with the available data on animal deaths in Brazil, crocodilians, in general, are involved in fewer cases than, for example, snakes, bees, scorpions, and spiders, the main venomous animals in Brazil, which can cause death by inoculation of toxins, but which may have their action aggravated by the victim's health characteristics (White, 2010). Crocodilian attacks are more like those of sharks due to large lacerations, excessive bleeding, and serious secondary infections due to the dentition, strength of the jaws and microbiota present in the animal's mouth (De Campos et al., 2013), and both have low numbers of cases during the period 2010–2020.

Although such animals are seen as villains by the general population, the number of deaths caused by them in 10 years is extremely low when compared, for example, to the number of homicides that occurred in Brazil, for only 2019. This shows that the Brazilian population still lacks basic knowledge about the function of the natural environment that surrounds it. Humans have a natural fear of the unknown, that is, a propensity to feel fear, caused by the absence of information perceived at any level of consciousness or point of processing (Carleton, 2016) and, from that fear, actions against the components of nature, in an act that it considers as defense, causing damage that is often irreparable. In this sense, animals are killed because they are considered 'extremely' dangerous, even those that science considers harmless. This commonly occurs with snakes, which makes the act of encouraging the general population to accept conservation programs for snakes, for example, become a difficult task due to the bad reputation that snakes have in various regions of the world (Santos-Fita et al., 2010; Maschio et al., 2016), even though most snakes are admittedly harmless.

Environmental education practices carried out with local populations should be an alternative for people to be properly educated and instructed on the importance of the different components of nature (Silva & Junqueira, 2007), including crocodilians. Likewise, it is necessary to instruct them in relation to the necessary care that they must take when they live in the same environment in which these animals are found. This could certainly reduce the conflict between humans and crocodilians (Moura et al., 2010), while also reducing potential incidents caused by this encounter (Pooley et al., 2021).

The relationship between crocodilians and humans is still poorly documented in Brazil. The lack of systematisation and detailing of incident records ends up hindering accounting, and analysis. The use of data from local news and newspapers, although they do not have the same degree of rigor as scientific articles, can be an important source of data, in topics with a lack of studies. Adequate documentation is important for species conservation strategies, and for infrastructure development and the well-being of the human population, who live in areas that overlap with the distribution of crocodilian species. The coexistence between animals and humans is part of the global theme of sustainable development and should be encouraged (König et al., 2020). For this it is necessary to understand and analyse the real conflicts, with greater incentives for public policies, with the use of protocols, studies aimed at the conservation and preservation of crocodilians (Pooley et al., 2021).

Crocodilian incidents are rare in Brazil, and the number of deaths caused by them is very small when compared to incidents involving other animals. Most incidents are related to the possibility of facilitating feeding, such as catching fish already caught by humans, or as a defense mechanism. We emphasise the need for further studies and greater systematisation of incidents involving crocodilians. Furthermore, studies on the impacts of habitat destruction and fragmentation on these animals are essential to propose environmental education programs that help to minimise ecological and behavioral changes in animals, allowing for a more harmonious coexistence between humans and crocodiles.

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SHORT NOTE

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You are what your ancestors ate: retained bufadienolide resistance in the piscivorous water cobra *Naja annulata* (Serpentes: Elapidae)

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Predators exploiting chemically defended prey are generally resistant to prey toxins. However, toxin resistance usually incurs a fitness cost and is therefore often lost when no longer needed. Bufonid toads are a frequently abundant food resource, but chemically defended by a group of cardiotonic steroids, bufadienolides. Bufophagous predators have evolved a specific and near-universal mechanism of resistance to these toxins, consisting of two amino acid substitutions in the Na^+/K^+-ATPase H1–H2 extracellular domain. The dynamics of loss or retention of this adaptation in secondarily non-bufophagous lineages remain inadequately understood. Here we explore this topic by showing that the piscivorous banded water cobra *Naja annulata* retains the bufadienolide-resistant genotype of the otherwise toad-eating cobra clade. This confirms a trend for secondarily non-toad-eating snakes to retain bufadienolide resistance.

