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Front cover: The showy leopard frog *Lithobates spectabilis.* There is an article about this species on page 92.
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An improved habitat suitability index for the great crested newt *Triturus cristatus*

Emily Seccombe[1,2] & Roberto Salguero-Gómez[2]

[1]School of Geography and the Environment, Oxford University Centre for the Environment, University of Oxford, South Parks Road, Oxford OX1 3QY, UK

[2]Department of Biology, 11a Mansfield Rd, Oxford 3SZ OX1, UK

Great crested newts *Triturus cristatus* are a European protected species whose conservation depends on the provision and protection of their breeding habitat. The species is in decline internationally, partly due to loss of suitable breeding habitat (European Environment Agency, 2019). A habitat suitability index (HSI) developed in 2000 is extensively used in great crested newt conservation to assess breeding habitat quality in the UK. Here, we introduce a new HSI with an improved ability to reflect *T. cristatus* presence/absence in UK ponds. This proposed HSI is easier to use, requires less data and predicts *T. cristatus* presence/absence better than the previous index. To inform the new index, we used a dual approach to identify the relative importance of environmental criteria to predict *T. cristatus* presence/absence. Firstly, we conducted a survey of 288 HSI users to assess the perceived strengths and limitations of the existing index. Secondly, we analysed national datasets of *T. cristatus* presence/absence and associated environmental data. Using these findings, we then tested various index modifications. The final modifications of the new HSI include (i) using an arithmetic (instead of geometric) mean, to reduce calculation errors and allow compensation between variables; (ii) excluding water quality and waterfowl impact, as these lacked significant power to predict *T. cristatus* presence/absence and were deemed inaccurate by HSI users; and (iii) changing the scoring relationship for pond area to better reflect current data and provide scores for ponds over 2000m^2. We compared scores from the new and original HSIs using an independent dataset for validation, showing that the new HSI better reflects *T. cristatus* presence/absence (larger effect sizes and R-squared values) in comparison to the old HSI. Adopting this improved HSI will enable more effective conservation of the protected species via better-informed decision-making and monitoring.

Keywords: HSI, monitoring, *Triturus cristatus*, amphibian

INTRODUCTION

Britain hosts populations of international importance for great crested newts *Triturus cristatus* (Salamandridae, Dunford & Berry, 2013; Haysom et al., 2018). However, populations of this species are in serious decline (Beebee & Griffiths, 2000). The Joint Nature Conservation Committee's report on UK *T. cristatus* status (2013–2018) concluded that there was an insufficient area and quality of occupied and unoccupied habitat for their long-term viability, and that habitat quality was decreasing (JNCC, 2019). The UK status report echoes the reports of other countries (European Environment Agency, 2019). Consequently, quantifying and identifying *T. cristatus* habitat suitability is an important task for their conservation.

Due to their decline and international rarity, *T. cristatus* is protected in the UK under the Wildlife and Countryside Act (UK Government, 1981) and is a European Protected Species (The Council of the European Committees, 1992). Consequently, actions that may harm individuals or their habitat may require a licence, detailing mitigation requirements (English Nature, 2001). However, these mitigation measures are often suboptimally implemented and monitored (Edgar & Griffiths, 2004; Lewis et al., 2007; 2014b). This mitigation-based approach relies on having sufficient understanding of the species' habitat preferences to ensure the replacement habitat is suitable.

Habitat Suitability Indices (HSIs hereafter) provide a process-based approach to model habitat suitability. For an HSI to be useful, it needs to accurately predict presence/absence of a given species based on limited data (Zajac et al., 2015). HSIs are widely used (e.g. Soniat et al., 2013; Bender et al., 1996), yet there are key concerns over their use. These include an overreliance on variable expert opinion (Johnson & Gillingham, 2004), a lack of output validation (Brooks, 1997) and a lack of suitable frameworks for objective evaluation (Roloff & Kernohan, 1999). Nonetheless, HSIs are often seen as a pragmatic solution for situations requiring management action (Brooks, 1997).

The current method for assessing a site's *T. cristatus* suitability is the HSI developed by Oldham et al. (2000). This index was created as "a simple model for use by the non-specialist, which provides conservationists with an informed view of the value of a site" (Oldham et al., 2000). In this HSI, scores for ten Suitability Indices (SIs hereafter)

Correspondence: Emily Seccombe (emily_rose@btinternet.com)

are calculated then combined by taking their geometric mean, resulting in a score from zero (poor habitat) to one (suitable habitat) - see Table 1 and Oldham et al. (2000) and ARG UK (2010) for full details. The HSI relies upon data from the 1991 National Amphibian Survey (Swan & Oldham, 1993; Oldham et al., 2000), now 30 years outdated. This HSI underwent limited validation - its parameterisation used only 72 ponds, predominantly located in only two of England's 48 counties.

This HSI is used in a variety of key applications. These include site assessments for developments (Natural England, 2015) and assessment of mitigation success (Lewis et al., 2007; 2014a). Importantly, the HSI is used in the UK's statutory reporting on European Protected Species' Favourable Condition Status (Reason, 2013) and in national reporting efforts (Wilkinson & Arnell, 2013). Furthermore, the HSI has been adapted for monitoring schemes in continental Europe too (Jehle et al., 2011; Unglaub et al., 2015; Bełcik et al., 2019).

Despite the extensive applications of Oldham et al.'s (2000) HSI, there are significant concerns over its accuracy (Reason, 2013; O'Brien et al., 2017; Buxton et al., 2021; Priol et al., 2022). Oldham et al. (2000) acknowledged the conjecture in the production of their HSI, noting that the index "can be upgraded easily as knowledge of crested newt habitat requirements improves".

Several studies have assessed the HSI and found a lack of a relationship with *T. cristatus* occupancy. This research includes studies in England (Reason, 2013; Buxton et al., 2021; Lewis et al., 2007), Scotland (O'Brien et al., 2017; Harper et al., 2019a) and in the Mediterranean (Priol et al., 2022). Buxton et al. (2021) found that only waterfowl (SI6) and fish (SI7) indexes in the HSI were significantly correlated with the species' presence/absence. O'Brien et al. (2017) suggested no significant relationship between *T. cristatus* presence/absence and scores for pond area (SI2), waterfowl (SI6), fish (SI7) or pond density (SI8). Priol et al. (2022) suggested the only SI that significantly affected *T. cristatus* occupancy was pond drying (SI3). In contrast, two studies support the use of the HSI: Harper et al. (2019b) suggest that the HSI can predict eDNA-based *T. cristatus* detection in the UK, and Bełcik et al. (2019) propose that *T. cristatus* occurrence could be predicted by HSI scores in central Poland. Bełcik et al. (2019) found that pond area (SI2), water quality (SI4) and fish (SI7) were the most important factors influencing site occupancy.

Despite advances in data availability since its creation, the HSI has remained largely unchanged. In 2007, the HSI was conservatively amended following a user-group workshop (ARG UK, 2010) to improve standardisation and usability. O'Brien et al. (2017) suggest a change to SI1 boundaries in Scotland. Additionally, Buxton & Griffiths (2022) propose a change to how the HSI scores relate to categories. However, the index remains largely as created in 2000.

Motivated by the widespread application of Oldham's HSI, here we develop an improved great crested newt HSI that increases its accuracy and simplicity. To do so, we surveyed HSI users and analysed national ecological datasets to identify the importance of different environmental factors in predicting *T. cristatus* presence/absence across the UK. We then tested modifications and cross-validated the proposed HSI against the original HSI using an independent dataset. Our new HSI outperforms the original one as the goodness-of-fit is higher when using the new HSI compared to the original HSI, for both the original dataset and new independent validation dataset. We hope that the adoption of this HSI will allow more accurate estimation of this species' habitat suitability, thereby enabling more effective protection of this endangered species via better informed decision-making and monitoring.

Table 1. Suitability indices and their corresponding environmental criteria. This is a summary of the information provided in Oldham et al. (2000) and ARG UK (2010).

Suitability Index	Environmental criteria	Details
SI1	Geographic location	3 categories giving scores of 0.01, 0.5 or 1
SI2	Pond area	Graph gives scores for pond areas measured in m^2
SI3	Pond permanence	4 categories giving scores of 0.1, 0.5, 1 or 0.9
SI4	Water quality	4 categories giving scores 0.01, 0.33, 0.67 or 1, based on invertebrate diversity
SI5	Shoreline shade	Graph gives scores for percentage of shoreline shaded
SI6	Waterfowl	3 categories giving scores of 0.01, 0.67 or 1
SI7	Fish	4 categories giving scores of 0.01, 0.33, 0.67 or 1
SI8	Pond density	Graph gives scores using the number of ponds within 1km divided by π
SI9	Terrestrial habitat	4 categories giving scores of 0.01, 0.33, 0.67 or 1
SI10	Macrophyte cover	Graph gives scores for percentage of surface area covered by macrophytes

MATERIALS & METHODS

All statistical analyses were performed with RStudio (v. 1.2.5001, R version 3.6.1, R Core Team, 2017) with the 'tidyverse' (Wickham et al., 2019) and 'modeest' (Poncet, 2019) packages for data preparation and description. The code used and a copy of the data with confidential information removed can be found at https://osf.io/2yt6r/.

User survey

We ran a survey targeting users of Oldham et al.'s HSI to assess perceptions of HSI recording accuracy, to elicit suggestions to improve the HSI, and to identify the extent of user support for the existing HSI. Firstly, we conducted a pilot survey (Supplementary material 1) to identify the required minimum sample size and check the survey design viability, after which minor formatting changes were made. The final survey (Supplementary material 2), published on SurveyMonkey, was sent to 139 UK-based conservation organisations whose members are likely to use the HSI, such as ecology consultancies, biological record centres and conservation organisations. Quantitative questions asked for ratings of accuracy of records of the suitability indexes (SIs) and the HSI itself. Ten qualitative questions asked for information on limitations that reduced accuracy of SI records. Two further qualitative questions asked about additional factors for inclusion and other suggestions for improving the HSI.

The survey was completed by 288 respondents. Most respondents (86%) were professional ecologists. There was a good level of response from experienced users (median of 14 years of HSI experience, ± 0.56 S.E.). Additionally, there was a reasonably high frequency of HSI use amongst the respondents, with 50.4% using it over ten times per year. Neither years of experience ($\chi^{2df=2} = 1.386$, P = 0.500) nor frequency of use of the index ($\chi^{2df=4} = 1.748$, P = 0.782) significantly impacted scores of HSI accuracy.

We analysed the numeric questions through chi-square tests using the chisq.posthoc.test package (Ebbert, 2019). We applied content analysis in NVivo (QSR International Pty Ltd. 2018) to systematically examine the qualitative sections. We coded all answers, then joined codes together into themes based on frequency and similarity, following the practice set out in Bryman (2012). We used open coding rather than a pre-specified coding manual because of the exploratory nature of the survey (Bryman, 2012).

Statistical analysis of ecological datasets

Next, we conducted a series of statistical analyses on UK ecological datasets of environmental correlates of *T. cristatus* population presence/absence. We sourced three datasets containing sites with data on *T. cristatus* presence/absence and HSI information, see Table 2. An additional dataset from NatureSpace was used for verification of the new model. Prior to analysis, we performed data merging, homogenisation and cleaning, using the R packages 'EnvStats' (Millard, 2019) and 'lubridate' (Grolemund & Wickham, 2011). For sites with multiple survey records over time, we chose the most recent record only, to avoid unequal representation of sites in the combined dataset. We checked for independence between the datasets by searching the data for duplicate geographic information. The verification dataset contained no duplicated locations from the original dataset.

We performed a range of tests to identify statistically significant relationships between individual environmental variables and *T. cristatus* presence/absence. We first fitted logistic regressions in a generalised linear model framework (R package 'glmm', Knudson, 2020) to test the relationship between each of the continuous variables (e.g. pond area, macrophyte cover) and *T. cristatus* presence/absence (Skei et al., 2006). Next, we used chi-square tests to test the relationship between each of the categorical variables (e.g. waterfowl impact, fish presence) and *T. cristatus* presence/absence. We used a Fisher's test in place of chi-square test for location due to imbalanced data (most samples being from 'Area A'). For those variables that were significantly correlated with *T. cristatus* presence/absence (P < 0.001), we then performed post-hoc tests of pairwise comparisons using the Bonferroni method (R package 'chisq.posthoc.test'; Ebbert, 2019). Additional statistical details from the post-hoc tests are provided in Supplementary material 3.

Combining and testing modifications to the HSI

By combining the results of the survey and the statistical analyses regarding the environmental correlates of *T. cristatus* presence/absence, we produced a list of candidate modifications to Oldham et al.'s HSI. We tested some of these modifications where sufficient data were available to explore potential improvements in the HSI's predictive ability. When considering the final modifications to test, our primary aim was to create an improved HSI that would be most useful to land managers, ecologists and policy makers while remaining statistically grounded, rather than to just create a model that best explains the great crested newt presence/absence data, but would be of limited usability by our target users. Accordingly, we considered the findings of the survey in conjunction with the ecological data analyses, to inform the modifications to test, such as which SIs to exclude. 37.78% of records had at least one SI value recorded as 'NA', which reduced the

Table 2. Information on the datasets used in this study. All datasets contain information on *T. cristatus* presence/absence or abundance and environmental variables, including HSI scores.

Dataset	Cofnod great crested newt project	National amphibian and reptile recording scheme	Natural England eDNA research project	Natural England evidence enhancement project	NatureSpace south Midlands district level licencing
Records	574	852	5866	3137	153
Geographic Spread	Wales	UK	England	6 pilot areas across England	South Midlands, England
Years	2011–2019	2013–2019	2017–2019	2013	2021
Surveyors	Professionals and citizen scientists	Citizen scientists	Professionals and citizen scientists	Professionals	Professionals
Newt survey method	Mixed	Mixed. See Wilkinson & Arnell (2013)	Mixed. See Natural England Open Data (2019)	eDNA. See Natural England Open Data (2017)	eDNA

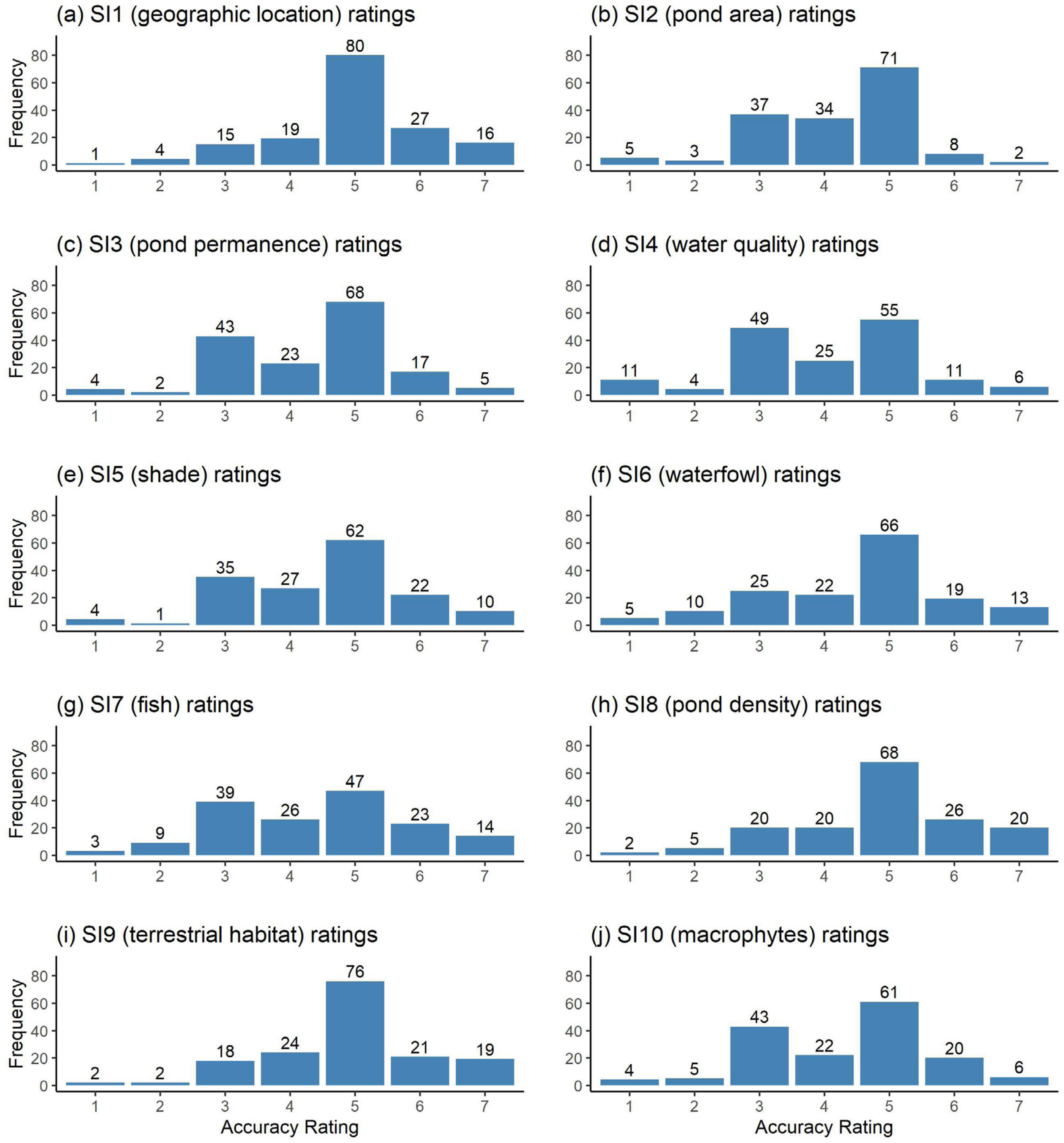


Figure 1. The perceived accuracy of suitability index (SI) records by survey respondents varies across all ten categories (Table 1). Bar charts show the frequency of our sampled 288 respondents selecting each rating option (from 1 to 7, where 1 is the lowest) in answer to the questions on the accuracy of records of SIs 1–10 **(a–j)**.

validity of conducting stepwise regressions to determine which SIs to include.

We then quantitatively assessed the potential improvement of the modifications on the HSI by adopting the approach implemented by Buxton et al. (2021). This involved comparing the distribution of *T. cristatus* presence and absence amongst the five habitat suitability categories (Poor, Below Average, Average, Good, Excellent) using the new and modified HSIs. We used chi-square tests and the pertinent post-hoc tests of significant models on the frequency tables of HSI category and *T. cristatus* presence/absence to identify if modified HSIs were more accurate in differentiating habitat quality than Oldham et al.'s HSI. For these tests, we used the R packages 'dplyr' (Wickham et al., 2020), 'EnvStats' (Millard, 2019), 'forcats' (Wickham, 2023) and 'chisq.posthoc.test' (Ebbert, 2019).

Index cross-validation

To compare the original and new HSIs, we used the independent dataset (provided by NatureSpace, as noted above). The new HSI scores were calculated for each pond. We fitted logistic regressions in a generalised linear model framework (R package 'glmm'; Knudson, 2020) to test the relationship between the original HSI scores and *T. cristatus* presence/absence. Next, we used chi-square tests and post-hoc tests to test the relationship between original HSI category scores (Poor to Excellent) and *T. cristatus* presence/absence, also using the Bonferroni method as above (R package 'chisq.posthoc.test'; Ebbert, 2019). We then repeated both methods using the new HSI scores.

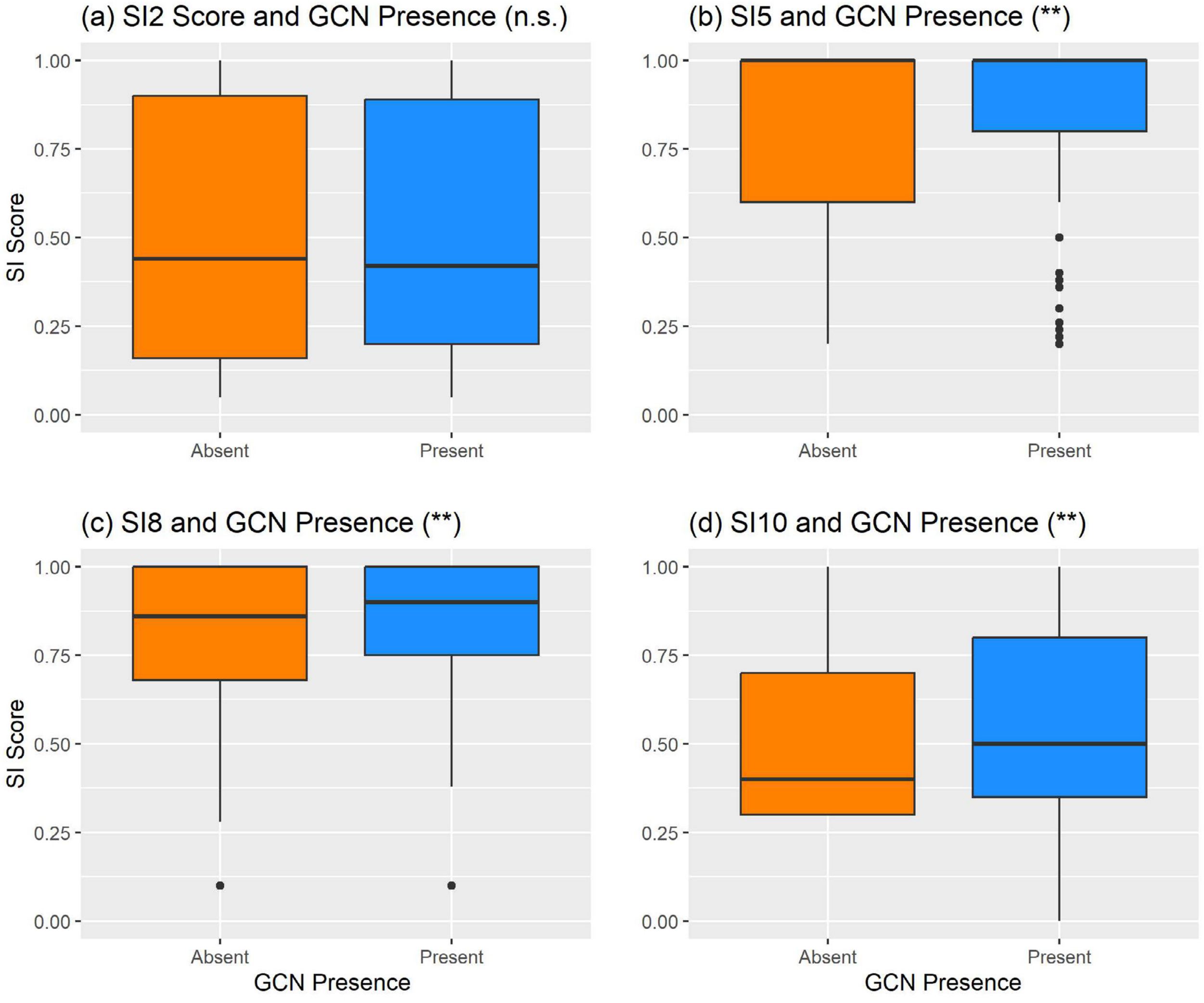


Figure 2. Great crested newt (GCN) presence/absence and continuous suitability index (SI) scores. Minimal differences in scores of continuous suitability indexes (SIs) are apparent between sites with great crested newt absent (orange) or present (blue). Box and whisker plots show **(a)** SI2: pond area, **(b)** SI5: shade, **(c)** SI8: pond density, and **(d)** SI10: macrophyte scores for sites with *T. cristatus* present or absent. "n.s." indicates statistically non-significant differences between SI scores in ponds with *T. cristatus* absent or present; "**" indicates statistically significant differences at P < 0.001 between SI scores in ponds with *T. cristatus* absent or present.

RESULTS

Survey

The survey's rating scale section (Supplementary material 2) on perceived SI recording accuracy revealed much variation between SIs (Fig. 1). The chi-square tests showed that different SIs had significantly different perceived accuracy ratings ($\chi^{2df=18} = 77.454$, $P < 0.001$). Post-hoc tests showed that SI1 (geographic location) and SI9 (terrestrial habitat) had higher perceived accuracy scores ($P < 0.001$ and $P < 0.03$ respectively), and that SI4 (water quality) had lower accuracy scores ($P < 0.001$). The use of the HSI for *T. cristatus* abundance estimations was rated significantly lower than for presence/absence estimations ($P < 0.001$; Fig. S1). Overall, a quarter (26%) of respondents rated the HSI as 'very inaccurate' for estimating abundance.

Common themes arising from qualitative comments on the limitations of SI1–10 accuracy were identified. These themes included the subjectivity of SI measures, seasonal limitations of the HSI, the difficulty interpreting SI graphs (particularly for SI2 [pond area] and SI8 [pond density]) and criticism of surveyor skills. Respondents also suggested a range of additional factors of potential importance for *T. cristatus* habitat suitability that are not in the current HSI. The most frequent suggestions (with 17 comments each) were proximity to local *T. cristatus* populations, chemical water tests and disturbance/predation (predominantly in relation to dogs or people).