Keywords: Antipredator adaptation, cardiotonic steroid, cardiac glycoside, evolution, piscivory

Cardiotonic steroids, also known as cardiac glycosides, are a common chemical defence among many plant and animal taxa. These toxins bind to and disable the ubiquitous and vital animal enzyme Na^+/K^+-ATPase, causing potentially lethal poisoning. However, many animals have evolved resistance to cardiotonic steroids. The main mechanism of resistance, which has evolved multiple times across the animal kingdom, consists of replacements of two of the amino acids comprising the Na^+/K^+-ATPase H1–H2 extracellular domain (Ujvari et al., 2015). This single, near-universal mechanism has made it possible to assess the likely susceptibility of a wide variety of animals to cardiac glycosides by simply sequencing the short segment of the Na^+/K^+-ATPase gene containing the H1–H2 extracellular loop (Ujvari et al., 2013; 2014; Mohammadi et al., 2016; Marshall et al., 2018).

Bufonid toads are abundant components of many freshwater and terrestrial ecosystems, and hence a potentially valuable food source for predators. However, most species are chemically defended by the group of cardiac glycosides known as bufadienolides, which make them lethal to unadapted predators (Shine, 2010). However, amphibian predators likely to encounter toads, including multiple snake lineages (Ujvari et al., 2015; Mohammadi et al., 2016), have evolved resistance to these toxins, allowing them to exploit this food source.

Since most antitoxin defences carry a cost, there may be selection against their retention when the drivers of their initial evolution are no longer present, as may have happened in Australo-Papuan varanid lizards (Ujvari et al., 2013). On the other hand, in snakes, Mohammadi et al. (2016) found that many species that have secondarily switched away from preying on bufonid toads nevertheless retain their ancestral resistance to bufadienolides. This suggests that these mutations either carry a very limited cost, or provide other selective advantages, or that reversal would be costly, perhaps due to epistatic intramolecular effects or pleiotropic interactions with other, possibly co-evolved, traits.

Cobras (*Naja* and relatives - Kazandjian et al., 2021) are common predators of toads (Luiselli et al., 2002; Shine et al., 2007), and all members of the clade tested to date display the bufadienolide-resistant genotype of the alpha 3 isoform of the Na^+/K^+-ATPase H1–H2 domain (Mohammadi et al., 2016). The main exception to the generalisation of toad-eating in cobras appear to be the water cobras: these consist of a clade of three species of the subgenus *Boulengerina*, *Naja annulata*, *N. christyi* and *N. nana*, and are phylogenetically nested deep within the genus *Naja* (Wüster et al., 2007; Wallach et al., 2009; Kazandjian et al., 2021). Water cobras are generally regarded as exclusively piscivorous (Chirio & LeBreton, 2007; Pauwels & Vande weghe, 2008; Collet & Trape, 2020; Spawls & Branch, 2020), although systematic studies of their diet are lacking (Grundler, 2020). Captive specimens of *N. annulata* have been reported to consume a *Rana temporaria* and a *Lycodonomorphus bicolor* (Kratzer, 1965; Madsen & Osterkamp, 1982), and there has been speculation that the species may eat amphibians, but there are no confirmed records of this occurring in the wild (Spawls et al., 2002). In keeping with much of the available literature, we therefore treat *N. annulata* as more or less exclusively piscivorous in nature.

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The apparent loss of bufophagy in *N. annulata* raises the question whether this species has retained the resistance to bufadienolides that arose during the initial divergence of the cobra clade (Mohammadi et al., 2016), or whether, in the absence of selection for cardiac glycoside resistance, this species has reverted to the sensitive genotype. To add to our understanding of the dynamics of gain and loss of this adaptation in cobras and their relatives, we here report on additional sequences of the Na$^+$/K$^+$-ATPase H1–H2 domain in elapid snakes, with particular focus on *N. annulata*.

Our methods largely follow Mohammadi et al. (2016) and Marshall et al. (2018). We extracted DNA from tissue samples collected from pet trade specimens or by colleagues at Liverpool School of Tropical Medicine in the course of other work (Kazandjian et al., 2021), using Qiagen Dneasy Blood and Tissue Kits following the manufacturer's instructions. The alpha 3 isoforms of the Na$^+$/K$^+$-ATPase H1–H2 domain were amplified using the primers ATP1a3Fwd (CGAGATGGCCCCAATGCTCTCA) and ATP1a3Rvs (TGGTAGTAGGAGAAGCAGCCGGT) (Mohammadi et al., 2016). For details of PCR conditions and the cleaning of PCR products, see Marshall et al. (2018). PCR products were sent to Macrogen (Seoul, South Korea) for sequencing using both forward and reverse primers.

The sequence trace files were checked for errors and aligned in MEGA 11 (Tamura et al., 2021). We then aligned them with the relevant sequences from Mohammadi et al. (2016), and used Genome BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to obtain corresponding sequences from the published genomes of *Bungarus multicinctus*, *Laticauda colubrina*, *L. laticaudata*, *Naja naja* and *Ophiophagus hannah* (Vonk et al., 2013; Kishida et al., 2019; Suryamohan et al., 2020; Zhang et al., 2022). The coding sequences for the Na$^+$/K$^+$-ATPase H1–H2 domain were translated into amino acid sequences in MEGA 11 and checked for the key Q111L and G120R substitutions that confer resistance to cardiac glycosides (Ujvari et al., 2015; Mohammadi et al., 2016).

We obtained new sequences of *Aspidelaps scutatus*, *Hemachatus haemachatus*, *N. annulata*, *N. kaouthia*, *N. naja*, *N. nigricollis*, *N. nivea* and *N. subfulva*. With existing sequences, we aligned a total of 19 sequences of 16 species (Table 1). The translated amino acid sequences show that all members of the cobra clade share the resistance-conferring Q111L and G120R substitutions. This includes the piscivorous *N. annulata* as well as the most basal lineage of the group, *Aspidelaps*. All other elapids analysed lack these resistance-conferring mutations, except that *B. multicinctus* has Q111L but not G120R. The amino-acid sequences of the H1–H2 domain mapped onto the phylogeny of the Elapidae are shown in Figure 1. All new sequences were deposited in GenBank (Accession numbers OQ338149-OQ338156 – Table 1)

Our results shed new light on the dynamics of the evolution of cardiac glycoside resistance in elapid snakes. With increasing phylogenetic breadth and depth (i.e. representation of *Aspidelaps* and the subgenus *Uraeus* through inclusion of *N. nivea*) and additional species of the other subgenera of *Naja* (Wallach et al., 2009), our results strongly support the inference that bufadienolide resistance most likely evolved once at the base of the cobra radiation (Mohammadi et al., 2016). Virtually all members of this clade are occasional or frequent toad predators (Luiselli et al., 2002; Shine et al., 2007; Grundler, 2020), including its most basal lineage, *Aspidelaps* (Broadley & Baldwin, 2006). For the most part, bufadienolide resistance is thus restricted to toad-feeding elapid snakes.