The respondents provided diverse suggestions for improvement of the HSI. These responses were grouped into themes which included improving guidance to reduce subjectivity, making the HSI easier to use, using HSI results more appropriately and adapting the HSI to fit other circumstances (e.g. to apply to ditches). Many comments

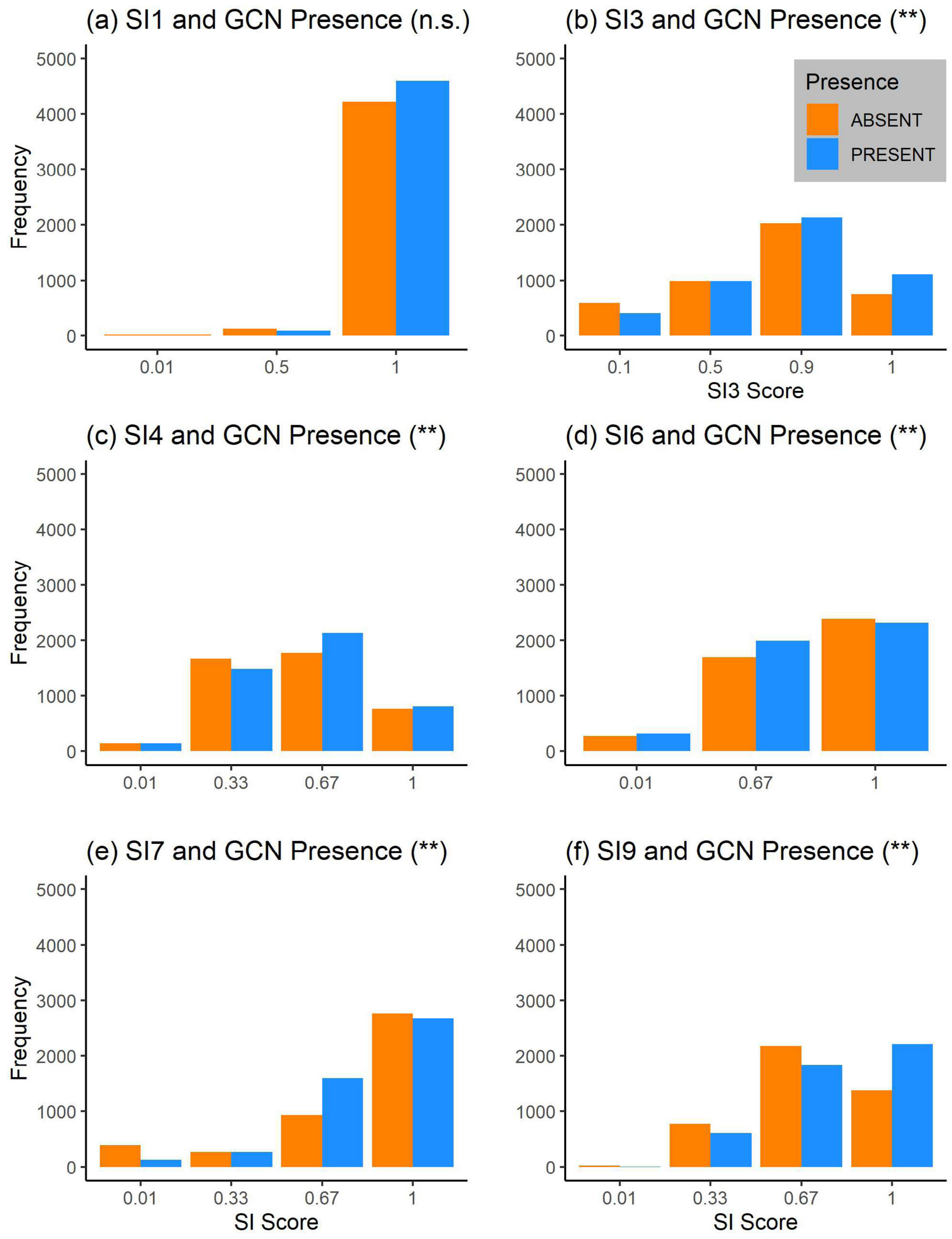


Figure 3. Great crested newt (GCN) presence/absence and categorical suitability index (SI) scores. Most categorical SIs (Table 1) show marginally higher frequency of ponds with *T. cristatus* present (blue) for higher score categories, and lower frequency of ponds with *T. cristatus* absent (orange) for lower score categories. This pattern is not apparent for waterfowl (SI6), and is hard to assess for pond location (SI1) due to the predominance of a score of 1. Bar charts show the frequency of score levels for sites with *T. cristatus* present or absent for **(a)** SI1: geographic location, **(b)** SI3: pond permanence, **(c)** SI4: water quality, **(d)** SI6: waterfowl, **(e)** SI7: fish, and **(f)** SI9: terrestrial habitat. "n.s." indicates statistically non-significant differences between SI scores in ponds with great crested newts absent or present, "**" indicates the statistically significant differences at P < 0.001 between SI scores in ponds with *T. cristatus* absent or present.

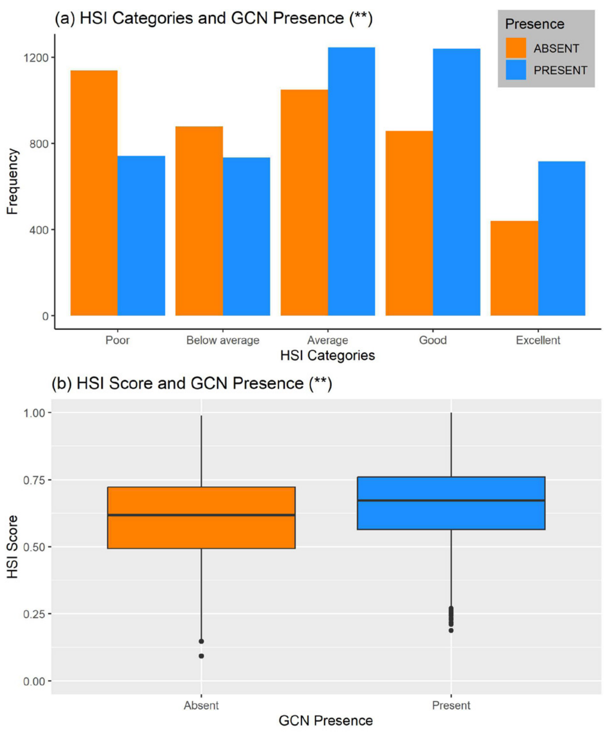


Figure 4. Great crested newt (GCN) presence/absence and habitat suitability index (HSI) scores. HSI scores for great crested newts are higher in ponds where the species is present. HSI **(a)** categories and **(b)** scores are shown for sites with (orange) or without (blue) great crested newts. **(a)** The bar chart shows higher frequencies of ponds with *T. cristatus* present and lower frequencies of ponds with *T. cristatus* absent for the higher HSI categories (Average, Good and Excellent). The panel also shows lower frequencies of ponds with *T. cristatus* present and higher frequencies of ponds with T. *cristatus* absent for the lower HSI categories (Poor and Below Average). **(b)** Box and whisker plot of scores showing higher HSI scores for sites where *T. cristatus* are present.

suggested misuse of the HSI, such as application outside of the recommended season and incorrect geometric mean calculation. Hierarchy plots of the qualitative question responses are given in Supplementary material 4.

Ecological determinants of *T. cristatus* presence

Scores of continuous SIs were significantly higher in sites with *T. cristatus* present than sites with *T. cristatus* absent for SI5 (shade), SI8 (pond density) and SI10 (macrophytes) (all P < 0.001; Fig. 2). Logistic regressions found no significant difference in SI2 (pond area) scores for ponds with *T. cristatus* present or absent.

Of the categorical SIs, significant differences in scores at ponds with *T. cristatus* present or absent were apparent for SI3 (pond permanence), SI4 (water quality), SI6 (waterfowl), SI7 (fish) and SI9 (terrestrial habitat) (P < 0.001, for full residuals, please see Supplementary material 3a) - see Figure 3. The post-hoc tests showed that these differences were in the directions expected (i.e. higher SI values correlated with presence of the species) for most of these SIs, except for SI6 and SI7.

Table 3. Potential modifications to improve the great crested newt suitability indices (SIs) within the habitat suitability index (HSI), inferred from peer-review literature, user survey results and ecological data analysis performed in this research.

Aspect of the HSI	Potential modifications to HSI or associated guidance
SI1	Increase the number of zones, use more up-to-date data and better define borders Include new map with new Scotland zones (O'Brien et al., 2017) Make an easier-to-read map or an online look-up system
SI2	Create SI2 scores for ponds over 2,000 m^2 Removing or rescoring SI2 based on underlying environmental data (Denoel & Ficetola, 2008)
SI3	Test relationship between great crested newts presence/absence and desiccation (Griffiths & Williams, 2000)
SI4	Remove SI4 from HSI Replace with chemical testing of water
SI6	Remove SI6 from HSI
SI7	Test importance of fish presence for great crested newts; try removing from HSI
SI8	Trial excluding SI8 from HSI if HSI < 0.75 (Oldham et al., 2000) Ensure SI8 scores correctly divided pond numbers by pi; create new graph to avoid need to divide by pi Test importance of pond density for great crested newts Remove from HSI
SI10	Change focus from living plant material to any egg-laying material

Surprisingly, the post-hoc test for SI6 showed that ponds with *T. cristatus* absent had significantly more scores of 1 (indicating waterfowl absence) and fewer 0.67 scores (indicating minor waterfowl impact) and vice versa for ponds with *T. cristatus* present (P < 0.001). The post-hoc test for SI7 also showed an unexpected pattern; ponds with *T. cristatus* absent had more 0.01 scores (indicating major fish impact), more 1 scores (indicating fish absence) and fewer 0.67 scores (indicating possible fish impact) and vice versa (P < 0.001). No significant difference was found for SI1 scores (geographic location) at ponds with *T. cristatus* present than ponds with the species recorded as absent.

Logistic regression found a significant relationship (slope = 0.703, d.f. = 9,046, P < 0.001) between HSI scores and *T. cristatus* presence/absence (Fig. 4). We found significant differences in the HSI category frequencies for ponds with *T. cristatus* present or absent ($\chi^{2df=4}$ = 245.04, P < 0.001). As expected, post-hoc tests showed that ponds without *T. cristatus* had more Below average and Poor scores and fewer Excellent and Good scores than ponds with *T. cristatus* (P < 0.001 in all cases). This reflects the patterns shown in Figure 4a.

HSI improvements

Informed by the previous results, a list of potential modifications is presented in Tables 3 & 4. Some of these

Table 4. Potential modifications to improve the great crested newt habitat suitability index (HSI), inferred from peer-review literature, user survey results, and ecological data analysis performed in this research.

Improve guidance to reduce subjectivity and emphasise appropriate use (not as substitute for population survey) (Buxton et al., 2021)
Weight SIs according to importance (Oldham et al., 2000)
Add measures of uncertainty to output (Bender et al., 1996; Burgman et al., 2001; Zajac et al., 2015; Biggs et al., 2014)
Test predictive ability of HSI scores on great crested newt presence/absence, abundance and breeding success (Reason, 2013; Buxton et al., 2021; Lewis et al., 2007)
Create clearer graphs and provide formulae for the relationship between the SI score and the underlying environmental variable shown in the graph
Check if HSI scores provided correctly uses the geometric mean of SIs; try arithmetic mean or cumulative score of SIs to reduce mistakes of miscalculation (U.S. Fish and Wildlife Service, 1981; Burgman et al., 2001)
Increase the number of categories for categorical SIs
Investigate additional factors for potential inclusion (Langton et al., 2001; Skei et al., 2006; Marklund et al., 2002; Denoel & Ficetola, 2008; Gustafson et al., 2011; Cresswell & Whitworth, 2004; JNCC, 2019; Dunford & Berry, 2013)
Provide greater clarity on detailed, reproducible methods for recording SIs

modifications require additional data or involve changes to the written guidance. The modifications that could be tested with the data available for this study were explored and results presented below. Additionally, new graphs were produced to aid reading of continuous SIs (see Figs. S4–S6), and formulae are provided in Supplementary material 5.

Two of the datasets were found to have incorrect HSI calculations due to not adjusting the exponent in the geometric mean calculation to take account of the number of SIs recorded. To overcome this issue, new HSI scores were calculated using the arithmetic mean. The distribution of HSI score categories remained significantly different between sites with *T. cristatus* present or absent ($\chi^{2df=4}$ = 261.85, P < 0.001), see Supplementary material 3b for residuals from the post-hoc test. Therefore, the arithmetic mean-based HSI is comparable in terms of reflecting *T. cristatus* presence/absence, but avoids the likelihood of calculation mistakes as it is simpler to calculate.

SI8 (pond density) scores were not calculated correctly in the NARRS dataset (SI8 scores were not divided by π, resulting in no sites scoring between 0.1 and 0.7). The risk of error by forgetting to divide by π was also noted by survey respondents. To prevent these errors, a modified graph was produced (Fig. S5) that avoids the need for division by π.

New HSI scores were created by excluding the SIs that received low accuracy ratings in the survey. Independent exclusion of SI4 (water quality) and SI6 (waterfowl) were found to be beneficial (see Supplementary material 3c & 3d), so these SIs were then excluded simultaneously. The distribution of sites between HSI categories remained different for ponds with *T. cristatus* present or absent ($\chi^{2df=4}$ = 369.83, P < 0.001). The post-hoc test showed the modified HSI was better than the original (larger residuals) for distinguishing Excellent and Poor ponds, and comparable for other categories (Supplementary material 3e).

Table 5. Habitat suitability index (HSI) categories (left column) and corresponding values (centre column) under the original habitat suitability index (ARG UK, 2010) and with the new modified habitat suitability index (right column).

Category	HSI value cut-offs for original HSI, as given in ARG (2010)	HSI value (x) cut-offs for new HSI combining useful modifications
Poor	< 0.5	x < 0.70
Below average	0.5–0.59	0.70 ≥ x < 0.77
Average	0.6–0.69	0.77 ≥ x < 0.80
Good	0.7–0.79	0.80 ≥ x < 0.87
Excellent	>0.8	x ≥ 0.87

A new scoring method for SI2 (pond area) was created by plotting new data to create a new scoring graph (see Figs. S2 & S3). HSI scores were recalculated with the new SI2 scores. The distribution of sites between HSI categories remained different for ponds with *T. cristatus* present or absent with the new SI2 scores ($\chi^{2df=4}$ = 297.51, P < 0.001). The post-hoc test showed the modified HSI was better at distinguishing between ponds in the Below Average, Excellent and Good categories, although very slightly worse for the Poor category (see residuals in Supplementary material 3f) in comparison to the original HSI.

The modifications detailed above were then combined and new HSI scores were calculated using these modifications. The scores were grouped in categories with new boundaries to create more equal splits to facilitate interpretation (Table 5). The new scores and category frequencies are shown in Figure 5, which can be compared to the original HSI scores and category frequencies in Figure 4.

The chi-square test of the new HSI categories and *T. cristatus* presence/absence showed strong significance ($\chi^{2df=4}$ = 533.11, P < 0.001). The residuals for all categories are larger than for the original HSI (see Supplementary material 3g for residuals).

Cross-validation

Both the original and new HSIs lacked predictive power for *T. cristatus* presence/absence at high significance values with the independent dataset. However, the new HSI had higher effect sizes and lower residuals from the logistic regression than the original HSI scores (see Fig. 6 and Supplementary material 3h for full residuals). Following chi-square tests to look at HSI category scores, post-hoc tests were carried out, but no significant differences in *T. cristatus* presence/absence between HSI categories were found (see Supplementary material 3i).

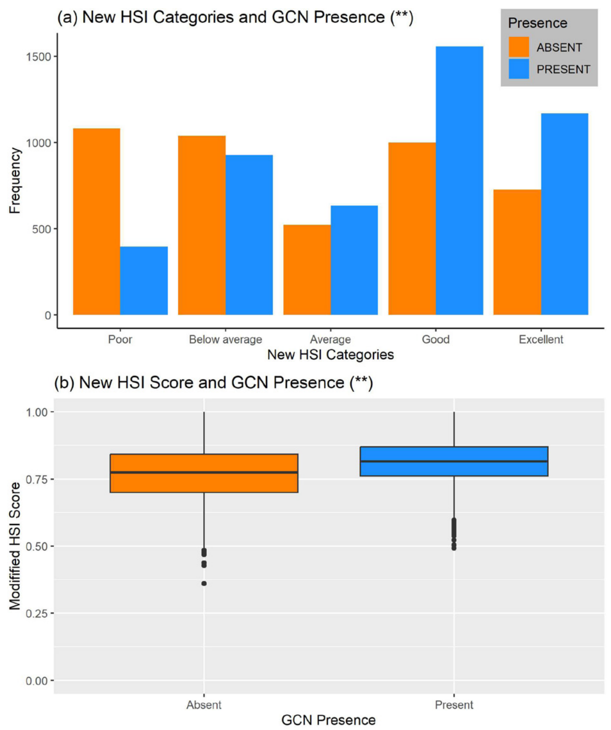


Figure 5. Distribution of **(a)** HSI categories and **(b)** HSI scores for sites with great crested newts (GCNs) present or absent under the newly proposed habitat suitability index (HSI). HSI scores are statistically higher in ponds with *T. cristatus* present (P < 0.001) - see Supplementary material 3g. **(a)** Bar chart showing higher frequencies of ponds with *T. cristatus* present and lower frequencies of ponds with *T. cristatus* present for the higher HSI categories (Average, Good and Excellent). This panel shows lower frequencies of ponds with *T. cristatus* present and higher frequencies of ponds with *T. cristatus* absent for the lower HSI categories (Poor, Below Average). **(b)** Box and whisker plot of the newly proposed HSI scores showing higher HSI scores for sites with *T. cristatus* present (P < 0.001) - see Supplementary material 3h. The interquartile range of modified HSI scores in Figure 5b is higher and more compact than that of Oldham's HSI shown in Figure 4b, necessitating the new category divisions proposed in Table 5.

DISCUSSION & CONCLUSION

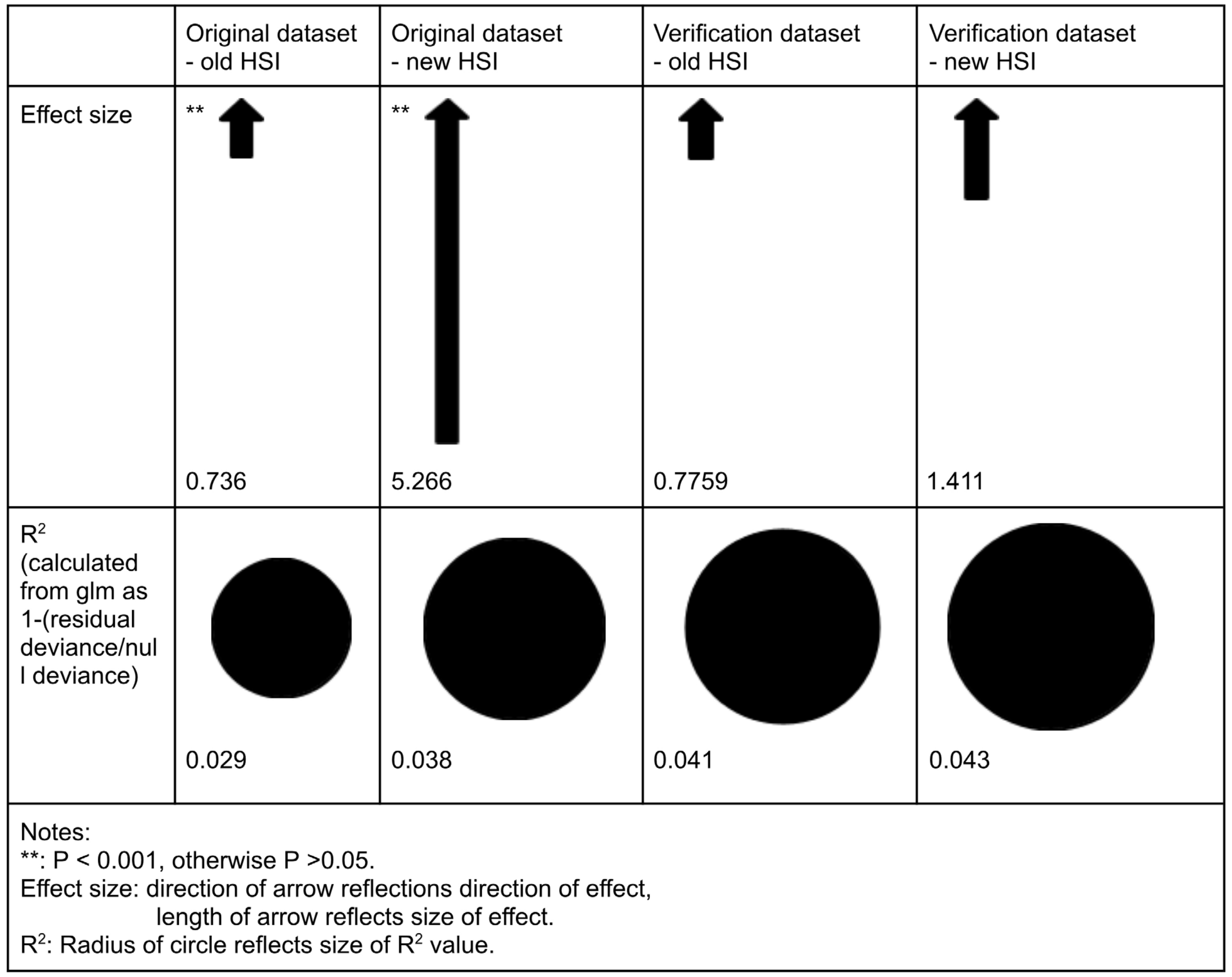
We examined potential improvements to the existing habitat suitability index (HSI) for the great crested newt proposed by Oldham et al. (2000), as adapted by ARG UK (2010). Through a combined approach using a HSI-user survey and robust statistical analyses of presence/absence and environmental data across the UK, we found that the HSI could be improved by removing the indices for SI4 (water quality) and SI6 (waterfowl), combining the index values with an arithmetic (rather than geometric) mean, and creating a new scoring scheme for SI2 (pond area). Thus, we introduce an improved HSI for great crested newts in the UK.

This study identified a range of limitations of the original HSI which builds upon a growing evidence base suggesting the HSI needs improvement. The findings echo wider literature about HSIs, such as concerns about a lack of objectivity (Brooks, 1997). HSIs are intended as applicable management tools and thus their accuracy will pragmatically always be limited (Johnson & Gillingham, 2004). Nonetheless, the limitations to the HSI given in the survey and the criticism in the existing literature justify further work to improve the HSI, particularly given its conservation importance for the species. Indeed, the original HSI paper noted that the HSI "can be upgraded easily as knowledge of crested newt habitat requirements improves" (Oldham et al., 2000), demonstrating the expectation of ongoing modification to the HSI.

Concern over application of the HSI to reflect great crested newt abundance was a key theme arising from the survey. The HSI guidance (NARRS, n.d. & ARG UK, 2010) and Natural England's great crested newt web page (Natural England, 2015) specify that the HSI should not be used as replacement for population surveys or to predict abundance. Our survey found that this practice was continuing despite the guidance, which corroborates concerns raised by Lewis et al. (2007) and Buxton et al. (2021). This finding demonstrates the importance of clearer HSI guidance to ensure practitioners are not misusing the HSI to absolve the need for population surveys.

The ecological data analysis suggested that the original HSI has limited ability to differentiate *T. cristatus* suitability as inferred from presence/absence. Our results showed that many ponds in low HSI categories (Poor or Below Average) have *T. cristatus* present, and many ponds in high HSI categories (Good or Excellent) have *T. cristatus* recorded as absent. This finding echoes the results of Buxton et al. (2021) at a national stage. This demonstrates the need to continue investigating HSIs after their creation - such research is lacking for other species' HSIs too, as highlighted in Brooks (1997). These findings provide clear evidence that the existing HSI should not be overly-relied upon for conservation and mitigation decisions.

A limitation of the HSI identified by the ecological analysis is that only some SIs were significantly correlated with *T. cristatus* presence/absence. This finding corroborates wider evidence, such as that of O'Brien et al. (2017) and Priol et al. (2022). SI1 (location) and SI2 (pond size) were not significantly correlated with *T. cristatus* presence/absence. SI5, SI8, SI9 and SI10 (shade, pond density, terrestrial habitat quality and macrophytes) scores were positively correlated with *T. cristatus* presence. SI3 (pond permanence), SI4 (water quality), SI6 (waterfowl) and SI7 (fish) had less clear results. The lack of clear correlation between SI7 and *T. cristatus* presence/absence in this study reflects the findings of Denoël & Ficetola (2008), who found a negative impact of fish on other newt species, but no significant impact on great crested newts specifically. Wider research suggests that fish do have a negative impact on great crested newts (e.g. Skei et al., 2006; Miró et al., 2017; Harper et al., 2019b; Bełcik et al., 2019), so the lack of significant correlation in our study may be due to the difficulties in determining fish presence as part of the HSI methodology, a challenge noted by survey respondents and in O'Brien et al. (2017).



	Original dataset - old HSI	Original dataset - new HSI	Verification dataset - old HSI	Verification dataset - new HSI
Effect size	** 0.736	** 5.266	0.7759	1.411
R^2 (calculated from glm as 1-(residual deviance/null deviance)	0.029	0.038	0.041	0.043

Notes:
**: P < 0.001, otherwise P >0.05.
Effect size: direction of arrow reflections direction of effect,
length of arrow reflects size of effect.
R^2: Radius of circle reflects size of R^2 value.

Figure 6. Effect sizes and goodness-of-fit for the new and original Habitat Suitability Indexes (HSIs). The newly proposed HSI outperforms the old HSI in terms of effect sizes and goodness-of-fit when *T. cristatus* predicting presence/absence. The length of the arrows is proportional to the effect size for the output of the logistic regression, under the two HSIs, firstly tested on the original dataset and secondly on the independent verification dataset. The circles show the R^2 values - with the new HSI, these values are larger (more so for the original dataset) suggesting a better goodness-of-fit between the new HSI and the *T. cristatus* presence/absence data. The effect sizes are only statistically significant when using the larger, original dataset.

This study also identified errors in HSI calculation. These errors include incorrectly calculating pond density scores (as in the NARRS dataset) or not correctly adjusting the geometric mean calculation for the number of SIs recorded (as in the NARRS dataset and the Natural England eDNA dataset). This finding adds to the need for increased validation of HSIs (Brooks, 1997), to ensure that not only does the HSI provide an accurate reflection of the species' habitat requirements, but that the HSI is easy to calculate correctly.

This study produced a range of potential modifications to improve the HSI (summarised in Tables 3 & 4). Some of these require further ecological data collection and analysis, and so are out of the scope of this study, but provide a starting point for further research. However, additional variables and complex methodologies could make the HSI harder to use. Future research could also investigate how uses and perceptions of the HSI vary across the geographical range in which the HSI is applied, including outside of the UK.

Those modifications which could be tested with the available data and would not add to calculation complexity were investigated in this study, leading to a new HSI formulation. The proposed HSI has the following modifications: an arithmetic mean is used to combine SIs instead of a geometric mean, a new SI2 (pond size) scoring relationship is provided, SI4 (water quality) and SI6 (waterfowl) are removed, and new graphs are provided for SI calculation. Following the approach of Buxton et al. (2021), the distribution of ponds with *T. cristatus* present or absent between different HSI categories was used to assess HSI accuracy. According to this method, the new HSI is better than the original at distinguishing great crested newt habitat suitability, assuming that suitability can be inferred from presence/absence (Lewis et al., 2007).

The new HSI should be easier to conduct due to simpler calculation and having fewer SIs. The exclusion of SI4 (water quality) and SI6 (waterfowl) avoids the problems in assessing water quality and waterfowl impact noted by survey respondents. The new graphs (Figs. S4–S6) and formulae (Supplementary material 5) also support ease-of-use of the new HSI. By not incorporating any new SIs, the new HSI has backwards compatibility with existing records; the changes to the HSI would allow the recalculation of scores on existing data, where underlying data (e.g. pond scores not just SI2 scores) are available. This ability is only possible due to not adding any additional SI variables. Although other factors may have significant influences on *T. cristatus* occupancy, we focussed on amendments that would make backwards comparisons possible. The improved HSI presents a way forward for great crested newt habitat monitoring, which could provide more indicative results (better reflection of likely presence/absence), with fewer errors.

The results of the cross-validation logistic regression using generalised linear models suggests that the new HSI scores better reflect *T. cristatus* presence/absence. Both indexes are limited in predictive power, although the new index has higher effect sizes and R^2 values, whilst importantly using fewer variables. The lack of significance when using either HSI on the verification dataset may be due to the small sample size, or irregularities of the data.

The complex nature of the ecological data used introduces limitations, such as non-random site selection and varied sampling effort. One difficulty comes from the false assumption that populations are in equilibrium with the environment, thereby creating an issue with using presence/absence as a proxy for habitat suitability (Latimer et al., 2006). This assumption is particularly problematic for species with metapopulation dynamics (Kupfer & Kneitz, 2000).

The research presented here provides an evidence-based starting point for adapting the great crested newt HSI and the accompanying guidance. In the context of the international decline in their populations and suitable habitat (European Environment Agency, 2019), the HSI is a crucial tool for monitoring and for determining mitigation requirements, being used in UK status reporting (JNCC, 2019) and relied upon in mitigation practices (National England, 2015). Accordingly, the issues with the existing HSI highlighted in this study are of importance to the conservation of this species, as they present a notable barrier to accurate habitat assessment. The modified HSI delivers upon the original HSI creator's intention to update the HSI when improved knowledge was available (Oldham et al., 2000). With the modified HSI and appropriate usage guidance, we believe the assessment of great crested newt habitat can be improved, thereby increasing the potential of conservation and mitigation works to benefit the species.

Our multidisciplinary approach, which combines expert great crested newt elicitation together with robust environmental statistical analyses, shows tangible ways to improve the existing great crested newt HSI. We presented greater insight into the current HSI's limitations, and proposed a modified HSI which better reflects *T. cristatus* presence/absence and is easier to use. Our proposed new HSI has three key modifications: i) using an arithmetic (rather than geometric) mean to combine variables, to reduce calculation errors and allow compensation between variables; (ii) excluding water quality and waterfowl impact as these lacked significant power to predict *T. cristatus* presence/absence and were deemed inaccurate by HSI users; and (iii) changing the scoring relationship for pond area to better reflect current data and provide scores for ponds over 2000m^2. We argue that the improved HSI developed in this study is better able to inform accurate assessment of the habitat suitability of ponds for great crested newts, and thereby provide more accurate monitoring of habitat trends and guide their conservation and development-mitigation. This research is important due to the extensive use of the HSI in great crested newt conservation and we hope that a new HSI will be implemented as a result of this research.

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DATA ACCESSIBILITY

The data, code and supplementary materials can be found here: https://osf.io/2yt6r/. The Natural England data can be found at https://naturalengland-defra.opendata.arcgis.com/ - see references for Natural England Open Data 2017 and 2019.

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Nesting of *Caretta caretta* in Tuscany area (north-western Mediterranean Sea): insights of a recent colonisation phenomenon

Cecilia Mancusi[1,2], Chiara Caruso[3], Giuliana Terracciano[4], Giovanni Raimondi[5], Lucia Venturi[3], Laura Tonelli[3], Alessandra Neri[2,6], Ilaria Ceciarini[2], Enrica Franchi[2], Sergio Ventrella[2], Letizia Marsili[2] & Marco A.L. Zuffi[7]

[1] Environmental Protection Agency - Tuscany Region (ARPAT), Via Marradi 114-57126, Livorno, Italy

[2] Department of Environment, Earth and Physical Sciences, University of Siena, Via Mattioli 4, 53100 Siena, Italy

[3] Maremma Regional Park, "Centro Recupero Tartarughe Marine di Talamone", Talamone, Grosseto, Italy

[4] Istituto Zooprofilattico Sperimentale Lazio e Toscana, sede di Pisa, Pisa, Italy

[5] Acquario di Livorno, Costa Edutainment S.p.A Livorno, Italy

[6] CIBM, Centro Interuniversitario di Biologia Marina, Livorno, Italy

[7] Museo di Storia Naturale - Università di Pisa, Calci, Pisa, Italy

From 2013 to 2021 twenty-three *Caretta caretta* (Loggerhead turtle) nests were found along the coast of Tuscany (Italy, north-western Mediterranean Sea). Loggerhead nesting is a new event for this part of the Mediterranean Sea, which occurred only recently. Laid eggs were 2081 with 943 hatchlings, for an average hatching success of 45.31%, ranging from 0.1 to 95%, depending on the year and the site. After hatching, nest inspection was carried out and, when possible, eggs and hatchlings (dead and alive) were measured (e.g. curved carapace length with calipers in mm, body mass, with an electronic balance in grams) and standard environmental parameters at the nests were registered (e.g. nest chamber depth and width, grain size, sand temperature). We strongly recommend accurate monitoring and observations of the beaches along these coastlines to establish other nesting occurrences of loggerhead turtles in this region.

Keywords: loggerhead turtle, reproduction, clutch size, Tyrrhenian Sea

INTRODUCTION

Sea turtles are included in Directive 92/43/EEC "Habitat", implemented with DPR 357/97 and modifications, which provides specific rules on capturing, handling and detaining these animals. The inclusion of sea turtles in Annex IV of the Directive also provides the obligation on the part of the Member State to carry out monitoring and surveillance activities of their state of conservation, as well as their accidental capture or killing (Mo et al., 2013). From a conservation point of view, *Caretta caretta* is listed in Appendix 1 of CITES and in appendix II of Bern convention (1979). According to the IUCN it is Least Concern at Mediterranean subpopulation level (IUCN Red List; www.iucnredlist.org: Table 1, download 25 november 2020).

Nesting of marine turtles in the Mediterranean basin has been verified and monitored for decades. Main areas of nesting are in Greece, Turkey, Libya and Cyprus, but also reported in the western basin (Tomas et al., 2002), while feeding areas are the Tunisian continental platform, the Adriatic and Ionian seas, between the Balearic Islands and the Alboran Sea, the Egyptian continental platform and the Turkish coast (Casale et al., 2018). The species occurs also in all the other marine areas of the Mediterranean, even if with lower frequency (Argano, 1992; Mingozzi et al., 2007; Casale et al., 2018). Available information regarding two breeding populations of marine turtles, the loggerhead turtle *Caretta caretta* and the green turtle *Chelonia mydas*, nesting in the Mediterranean has been greatly improved during the past two decades (Margaritoulis et al., 2003; Casale & Margaritoulis, 2010). Nevertheless, there are major knowledge gaps that still limit our capacity to accurately assess the conservation status of both species and predict the consequences of anthropogenic stressors, particularly fisheries bycatch and climate change.

In the Mediterranean Sea, loggerhead turtle *C. caretta* nesting sites are concentrated in the eastern and central basins, whereas the waters of the western basin host a large number of turtles for feeding activities in pelagic and coastal habitats (Marquez, 1990; Margaritoulis et al., 2003).

Sea turtle nesting in the western Mediterranean (i.e. southern Tyrrhenian Sea, as in northern Sicily, southern Calabria) has been reported since the early 1990s, but it was always referring to single sporadic events, widely scattered over the coasts bordering the basin. However, since the 2010s, reports of nesting activity have been increasing and, at least in some areas (i.e. northern Campania, Latium, Tuscany), new nesting sites have been reported (Mingozzi et al., 2007; Hochscheid et al., 2022).

Available data do not allow demonstrating historic nesting of the loggerhead on the Tuscany coasts. According to anecdotal information and from oral enquires and testimonies, it is thought that no one marine turtle has ever nested on the sandy coasts of this region in the last century. Also after widespread literature check, no substantiating evidence could be unearthed.

We aimed at describing the new *C. caretta* nesting phenomenon for the Tuscany area, as for nest geographical distribution, nest characteristics, clutch, egg and hatchling size, hatching success and incubation temperatures.

MATERIALS & METHODS

A probable loggerhead turtle hatching was registered in Tuscany for the first time on 3 October 2013 when 22 hatchlings were seen on the beach in Scarlino (Grosseto). On that occasion, no nest was found. Another loggerhead turtle nest was discovered accidentally on a beach in Giannella (Grosseto) on 6 September 2015, 50 km south from the 2013 nest. About 30 hatchlings were seen on the beach crawling seaward in the early morning. Later also the emergence point was discovered, where hatchlings tracks originated. It was monitored for some days and it was inspected on 10 September.

Based on these sporadic events, from 2017 on, we carried out a standardised beach monitoring routine from early June to late August, along the several beaches located along the southern and central part of Tuscany coasts. We verified nesting attempts by checking the presence of loggerhead tracks on the sand, characterised by the typical 'U' shaped trace, as outcome and income from the sea. Depending on positive or negative results from inspection at the direction change of the turtle track, we assessed the false or actual nesting crawl. Due to the intensification of the nesting events during the recent years, beach monitoring (daily on foot or with fat bikes, and weekly with drones), was carried out on along the entire Tuscany coast, including the northern part, according to rules established by the authorisation of the Italian Ministry for Ecological Transiction in derogation to DPR 357/97 (Mo et al., 2013).

As soon as a track or a turtle is detected along the beach, either during the night or early morning, the Sea Turtle Stranding Network (STSN) is in charge of verifying the presence of the nest. Location data (e.g. latitude and longitude, distance from the sea and the dune in m) were registered. We placed data logger (EasyLog USB EL-USB, www.lascarelectronics.com), with USB interface for configuration and download, under the sand at a depth of about 40 cm, close to the nest chamber, for continued registration of the temperature. The user-friendly control software window allowed setting the registration of temperature at regular intervals, every 30 minutes. The data could then be graphed, printed and exported to other applications for detailed analyses. We also registered surface sand temperature using a digital thermometer (Hanna Checktemp; www.hanna.it), with a resolution of 0.1 °C and an accuracy of ± 0.2 °C. Sampling of sand for grain-size investigation was conducted near the nesting site. A 1000 g sand sample was collected within a plastic jar. The analysis was conducted following ISPRA guidelines (Romano et al., 2018). Three-dimensional classes were determined: gravel (> 2 mm), sand (< 2 mm > 0.063 mm), silt (< 0.063 mm) (Krumbein, 1934).

If the exact moment of the deposition is known, we consider a period for hatching between 45.6 to 70.0 days (6.4 to 10 weeks), as estimated for the Italian rookeries (Casale et al., 2018); 6-10 weeks of incubation, depending on the temperature, was reported also by Miller (1985).

Hatching is expected to occur during night-time, when an opposite temperature gradient is recorded: temperature under the sand is a little bit higher than on external sand (Matsuzawa et al., 2002). Therefore, from the 42nd day the nest will be surrounded by a fence against any possible disturbance or damage, and a corridor for hatchlings to reach the sea will be realised. From this moment, the nest has been watched and monitored by volunteers for 24 hours. During this period, we also registered superficial temperatures periodically during day and night. When a little subsidence does appear on the sand surface, it means that hatchlings are outside their eggs, provoking the sand to collapse among and around the clutch, and indicating they are close to emerging on the sand surface (pers. obs.). Within a maximum of 24 hours, the little turtles will be on the beach, crawling seaward. They perhaps will continue coming out from the sand, during the night, for the following two–three days. Each night, the time and number of hatchlings were recorded. No hatchlings were handled, nor disturbed during their crawling seaward. Only red light was used for observation, to avoid any stress or disturbance to the hatchlings.

We calculated incubation times (in days) for nesting events whose exact date of deposition and emersion were known (Ackerman, 1997; Matsuzawa et al., 2002; Miller et al., 2017; Watson & Lamont, 2022).

After three–four days from the last emergence of the last hatchling from the sand, the nest was inspected, digging it out to recover the hatched eggs, annotating the date of inspection. All the content of the nest was catalogued and the number of hatched and unhatched eggs, pipped eggs with dead hatchlings, and eggs with partially developed embryo and eggs with no discernible embryo were registered (Eckert et al., 1999). With a calliper, the diameter of unhatched eggs was measured in cm and their mass registered using a digital electronic scale with a precision of 0.1 g. In addition, the curved carapace length (cm) and total body mass (g) of dead and the still living hatchlings found in nest were recorded. Physical characteristics of the eggs chamber (e.g. depth and width) were measured.

For each nest, emersion success (ES percentage, number of emerged hatchlings/total number of eggs), and hatching success (HS percentage, hatched eggs/total number of eggs) were evaluated (Eckert et al., 1999).

Correspondence: Marco A.L. Zuffi (marco.zuffi@unipi.it), Cecilia Mancusi (c.mancusi@arpat.toscana.it)

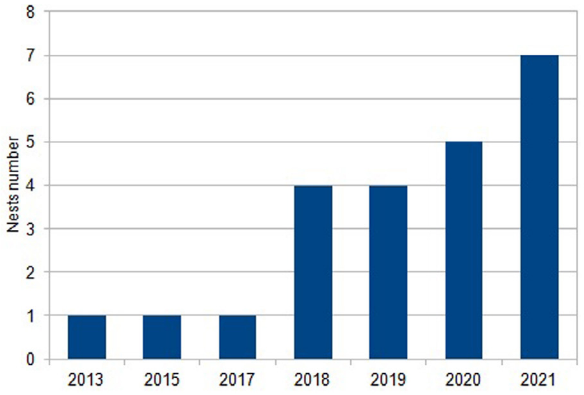

Figure 1. Number of nests per year

In most of the nesting events, we collected samples to perform several analysis: bacteriological, virological, pollutants and contamination on unhatched eggs; others on morphology (Zuffi et al., 2023) and genetic (Tolve et al., 2023) were conducted on dead embryos and pipped. Development of whole genome sequencing techniques now allows the characterisation of whole mtDNA genome sequences, which can provide a much more comprehensive scenario of loggerheads' phylogeographic history.

Considering chemical contamination among possible threats to hatching success, toxicological investigations were conducted on biological material sampled from four nests laid along the Tuscan coast during summer 2019. Egg contents, embryos, and embryonal annexes were analysed to evaluate the presence of organochlorine compounds such as DDTs, 30 of 209 congeners of PCBs and HCB using Marsili & Focardi (1996) analytical method (Ceciarini et al., pers. obs.).

All the operations were carried out within the Cetaceans, Elasmobranchs and Sea Turtle Stranding Network co-ordinated by Tuscany Observatory for Biodiversity and following ISPRA Guides Lines (Mo et al., 2013).

RESULTS

During the period 2013–2021 along Tuscany coasts, we detected 23 *C. caretta* nests. Despite having been a sporadic and limited phenomenon (only one nest per year in 2013, 2015, 2017, no nests in 2014 and 2016), from 2018 nesting events established in Tuscany with an increased number of nests in each year (Fig. 1). All the data recorded are summarised in Table 1. Geographical distribution of the beaches chosen by turtles along the Tuscany coast indicates a preference for the southern part and the Elba Island (Fig. 2). From 2016 on, we also registered 29 attempts of nesting with an extension toward the north of the region (Fig. 3). Nine out of the 23 nests, potentially in danger because of the short distance from the shoreline, were relocated on the same beach or the nearest beach with similar characteristics at a greater distance from the maximum known line of storm surge. Distance from shoreline was registered for 15 nests and it ranged from 7.7 m to 24.2 m (mean 14.5 ± 5.5 m). The relocated nests were initially located at 7.7 m and 15 m from the water.

Grain size analysis performed for ten nests showed that sand class ranges from < 2mm to > 0.063 mm were predominant, with 67% of occurrence. Temperature profile recorded for seven nests are shown in Figure 4. Temperature for eggs incubation ranged from 26.8 to 32.5 °C. Temperature has no normal distribution (Kolmogorov Smirnov test = 17.360, $P < 0.0001$), with significant differences among nests (Kruskall-Wallis = 4602.809, 6 df, $P < 0.0001$) (Fig. 4). Pair-wise comparisons underlined that only Santa Lucia1 2018, Rimigliano 2018, and Cecina 2019 nests were similar (Santa Lucia1_2018 vs Rimigliano_2018 $P = 0.458$; Santa Lucia1_2018 vs Cecina_2019 $P = 0.997$; Rimigliano_2018 vs Cecina_2019 $P = 0.520$).

We showed nest temperatures and incubation period, calculated for seven nests (according to Watson & Lamont, 2022) in Table 2a. Incubation period varied from a minimum of 47 days in 2019 to a maximum of 74 days in 2020, with a mean duration of 59 days; the nesting period ranged from 10 June to 6 October (Table 2b). As to the nests from which we collected temperature data (n = 7), we applied the algorithms by Ackerman (1997), Matsuzawa et al. (2002), Miller et al. (2017) and Watson & Lamont (2022) to calculate the expected date of emergence (Table 2b), verifying the suitability of the used algorithms.

Table 1. Data of the Tuscany nests of *Caretta caretta* (NA = not available; measures of hatchlings and eggs were presented as mean ± 1 SD)

	2013	2015	2017	2018	2019	2020	2021	Total
Number of nests	1	1	1	4	4	5	7	23
Number of eggs	NA	72	118	372	375	479	665	2081
Number of hatchlings	NA	60	99	55	164	131*	68**	378
Hatchling success (%)	NA	87.5	87.3	46.5	47	25.4	12.0	
Emergence success (%)	NA	83.33	83.90	13.39	44.96	31.00	80.00**	
Hatchlings measured	NA	3	4	17	3		4	31
Hatchlings carapace curve length (cm)	NA	3.6 ± 0.06	4.6 ± 0.31	4 ± 0.57	3.7 ± 0.25		4.1 ± 0.20	30
Hatchlings mass (g)	NA	15.3 ± 0.58	15.6 ± 1.21	13.3 ± 1.60	14.2 ± 3.27		14.8 ± 0.06	24
Number of eggs measured	NA	9	14	197	204	125	13	569
Egg diameter (cm)	NA	3.6 ± 0.07	3.4 ± 0.60	3.4 ± 0.21	3.7 ± 0.19	3.9 ± 0.27	3.6 ± 0.17	535
Egg mass (g)	NA	25.4 ± 3.36	22.1 ± 6.80	23.2 ± 2.60	22.5 ± 4.73	18.9 ± 3.12	19.6 ± 5.89	544

*partial, only for 3 nests **partial, only for 1 nest

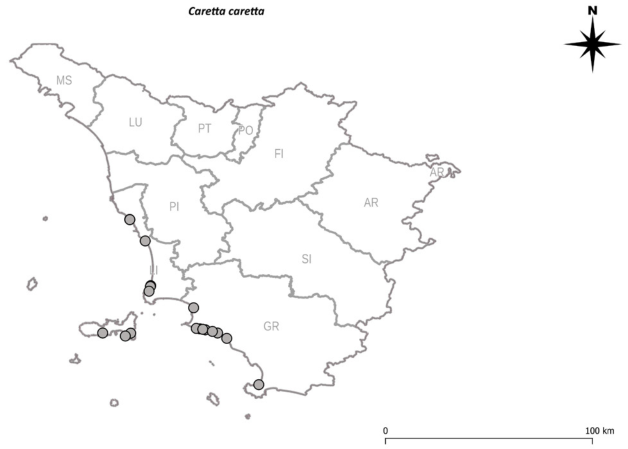

Figure 2. Geographical distribution of *Caretta caretta* nesting in Tuscany (and provinces) during the period 2013–2021

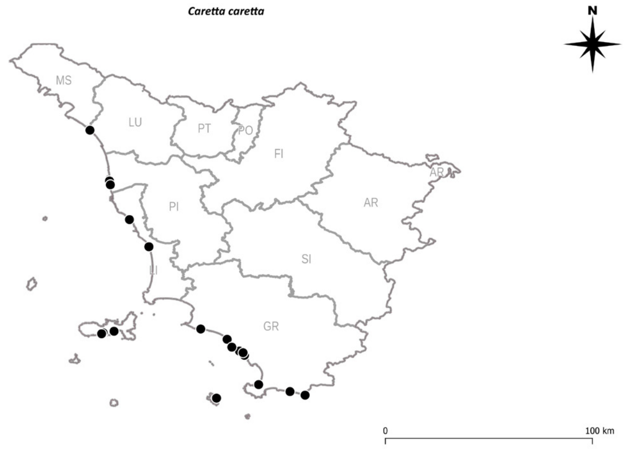

Figure 3. Geographical distribution of *Caretta caretta* nesting attempts in Tuscany area (and provinces) during the period 2016–2021

No hatching was recorded in six nests. Hatchings success (HS) averaged 51% (range 0% to 95%) and emergence success (ES) averaged 40% (range 0—84%). It was not possible to access to the material contained inside the eggs chamber during or after the inspection procedure for six out of the seven nests laid in 2021.

We had a total of 2081 eggs laid and 943 hatchlings. The number of eggs laid ranged from 64 to 127 with a mean value of 95 eggs. For the ten studied nest chambers, eggs features were as follows. Diameter of eggs was on average 3.59 ± 0.27 cm (n = 410, from 3.43 ± 0.15 cm for Santa Lucia1 nest to 4.11 ± 0.21 cm for Rimigliano nest; min range 2.4 cm, max 4.9 cm). Egg size was significantly different among nests (ANOVA, F = 56.299, 9 df, $P < 0.0001$). Egg mass was 22.7 ± 4.04 g (n = 419, from 19.61 ± 5.89 g for Morcone nest to 31.42 ± 2.58 g for Capoliveri nest; min range 18 g, max 35 g). Egg mass was significantly different among nests (ANOVA, F = 6.333, 9 df, $P < 0.0001$).

Hatchling carapace curve length (CCL) was 4.05 ± 0.52 cm (n = 31, 21 alive and 10 dead; min 3.4 cm, max 6 cm) and mass was 12.35 ± 4.61 g (n = 24, min 9.9 g, max 17.8 g). On the dead specimens and on the term embryos found inside the eggs, 34 samples were taken for genetic analyses and 102 for insights about development stage (Miller, 1999; Zuffi et al., 2023).

The genetic analyses conducted has revealed two different mitochondrial haplotypes: one is CC-A2.1, which is predominantly Mediterranean strain, but also Atlantic CC-A3.1, which is a newly identified strain. HCB, DDTs and PCBs were present in all samples analysed. Even though the mean values of the contaminant classes were different in each nest, all samples had the same concentration ranking: PCBs > DDTs > HCB. Even if these xenobiotic contaminants are strongly banned and regulated by several laws, it is important to investigate their presence in the marine biota, mostly because they can potentially affect with adverse effects living organisms, due to their persistence in marine environments. Nevertheless nowadays, a direct correlation between contaminant levels and hatchling success does not exist (Ceciarini et al., pers. obs.).

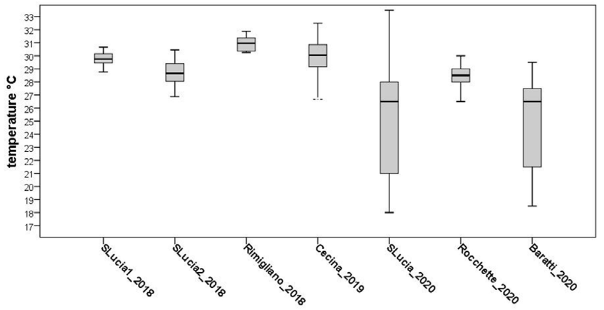

Figure 4. Temperatures values (median, interquartiles and extremes) in the studied nests

DISCUSSION & CONCLUSION

The Cetaceans, Elasmobranchs and Sea Turtle Stranding Network (CESTSN) in Tuscany (Italy, north-western Mediterranean) has been operating since 1980. More recently, it was implemented thanks to the former Tuscany Observatory for Cetacean, now Tuscan Observatory for Biodiversity (Osservatorio Toscano Biodiversità, OTB) sensu art. 11 L.R. 30/2015.

This experience of the Tuscany region represents a real contribution to the international effort for the conservation of the sea and its resources. This region has created a co-ordinated and synergic network among its members, which are ARPAT (Environmental Protection Agency, Tuscany Region), IZSLT (Experimental Zooprophylactic Institute, Latium and Tuscany) universities, research centres, museums, aquaria,

Table 2a. Average nest temperature (nest T), standard deviation (SD), range (minimum and maximum) values, and the average nest temperature within the 5–50 incubation days range (Watson & Lamont, 2022) (* = data for the 5–50 days interval not available)

Nest id	Year	Nest T (°C)	SD	Range °C	Average T, 5-50 day (°C)
SLucia1	2018	29.27	0.66	27.37-33.22	*
SLucia2	2018	28.69	0.86	26.9-30.5	*
Rimigliano	2018	30.59	1.26	27.1-31.9	*
Cecina	2019	29.83	1.46	25.3-32.5	29.96
SLucia	2020	25.35	3.59	18.0-33.5	27.07
Rocchette	2020	28.14	1.33	23.0-33.0	28.70
Baratti	2020	25.08	3.41	18.5-29.5	27.10

environmental associations and fishermen. In Tuscany, the presence of the three species of sea turtle (*Caretta caretta*, *Chelonia mydas*, *Dermochelys coriacea*) is reported and a great effort has been made during past and recent years to recover stranded and by-caught animals, dead or alive (Meschini, 1998; Meschini et al., 1998; 2000; 2006). Our data integrate a much recent assessment of nesting expansion of *C. caretta* in north-western Mediterranean waters (Hochsheid et al., 2022). They support the speculation that this region may have once hosted a nesting population of loggerhead turtles. These records are some of the very rare nesting occurrences in the western Mediterranean (Tomàs et al., 2002; Gonzalez-Paredes et al., 2021; Hochsheid et al., 2022). Among the Italian records of this study, they represent the northernmost-recorded nesting area in the Mediterranean until 2021 (Delaguerre & Cesarini, 2004; Sénégas et al., 2008; Hochsheid et al., 2022). Our records of nesting attempts, many of them failed because of human disturbance during the turtle activity on the beach, should be considered as a recent and new behavioural pattern shown by loggerhead turtles in this part of the Tyrrhenian Sea. This behaviour corroborates the recorded nesting activities. Further observations of beaches are strongly recommended to establish other nesting occurrences of loggerhead turtles in this region. Reports on occasional nests in the western Mediterranean appear to have become more frequent recently and although the available data are still too few, a possible connection to global warming should not be excluded: a rise in temperature of the western basin can modify known sea turtle behaviours.

Different incubation temperatures among nests suggest that, at least from this dataset and considering the pivotal temperature for sex determination (e.g. 29.3 °C, Mrosovsky et al., 2002; 28.7 °C, Ackerman, 1997), that some nests could have produced more males than females and some others more females than males.

Comparing the observed vs the expected incubation period (Table 2b), it is evident there are discrepancies among the several estimation algorithms.

Incubation period varies among populations and with beach latitude. For the Tuscany nests, the range was between 47 and 74 days, and fell within that reported by Casale et al. (2018) for Italian seas (45.6–70 days). However, these authors report a hatchling emergence success ranging between 24.7% and 86%, while our data represent higher maximum values and the lowest minimum value (see Hochsheid et al., 2022).

Diameter of eggs of 3.6 cm was a little bit smaller than reported in literature for Mediterranean basin (Turkey: 3.9 cm, Marquez, 1990) and elsewhere (4.5 cm, Marquez, 1990; 4.1 cm, Miller, 1997). Egg weight measurements reported in literature are less frequent than those regarding diameter, and available data outside the Mediterranean area range from 26.2 to 46.8 g (Marquez, 1990; Miller, 1997), higher than our mean value of 22.7 g. This could be related to a smaller reproductive female size in this geographical area. Size and weight of hatchlings are considered to be correlated directly with the size of the eggs. In Turkey, straight carapace length (SCL) of 3.99 mm (from 3.7 to 4.2 cm, n = 50) have been reported (Marquez, 1990); for Atlantic and Pacific Oceans total mass range was 18.8–21.2 g (Marquez, 1990; Miller, 1997). Our data show slightly bigger hatchlings (4.05 cm CCL) with a lower mass (12.3 g).