Table 1. Taxa included in the analysis, origin of new material, GenBank accession numbers and original reference

Species	GenBank Accession number	Origin of new material	Source
Laticauda colubrina	BLBF01000071		Kishida et al. (2019)
Laticauda laticaudata	BHFT01056349		Kishida et al. (2019)
Bungarus multicinctus	CM042835		Zhang et al. (2022)
Ophiophagus hannah	AZIM01007862		Vonk et al. (2013)
Dendroaspis angusticeps	KU738076		Mohammadi et al. (2016)
Dendroaspis polylepis	KU738077		Mohammadi et al. (2016)
Aspidelaps scutatus	OQ338149	UK pet trade specimen, locality of origin unknown	This study
Hemachatus haemachatus	KU738087		Mohammadi et al. (2016)
Hemachatus haemachatus	OQ338153	LSTM Hem Hae ZAF003, South Africa	This study
Naja (Uraeus)nivea	OQ338155	LSTM Naj Niv ZAF001, South Africa	This study
Naja (Naja) naja	OQ338151	UK pet trade specimen, Sri Lanka	This study
Naja (Naja) naja	CM019160		Suryamohan et al. (2020)
Naja (Naja) atra	KU738098		Mohammadi et al. (2016)
Naja (Naja) kaouthia	OQ338152	UK pet trade specimen, Malaysia	This study
Naja (Afronaja) nigricollis	OQ338154	LSTM Naj Mos TZA004, Tanzania	This study
Naja (Afronaja) nigricollis	KU738100		Mohammadi et al. (2016)
Naja (Boulengerina) subfulva	OQ338156	LSTM Naj Mel CMR002, Cameroon	This study
Naja (Boulengerina) melanoleuca	KU738099		Mohammadi et al. (2016)
Naja (Boulengerina) annulata	OQ338156	UK pet trade specimen, locality of origin unknown	This study