Table 2b. Deposition date, emergence date, incubation period and the expected incubation period, according to main references (with % estimated error vs observed incubation days, where possible), among the studied nests

Nest id	Year	Deposition date	Emergence date	Incubation period (days)	Expected incubation	Expected incubation	Expected incubation	Expected incubation
					Ackerman, 1997	Watson & Lamont, 2022	Matsuzawa et al., 2002	Miller et al., 2017
SLucia1	2018	28 July	23 September	57	58.7 (2.98)	/	54.8 (3.86)	51 (10.52)
SLucia2	2018	9 August	-----	/	62	/	57.6	54
Rimigliano	2018	10 June	28 July	49	54.2 (10.6)	/	49.2 (0.4)	48 (2.04)
Cecina	2019	25 June	-----	/	52	64.5	52.4	50
SLucia	2020	15 August	-----	/	/	70.9	82.5	83
Rocchette	2020	25 July	6 October	74	65 (12.16)	67.3 (9.05)	60.9 (17.70)	60 (18.91)
Baratti	2020	11 August	-----	/	/	70.8	85.3	84

Mean number of laid eggs was 95 (range of 64–127 eggs) for our geographical area, while Marquez (1990), for the Mediterranean basin, reported 83 for Greece and 93 for Turkey. Casale et al. (2018) reported 97.9–99 eggs as range of means for Italy; for the Mediterranean basin, the minimum registered was 64.3 eggs and the maximum 126.8. For oceans, Miller (1997) and Marquez (1990) reported 106.5 and 112.4 eggs respectively.

In conclusion, this study relates to a new nesting area of loggerhead turtle *C. caretta*, and shows how important it is investigate this marine reptile and its nesting activity in areas with little information, to undertake appropriate conservation management measures in the near future.

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Are plasticine models efficient to test defensive colouration of snakes?

Edelcio Muscat[1], Matheus de Toledo Moroti[1,2], Ivan Sazima[1,3], Luís Felipe Toledo[3], Raoni Rebouças[3,4,5]

[1]Projeto Dacnis, Estrada do Rio Escuro, 4754, Sertão dasCotias, CEP 11680-000, Ubatuba and São Francisco Xavier, São Paulo, Brazil

[2]Programa de Pós Graduação em Ecologia e Conservação, Universidade Federal de Mato Grosso do Sul. Cidade Universitárias, Campo Grande, Mato Grosso do Sul

[3]Museu de Diversidade Biológica, Universidade Estadual de Campinas, Campinas, São Paulo, Brazil

[4]Laboratório de História Natural de Anfíbios Brasileiros, Departamento de Biologia Animal, Instituto de Biologia, Universidade Estadual de Campinas. Cidade Universitária Zeferino Vaz, Campinas, São Paulo

[5]Programa de Pós Graduação em Biologia Animal, Departamento de Biologia Animal, Instituto de Biologia, Universidade Estadual de Campinas. Cidade Universitária Zeferino Vaz, Campinas, São Paulo

[6]Laboratório de Ecologia Evolutiva de Anfíbios, Departamento de Zoologia, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora, Juiz de Fora, Minas Gerais, 36036-900, Brazil

Plasticine models are often used to test hypotheses related to defensive colourations. However, the behaviour of natural predators is hardly ever examined in these experiments, which can lead to imperfect conclusions about the interactions. Accordingly, we ran an experiment to test whether plasticine models are an efficient tool to test Batesian mimicry, aposematism and camouflage hypotheses, using Atlantic forest snakes as a model group. We made 150 non-toxic simulacra of four snake species and two generic plasticine models. We placed cameras traps at the study sites to record the behaviour of potential predators towards the models. We classified the predator-simulacrum relations as physically interactive, interactive with no contact, or not visualised, and we examined whether mammals behaved differently from birds towards the plasticine models. We recorded 110 instances of birds or mammals approaching the plasticine models, most of them with models. Most birds presented an interaction with no contact during the day, and mammals presented physical interaction during the night. None of the model types influenced the interaction with predators, but we observed that mammals interacted significantly more with models than birds. While mammals clearly did not behave protectively when interacting with the models, some birds did behave with caution when approaching them. Our results showed that the use of plasticine models may not always result in reliable data to test predator-prey hypotheses.

Keywords: Mimicry, predation, Atlantic forest, aposematic colouration, Serpentes

INTRODUCTION

Predator-prey relationships provide a classic paradigm in animal ecology studies, frequently involving the diversification of behavioural strategies, such as defensive colourations (Barnett et al., 2018; Postema et al., 2023; Toledo & Haddad, 2009). These aposematic colouration patterns, which can be a result of sexual selection (Santos et al., 2014) acts as a warning to would-be predators by signaling the presence of toxins, unpalatable substances, or the possibility of physical counter-attack (Stevens, 2007; Toledo et al., 2011). On the other hand, some harmless or profitable organisms are specialised in production of information sought by a predator as a warning signal (Bates, 1862; Vane-Wright, 1980). These patterns were already observed in diverse taxa, such as plants (Launchbaugh & Provenza, 1993), invertebrates (Mallet & Gilbert Jr, 1995), and vertebrates (Prates et al., 2012; Toledo & Haddad, 2009). Some non-venomous snakes, such as *Erythrolamprus aesculapii*, are hypothetical colour mimics of coral snakes, such as those from the genus *Micrurus*, which potentially confuses the predator (Banci et al., 2020). From this perspective, several methods were adopted to test the function of colours, and the use of plasticine models is one of the most common approaches (e.g. Banci et al., 2020; Brodie III, 1993; França et al., 2017; Kakazu et al., 2010; Rebouças et al., 2019).

Brodie III (1993) was likely the first to employ plasticine models of snakes to evaluate predator-prey relationships. Several studies have since deployed similar experiments to explore how variations in colour aposematism (Banci et al., 2020; Husak et al., 2006; Rebouças et al., 2019; Saporito et al., 2007), body shape (Guimarães & Sawaya, 2011; Shepard, 2007) and crypsis (Kakazu et al., 2010; Vignieri et al., 2010) affect predation rates. This approach is attractive as it is low cost, and allows for identification and quantification of predation marks left on the models,

Correspondence: Raoni Rebouças (raonisreboucas@gmail.com)

such as bites, beak indentations and claw imprints (see Brodie III, 1993; Kakazu et al., 2010; Rebouças et al., 2019). However, this technique fails in some cases as the models are motionless and therefore problematic for studies involving predators that respond to kinetic cues (Paluh et al., 2014). The cognitive ability and behaviour of prey and predator are also frequently disregarded in this type of experiment (Beckers et al., 1996).

Since the accuracy of experiments depends on how simulated conditions reflect the natural history of the organisms involved, an observational approach is recommended for experiment construction and hypothesis testing (Guidetti et al., 2014). In experiments using plasticine simulacra, it is common to use methodically disposed models of potential prey in the field, and the marks left on these models are used as evidence of interaction to answer ecological questions (Banci et al., 2020; Bordignon et al., 2018; Paluh et al., 2014), and, consequently, can only be accounted when marks on models are visible. However, the interactions are generally not recorded at the moment of the potential predation (Beckers et al., 1996; Harmel et al., 2020), although the behaviour of predators when finding the models could provide relevant information. Consequently, we conducted a traditional experiment with plasticine prey models to answer evolutionary hypotheses about colouration patterns in snakes (e.g. Akcali et al., 2019; Banci et al., 2020), with the difference that we set camera traps to record behaviours of predators when detecting, approaching and interacting with the models. Thus, we could also test the reliability of the use of plasticine models in simulacra-predation studies.

MATERIALS & METHODS

The study was conducted between August and September 2020 in the Projeto Dacnis private reserve in the sub-district of São Francisco Xavier, municipality of São José dos Campos, São Paulo, Brazil (-22.89, -45.94; WGS-84; 700–800 m a.s.l.). The area within Atlantic forest domain presents marked seasonality, with a dry winter (between June and August) and humid summer (between December and February). The transect where the experiment was carried out is surrounded by secondary forest, with creeks and native palm trees *Euterpes edulis*, which is a common habitat for diverse snake species.

The snake species to be modelled were selected as they occur in the study site, and would be familiar to local potential predators. We used non-toxic plasticine models of the four snake species: 1) *Bothrops jararaca*, venomous and hypothetically cryptic; 2) *Xenodon neuwiedii*, non-venomous and hypothetically mimic of *B. jararaca*; 3) *Micrurus corallinus*, venomous and hypothetically aposematic; and 4) *Erythrolamprus aesculapii*, slightly venomous and hypothetically mimic of *M. corallinus*. Additionally, we tested two generic plasticine models: a brown, cryptic with the background, and a green, conspicuous with the background (Fig. 1). In total, we used 150 plasticine models with 60 cm total length, 25 for each species. We selected three sampling sites along a 3 km transect, spaced from each other by 500 m, to install the camera traps (Bushnell Trophy Cam HD-E-3), based in environmental setting and whether the camera suited to record. Once triggered by a movement, the cameras were set to record predator-model interaction for 30 seconds. Simulacra were divided in three groups to be tested separately in each point: group 1, with *B. jararaca* and *E. aesculapii* models; group 2, with *X. neuwiedii* and *M. corallinus* models; and group three, with the generic green and the generic brown. A plasticine model of each group was placed in each site about 50 cm in front of the camera trap among the leaves on the ground, with three simulacra per point. In each site, we inserted a random simulacrum of each group (e.g. point 1: *B. jararaca*, *X. neuwiedii* and generic green; point 2: *E. aesculapii*, *X. neuwiedii* and generic brown; point 3: *B. jararaca*, *E. aesculapii* and generic brown), and the model was replaced by another of the same group during the daily checking, which lasted ~5 minutes. In total, each plasticine model was randomly placed 30 times. Those models physically modified were replaced to avoid that their appearance influenced the results. Camera-recorded predator behaviour was placed in one of three categories: (1) physical interactions, when a potential predator interacted physically with the model; (2) interactions with no contact, when predators detected, visualised and ignored the model; and (3) approaches with no visualisation, when the model apparently was not seen by predator. Furthermore, we corresponded video data with marks in models and all interactions were classified in diurnal and nocturnal activity. All videos were deposited at Fonoteca Neotropical Jacques Vielliard, Museu de Diversidade Biológica (ZUEC-VID 953-967), Universidade Estadual de Campinas, Campinas, SP, Brazil (Dena et al., 2018; 2020).

To evaluate whether mammals and birds preferred any model, we employed Ivlev’s electivity index (Lechowicz, 1982), through the R package 'electivity' (Quintans, 2019), which uses the availability and the interaction with each prey to evaluate avoidance (when the index range is between -1 and 0) and preference (when the index ranges from 0 to +1). We considered as “availability” the sum of non-interactive and interactions, since we were unable to evaluate if all the models in each treatment were perceived by predators, and “use” when potential predators actively interacted with the models. To avoid the influence of intrinsic characteristics of predator species in interaction frequency with the models, we separated predators into two general categories: birds and mammals. Furthermore, to evaluate if there were differences in interaction with any model, we performed a Generalised Linear Model analysis (GLM), with a binomial distribution and a logit link, considering the interaction as a response variable (non-interactive = 0, interactive = 1), and the model type (*B. jararaca*, *X. neuwiedii*, *M. corallinus*, *E. aesculapii* and those hypothetically cryptic and conspicuous, the brown and green models, respectively). We used the class of predator (bird or mammal) as response variables. To determine the size effect of each variable, we used the analysis of variance type II through the function “Anova” of the R package 'car' (Fox et al., 2012). All analyses were performed in R 4.0.4 (R Core Team, 2023) with a confidence interval of 95%.

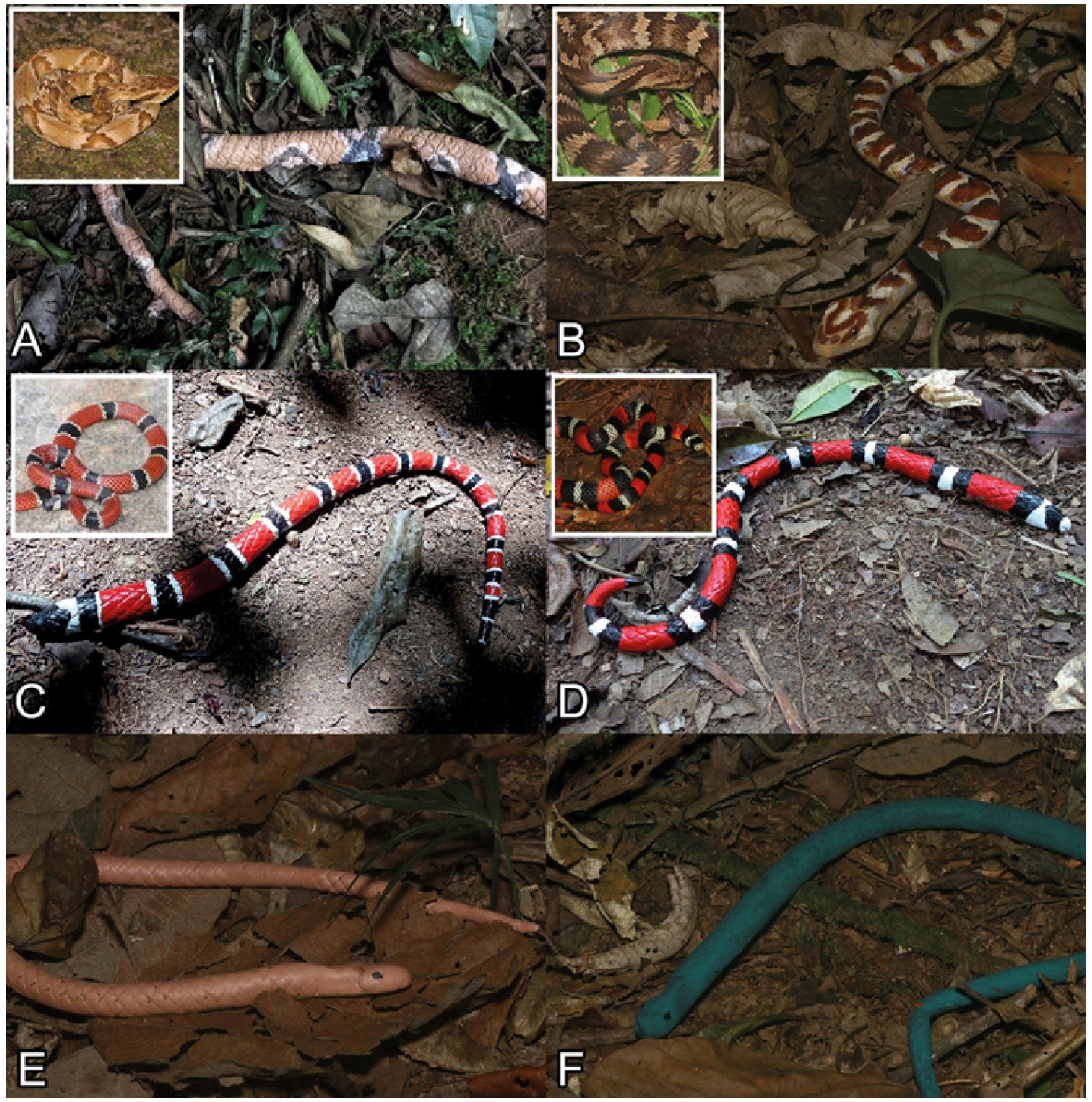

Figure 1. Plasticine models of snake species- **(A)** the venomous pitviper *Bothrops jararaca*, **(B)** Neuwied’s false pitviper *Xenodon neuwiedii*, hypothetical mimic of *B. jararaca* **(C)** the venomous painted coral snake *Micrurus corallinus*, **(D)** South American false coral snake *Erythrolamprus aesculapii*, hypothetical mimic of *M. corallinus*; generic models, **(E)** brown cryptic and **(F)** green conspicuous.

RESULTS

We recorded 110 events in which birds or mammals approached the plasticine models (Fig. 3; Table 1), of which 69.8% (n = 74) interacted with models, 28.3% interacted, but with no contact (n = 30) and 1.89% (n = 2) did not visualise the simulacra. Among interactions, 87.83% (n = 65) were displayed by mammals and 12.2% (n = 9) by birds. Among the videos where there was interaction but with no contact, 73.3% were displayed by birds (n = 22) and 26.7% (n = 8) by mammals. Only mammals did not visualise the simulacra in two instances (Fig. 2).

Most bird interactions with plasticine models occurred during the day, with 22 birds interacting with no contact (71%) and eight birds physically interacting with the models (25.81%). Only one individual presented interaction during the night, and none presented no visualising. Among mammal interactions, 71 occurred during the night, with 65 representing physical interactions (91.6%), five representing interactions with no contact (7.04%) and in only one interaction the individual did

Table 1. Models used in the experiment, their predators and observed behaviors and interaction

Plasticine prey model		Predator	Behaviour	Records	Interaction
Bothrops jararaca					
	Birds	*Baryphthengus ruficapillus*	Eating the model	2	Yes
		Crypturellus obsoletus	Foraging around the model	1	No
		Grallaria guatimalensis	Foraging around the model	2	No
		Leptotila rufaxilla	Foraging around the model	1	No
		Penelope obscura	Foraging around the model	1	No
		Trichothraupis melanops	Foraging around the model	1	No
		Turdus rufiventris	Foraging around and sitting on the model	1	No
	Mammals	*Didelphis aurita*	Eating the model	10	Yes
		Leopardus pardalis	Passed over the model	1	No
		Metachirus nudicaudatus	Eating the model	3	Yes
		Nectomys sp.	Eating the model	6	Yes
Xenodon neuwiedii					
	Birds	*B. ruficapillus*	Eating the model	1	Yes
		C. obsoletus	Foraging around the model	1	No
		Myiothlypis leucoblephara	Foraging around the model	1	No
		P. obscura	Foraging around the model	1	No
	Mammals	*D. aurita*	Eating the model	6	Yes
		Guerlinguetus ingrami	Foraging around the model	1	No
		Leopardus guttulus	Urinating on the model	1	Yes
		Nectomys sp.	Eating the model	1	Yes
Micrurus corallinus					
	Birds	*B. ruficapillus*	Eating the model	1	Yes
		G. guatimalensis	Foraging around the model	1	No
		L. rufaxilla	Foraging around the model	1	No
		P. obscura	Foraging around the model	2	No
		Turdus leucomelas	Foraging around the model	1	No
	Mammals	*D. aurita*	Eating the model	6	Yes
		M. nudicaudatus	Eating the model	7	Yes
		Nectomys sp.	Eating the model	2	Yes
Erythrolamprus aesculapii					
	Birds	*B. ruficapillus*	Eating the model	1	Yes
		G. guatimalensis	Foraging around the model	1	No
		L. rufaxilla	Foraging around the model	1	No
		P. obscura	Foraging around the model	1	No
			Eating the model	1	Yes
	Mammals	*Canis lupus familiaris*	Passed over the model	1	No
		D. aurita	Eating the model	2	Yes
		Leopardus pardalis	Passed over the model	1	No
		Nectomys sp.	Eating the model	8	Yes
Brown cryptic					
	Birds	*B. ruficapillus*	Eating the model	2	Yes
		C. obsoletus	Foraging around the model	1	No
		G. guatimalensis	Foraging around the model	1	No
		L. rufaxilla	Foraging around the model	1	No
		P. obscura	Foraging around the model	2	No
	Mammals	*C. lupus familiaris*	Passed over the model	1	No
		D. aurita	Eating the model	5	Yes
		L. pardalis	Passed over the model	1	No
		M. nudicaudatus	Eating the model	1	Yes
		Nectomys sp.	Eating the model	3	Yes
Green conspicuous					
	Mammals	*D. aurita*	Eating the model	3	Yes
		Herpailurus yagouaroundi	Passed over the model	1	No
		L. pardalis	Passed over the model	1	No
		Mazama gouazoubira	Sniffing the model	1	Yes
		Nectomys sp.	Eating the model	2	Yes

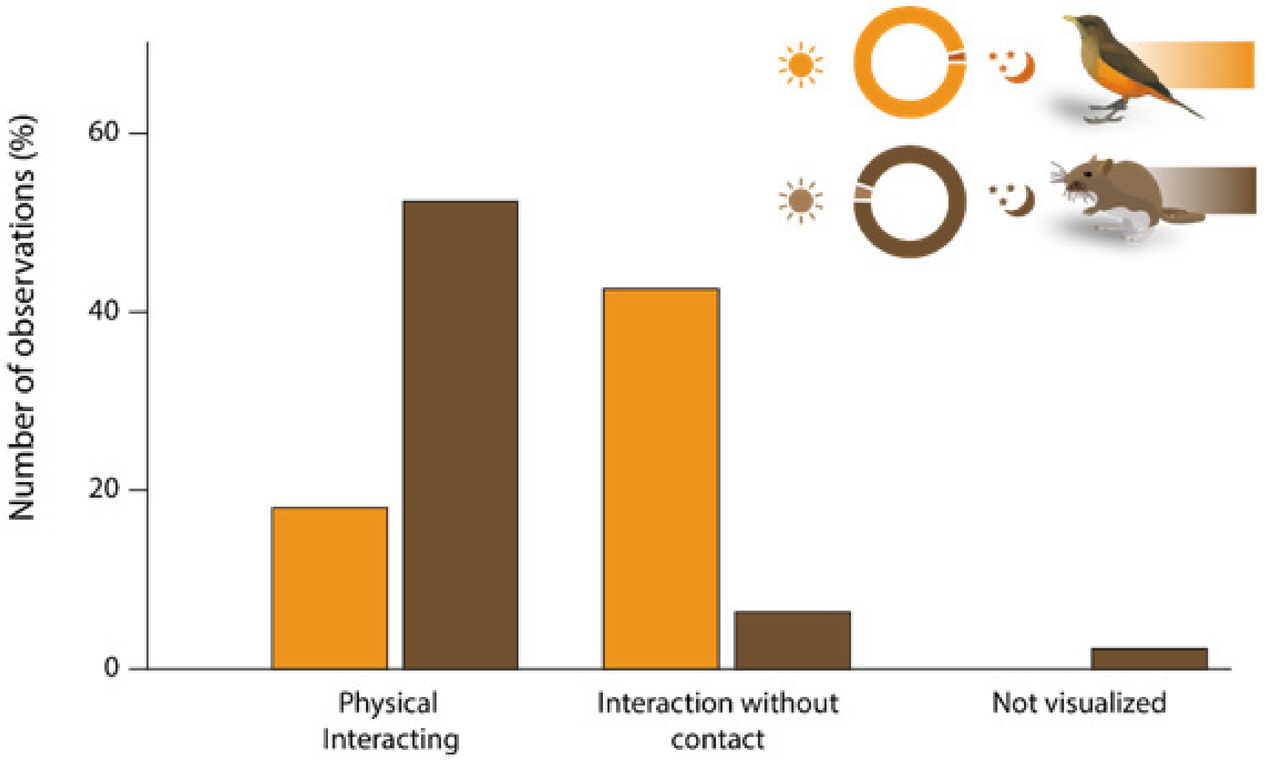

Figure 2. Number of individuals of birds (orange) and mammals (brown) observed in camera traps classified by type of interaction (interacting, interaction without contact, and not visualised). The donut chart indicates the total of interactions by period (diurnal: light colour; nocturnal: dark colour) for birds (orange) and mammals (brown).

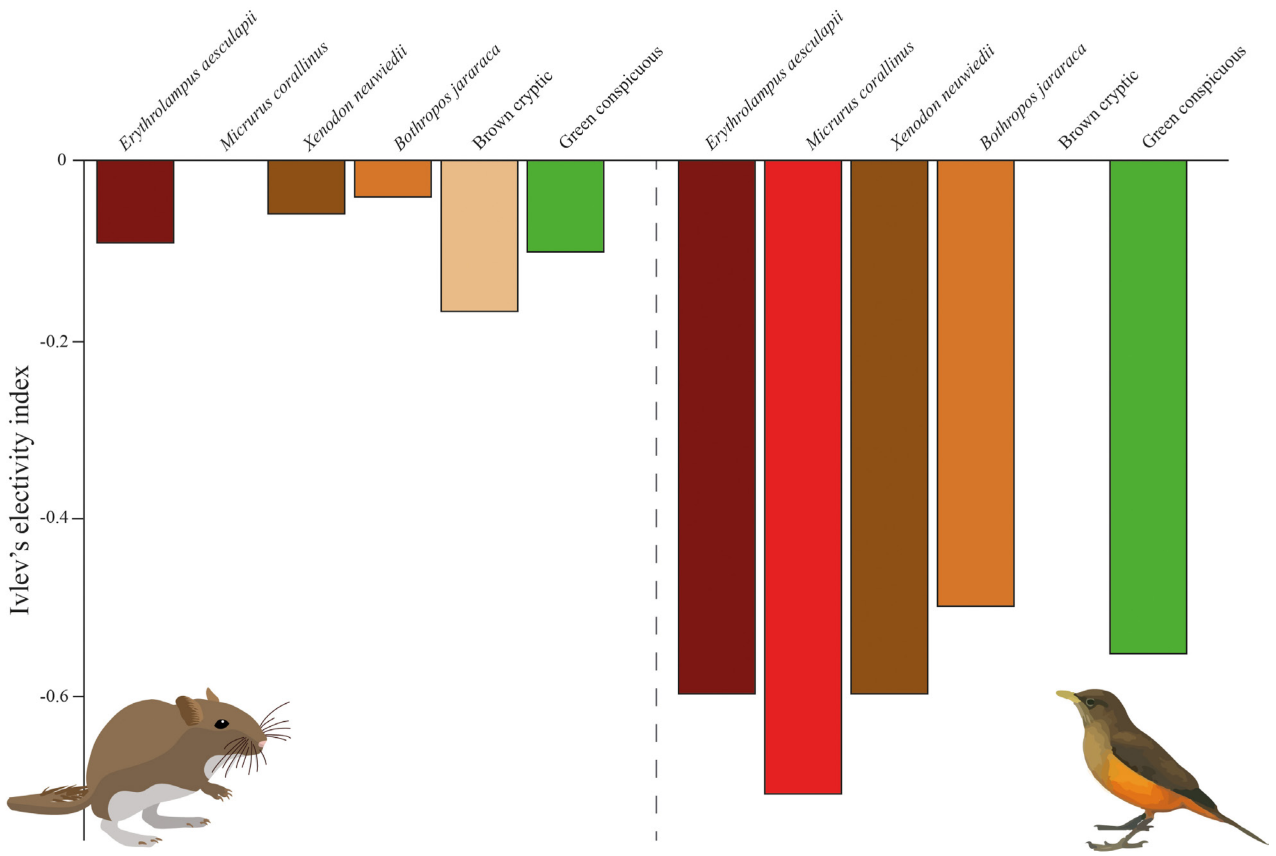

Figure 3. Ivlev's electivity index of predation by birds (left) and mammals (right) on each plasticine model

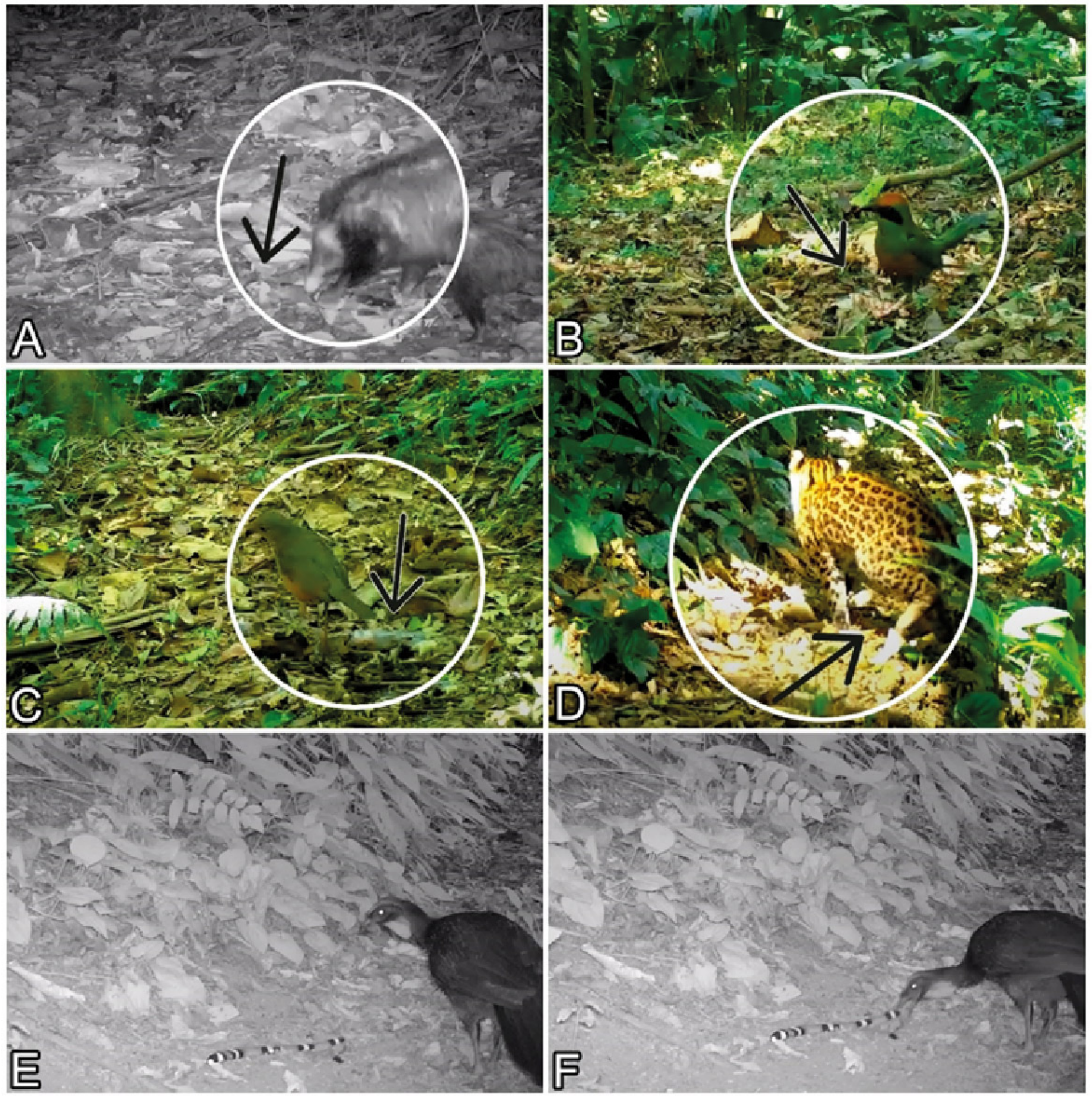

Figure 4. Images extracted from trail cameras depicting interaction with snake plasticine models.Within the white circles, the images enlarged. **(A)** Predation by the black-eared opossum *Didelphis aurita* on a model of the venomous pitviper *B. jararaca*; **(B)** rufous-capped motmot *Baryphthengus ruficapillus* examines and pecks at a *B. jararaca* model; **(C)** rufous-bellied thrush *Turdus rufiventris* lands over the same snake species model; **(D)** southern tiger cat *Leopardus guttulus* urinates on a *B. jararaca* model; **(E-F)** dusky-legged guan *Penelope obscura* examines and pecks at one of the aposematic models of the non-venomous *Erythrolamprus aesculapii*. Arrows indicate where models were placed.

not visualise the model. Only four interactions between mammals and the models occurred during the day, three with no contact (75%) and one with the individual not visualising the model. During the day we did not observe physical interaction between mammals and the models.

We detected six behaviours of potential predators: four “interaction” (chewing and/or eating, n = 72, 96%; urinating, n = 1, 1.3%; sniffing, n = 1, 1.3%; and landing on model/foraging in surroundings, n = 1, 1.3%) and two “not-interaction” behaviours, which were classified as “moving”, when the potential predators passed by the model or were foraging in the surroundings, with no physical interaction with the model (n = 11, 32%), or did not display a clear signal of perception (n = 23, 68%).

In general, all models were “avoided” by predators (Fig. 3), but Ivlev’s index showed difference among treatments: all values ranged from -0.13, for the *M. corallinus* model, to -0.24, for the generic green conspicuous model. When the two predator groups were considered individually, values for mammals remained close to 0 for all models, ranging from 0 for the *M. corallinus* model, to -0.16 for the generic brown cryptic model. For birds the indexes were lower, ranging from -0.5 for the *B. jararaca* model, to -0.71 for the *M. corallinus* model (Fig. 3). We observed no interaction with generic brown cryptic models by birds. Our results of the GLM analysis showed that none of the models influenced the predators’ interactions (models: χ^2 = 2.906, p = 0.71). However, we observed a significant difference between predator groups (χ^2 = 38.03, p < 0.001), whereby interactions with plasticine models were greater in mammals than in birds (Table 2).

We recorded several predation events on all the snake models for mammal and bird species (Fig. 4). The black-eared opossum *Didelphis aurita*, for example attacked and ate parts of the *B. jararaca* model (Fig. 4A), whereas the rufous-capped motmot *Baryphtengus ruficapillus* examined and pecked at the model of the same venomous snake (Fig. 4B). One individual of the dusky-legged guan *Penelope obscura* examined and pecked a model (Fig. 4 E–F), but apparently lost interest after an initial peck and subsequently foraged in the proximity of the model. Similarly to *P. obscura*, an individual of rufous-bellied thrush *Turdus rufiventris* alighted over the *B. jararaca* model and foraged in close proximity to the model (Fig. 4C).

Camera trap videos indicate olfactory location of models by mammals. For example, this behaviour was observed for an individual of the southern tiger cat *Leopardus guttulus*, which urinated on a model of *B. jararaca* (Fig. 4D). An individual of the grey brocket deer *Mazama gouazoubira* was observed sniffing a generic green conspicuous model for some time. The water rat *Nectomys* sp. and the brown four-eyed opossum *Metachirus nudicaudatus*, which are part of the *B. jararaca* diet (Sazima, 1992), did not avoid the models and ate parts of them.

DISCUSSION

Our results indicate that, although the use of plasticine models is readily available to test hypotheses, it is not entirely reliable for studies that aim to test the defensive colouration of snakes. We noted that the behaviour of potential predators toward the models differs from these relationships in natural encounters, as suggested by Beckers et al. (1996). While mammals clearly did not regard any models as a threat, birds appeared to avoid the interaction with the models. It was a clear pattern in our study, since we observed that most of the bird interactions occurred during the day, and with no physical contact, while interactions by mammals occurred during the night with physical contact. Since the defensive repertoire of a species against a predator is intrinsically related to its survival in the wild (Tozetti et al., 2009), the mechanisms of prey-predator interaction are important elements to be evaluated in experiments that aim to represent natural history of an interaction. Our study revealed potential caveats when using plasticine models to evaluate predation, especially in the context of snakes, and particularly when mammals are the predators.

Mammals commonly use olfaction for localisation, since odour is an important clue for this animal group for recognising potential prey (Apfelbach et al., 2005; Laska et al., 2005). The feeding on plasticine models by the marsupials and rodents is unsurprising, as both marsupials are omnivorous generalists and their diets range from fruits to vertebrates (Cáceres, 2004), which also applies to small rodents such as those from the genus *Nectomys* spp., excluding vertebrates as food (Lessa et al., 2019). However, we observed only a few interactions with plasticine models during the day, and consequently we cannot address if mammals tend to avoid the models in daylight. In relation to birds, it was expected that they would avoid snake models (Smith, 1976; Brodie, 1993), with exception of motmots (Brodie, 1993; present study) and some other bird species (Brodie, 1993). Our results suggest that most birds avoided the interaction with the snake models. However, our observations are not complete enough to highlight properly if birds avoid the contact by perceiving some danger signal, or if the activity predominantly during the day could improve their visualisation. Species that perceived the simulacra as a real snake would not approach the models, by the perception of some danger, and consequently would not be recorded by cameras. Those birds that approached the models but did not interact with models could (i) perceive that those were not real snakes; (ii) perceive the absence of movement, and consequently consider that the snake was dead, presenting no risk; or (iii) show no avoidance to snakes. Further studies with experimental approaches, such as experiments with multiple choice testing if birds could visualise the models during the night and mammals avoid during the day, and with recording of animal behaviour are necessary to evaluate these hypotheses.

Table 2. Coefficients of Generalised Linear Model analysis. Significant value is in bold.

	Estimate	Std. Error	z value	p
Intercept	-0.73	0.62	-1.17	0.24
Models				
Micrurus corallinus	0.05	0.89	0.06	0.95
Erythrolamprus aesculapii	-0.79	0.95	0.83	0.41
Xenodon neuwiedii	0.44	1.01	0.43	0.65
Brown cryptic	-1.66	1.11	-1.49	0.14
Green conspicuous	-0.65	0.89	-0.73	0.47
Predators				
Mammals	3.31	0.63	5.28	**< 0.001**

Among the 18 recorded species, the only potential predators that include vertebrates in their diet are a bird and a mammal. The rufous-capped motmot *B. ruficapillus* preys on rodents (Pagotto et al., 2019) and the big-eared opossum *D. aurita* preys on snakes, including the venomous ones as the pitviper *B. jararaca* (Almeida-Santos et al., 2000; Jared et al., 1998; Oliveira & Santori, 1999; Sazima, 1992). Both the motmot and opossum species showed more interactions with the model of *B. jararaca*, a snake that displays a varied defensive repertoire ranging from immobility to retaliation (Sazima, 1992). No predator species engaged into the predation sequence described by Oliveira & Santori (1999), and attacked several places of the model apparently with no discrimination at which end the head could be. Using plasticine coral snake-like models, Brodie (1993) recognised a few bill marks due to motmot attacks on some models, which agrees with our observations. However, a comparison between handling live prey and immobile plasticine models could be unduly speculative, though motionless rattlesnakes were eaten alive by the opossum *D. aurita* (as *D. marsupialis*), which preferably targets the tail first (Almeida-Santos et al., 2000).

The use of plasticine models is based on assumptions such as confirmation of 'attacks', the ability to identify predators, and the accuracy of models in representing prey to draw conclusions about predation (Bateman et al., 2017). Apparently, these assumptions are limited if the behavioural interactions between potential predators and their prey models are not considered. Thus, we recommend that future studies must consider behavioural aspects in their research, such as defensive strategies of the modelled prey, and also strive to emulate their natural movements. Additionally, the odour of models may be another confounding and relevant factor for mammals. In this sense, caution is needed since robust statistics may obscure flaws in primary data collection.

Plasticine models have long been considered useful for testing shapes (Guimarães & Sawaya, 2011) and colours (Rebouças et al., 2019). However, our observations indicate that using only predation marks on the models can lead to biased conclusions that do not portray real encounters between predators and snake prey. We should not underestimate the ability of predators to detect and identify its prey. Thus, it is essential that the behavioural and innate characteristics of prey (e.g. movement, defensive repertoire, odour) are also considered, or at least considered in this type of experimental trail.

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DATA ACCESSIBILITY

Data are available in Museu de Diversidade Biológica (MDBio), Unicamp, under request.

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One gecko's pain is another gecko's gain: is the Moorish gecko *Tarentola mauritanica* becoming invasive in France?

Julien Renet[1], Théo Dokhelar[2] & Nicolas Dubos[3]

[1] Fauna studium, scientific consulting, 04200 Sisteron, France

[2] CEFE, University of Montpellier, CNRS, EPHE, IRD, 34090 Montpellier, France

[3] The Jacob Blaustein Institutes for Desert Research, Sde-Boqer Campus, Ben Gurion University of the Negev, Midreshet Ben-Gurion 8499000, Israel

The Moorish gecko *Tarentola mauritanica* is currently expanding around the Mediterranean basin as a result of natural dispersal and anthropogenic spread. The species is observed at several sites in sympatry with other gecko species. To date, no impact has been observed on the native species and *T. mauritanica* is not considered invasive. We present an eight-year survey in southern France, where it lives in sympatry with the European leaf-toed gecko *Euleptes europaea*. The survey started when the Moorish gecko was rare which enabled us to observe an important increase in abundance. This increase was strongly correlated with a notable decline of *E. europaea*, explaining 49% of transect-specific temporal variation in abundance. We suspect that the increase in *T. mauritanica* density is causally related to this decline and recommend intensive monitoring of the species throughout the Mediterranean basin to determine whether or not the species should be classified as invasive.

Keywords: Euleptes europaea, *Tarentola mauritanica*, temporal trends, biotic interaction, competition, invasive alien species

INTRODUCTION

Global changes (e.g. international trade, climate change) have caused shifts in the distribution of biodiversity, especially in squamates (Pyšek et al., 2010; Moreno-Rueda et al., 2012; Ceia-Hasse et al., 2014; Bonino et al., 2015). This process leads or will lead to the contraction of many species ranges and the colonisation of new geographical areas for some species (Nasrabadi et al., 2018; Gómez-Cruz et al., 2021).

Native to North Africa and Iberian Peninsula, the Moorish gecko *Tarentola mauritanica*, currently expanding in southern Europe, appears to be particularly responsive to globalisation. The colonisation patterns of the species are probably a result of a combination of anthropogenically mediated-spread and natural dispersal (Harris et al., 2004a; 2004b; Perera & Harris, 2008; Rato et al., 2010; 2012; Silva-Rocha et al., 2022)thanks in part to the resilience of an Iberian core during the Pleistocene (Rato et al., 2010). It is also possible that the natural spread of the species is facilitated by climate change, since the species is present in warmer and more arid regions (Rato & Carretero, 2015; Rato et al., 2015).

The discovery of *T. mauritanica* in new localities is often attributed to recent accidental introductions by humans, particularly on islands (e.g. Jesus et al., 2008; Barreiros et al., 2010; Mačát et al., 2014; Deso et al., 2020; Rato et al., 2021; Strachinis et al., 2023). The species has become established in very distant areas from its native range, such as Mexico (Ortiz-Medina et al., 2019), Argentina (Baldo et al., 2008; Díaz-Fernández et al., 2019), Uruguay (Baldo et al., 2008), California (Mahrdt, 1998) and Florida (Rochford & Krysko, 2019). Within the Mediterranean basin, the species recently extended its distribution northward, where several colonisation fronts were identified (Fig. 1). One front is located in south-western France, along a major terrestrial transportation route (Garonne valley), already identified as a factor of spread in the past for several other Mediterranean species (Berroneau, 2014). Another one is located in the Rhône valley where *T. mauritanica* has reached the Isère French department (Grossi & Fonters, 2015). A northward spread was also recorded in the Durance valley and in the Var valley (Alpes-Maritimes) (Renet, J., unpublished data).

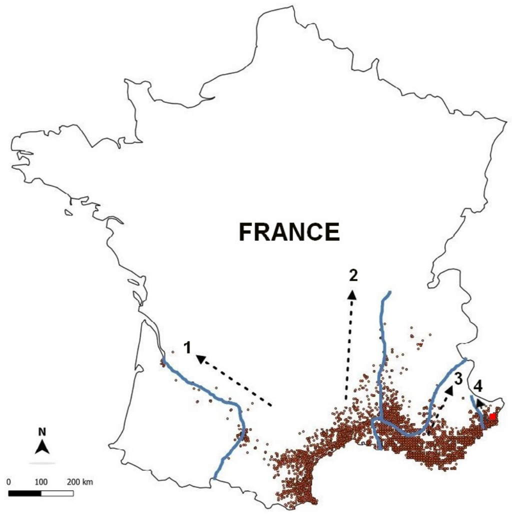

Figure 1. Distribution of the Moorish gecko *Tarentola mauritanica* in mainland France. Orange points correspond to species occurrence. Black arrows correspond to the direction of its spread in the (1) Garonne, (2) Rhône, (3) Durance and (4) Var valleys. The red square shows the location of the study site in the Alpes-Maritimes. Data OpenObs (INPN–SINP) Leaflet–Map data © OpenStreetMap, imagery © CartoDB.

Correspondence: Julien Renet (julien.renet13@hotmail.fr)

Whether *T. mauritanica* has an impact on native species is currently subject to debate from a conservation point of view. For example, the decline of the common wall lizard *Podarcis muralis* in urban environments was attributed to the Moorish gecko's arrival, although no scientific evidence has been reported yet (Geniez & Cheylan, 2012). Conversely, Simbula et al. (2019) highlighted that co-existence with *P. muralis* (and other species) does not lead to competitive interactions but rather to a strong partitioning of ecological niches likely due to extensive separation in microhabitat (e.g. vertical surfaces) and the period of activity of the two species (i.e. catemeral vs. diurnal). Despite their activity being asynchronous, both species feed on terrestrial arthropods, which might reduce local resources. Additionally, *P. muralis* may share retreat sites for thermoregulation with *T. mauritanica*, which suggests possible competition for micro-habitats.

In the western Mediterranean, *T. mauritanica* co-occurs with two other species of nocturnal geckos, the Mediterranean gecko *Hemidactylus turcicus* and the European leaf-toed gecko *Euleptes europaea*. The latter is narrowly distributed and mainly occupies islands, which makes it a species of high conservation value (Salvidio et al., 2010). It is classified as Near Threatened at the global level (Temple & Cox, 2009) and in danger of extinction in south-eastern France (Marchand et al., 2017).

Several cases of co-occurrence between *T. mauritanica* and *E. europaea* have been documented throughout the natural range of *E. europaea*, notably on Corsica, Sardinia and some satellite islands (e.g. Girraglia, Finnocchiarola, etc.), on islands in Tuscany (e.g. Elba, Montecristo, etc.) (Delaugerre & Cheylan, 1992; Delaugerre & Guyot, 1995), in Provence (Ratonneau, Levant Island, Port-Cros, Porquerolles) (Delaugerre, 1981; Astruc et al., 2009; Deso et al., 2018; Deso et al., 2020) and on several mainland localities in the Alpes-Maritimes, Liguria and Tuscany (Renet et al., 2008; Salvidio et al, 2010; Radi & Zuffi, 2022). *Tarentola mauritanica* is suspected to have caused local extinctions in *E. europaea*. This is the case in the island of Bendor, which is now occupied solely by *T. mauritanica*, although the island was home to a *E. europaea* population, before *T. mauritanica* arrived (Mourgue, 1910; Jahandiez et al., 1933; Ineich et al., 2019). The presence of *T. mauritanica* was also suspected to cause the extirpation of *E. euleptes* on the largest island of the Galite archipelago (Tunisia), Galita. Indeed, the island is largely occupied by *T. mauritanica*, while *E. europaea* has never been observed despite several sampling campaigns (Lanza & Bruzzone, 1959; Abbes et al., 2008). This is supported by its presence on smaller peripheral islands which are free of *T. mauritanica* (Delaugerre et al., 2011; Corti et al., 2022). A similar situation has been observed on the Lérins archipelago (Cannes, France) where *T. mauritanica* largely occupies the two largest islands (i.e. Sainte-Marguerite and Saint-Honorat) while *E. europaea* is confined to adjacent islets (i.e. Saint-Féréol and La Tradelière) (Renet & Martinerie, 2011; Renet et al., 2013). In spite of this, no monitoring protocol has been set to study in detail the consequences of the co-existence of these two species.

This study presents an 8-year monitoring of a population of *E. europaea* at a site where it is found in sympatry with *T. mauritanica*, with both species being recorded during application of a standard transect protocol. We provide evidence for the collapse of *E. europaea* and the rise of *T. mauritanica*. The aim of this study is to quantify population trends for both species, discuss the causes that could explain this observation and to provide guidelines for further research and management.

MATERIALS & METHODS

Study site and data collection

The sampling site is located in a group of south-oriented limestone ledges in the Alpes-Maritimes, at 610 m a.s.l. on the communes of Eze and La Turbie. The site corresponds to the thermo-Mediterranean floristic stage (Quézel & Médail, 2003), with a shrub layer mainly composed of *Pistacia lentiscus* L., *Rhamnus alaternus* L., *Quercus ilex* L. and *Euphorbia dendroides* L. A strategic military track was integrated into the site by the end of the 19th century, which facilitated access and sampling campaigns from one year to the next. The track consists of structurally homogeneous rocky walls on one side and a partially jointed dry-stone wall about 80 cm high on the other side.

We recorded the number of reptiles along four geolocated 110 m-long transects, set up on the military track at least 100 m apart from each other. These were surveyed between 2009 and 2017 for 40 minutes each, systematically, by two experienced observers on two to three occasions in spring (April to June) and autumn (September to October) for a total of 40 sessions. We recorded *E. europaea* and *T. mauritanica* at night when weather conditions were appropriate (i.e. in the absence of precipitation and wind, with temperature ranging between 12 and 22 °C) using headlamps (500 lumens). The start times of the surveys varied from 20:00 h to 21:30 h depending on the season, and did not go beyond 00:45 h. We carefully inspected micro-habitats on both sides of the track. Each observer was assigned to only one edge of the runway to avoid double counting. Only sexually mature adults were counted. We did not count juveniles, because they are more difficult to detect due to their smaller size.

Statistical analysis

Assessing temporal trends

We fitted Poisson Generalised Linear Mixed models (GLMMs) to the count data using the lme4 package

(Bates et al., 2015) in R v.4.2.0 (R Core Team, 2021) (this R package was used for all analyses here). This allowed us to test for linear temporal trends in the number of observations of the two sympatric species *E. europaea* and *T. mauritanica*. We built a null model which included three adjustment variables: We accounted for differences in abundance between species using a 'species' fixed effect, while the transect number and the sampling session were treated as random effects. We built one model assuming a common temporal trend among both species (i.e. with year as an additive continuous effect). We then assessed a model assuming a different temporal trend between species using season as a fixed effect and an interaction term between species and year. We assessed these models using the dredge function from the MuMIn package (Barton, 2020) on the basis of information theory (second order Akaike Information Criterion, AICc ; Burnham & Anderson, 2002). We chose the model with the best trade-off between goodness-of-fit and the number of variables included, i.e. with the lowest AICc (Wagenmakers & Farrell, 2004; Bolker, 2008; 2021). Models within a delta AICc of two of the best model were considered to have substantial statistical support and given further consideration (Burnham & Anderson, 2002). We computed the Marginal R^2 (Rm^2), estimating the variance explained by the fixed factors, and conditional R^2 (Rc^2), estimating the variance explained by both the fixed and random factors (Nakagawa et al., 2017) using the R package performance (Lüdecke et al., 2021).

We performed pairwise post-hoc tests with the emmeans function from the emmeans R package (Lenth, 2021) in order to test the difference in the number of observations between the two species through the eight sampling years. All graphics were generated using the R package ggplot2 (Wickham, 2016).

Assessing non-linear temporal trends

We tested the robustness of our linear assumptions on temporal trends using Generalised Additive Mixed Models (GAMMs) with the R package mgcvv. 1.8-42 (Wood & Scheipl, 2014). GAMMs fit the data with optimal degrees of freedom and are appropriate to describe non-linear temporal trends or fluctuations. We built one model for each species separately, with species count as response variable and year with a spline effect as predictor. We included transect and sampling session as random effects.

Assessing the effect of *T. mauritanica* on *E. europaea*

We quantified the effect of *T. mauritanica* abundance on *E. europaea*. We used GLMM assuming a Poisson distribution with *E. europaea* counts as response variable and *T. mauritanica* counts as predictor (hereafter, m1). We inspected for differences in abundance between transects and sampling sessions using random effects. We took into account the temporal dependence between consecutive years in each transect by including a 'year' random intercept and slope effect, which we allowed to vary among transects with a random intercept term. We compared the AICc of the model with that of a null model (which includes adjustment variables only; hereafter m0) to verify that the inclusion of *T. mauritanica* counts is informative. Finally, we quantified the amount of temporal variance specific to each transect explained by *T. mauritanica* abundance. To do so, we computed the ratio between the between-year variance of m0 and the between-year variance of m1 estimated with the random effects.

Table 1. Model ranking for the effect of species and year on gecko count (*Euleptes europaea* and *Tarentola mauritanica*). Marginal (Rm^2) and conditional (Rc^2). R^2 values are indicating the goodness-of-fit of the models.

Explanatory variable	AICc	dAICc	df	Rm^2	Rc^2
Species*Year	1611.2	0.00	18	0.886	0.965
Season+Species*Year	1612.9	1.75	19	0.886	0.965
Species+Year	3182.1	1570.88	11	0.536	0.858
Season+Species+Year	3183.7	1572.53	12	0.536	0.857
Species	3190.1	1578.87	4	0.531	0.856
Season+Species	3191.9	1580.75	5	0.527	0.857
Season	4261.8	2650.63	4	0.026	0.700

Table 2. Summary of the pairwise emmeans post hoc test using *Euleptes europaea* and *Tarentola mauritanica* as contrast

Year	Estimate	SE	df	z.ratio	p.value
2009	102.286	38.8489	302	12.185	< 0.0001
2010	44.538	12.4907	302	13.537	< 0.0001
2011	8.776	1.0625	302	17.941	< 0.0001
2012	5.129	0.5814	302	14.423	< 0.0001
2013	2.075	0.1921	302	7.888	< 0.0001
2014	1.198	0.1089	302	1.989	0.0476
2015	0.562	0.0515	302	-6.290	< 0.0001
2017	0.296	0.0384	302	-9.379	< 0.0001

RESULTS

Between 2009 and 2017 *E. europaea* observations decreased by 78.49% while *T. mauritanica* observations increased by 7329%. Based on AICc scores, we found strong statistical support for species-specific temporal trends but no effect of season on species observation (Rm^2 = 0.886, Rc^2 = 0.965; Table 1). Pairwise emmeans post-hoc test showed that from 2009 to 2014 *E. europaea* were significantly more abundant than *T. mauritanica* (Table 2, Fig. 2) but that in 2015 and 2017 the opposite trend was observed (Table 2, Fig. 2). The GAMMs showed that the trends were non-linear (*E. europaea*: estimated degrees of freedom = 2.85; P > 0.0001; *T. mauritanica*: estimated degrees of freedom = 2.74; P > 0.0001; Fig. 3). The abundance of *E. europaea* started to decline slowly near 2011 and declined linearly between 2012 and 2017. *Tarentola mauritanica* showed a rapid linear increase between 2009 and 2012 approximately, which was less pronounced between 2012 and 2017. We found strong statistical support for a negative impact of *T. mauritanica* on the abundance of *E. europaea* (estimate ± SE = -0.06 ± 0.005, P < 0.00001; ΔAICc (m0-m1) = 28.5). The abundance of *T. mauritanica* explained 49% of the transect-specific inter-annual variance.