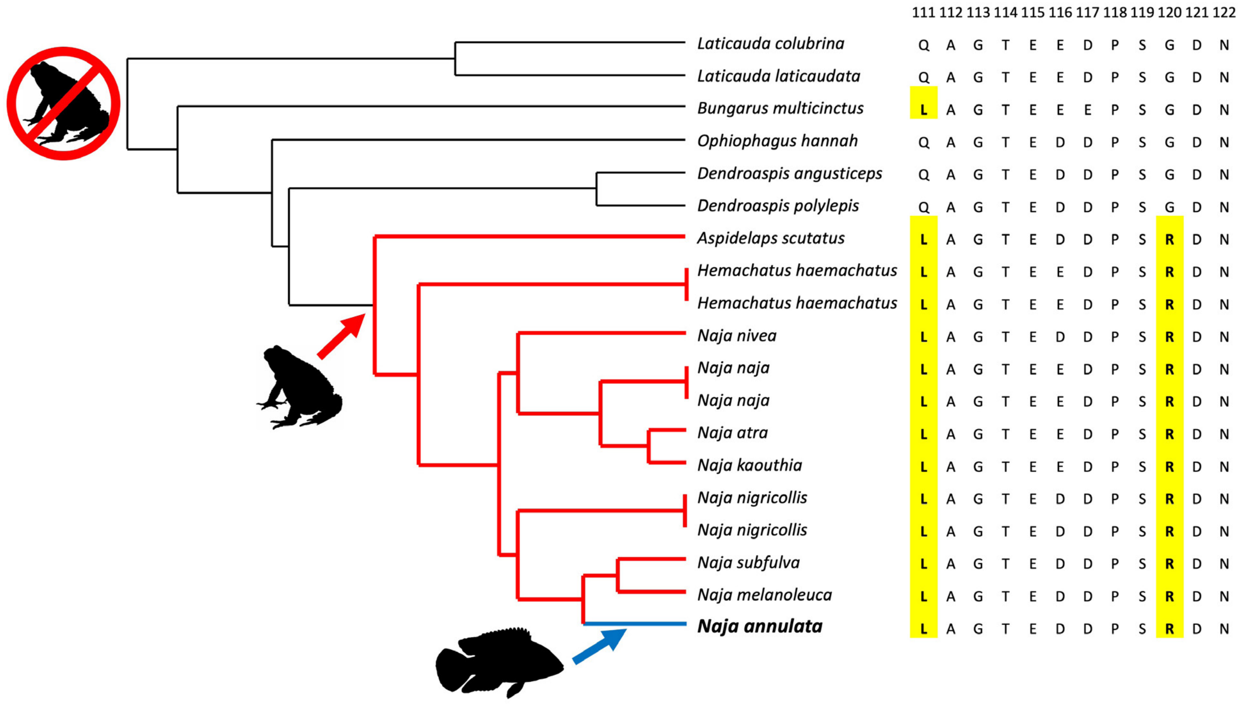

Figure 1. Amino acid sequences of the H1–H2 extracellular loop of all species analysed in this study mapped onto the phylogeny of the cobras and their relatives. Phylogeny redrawn from Kazandjian et al. (2021); the timing of the divergence between *Laticauda colubrina* and *L. laticaudata* is taken from Buehler (2020). Bold type on a yellow background indicates amino-acids likely to confer bufadienolide resistance, bold red branches indicate inferred bufophagous lineages, and the bold blue branch indicates the origin of piscivory in water cobras.

The notable exception to this pattern is *N. annulata*, which appears to be a highly specialised fish predator, but is nested deep within the bufophagous cobra clade and retains the resistant genotype. This species therefore appears to follow the pattern identified by Mohammadi et al. (2016), that while bufadienolide resistance evolves readily in toad-eating lineages, it is often retained in descendant lineages that no longer feed on bufonids. This provides additional impetus to the study of the costs and benefits of bufadienolide resistance in snakes.

Another point of interest in our results is that the many-banded krait *B. multicinctus*, a member of a largely snake-eating clade, displays one, but not both, of the two substitutions associated with cardiac glycoside resistance. It is questionable whether substitution Q111L alone confers significant resistance against cardiac glycosides, so the functional consequences of this mutation in *B. multicinctus* remain unknown (Mohammadi et al., 2016). The topic of bufadienolide resistance in kraits may repay further investigation, as there are several records of one species, *B. caeruleus*, eating bufonids (Slowinski, 1994). In addition, it is worth noting that ophiophagous snakes are potentially at risk of secondary cardiac glycoside poisoning through the consumption of recently-fed bufophagous snakes, especially if the latter sequester bufadienolides (Hutchinson et al., 2007). However, there are virtually no documented observations of such encounters, except that Hesed (2006) observed a non-resistant king cobra *Ophiophagus hannah* feeding on a *Rhabdophis nigrocinctus*, a species known to sequester bufadienolides in specialised nuchal glands (Takeuchi et al., 2018). Unfortunately, the king cobra was not followed after the event, so any ill-effects would have remained undocumented. In any case, the risk of secondary bufadienolide poisoning may provide a selective explanation for the retention of resistance in some of the non-bufophagous snake-eating species, such as *Clelia clelia*, *Erythrolamprus aesculapii* and *E. bizonus*, noted by Mohammadi et al. (2016). It should also be noted that only the alpha 3 isoform of the Na$^+$/K$^+$-ATPase H1–H2 domain has been sequenced for most snakes: resistance-conferring mutations in other isoforms are found in other clades (Ujvari et el., 2015), and could potentially also occur in squamates, providing additional pathways to resistance.

In conclusion, our results have shed new light on the dynamics of the evolution of bufadienolide resistance in the family Elapidae: they confirm the single origin of this adaptation at the base of the clade of cobra-like elapids, and the tendency for resistance to be retained in snakes descended from toad-eating ancestors, in this instance the water cobra *N. annulata*, and highlight the potential interest of the genus *Bungarus* for further research on this phenomenon. Taxonomically fine-grained studies of clades with variable diets, as here, are particularly helpful to better understand the selective advantages and costs of bufadienolide resistance in snakes, and whether retention of this adaptation is better explained by a low cost of resistance, or a high cost of reversal due to factors such as intramolecular epistasis (Mohammadi et al., 2021) or potential pleiotropic links to co-evolved physiological adaptations.

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