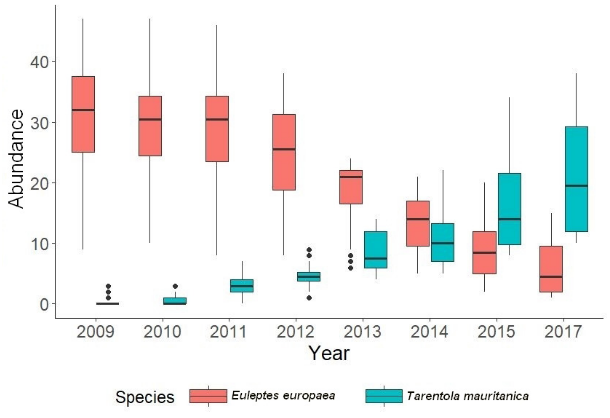

Figure 2. Abundance of *Euleptes europaea* and *Tarentola mauritanica* along four transects throughout eight sampling years. Boxes indicate the 25%, 50% (median) and 75% quartiles. Lines show minimum and maximum values excluding outliers (indicated as dots).

DISCUSSION

We provide evidence of a decline in the abundance of *E. europaea* which is worryingly correlated with a very large increase in the number of *T. mauritanica* observed within the same microhabitats. We believe that this may be linked to access to refuges. The small size of *E. europaea* allows it to select narrower crevices that are difficult for adult *T. mauritanica* to access. On the other hand, these narrow fissures could be accessible to immature *T. mauritanica*. At present, our data do not allow us to carry out a detailed analysis of habitat selection by these two species.

The cause of the increase in *T. mauritanica* is currently unknown. The species was already considered common in south-eastern France at the beginning of the 19th century (de Serre, 1822; Caziot, 1922) and no habitat change was recorded at the study site before or during the monitoring period. In addition, unrestrained urbanisation is likely to have facilitated the spread of individuals translocated from other areas (e.g. through the transportation of construction materials or ornamental plants). This may have led to increased interspecific competition, especially for access to food resources and refuges in rocky habitats (crevices).

Tarentola mauritanica is characterised by a strong ecophysiological plasticity (Carretero, 2008; Rato & Carretero, 2015), an efficient mode of reproduction (i.e. continuous spermatogenesis: Angelini et al., 1983; Picariello et al., 1989) likely to vary with climate change (e.g. possible extension of the ovarian seasonal cycle length) (Clarke & Zani, 2012), strong territoriality and frequent agonistic behaviours (Lisičić et al., 2012; Salvador, 2016). Although both nocturnal and diurnal, *T. mauritanica* is mainly nocturnal (Lisičić et al., 2012), and hence potentially overlaps with the activity of *E. europaea*. Overall, these traits suggest a high potential of invasiveness. Further research that focuses on determination of the climate niche of the species may therefore improve our understanding of climate change effects and its spread. Notwithstanding the high potential invasiveness of *T. mauritanica*, its impact may be mitigated in the case of niche partitioning.

Evidence of strong spatial segregation between *T. mauritanica* and *H. turcicus* was previously found without proving the existence of a mutual negative impact (Lisičić et al., 2012). Competition for spatial niches leading to habitat partitioning may occur at our study site. Olfactory marking induced by the presence of a potential competitor or predator can generate

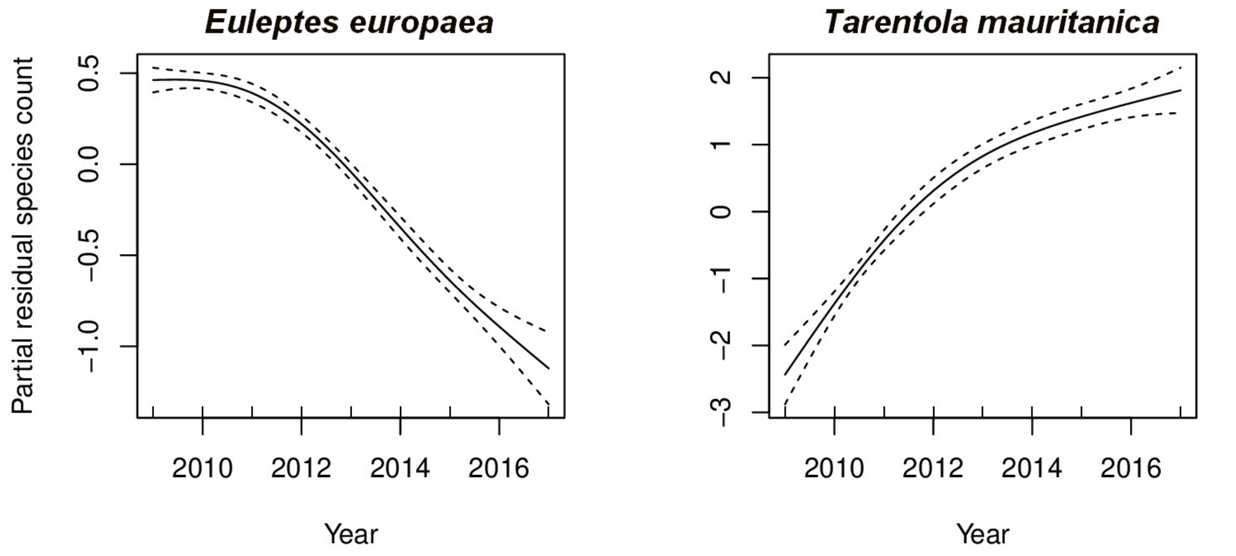

Figure 3. Temporal variation in abundance of *Euleptes europaea* (left panel) and *Tarentola mauritanica* (right panel). Curves represent fitted values from GAMM, with year as a spline effect. Dotted lines represent 95% confidence intervals.

physiological stress that leads to different behavioural responses (Febrer-Serra et al., 2023). In particular, in the presence of a nocturnal co-occurring species (i.e. the Black rat), *E. europaea* tends to remain sheltered in crevices (Delaugerre et al., 2019). This may have affected our ability to detect the species and led to a large underestimation of the number of individuals present. Nevertheless, in Cala Violina on the Tuscan coast, *E. europaea* did not show any atypical spatial behaviour (e.g. emerge from crevices less frequently) despite the presence of *T. mauritanica* in similar densities (Radi & Zuffi, 2022).

The question of high predation pressure also arises, but studies on the trophic ecology of *T. mauritanica* indicate that it feeds almost exclusively on arthropods (Gil & Perez-Mellado, 1994; Hódar et al., 2006; Martins et al., 2022). However, some cases of predation on several species of Lacertidae (Salvador, 1978; Franco, 1980; Pellitteri-Rosa et al., 2015) have been documented in other areas. For example, theDNA of the wall lizard *Teira dugesii* was detected in nearly 27% of *T. mauritanica* samples from Madeira Island (Martins et al., 2022). Predation of *H. turcicus* by *T. mauritanica* has also been observed (Rieppel, 1981; Gonzalès de la Vega, 1988; Bauer, 1990). Given the large difference in body size between *T. mauritanica* (total length 15 cm) and *E. europaea* (total length 8 cm), predation may occur occasionally on juvenile or adult *E. europaea*, but this alone cannot explain the extent of the observed decline. Apart from potential predation by *T. mauritanica*, competition for food resources cannot be ruled out, given the high similarity in trophic ecology between the two species (Oneto et al., 2008; Hódar et al., 2006; Martins et al., 2022).

Finally, the possibility of parasite transmission cannot be excluded. *Tarentola mauritanica* often carry intestinal parasites (Helminth) (El-Rghibi et al., 2022), hemoparasites (*Leishmania tarentolae*, haemogregarines of the Genera *Hepatozoon* ssp.) (Rioux et al., 1969; Tomé et al., 2016; Parejo-Pulido et al., 2023) and acari (geckobians) (Girot, 1968; Bertrand et al., 2012) which may impact the physiology, the immunity and the behaviour of their hosts (Oppliger et al., 1996; Oppliger & Clobert, 1997; Amo et al., 2005). The introduction of non-indigenous parasites transmitted to the local *T. mauritanica* population by fortuitous individual displacement is also to be considered, along with their potential impact on physiology and population dynamics.

Our study does not allow us to certify that the increase in the occurrence of *T. mauritanica* is causally related to the decline of *E. europaea*. Nevertheless, the strong correlation between the collapse of the population of *E. europaea* and the increase in the number of *T. mauritanica* raises questions regarding the conservation of *E. europaea*. *Tarentola mauritanica* was introduced very recently on two islands in Provence (Port-Cros, Ile du Levant) (Deso et al., 2021) which could provide a more rigorous test of a direct negative impact by *T. mauritanica* on *E. europaea* populations. Most of the islands of Provence are home to high-density *E. europaea* populations and benefit from a high level of protection (Port-Cros National Park and Calanques National Park). We encourage the urgent implementation of biocontrol strategy and robust monitoring at our study site and more widely at other sites where *T. mauritanica* co-occurs with other gecko species beyond its native range, especially on islands. This will avoid the entry of new specimens of *T. mauritanica* and improve the understanding of the colonisation dynamics of *T. mauritanica* and its biotic interactions. In the meanwhile, the invasive status of *T. mauritanica* remains an open question.

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Seasonal variation of the relationship between body condition and fluctuating asymmetry in two sympatric ranid frogs

Enrique Cante-Bazán[1] & Ricardo Luría-Manzano[2]

[1]Facultad de Ciencias Biológicas, Benemérita Universidad Autónoma de Puebla, Puebla, Mexico

[2]Departamento de Ecologia, Instituto de Biociências, Universidade de São Paulo, São Paulo, Brazil

Fluctuating asymmetry (FA) in organisms is an indicator of disturbances during development. This indicator has been associated with multiple causes, such as presence of metals, and organic and inorganic pollutants. However, the effect of FA on individual traits has been little explored. In this study, we examine the presence of FA in some morphological characters in two sympatric species of ranid frogs, *Lithobates spectabilis* and *Lithobates zweifeli*, predicting that *L. zweifeli* would exhibit more characters with FA and higher levels of FA. We also evaluated whether body condition in the two species differ between seasons and whether body condition is associated with FA of limb characters in two seasons. We predicted that this association would be more pronounced in the dry season. We found FA in tympanum and limb characters in both species, but *L. zweifeli* exhibited FA in one character more than *L. spectabilis*, as well as higher levels of FA in the horizontal length of the tympanum. Additionally, the characters exhibiting FA only in *L. zweifeli* are presumed to be more relevant for locomotor performance, fitness and survival than those exhibiting FA only in *L. spectabilis*. We did not find differences in body condition between seasons in either species, but we found a negative correlation between body condition and FA of the humerus in *L. zweifeli*. However, this correlation was detected in the rainy but not in the dry season, which could be related to depletion of energetic reserves associated with reproductive investment. This study highlights the importance of evaluating sensitivity of sympatric species through analyses of FA and its effects on individual traits, as well as the analysis of these effects in seasons with different environmental conditions.

Keywords: Atoyac basin, limb asymmetry, *Lithobates*, Mexico, tympanum asymmetry

INTRODUCTION

Developmental stability is the physiological process by which the expression of a predetermined phenotype is regulated by stimuli and environmental conditions during growth (Zakharov et al., 2020). This process can be altered under conditions of environmental and genetic stress, causing morphological characteristics to deviate from the standard, a phenomenon that has been called developmental instability or noise (Grillitsch & Chovanec, 1995; Glennemeier & Begnoche, 2002; Benítez & Parra, 2011). Bilateral variation has historically been measured through asymmetry, a morphometric pattern that reflects the small deviations that occur between the right and left sides in organisms with bilateral symmetry (Palmer, 1994; Niemeier et al., 2019; Gondim et al., 2020). There are three typical patterns of asymmetry: directional asymmetry (DA), antisymmetry, and fluctuating asymmetry (FA; Van Valen, 1962). Fluctuating asymmetry has been considered a useful indicator of the health of populations under conditions of anthropisation, pollution, food scarcity and increases in predators or competitors (Brown, 1996; Söderman et al., 2007; Benítez et al., 2020). Therefore, the analysis of FA is an alternative to long-term population studies. In some cases, the latter could take longer to be conducted than the time during which population declines occur, making studies of FA particularly important in groups highly susceptible to declines, such as amphibians (Stuart et al., 2004; Catenazzi, 2015).

Studies of FA in amphibians have been focused on species of the families Ranidae and Bufonidae (e.g. *Pelophylax ridibundus, Rana arvalis, Bufotes viridis*). These studies have found that deforestation, discharges from urbanised sites, heavy metals, pesticides and fertilisers affect the normal development of individuals, which is reflected in higher levels of FA in morphology. The most affected morphological traits are the limbs, digits and dorsal spot colouration patterns (Wright & Zamudio, 2002; Lauck, 2006; Söderman et al., 2007; Eisemberg & Bertoluci, 2016; Guo et al., 2017; Zhelev et al., 2017; Khattab et al., 2021). Despite the growing information about factors that cause FA, few studies have paid attention to the effects of this phenomenon on morphological, ecological and behavioural traits in amphibians. For example, FA of the tympanum in females of *Alytes obstetricans* reduces their accuracy in detecting sound sources, which could be detrimental to their survival and reproductive success (Bosch & Márquez, 2000). On the other hand, there is no effect of FA of hindlimbs on locomotor performance in *Pelophylax perezi* (Moreno-Rueda et al., 2020).

Correspondence: Enrique Cante-Bazán (cante662@gmail.com)

Another variable potentially affected by FA is body condition. For example, in *P. ridibundus* it was found that FA of the number of spots is high and body condition is low in disturbed sites (Zhelev et al., 2017). Additionally, individuals with higher levels of FA of the number of spots have lower values of body condition in the salamander *Ambystoma maculatum* (Davis & Maerz, 2007). However, in the frogs *Dendropsophus ebraccatus* and *Agalychnis callidryas* the results are not consistent. In the former species, body condition decreases in disturbed sites, but FA is higher in undisturbed sites. In *A. callidryas*, neither body condition nor FA decreases in disturbed sites (Matías-Ferrer & Escalante, 2015). Available evidence thus suggests that FA may have negative effects on body condition in some cases, but additional studies are needed in this regard. The inclusion of other variables that affect body condition in studies relating it to FA may help to identify such cases.

Since body condition is an estimate of the nutritional state of an individual, it can provide a snapshot of the physiological state (Jakob et al., 1996). Therefore, this variable is susceptible to temporal variations in environmental conditions. For example, increases in environmental temperatures and habitat disturbance over the years reduce body condition in some amphibian species (Reading, 2007; Tomašević et al., 2007; Vera-Candioti et al., 2019; Cogălniceanu et al., 2021). Additionally, it has been observed that body condition varies between seasons in amphibians such as *Lechriodus fletcheri* and *Bombina variegata*. This is possibly related to the reproductive season, when energy reserves are used and there is greater intraspecific competition for resources; alternatively, this variation may be related to the period after winter (Gould & Valdez, 2021; Băncilă et al., 2010). Therefore, seasonal variation in environmental conditions could strengthen the negative effect of FA on body condition, so it is relevant to consider this variation.

Amphibians exhibit interspecific variation in their sensitivity to environmental stressors, a variation that depends on the characteristics of each species and their ecological interactions (Angulo, 2002). This variation can be studied in amphibian species that live in sympatry, as they share the same environmental conditions (e.g. food, reproductive sites and climate), and thus may be under similar selective pressures. The anuran species *Lithobates spectabilis* and *Lithobates zweifeli* belong to the family Ranidae, are endemic to Mexico, distributed from north-east Jalisco to Guerrero, and live in sympatry in the Ameyalpa stream, which belongs to the Atoyac basin. The Atoyac river is the main water body of this basin, and has high levels of heavy metals such as Cd, Pb and Cr, which are commonly associated with FA (Mancilla-Villa et al., 2012; Ortiz-Alamilla et al., 2015; Guo et al., 2017). With this information in mind, the objectives of this study are: i) to examine the levels of FA in *L. spectabilis* and *L. zweifeli* in the Ameyalpa stream, ii) to compare body condition between seasons in both species, and iii) to evaluate the effect of FA on body condition in different seasons. Although neither species is considered to be under a risk category of extinction according to the IUCN (2022), *L. spectabilis* is commonly found in urbanised sites, whereas *L. zweifeli* is not (MacGregor-Fors et al., 2012), suggesting that the latter species is more sensitive to habitat modification. Accordingly, we predicted that *L. zweifeli* would exhibit FA in more characters and higher levels of FA than *L. spectabilis*. Furthermore, we predicted that body condition will be lower in the dry season, that it will be negatively affected by FA of the limbs, and that this latter effect will be more pronounced in the dry season. We predicted these patterns due to the increased use of energy reserves caused by resource scarcity during the dry season (Tomašević et al., 2007; Băncilă et al., 2010).

MATERIALS & METHODS

Fieldwork was conducted in the Ameyalpa stream, located in La Magdalena Cuaxixtla, municipality of Tecali de Herrera, state of Puebla, central Mexico (18.8835° N, 97.9526° W; 2041 m a.s.l). The predominant vegetation type is xeric shrubland, composed of succulent plants, some of which form colonies (Rzedowski, 2006). The study site is part of the Atoyac basin, which in turn belongs to the Río Balsas hydrological region (INEGI, 2009). The climate is temperate sub-humid with summer rains, with a mean annual temperature of 18.3 °C and an annual precipitation of 534 mm. In 2018, the mean temperature was 19.7 °C and precipitation was 155 mm in the months of the dry season covered in this study (March–May). The mean temperature was 19.7 °C and precipitation was 288 mm in the months of the rainy season covered in the study (June–September; CONAGUA, 2020).

We conducted nine three-day trips to the Ameyalpa stream, from March to September 2018. We collected postmetamorphic individuals of *L. spectabilis* and *L. zweifeli* manually or with a dip net and took them to the camp. We obtained body weight with a spring scale (± 0.1 g) and measured snout-vent length (SVL) with a digital caliper (± 0.1 mm) for each frog. Subsequently, individuals with a SVL ≥ 50 mm were anesthetised by placing them in a plastic bag with an ether-moistened cotton ball (Stetter, 2001; Zhelev et al., 2017). In *L. spectabilis*, the mean time of anaesthesia was 7.9 min and the mean recovery time was 20.21 min; in *L. zweifeli*, the mean time of anaesthesia was 4.91 min and the mean recovery time was 24.96 min. Once the frog was sedated, we measured the length of six morphometric limb characters: femur, tibia-fibula, humerus, radio-ulna, third finger and fourth toe (Matías-Ferrer & Escalante, 2015; Eisemberg & Bertoluci, 2016). We also measured the horizontal and vertical length of the tympanum (Bosch & Márquez, 2000). Additionally, we photographed the dorsum of individuals of *L. spectabilis* with a reflex camera at a standardised distance, to evaluate asymmetry in colouration pattern. It was not possible to evaluate asymmetry in colouration pattern in *L. zweifeli*, since this species does not present clearly defined spots that contrast with the background colouration. All measurements were taken with a digital calliper (± 0.1 mm), which was zeroed after each measurement, and were taken thrice by the same person (ECB) without access to values of previous measurements, to reduce

Table 1. Results of mixed-factor analyses of variance for individuals (genotype) and trait side of the two ranid frog species studied. Significant P values for the interaction of side vs. genotype are in bold, which indicates fluctuating asymmetry.

	Source of variation	*Lithobates spectabilis*			*Lithobates zweifeli*		
		MS	**F**	**P**	**MS**	**F**	**P**
Femur	Genotype	80.32	85.84	< 0.0001	176.06	204.46	< 0.0001
	Side	36.24	21.32	< 0.0001	72.86	48.76	< 0.0001
	Side vs. Genotype	1.7	1.82	**0.004**	1.49	1.74	**0.006**
	Error	0.935			0.86		
Tibia-fíbula	Genotype	92.46	153.7	< 0.0001	188.41	293.79	< 0.0001
	Side	5.35	6.92	0.011	0.0002	0	0.98
	Side vs. Genotype	0.77	1.29	0.135	1.57	2.46	**< 0.0001**
	Error	0.6			0.64		
Humerus	Genotype	18.26	1.18	0.237	10.09	49.29	< 0.0001
	Side	45.33	2.88	0.097	3.38	7	0.011
	Side vs. Genotype	15.72	1.01	0.46	0.48	2.36	**< 0.0001**
	Error	15.54			0.2		
Radio-ulna	Genotype	9.67	21.07	< 0.0001	28.28	88.71	< 0.0001
	Side	1.81	3.15	0.083	0.85	1.43	0.23
	Side vs. Genotype	0.57	1.25	0.164	0.59	1.88	**0.002**
	Error	0.45			0.31		
Third finger	Genotype	12.87	39.58	< 0.0001	28.99	78.83	< 0.0001
	Side	0.3	0.22	0.642	4.99	5.12	0.02
	Side vs. Genotype	1.4	4.32	**< 0.0001**	0.97	2.65	**< 0.0001**
	Error	0.325			0.36		
Fourth toe	Genotype	73.67	55.82	< 0.0001	267.51	1.69	0.01
	Side	23.38	1.68	0.202	66.88	0.43	0.517
	Side vs. Genotype	13.93	10.56	**< 0.0001**	156.85	0.99	0.495
	Error	1.31			158.3		
Tympanum horizontal length	Genotype	4	85.08	< 0.0001	4.98	49.25	< 0.0001
	Side	0.07	0.44	0.512	0.007	0.03	0.873
	Side vs. Genotype	0.17	3.65	**< 0.0001**	0.278	2.75	**< 0.0001**
	Error	0.04			0.101		
Tympanum vertical length	Genotype	3.94	49.38	< 0.0001	764.69	1.01	0.457
	Side	0.004	0.03	0.856	673.58	0.89	0.35
	Side vs. Genotype	0.131	1.64	**0.015**	757.03	1	0.473
	Error	0.079			753.93		

measurement error and increase precision (Palmer, 1994). Afterwards, the frogs were individually marked by toe-clipping to avoid pseudoreplication (Donnelly et al., 1994; HACC, 2004), vigorously washed with water after they recovered from the anaesthesia, and released at the capture site.

Data analyses

We used photographs of the dorsum of individuals to estimate total area covered by spots and number of spots on each side of the body by drawing a median line along the dorsum from snout to vent, using the Image J 1.51 program (Gallant & Teather, 2001; Wright & Zamudio, 2002). We estimated body condition with the residuals of the regression of the natural logarithm of weight against natural logarithm of SVL (residual index; Jakob et al., 1996). We classified positive values of body condition as indicating a good condition of the individuals, and negative values a poor condition (Peig & Green, 2009; Băncilă et al., 2010).

Normality of morphometric variables and body condition was verified with Kolmogorov-Smirnov tests. We evaluated the existence of FA in each morphometric character using a mixed-factor ANOVA. To determine if variation between sides (left and right) was higher than variation among replicates of the same measure (i.e. measurement error), sides and individuals were entered as fixed and random effects, respectively. A significant effect of the sides indicates the presence of DA. On the other hand, a significant effect of the interaction between sides and individuals indicates the presence of FA (Palmer & Strobeck, 1986). We estimated the levels of FA for those variables that exhibited this phenomenon with two indices: FA2, recommended because it corrects for dependence on body size, and FA10, because it describes the differences between sides after removing measurement error (Palmer, 1994; Villa, 2014; Matías-Ferrer & Escalante, 2015). We used the Levene test to determine if FA2 differed between species for those variables for which both exhibited FA (Palmer, 1994; Eisemberg & Bertoluci, 2016).

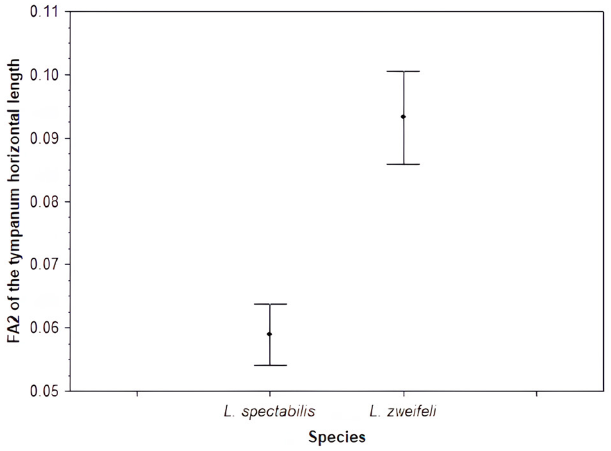

Figure 1. Mean ± SE of the level of fluctuating asymmetry (FA2; see Materials & Methods) of the horizontal length of the tympanum of the two species studied.

Table 2. Level of fluctuating asymmetry (FA10; see Materials & Methods) for both species. Cells without values correspond to morphological traits that did not show fluctuating asymmetry in a given species.

	AF10	
	Lithobates spectabilis	***Lithobates zweifeli***
Femur	0.255	0.211
Radio-ulna	-	0.093
Humerus	-	0.093
Tibia-fíbula	-	0.31
Third finger	0.36	0.202
Fourth toe	4.2	-
Tympanum horizontal length	0.041	0.059
Tympanum vertical length	0.017	-

We tested for the presence of DA in the area covered by spots and the number of spots in *L. spectabilis* with one-sample t-tests, in which we compared R–L against a mean equal to 0. We used a linear regression of |R–L| against SVL to evaluate whether there is a relationship of the area covered by spots and the number of spots with body size. Deviations attributed to FA occur randomly and independently on each side of the body, so it is expected that if these deviations are caused by developmental noise, they would present a normal distribution. We used Kolmogorov-Smirnov tests to determine whether the frequency distributions of FA2 of the area covered by spots and the number of spots followed a normal distribution, and thus confirm the presence of FA (Palmer, 1994; Gallant & Teather, 2001).

We did not consider sex in the analyses due to the inconsistencies in the available evidence regarding the impact of this variable on fluctuating asymmetry (Zhelev et al., 2012; 2017), and since its inclusion would significantly reduce the statistical power of our tests. We compared body condition between seasons for each species with a one-way ANOVA. We conducted multiple regressions of body condition against FA2 of those variables of the limbs that exhibited FA for each species by season. We tested for multicollinearity among predictors in multiple regressions with variance inflation factor (VIF) analyses (Midi et al., 2010). The maximum VIF value we obtained was 1.36, which indicates sufficient independence among predictors. Statistical analyses were conducted in NCSS 12 and Minitab 19. Results are shown as mean ± 1 SE unless otherwise indicated.

RESULTS

Forty-two individuals of *L. spectabilis* ($\overline{x}$ = 54.48 ± 1.98 mm SVL) and 45 individuals of *L. zweifeli* ($\overline{x}$ = 58.97 ± 2.0 mm SVL) were considered for the analyses. We found DA in the length of the femur and tibia-fibula in *L. spectabilis*, and in the length of the femur, humerus and third finger in *L. zweifeli*. We found FA in the length of the femur, third finger and fourth toe, and the horizontal and vertical length of the tympanum in *L. spectabilis*; and in the length of the femur, tibia-fibula, humerus, radio-ulna and third finger, and the horizontal length of the tympanum in *L. zweifeli* (Table 1). The level of FA2 in the horizontal length of the tympanum was higher in *L. zweifeli* than in *L. spectabilis* ($F_{1,83}$ = 5.91, P = 0.02; Fig. 1); but there were no differences in FA2 of femur ($F_{1,83}$ = 0.04, P = 0.82) or third finger length ($F_{1,83}$ = 0.65, P = 0.42) between species. According to the FA10 index, the fourth toe was the most asymmetric character in *L. spectabilis*, and the tibia-fibula was the most asymmetric in *L. zweifeli* (Table 2).

We did not find DA in the variables of spots in *L. spectabilis* (t = 1.08, P = 0.28 for the area covered by spots; t = 1.17, P = 0.25 for the number of spots). We did not find a relationship between asymmetry in the variables of spots and SVL (R^2 = 0.07, P = 0.84 for the area covered by spots; R^2 = 0.005, P = 0.65 for the number of spots). The area covered by spots and the number of spots did not follow a normal distribution (P < 0.01 in both cases), so we ruled out the presence of FA in these variables.

We did not find significant differences in body condition between seasons in *L. spectabilis* ($F_{1,40}$ = 0.14, P = 0.70, n = 42), nor in *L. zweifeli* ($F_{1,43}$ = 0.86, P = 0.35, n = 45). In *L. spectabilis*, body condition was not associated with FA2 of the femur, third finger or fourth toe in either season (r = 0.34, P = 0.42, n = 25 for the dry season; r = 0.45, P = 0.45, n = 15 for the rainy season). In *L. zweifeli*, body condition was not associated with FA2 of the femur, tibia-fibula, humerus, radio-ulna, nor third finger in the dry season (r = 0.41, P = 0.40, n = 32). However, this association was significant in the rainy season (r = 0.85, P = 0.05, n = 13), particularly with body condition being negatively correlated with FA2 of the humerus (r = −0.70, P = 0.007; Fig. 2).

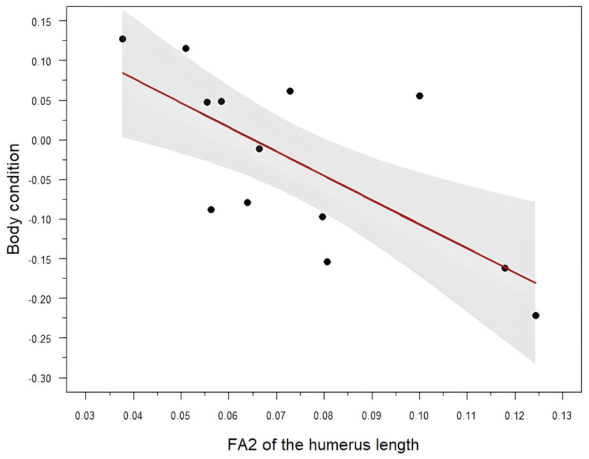

Figure 2. Linear regression between body condition and level of fluctuating asymmetry (FA2; see Materials & Methods) of the humerus length in *L. zweifeli* during the rainy season. Shadow areas represent the 95% confidence intervals.

DISCUSSION

The results of this study suggest that normal development of the two ranid frog species analysed could be affected by some stressor agent in the Ameyalpa stream. In line with our predictions, *L. zweifeli* exhibited FA in one more character than *L. spectabilis*, and also higher levels of FA in the horizontal length of the tympanum. Furthermore, we consider that the characters exhibiting FA only in *L. zweifeli* (i.e. tibia-fibula, humerus and radio-ulna) could be more relevant for locomotor performance, fitness and survival than the characters exhibiting FA only in *L. spectabilis* (i.e. fourth toe and vertical length of the tympanum). Based on these results, the response to the stress conditions is different for each of these sympatric species, with *L. zweifeli* being more susceptible to these conditions, making it a more reliable indicator of environmental health than *L. spectabilis*. We predicted that body condition would be lower in the dry season, but we did not find differences in this variable between seasons in either species. Finally, we found a negative correlation between body condition and FA of the humerus only in *L. zweifeli*, which partially supports our prediction. However, this correlation was detected in the rainy but not in the dry season, contrary to our expectation.

Origins of FA are diverse (e.g. organic and inorganic pollution, habitat modification and disturbance of ecological interactions). Many studies have evaluated the state of organic pollution in the Atoyac basin, to which the Ameyalpa stream belongs. These studies have found high concentrations of metals such as Pb, Cd, Hg, Fe, Al and Cu, as well as As, in both water and sediment (García-Nieto et al., 2011; Mancilla-Villa et al., 2012; Pérez-Castresana, 2019; Castro-González et al., 2019). Studies have found that Cd, Cr, Cu and As have negative effects on the survival and mobility of amphibians (Parris & Baud, 2004; Grillitsch & Chovanec, 1995), and that a first indicator of this detrimental effect is the appearance of FA (Khattab et al., 2021). Another detrimental effect is that organisms exposed to these metals accumulate high quantities in the kidney and liver, which may be related to high levels of FA (Guo et al., 2017; Zhelev et al., 2022). Considering that several of the contaminated localities and with high concentrations of metals in the Atoyac basin are located upstream of our study area (García-Nieto et al., 2011; Mancilla-Villa et al., 2012; Pérez-Castresana, 2019; Castro-González et al., 2019), it is possible that the Ameyalpa stream also contains high levels of metals. Therefore, it would be important to conduct further studies to evaluate whether FA of morphometric characters in *L. spectabilis* and *L. zweifeli* in this stream is related to heavy metals dissolved in the water.

Many studies have found that climate conditions cause variations in body condition (Reading, 2007; Băncilă et al., 2010; Matías-Ferrer & Escalante, 2015). However, we found that body condition did not differ between seasons in the populations studied, which suggest that environmental conditions were not inadequate for the organisms in either season. We must consider that body condition is also associated with other factors such as availability of resources and population density (Gómez-Hoyos & Maya, 2014; Unglaub et al., 2018; Vera-Candioti et al., 2019; Cogălniceanu et al., 2021). Therefore, it is possible that body condition in these populations is related to these variables, which were not considered in this study. Additionally, it has been suggested that species with generalist dietary habits are less vulnerable to disturbance than species with specialist dietary habits. Since the two species studied here have generalist diets (Mendoza-Estrada et al., 2008; Woolrich-Piña et al., 2017), this may partially explain the scant seasonal variation in body condition we found. Finally, we cannot rule out the possibility that body condition decreases in colder periods such as the winter, which was not sampled in this study.

We also predicted that FA would have a more pronounced negative effect on body condition in the dry season, because availability of resources could be lower in this season than in the rainy season. Contrary to this prediction, we found a negative association between body condition and FA of the humerus during the rainy season in *L. zweifeli*. This result may be related to the peak of the reproductive season or the period immediately following it, which presumably occurs in the rainy season as in other species of the *tarahumarae* group (Goldberg, 2020). Some anuran species decrease their food intake during the reproductive season (Díaz-Páez & Ortiz, 2003), males commonly deplete their energetic reserves at the end of this season (Eggert & Guyétant, 2003), and females of ranid frogs have large clutch sizes (Wells, 2007). Therefore, reproductive investment by both sexes is very high, which would explain our result of an effect of FA on body condition only in the rainy season. It is possible that the effort required to move between breeding sites and ultimately to clasp a female and sustain amplexus is higher in individuals with more asymmetric forelimbs, which would deplete their energetic reserves and decrease their body condition.

Amphibians in general exhibit FA in structures theoretically related to important aspects of their life, such as forelimbs, hindlimbs and tympanum (Vershinin et al., 2007; Matías-Ferrer & Escalante, 2015; Eisemberg & Bertoluci, 2016; this study). However, the consequences of this phenomenon are little known. In addition to the negative effect of FA of the humerus on body condition we found, it has been suggested that FA in the forelimbs also could negatively impact landing performance, since these structures are important to absorb the impact of jumps in ranid frogs (Petrović et al., 2017; Citadini et al., 2018; Didde & Rivera, 2019). On the other hand, there was no association between locomotor performance and FA of the hindlimbs in *P. perezi* (Moreno-Rueda et al., 2020), but this may be partially explained by the low levels of FA found. Finally, the tympanum plays a determining role in the reception of sound emissions from predators, prey and conspecifics. Therefore, FA in this structure decreases the ability of anurans to detect mates (St-Amour et al., 2010), and could even make them more susceptible to predators (Bosch & Márquez, 2000).

Although we did not find FA in the area covered by spots and the number of spots in *L. spectabilis*, previous studies have found that organisms develop FA in these variables in disturbed sites (Wright & Zamudio, 2002; Guo et al., 2017; Zhelev et al., 2019). On the other hand, studies that have found low levels of FA in colouration traits have suggested that environmental conditions during the initial stages of development of the organisms have been adequate, representing a minimal perturbation on the stability of individuals (Zhelev et al., 2021). Our finding of absence of FA in colouration variables but presence of FA in some morphometric variables in *L. spectabilis* can be explained as follows: Bone growth takes place from birth onward throughout the lifetime of an organism, unlike colouration patterns, which start to develop a few weeks before metamorphosis (Davis & Maerz, 2007; Thibaudeau & Altig, 2012). Therefore, asymmetry in traits such as colouration patterns could provide information on the stress that organisms experience during specific stages of their growth. Thus it is likely that stress factors affecting colouration (e.g. UV radiation) are different from those affecting bone growth (Gallant & Teather, 2001) or act at different stages of development.

In summary, our study revealed that the two species of ranid frogs exhibited FA in morphometric characters in accordance with the presence of a stressor agent at the study site. The species exhibited different degrees of sensitivity to the presumed stressor agent, which is reflected in differences in FA. These differences are expressed as a higher number of characters with FA and higher levels of FA in the horizontal length of the tympanum in *L. zweifeli*, but also as a negative effect of FA of the humerus on the body condition of this species in the rainy season. The latter result was unexpected due to the theoretically higher availability of resources in this season, as well as the similar body condition between seasons in the two species. Therefore, seasonality is enough to modulate the effect of FA of the humerus on body condition, but not to decrease it directly. Overall, our results highlight the importance of evaluating sensitivity of sympatric species through analyses of FA and its effects on individual traits, as well as the analysis of these effects in seasons with different environmental conditions.

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Are fibrocement slabs a useful tool for monitoring reptiles? The case of *Hierophis viridiflavus* in its south-western limit of distribution

Iñaki Romero-Iraola, Xabier Rubio, Alberto Gosá & Ion Garin-Barrio

[1]Sociedad de Ciencias Aranzadi. Departamento de Herpetología, Paseo de Zorroaga 11, 20014 Donostia-San Sebastián, España

The established methodology in detection and long-term reptile monitoring programmes has so far focused on visual encounter surveys (VES), a method that can yield very high capture rates for snakes, but, even when carried out in favourable habitats, can also be very limited. Therefore, one passive sampling method was tested: fibrocement slabs. A total of 81 slabs were placed in nine sampling stations in the southern part of Irún (Basque Autonomous Community, Spain). During the two years of sampling (2020–2021) a total of 79 reptile records were obtained, of which 33 were records of the European whip snake corresponding to 28 individuals. There was at least one reptile record in each transect and 75% of the reptile species present in the study area were detected. However, we did not identify any trend over time that indicated an increase in the number of individuals captured during the second year. All in, the data obtained have provided positive results; therefore, it could prove to be a very useful methodology for reptile studies, in particular for ophidians.

Keywords: European whip snake, conservation, methodology, artificial refuge, capture, detection, Basque Autonomous Community, Spain

INTRODUCTION

Visual encounter surveys (VES) are a recurring methodology in long-term reptile monitoring programs (Zuiderwijk et al., 1999; Graeter et al., 2013; Andreu et al., 2022), being used in the monitoring programs with the longest tradition in Europe, such as the one led by the RAVON Foundation in the Netherlands 'Monitoring Network of Reptile, Amphibian & Fish Conservation', or the one developed by the Amphibian and Reptiles Conservation (ARC) in collaboration with volunteers in the United Kingdom, 'The National Amphibian and Reptile Recording Scheme' (NARRS). The Spanish Herpetological Association, following these guidelines, set up the Spanish Amphibian and Reptile Monitoring Programme (SARE Programme in Spanish) in 2006, defining the same methodology for the sampling of terrestrial reptiles, based on pre-set itineraries within the most representative habitats of the study area. VES can yield very high capture rates for snakes (e.g. Brown et al., 2013), however, even when carried out in favourable habitats, can also be very limited (e.g. Zuiderwijk et al., 1999; Santos, 2014; Gosá, 2019). The size and/or density of the focused population, detections and counts may depend on a wide range of other variables, as detection rates may be influenced by the organism itself (such as sex effects, size effects or other sources of individual heterogeneity in activity and microhabitat use (Tyrrell et al., 2009; Christy et al., 2010), or by spatiotemporally varying environmental conditions (Vogt & Hine, 1982; Williams & Berkson, 2004; Lardner et al., 2015).

The difficulty in taking records for a rigorous analysis of the conservation status of snakes extends to the struggle that specialists encounter in arguing the inclusion of a species in a threat list (at local scale, Romero-Iraola et al., 2020; or broad scale, Böhm et al., 2013). Faced with this complicated situation and given the good results shown by passive sampling elements (water cisterns, García-Cardenete et al., 2014; cover boards, Halliday & Blouin-Demers, 2015; pitfall traps, Bateman et al., 2009), the application of another passive sampling method was tested: the placement of fibrocement slabs. They have already been used satisfactorily in the monitoring of reptiles in other European countries (e.g. *Hierophis viridiflavus*, Bonnet et al., 2021; *Malpolon monspessulanus* and *Zamenis scalaris*, Ballouard et al., 2016; *Testudo hermanni*, Ballouard et al., 2013). The slabs corrugations ease shelter and provide environments conducive to the thermoregulation of ectothermic animals such as reptiles, and snakes in particular (Lelièvre et al., 2010a), encouraging them to take refuge beneath them and facilitating their detection, as well as the capture of individuals. Given the potential negative consequences caused by trapping animals, cover objects, such as fibrocement slabs, are preferred to funnel and pitfall traps (Grant et al., 1992). Besides, fibrocement slabs have alredy been tested succesfully for *Hierophis viridiflavus* (Bonnet et al., 2021; Romero-Iraola et al., 2022), and cover boards have appeared to be underused by snakes (e.g. Eye et al., 2018).

Correspondence: Iñaki Romero-Iraola (iromero@aranzadi.eus)

The European whip snake *Hierophis viridiflavus* (Lacépède, 1789) is a medium-sized diurnal colubrid snake (snout-vent length 1089 mm on males and 1023 mm on females, and tail-length 381 mm on males and 354 mm on females; Schätti & Vanni, 1986), with a south-central European para-Mediterranean distribution, occurring across a variety of open environments characterised by temperate climatic conditions (Lelièvre et al., 2010a; Santos et al., 2015; Vanni & Zuffi, 2011; Sillero et al., 2014); whose optimum preferred temperature stands around 30 °C (Lelièvre et al., 2010b).

Hierophis viridiflavus is catalogued as Low Concern (LC) by the IUCN (Vogrin et al., 2009), however, it is indexed within the regional catalogue of endangered species (see Romero-Iraola et al., 2020), as it is the snake species with the lowest occupancy in the Basque Autonomous Community (northern Spain) (Bea, 1985). This population located in the Basque Autonomous Community is the south-western population. Populations along the range edge frequently display high levels of local adaptation and contain distinctive genotypes that may be required for the persistence of the species under future climatic conditions (Hampe & Petit, 2005). Studies focusing on *H. viridiflavus* have a reduced number of occurrence records, which are not representative of the species range, as they did not include this edge population (Rato et al., 2009; Mezzasalma et al., 2015; 2018; Senczuk et al., 2021), making *H. viridiflavus* a compelling target species.

In this study, we set up a network of fibrocement slabs, as we aimed to: i) test them for reptile studies in the local reptile community focusing in *H. viridiflavus´* south-westernmost population and ii) assess if catchability increases in time using this method.

MATERIALS & METHODS

Study area

The study area was located in Irún (Basque Autonomous Community, Spain), distributed in peripheral districts of the same municipality and on land belonging to the Special Area of Conservation (SAC) of Aiako Harriak (ES2120016), on land belonging to the same municipality (Fig. 1). According to the Köppen classification, this Natura 2000 Network protected area, like the municipality of Irún, has an oceanic climate cfb (mid-Atlantic), with annual rainfall of 2800 mm and an average temperature of 12 °C (Meteorological Station of Jaizubia [Agencia Vasca de Meteorología, 2023]).

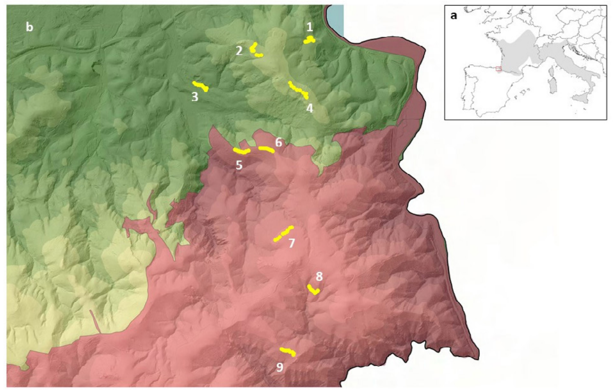

Figure 1. **a)** Global distribution of the European whip snake according to the IUCN (Vogrin et al., 2009) (in grey) and the study area (square in red). **b)** The nine sampling stations spread over the southern part of Irún (Spain) (1–4), five of them (5–9) within the Aiako Harriak Natural Park (in red).

Figure 2. Corrugated fibrocement slabs of 0.99 m² in an ecotonal area within the Aiako Harriak Natural Park

Regarding vegetation, coniferous plantations are the most abundant, with the most dominant being those of insignis pine *Pinus radiata*, Norway spruce *Pinus nigra* and Japanese larch *Larix kaempferi*. In addition, there are also plantations of deciduous trees such as American red oak *Quercus rubra* and Japanese chestnut *Castanea crenata*. Although in smaller quantities, native flora can also be found, such as common oak *Quercus robur*, beech *Fagus sylvatica* or honey oak *Quercus pyrenaica* forests. Furthermore, in areas with abundant disturbances such as those caused by human activity or fire, there are abundant populations of gorse *Ulex* spp., heather *Erica* spp. and ferns (Arbelaitz et al., 2002). Concerning the reptile fauna of the study area, seven snake species; southern smooth snake *Coronella girondica*, smooth snake *Coronella austriaca*, red-eyed grass snake *Natrix astreptophora*, viperine snake *Natrix maura*, Seoane`s viper *Vipera seoanei*, Aesculapian ratsnake *Zamenis longissimuss* and European whip snake *H. viridiflavus*, and five lacertids can be found; Catalonian wall lizard *Podarcis liolepis*, Common wall lizard *Podarcis muralis*, viviparous lizard *Zootoca vivipara*, slow worm *Anguis fragilis* and Western Green lizard *Lacerta bilineata* (SIARE, 2022).

Transects with fibrocement slabs (hereafter referred to as slabs) (Fig. 2) were established at nine sampling stations spread throughout the study area; four in the southern part of the municipality of Irún and five within the Aiako Harriak Natural Park (Fig. 1; supplementary material). These stations were selected in areas where the species had already been detected in the past, and included other areas with a high potential for its presence even if it had not been recorded so far. A limiting factor in defining the areas was land ownership, and only publicly owned lands were selected to facilitate sampling.

Field procedure

Fieldwork was carried out between April and September 2020 and 2021, although earlier information collected by rangers in the study area (2015–2019) was also taken into account (presented in the discussion and used to select sites). Slabs were checked every 3–4 days, i.e. twice a week during the first four months of sampling (April, May, June and July), the period of highest ophidian activity, and once a week in the following two months (August and September), for a total of 98 days of fieldwork. The time between samplings was not constant, because the most favourable days were chosen, avoiding the hottest days and discarding rainy days. Likewise, the order in which the slabs were examined was alternated so that the same area was not always the first or the last to be examined. At the same time, the sampling times were modified according to the duration of sunlight, starting between 10:00–11:00 h in spring and 09:00–10:00 h in summer, or according to the temperature of the chosen day. The transect sampling could last for a maximum of 300 minutes, as each series of nine transects was carried out in one day of fieldwork.

At each sampling station, the transects were made on foot, with an average length of 220 ± 55 metres (n = 9; range: 170–326 m), establishing an approximate separation between the slabs of 25 metres, with each transect consisting of nine slabs, so that the total number of installed slabs was 81. These had an undulating shape, which facilitates access and shelter for the animals, with an approximate surface area of 0.99 m^2 (1.1 x 0.9 m), and were arranged in preferred environments for the target species (*H. viridiflavus*): ecotonal areas between grasslands, shrublands and woodlands (Capula et al., 1997); all slabs were placed in the open and slightly covered by shrubs.

The protocol established to record the catch data was as follows:

1. Temperature measurement with a digital laser thermometer, both above and below the slabs.
2. Obtaining biometric records of the snakes captured (total length, snout-vent length, tail length and weight), counting the number of ventral scales (data collected for future studies) and noting the age class (juvenile, < 300 mm; subadult, 300–1000 mm; and adult, > 1000 mm). Before release, the individuals were marked using the cutting system on the lower edge of the ventral scales (see Brown & Parker, 1976), and any identifying marks (wounds or malformations) of the individual were photographed. Moults were treated as observations.
3. Similar data were collected for the other snake species captured, and the potential prey of the European whip snake (micromammals and lizards) were also censused; any incidents that may have occurred on the slabs, such as marks, displacements, breakages or deterioration, were also noted.

Placement of the slabs began during January and February 2020, 2–3 months before the proposed start of the species' activity period (Santos et al., 2015) and the start of the planned surveys (Naulleau, 2002). Due to the limitations imposed by the state of alarm resulting from the Covid-19 pandemic, only 45 of the slabs were placed in winter (January–February); the remaining slabs were placed during the month of April, in early spring, 16 days before the start of the fieldwork.

Statistical analyses

To assess if catchabily increases in time, we only used *H. viridiflavus* records, due to slabs that were placed in areas to target *H. viridiflavus*, and the detection of other species (particularly the specialist species) could be biased by the habitat where slabs were placed. For the analyses we discarded recaptures to avoid pseudo-replicates. Since the data did not show normal distribution, non-parametric Mann-Whitney tests were used (as previously seen in Ballouard et al., 2016). All the statistical tests were performed using RStudio (R Core Team, 2022).

RESULTS

Reptile diversity

A total of 79 reptile records of nine different species were obtained during the sampling years (2020–2021), beneath 27 different slabs (33.3%) and with at least one record on each transect. Seventy-five percent of the reptile species present in the Aiako Harriak Natural Park and neighbouring areas were counted. Fifty-five records were of snakes (European whip snake *H. viridiflavus*, n = 33; Red-eyed grass snake *N. astreptophora*, n = 13; Smooth snake *C. austriaca*, n = 5; Aesculapian ratsnake *Z. longissimus*, n = 3; and Seoane´s viper *V. seoanei*, n = 1), while 24 belonged to lacertids (Slow worm *A. fragilis*, n = 9; Western green lizard *L. bilineata*, n = 3; Common wall lizard *P. muralis*, n = 6; and Viviparous lizard *Z. vivipara*, n = 6) (Fig. 3).

Hierophis viridiflavus success

The 33 records of *H. viridiflavus* obtained in 2020–2021 corresponded to 28 individuals, including one juvenile, one subadult and one adult specimen identified by its moult. Of all records, five were recaptures (15% of the total). Broken down by year; in 2020, 12 records were obtained from ten individuals (one of them a moult), two of them recaptures (16%); in 2021, 21 records were obtained from 18 individuals, three of them recaptures (14%) (Table 1).

In 43% of the sampling days in 2020 at least one specimen was caught, while in 2021 catches increased to 84%. Despite this, it cannot be affirmed that there has been an increase in the number of individuals caught during the second year (Mann-Whitney U-test; U = 12; Z = - 0.81354; p-value = 0.4459). Ninety-four percent of the captures corresponded to adults, with the capture of subadults and/or juveniles being residual. The first capture of *H. viridiflavus* was made 14 days after the slabs were placed, while for the rest of the species identified it was necessary to wait more than 50 days after their placement (Table 2).

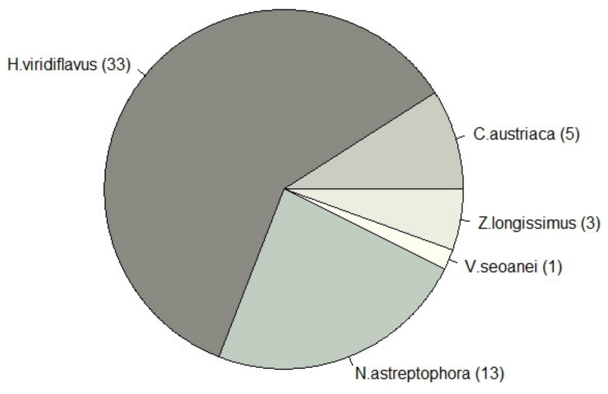

Figure 3. Number of snake records in fibrocement slabs during the years of sampling (2020–2021)

Table 1. Number of *Hierophis viridiflavus* recorded on the total number of fibrocement slabs installed, in the two sampling years

		Age		
		Adults	**Sub-adults**	**Juveniles**
2020	Captures	**11**	1	0
	Recaptures	**1**	1	0
2021	Captures	20	0	1
	Recaptures	3	0	0
Total	**Captures**	**31**	**1**	**1**
	Recaptures	**4**	**1**	**0**

Table 2. Days elapsed from the start of the sampling and the placement of the slabs before the first capture by each snake species

Species	Days
Hierophis viridiflavus	14
Natrix astreptophora	51
Coronella austriaca	63
Vipera seoanei	93
Zamenis longissimus	101

In 2020, the month with the highest number of captures was July, with 71% of sampling days with at least one specimen captured. In 2021, the most successful months were May and August with 100% of records, i.e. a minimum of one individual was caught on each sampling day. Even so, in 2021 June had a success rate of 80% and July 71% (Fig. 4).

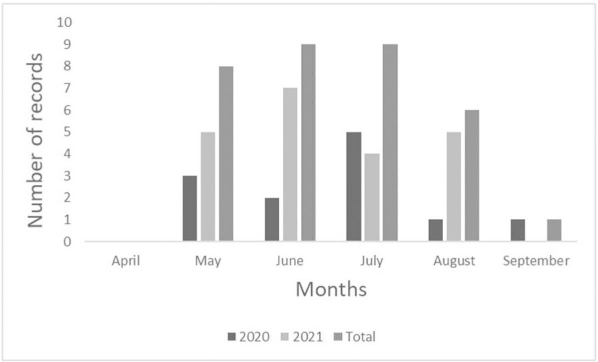

Figure 4. Records per year (2020 and 2021) and monthly totals of *Hierophis viridiflavus* in the set of transects carried out

External factors: Temperature and micromammals

The temperature recorded on the slabs depended on the amount of radiation received during the day, and thus varied greatly from day to day, with maximum temperatures of 56.3 °C above the slab and minimum temperatures of 9.1 °C, while under the slab the maximum temperature was 46.3 °C and the minimum was 8.6 °C.

Ninety percent of the slabs showed signs of micromammal activity (genera *Apodemus*, *Myodes* and *Sorex*), detectable through the identification of traces (mostly food remains) and the presence of nests. Furthermore, in 34.5% of the slabs, adult individuals were detected at the time of lifting them.

DISCUSSION & CONCLUSION

Nine of the possible 12 reptile species in the study area were found. Only the most specialist reptile species were not detected (Sanz-Azkue & Alkorta, 2017; Fernández-Arrieta et al., 2019; Bea, 1985), such as *N. maura* (linked to rivers and wetlands) and *P. liolepis* (characteristic of areas with high insolation, especially rocky outcrops), as well as *C. girondica* (an ophidian with similar requirements to the previous lizard, on which it preys). Given that slabs were placed in areas to target *H. viridiflavus*, it was predictable not to detect specialist species. However, these data indicate the use of the slabs by different groups of reptiles (e.g. Bonnet, 2013), giving the method a generalist character. Therefore, it may be useful for herpetological inventories, because explanatory results were obtained for the composition of the reptile community in the area, whereas previous studies required between four and seven years to achieve similar results (Sanz-Azkue & Alkorta, 2017; Fernández-Arrieta et al., 2019). The use of slabs would help in the task of locating terrestrial herpetofauna, such as snakes and lizards, which are cryptic and are often difficult to detect (Gibbons, 1988), reducing the time and effort required to do so, as well as providing the option of capturing the individual to collect data. However, our data have shown that they are more efficient at capturing ophidians and would, therefore, be more useful in studies focused on these species (e.g. Romero-Iraola et al., 2022).

Records of *H. viridiflavus* obtained until the beginning of the study have been scarce, with a total of eight individuals identified between 2015 and 2019 (Romero et al., 2020; X. Rubio, pers. obs.). This is because there was not an adequate methodology for their study, given that VES show very scarce results for snakes in the region (e.g. Gosá, 2019). Furthermore, despite that active searches of natural habitat can yield high snake capture rates (e.g. Rodda & Fritts, 1992), in the study area there is a lack of natural shelters like logs or rocks, so species find shelter in shrubs and burrows where active search is very difficult to carry out. By placing the slabs both in areas where *H. viridiflavus* had previously been detected and in areas with unknown presence to date, but presenting high potential for it, it was possible to verify that it was a relatively common species in the area, due to the number of records obtained. Using this method, the species was detected in nine new 1 x 1 grids, going from seven to 16 (see Romero-Iraola et al., 2022). The 28 individuals detected in the two years of sampling compared to eight during the last five years before the study, would indicate that the slabs can function as a complementary methodology to the visual transects when carrying out snake studies.

The early captures of the first records of *H. viridiflavus* individuals, without the need for a period of 'naturalisation' of the slabs to the environment, contrasts with the results obtained by Naulleau (2002), who suggests a period of 2–3 months for the slabs to homogenise with the environment. This suggests that in the censused population, the first records may be more influenced by the densities of the species (Bonnet et al., 2013), although the time elapsed since the slabs were placed may also be a determining factor, as the data obtained for the rest of the snake species might indicate. Nevertheless, lack of shelter may be a determining factor for species when using the slabs (e.g. *Testudo hermanni*, Ballouard et al., 2013).

As observed by Ballouard et al. (2016) over three years in other snakes such as the Montpellier snake *Malpolon monspessulanus* and the Ladder snake *Zamenis scalaris*, this two-year study also failed to confirm an increase in the number of individuals captured during the second year, i.e. it was not possible to confirm that the effectiveness of the slabs increased over time (Naulleau, 2002). Besides providing protection from predators, fibrocement slabs can facilitate thermoregulation (Lelièvre et al., 2010a). Therefore, the use of the slabs may be influenced by the temperature of the environment and the physiological needs of the snake rather than by the increasing passage of time (Bonnet, 2013). The months in which there have been the greatest number of captures are times that correspond to peaks of activity of *H. viridiflavus* (Santos et al., 2015); even so, individuals were captured up to mid-September 2020.

The high and low temperatures that can be reached by the slabs led to the need to choose a specific day and time to carry out the fieldwork, at times and hours when the temperature recorded by the slabs is within the optimum levels for *H. viridiflavus*, which stands around 30 °C (Lelièvre et al., 2010b). It should also be taken into account that the species changes its activity habits depending on the time of year. In spring and late autumn, daily activity has a unimodal pattern (peaking at midday), whereas in late spring and summer, it has a bimodal pattern (peaking in the early morning and late afternoon) (Capula et al., 1997). It is therefore recommended that rainy and very hot days, as well as the central hours of the day in summer, be ruled out.

The presence of micromammals under the slabs could be an indicator that snakes also use them to hunt potential preys. Such would be the case of *H. viridiflavus*, of whose diet micromammals form part (Vanni & Lanza, 1977).

Given the need for a new methodology to monitor reptile populations (Cox & Temple, 2009), this study of fibrocement slabs to census populations of *H. viridiflavus* in the mid-Atlantic environment, and other accompanying reptile species, has provided positive results. This methodology should be extended in future campaigns in order to carry out a more precise analysis of their effectiveness, which could be very useful for recording the abundance of ophidians and the necessary decision-making for their management. In addition, there were very few incidents related to the slabs. Ruptures/displacements were only recorded in areas with high livestock activity. The results presented in this study have led Navarre (northern Spain) to initiate in 2021 a similar monitoring programme of transects using the same methodology of fibrocement slabs, combining them with VES, in order to carry out snake censuses throughout its territory. All the same, considering the wide range of artificial refuges that could be used, other materials might be as effective as fibrocement slabs. Microhabitat preferences vary between reptile species and are influenced by environmental elements like temperature and moisture. As a result, the ideal environmental parameters for a given species may be met by a specific kind or size of artificial refuge (Engelstoft & Ovaska, 2000; Hecnar & Hecnar, 2011).

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Eastern Black Kingsnake *Lampropeltis nigra* dorsal scale colouration primarily adapted for thermoregulation

Caleb A. Aldridge[1,2] & Scott A. Rush[1,3]

[1]Department of Wildlife, Fisheries and Aquaculture, Mississippi State University, Mississippi State, Mississippi, 39762 USA

[2]Lower Mississippi River Fish and Wildlife Conservation Office, U.S. Fish and Wildlife Service, Tupelo, Mississippi, 38804 USA

The Eastern Black Kingsnake *Lampropeltis nigra* exhibits variation in colouration and pattern throughout its range. Drivers behind this colour variation remain largely unknown. To elucidate patterns in the percentage of light pigmentation in *L. nigra* dorsal scales (% light) we compared two primary hypotheses. Our first hypothesis posited that *L. nigra* colouration has been adapted for thermoregulation (thermoregulation). Alternatively, we hypothesised that *L. nigra* colouration has been adapted to avoid detection by predators and prey (crypsis). To test our hypotheses, we modeled *L. nigra* % light from 46 counties within Mississippi as a function of average temperature, average maximum temperature (thermoregulation) and soil value (darkness of soil colour; crypsis). We included percentage forest cover as a modifying variable in the thermoregulation hypothesis and an interacting variable in the crypsis hypothesis. The best competing model included average temperature and percent forest cover as explanatory variables (LOOIC = -291.3, weight = 0.84, Bayesian R^2 = 0.37 [fixed] and 0.55 [total]). There was a positive relationship between % light and average temperature (β = 0.23, 95% CIs = 0.13, 0.34) and average maximum temperature (β = 0.11, 95% CIs = -0.00, 0.21), and a negative relationship with percent forest cover (β = -0.14, 95% CIs = -0.23, -0.04). These results support the thermoregulation hypothesis. The climate in Mississippi, as in most of the world, is expected to experience shifts over the next century. If ambient temperatures experienced through these changes are outside of *L. nigra*'s thermal optima, as based on colour patterns and relationships observed in our study, then behavioural adaptations may result in some individuals experiencing thermal advantages that influence this species' distribution. The patterns observed in our study, and expected changes in *L. nigra* behaviour and distribution, are likely to occur among other ectothermic species with relatively static colouration.

Keywords: Colour adaptation, homeostasis, Mississippi

INTRODUCTION

An organism's colouration can afford multiple benefits through communication (e.g. aposematism), crypsis or camouflage (e.g. antipredator adaptation), and homeostasis (e.g. disease resistance, thermoregulation, and protection from ultraviolet radiation; Mappes et al., 2005). Colour can vary significantly among individuals within a population due to local abiotic and biotic conditions (Means & Krysko, 2001; Langkilde & Boronow, 2010; Westphal et al., 2011). For instance, individual colour may vary due to prey availability and competition, predator assemblage and climate and landscape characteristics (Merilaita et al., 1999; Means & Krysko, 2001; Langkilde & Boronow, 2010; Westphal et al., 2011; Cox & Davis Rabosky, 2013). Subsequently, colour variation can reflect individual and population benefits across ecosystems (Farallo & Forstner, 2012).

Colouration in reptiles is controlled by chromatophores (Huey, 1982; Cox & Davis Rabosky, 2013). Some squamates can alter pigments within chromatophores to quickly change their colouration in response to changes in conditions (e.g. predator presence; Stuart-Fox & Moussalli, 2009; Vroonen et al., 2012) – species of chameleons (Chamaeleonidae) and anoles (Dactyloidae) are prominent examples. Snakes, however, have relatively static and generally immutable colouration that cannot be changed rapidly but may provide benefits all the same (Cuthill et al., 2005). The deepest layers of chromatophores in snakes, melanophores, produce the dark pigment melanin that largely contribute to their colouration and pattern (Bechtel, 1978; Clusella-Trullas et al., 2007; Goiran et al., 2017). Melanin is synthesised from the amino acid tyrosine, and the resulting black, brown or reddish pigment that is stored in the melanosomes (Ullate-Agote & Tzika, 2021).

While environmental stimuli can affect colouration, there is a genetic basis regulating melanin production in snakes (Bechtel & Bechtel, 1962; 1978). For instance, albinism, or amelanistic colour variations result from mutations in genes that regulate the synthesis of melanin (Rosenblum et al., 2004). Melanism, on the other hand, can occur through an increase in the relative amount

Correspondence: Scott A. Rush (scott.rush@msstate.edu)

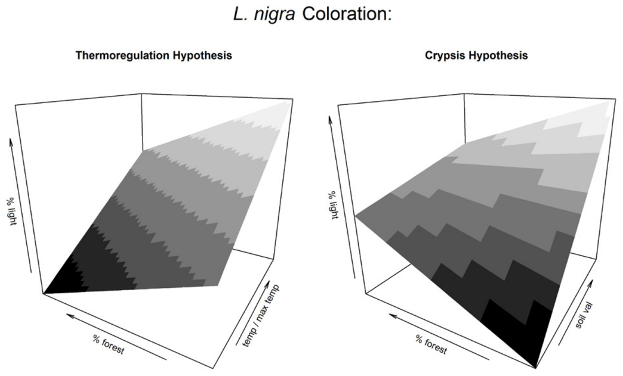

Figure 1. Graphical representation of competing hypotheses. The left plot represents our thermoregulation hypothesis – *Lampropeltis nigra* colouration (percentage of light colouration on dorsal scales; % light) is mostly explained by average temperature (temp) and/or average maximum temperature (max temp) and moderated by forest cover (% forest). The right plot represents our crypsis hypothesis – *L. nigra* colouration (% light) is mostly explained by soil value (darkness to lightness; soil val) and has an interactive effect with forest cover (% forest).

of melanophore density, or a lack of pigment cells other than melanophores in the skin (Kuriyama et al., 2016). A review of some of the processes that influence the synthesis of melanin, the migration of chromatoblasts to the dermis, and the density of these cells, is provided in Kuriyama et al. (2020).

Melanin-based colouration has been associated with a variety of behaviours and traits and linkages between this colouration and these traits is thought to have an embryonic origin (San-Jose & Roulin, 2020). For colouration among snakes, it has been shown, through a theoretical approach, that cell-to-cell and cell-to-environment interactions can affect the motility of pigment cells to sites within the skin during embryonic development (Kuriyama et al., 2020). Colouration is therefore sensitive to internal and external drivers and the evolution of melanistic-based colour patterns can involve balance between lethal and non-lethal mutations in the development of novel colour patterns (Kuriyama et al., 2020). Subsequently, it is expected that the rate of mutation among the suite of physiological conditions that influence colouration can vary with selection pressure (Rosenblum et al., 2004; Wan et al., 2021).

Environment can influence colouration and affect ectotherms, including snakes' ability to thermoregulate (Bechtel, 1978; Geen & Johnston, 2014). Darker individuals can absorb more radiation over a shorter period and reach operative temperature faster than lighter-coloured individuals or those with greater contrast between elements of disruptive colouration (Lattanzio & Buontempo, 2021). However, as body colouration is not plastic within generations of most snakes, rapid changes in ambient temperatures without access to thermal refugia can result in increased mortality rates in populations that cannot adapt (Sears et al., 2011), influencing the demographics of these populations (Lelièvre et al., 2013). Such effects can induce changes to proximate and ultimate factors affecting the distributions of these organisms (Shine & Harlow, 1993; DeGregorio et al., 2015; Tully et al., 2020).

Lampropeltis nigra (Eastern Black Kingsnake; Serpentes: Colubridae; Pyron & Burbrink, 2009) is a large (maximum total length 208 cm) constrictor found in North America east of the Mississippi River, ranging from Illinois to Ohio following the Appalachian Mountains south-westerly to the Gulf of Mexico in Alabama to Louisiana (Conant & Collins, 1991). It inhabits a variety of ecosystems from upland forest, river floodplains, coastal marshes, prairieland and agricultural periphery. Colouration of *L. nigra* is characterised by black dorsal scales with a white to yellow dot in the centre – a pattern that appears weaker in higher latitudes where scales of adults may be almost completely black (Conant & Collins, 1991). The diet of *L. nigra* is diverse, including reptile eggs, snakes, mammals and birds (Ernst & Ernst, 2003). Predators of *L. nigra* are primarily raptors and meso-predators (e.g. raccoons, skunks and opossums; Ernst & Ernst, 2003). Colour and pattern variation in *L. nigra* has, presumably, been adapted to benefit individuals across biotic and abiotic conditions (Means & Krysko, 2001; Krysko & Smith, 2005).

Hypotheses

We hypothesised that *L. nigra* colouration has been adapted for thermoregulation (thermoregulation hypothesis). Alternatively, we hypothesised that *L. nigra* colouration has been adapted to avoid detection from predators and prey (crypsis hypothesis). To test which of our hypotheses is more likely, we related spatial patterns in *L. nigra* colouration (percentage of light area on dorsal scales) within Mississippi to average temperature, average maximum temperature, percentage forest cover and soil value (darkness to lightness of soil based on organic matter content). If these data supported the thermoregulation hypothesis, we expect colour variation in snakes to be explained mostly by average temperature and average maximum temperature, with darker specimens in counties with lower average temperatures and average maximum temperatures. If data supported the crypsis hypothesis, we expect colour variation in snakes to be explained mostly by soil value, with darker specimens in counties with lower soil values (darker soils). We expect percentage forest cover to moderate the effects of average temperature and average maximum temperature, influencing the efficiency of basking (Harvey & Weatherhead, 2010) and ultimately, the thermoregulation hypothesis. Here, given similar temperatures, darker snakes would be found in counties with higher forest cover. However, we would also expect percentage forest cover to have an interactive effect in the crypsis hypothesis where snakes would become less monochromatic (i.e. more speckled) with increased forest cover across soil values, pattern matching dappled sunlight and the litter mosaic of the forest floor. Generically, the thermoregulation and crypsis hypotheses can be visualised in Figure 1.

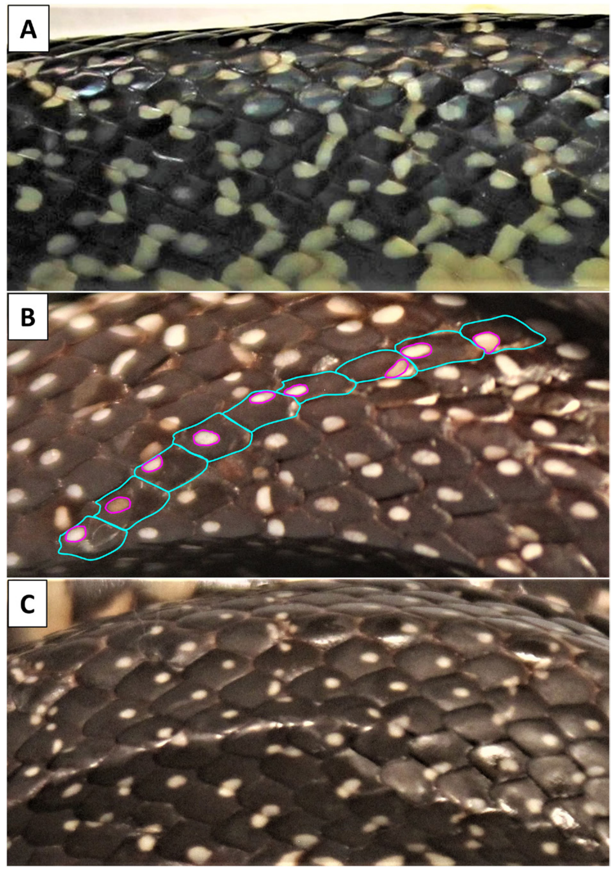

Figure 2. Example photos of an Eastern Black Kingsnake *Lampropeltis nigra* from which percentage area of colouration of nine dorsal scales was calculated (% light). Magenta lines encircle light pigmented areas while cyan lines encircle dark pigmented areas. Specimens are ordered from lightest (**(A)**; MMNS 9284) to darkest (**(B)** then **(C)**; MMNS 2392 and 9793) with % light values of 19.9%, 11.2% and 7.0%, for A, B, and C respectively. Specimens archived at the Mississippi Museum of Natural Science.

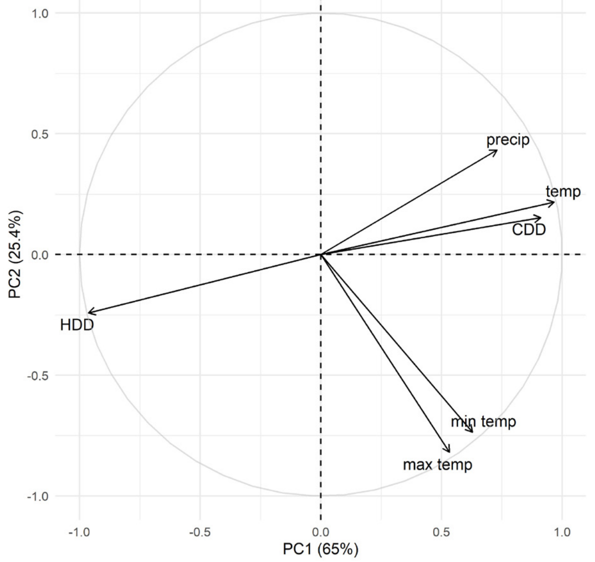

Figure 3. Variable loadings of six climate variables screened for inclusion in model building and comparisons. The x-axis represents the first principal component (PC1) which accounts for 65% of the variation among climate variables. The y-axis represents the second principal component (PC2) which accounts for an additional 25.4% of the variation among climate variables. Angle of arrows represents the association between climate variables and principal components (PC1 and PC2). The length of arrows represents loadings for climate variables which are also represented in the inset table. Data was retrieved from National Centers for Environmental Information county climate database (ncei.noaa.gov) for the 46 Mississippi counties where *Lampropeltis nigra* were collected. Note: temp = average temperature; max temp = average maximum temperature; min temp = average minimum temperature; CDD = cooling degree days; HDD = heating degree days; and precip = average precipitation.

MATERIALS & METHODS

We characterised *L. nigra* colouration as the percentage of area with light pigmentation in nine dorsal scales (% light) of specimens (n = 78) collected from 46 Mississippi counties. These nine dorsal scales were from a scale row along the dorsum of each specimen (Fig. 2B). While location of this row was approximately the mid-point between the snout and vent of each snake, variation among individuals may have occurred.

Snake specimens were part of archived collection housed at the Mississippi Museum of Natural Science (Jackson, Mississippi), a collection that spanned 70 years (1936–2006). We photographed scales mid-dorsum of each specimen using a single-lens reflex camera (Canon EOS 60 D) outfitted with a macro lens (Canon EFS 60 mm f/2.8) within a 61 × 61 × 91 cm (24 × 24 × 36 in) light shed (Impact Digital). Photos were taken at a distance of approximately 500 cm from each specimen, with the camera mounted on a tripod and positioned above the specimen. Photographs were imported into ImageJ v. 1.44 (Schneider et al., 2012). For each specimen we: 1) drew polygons around light and dark pigmented areas of scales (Fig. 2), 2) calculated the total area surveyed (light and dark pigmented areas), and 3) calculated the total area of light pigmentation and divided by the total area surveyed, multiplied by 100. We repeated this process for a subset of snakes considered as means to ensure consistency in measurement among specimens.

We conducted preliminary analysis to test if colouration of snakes differed with year of collection faded, as older specimens could have faded or become damaged. We used a logit linear model with a beta error distribution to test for an association between % light and year. The model indicated no relationship between % light and year (β = 0.00, 95% CIs = -0.01, 0.00) so we proceeded without including year as a covariate (Supplementary material Fig. 1).

Also, as a preliminary analysis, we screened climate variables shown to be associated with colouration and thermoregulation (Blouin-Demers & Weatherhead, 2001; Rozen-Rechels et al., 2019; Mack & Beaty, 2021).

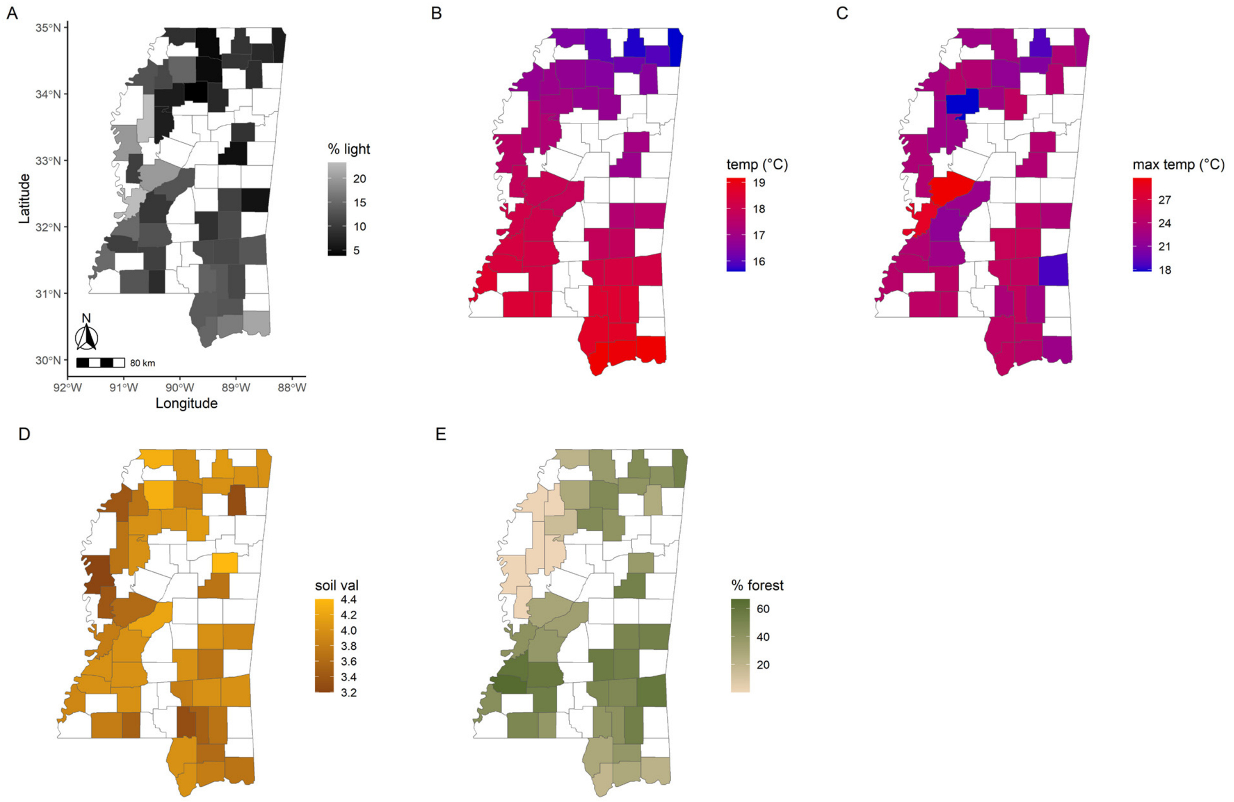

Figure 4. Distribution of *Lampropeltis nigra* percentage of light colouration on dorsal scales (% light; **A**), average temperature (temp [°C]; **B**), average maximum temperature (max temp [°C]; **C**), soil value (lightness to darkness; soil val; **D**), and percentage forest (% forest; **E**) in the 46 Mississippi counties where specimens were collected.

We retrieved average temperature, average minimum temperature, average maximum temperature, heating degree days, cooling degree days and precipitation for counties from which specimens were collected from the National Centers for Environmental Information county climate database (ncei.noaa.gov), which reports averages using data from 1901 to 2000. Using principal components analysis, we selected orthogonal variables with the highest principal components loadings (James et al., 2013). Average temperature (temp) had the highest loading (0.49) on the first principal component (Fig. 3; Supplementary material Table 1) and had high correlations with heating degree days (ρ = -0.99), cooling degree days (ρ = 0.95), and precipitation (ρ = 0.73; Supplementary material Fig. 2). Average maximum temperature (max temp) had the highest loading (-0.66) on the second principal component and had a high correlation with average minimum temperature (ρ = 0.90). Additionally, max temp was chosen over average minimum temperature because our study organism retreats to hibernaculum during winter months, avoiding selection pressures of cold temperatures (Wund et al., 2007). The first two principal components explained 90.4% of the variation in climate variables. We considered average temperature and average maximum temperature together in models representing thermoregulation.

We determined the average soil value (dark to light, lower to higher values respectively) for the three dominant soil classes of each county from which specimens had been collected (soil val). These data were extracted from the United States Department of Agriculture's Soil Survey (websoilsurvey.sc.egov.usda.gov). Soil colour has been shown to influence the distribution of some herpetofauna as a process of crypsis (Heinen, 1993; Isaac & Gregory, 2013). We applied similar logic in our expectation that *L. nigra* would distribute to areas of soil value most akin to their colouration.

The percentage of forested area in each county was derived from 2011 land use/land cover data retrieved from the Mississippi Automated Resource Information System (maris.mississippi.edu; % forest). We assumed that the percentage of forested area in counties has changed little between the time when specimens were collected and when data were collected for 2011 land use/land cover dataset. We acknowledge that some of our samples are before and after this assessment but also recognise that there has been limited change in land cover identified within much of Mississippi during the last 70 years (Karstensen & Sayler, 2009; English, 2011). Though there is a theoretical basis for including elevation and geology in studies of thermoregulation (radiative, convective and conductive heating) and crypsis (pattern matching), we did not consider them here as Mississippi has little variation in elevation (elevation range approx. 245 m) and most of its surface geology is unconsolidated sediments (Blouin-Demers & Weatherhead, 2001; Mappes et al., 2005; Dockery & Thompson, 2016). The spatial distribution of *L. nigra* % light, temp, max temp, soil val, and % forest are displayed in Figure 4.

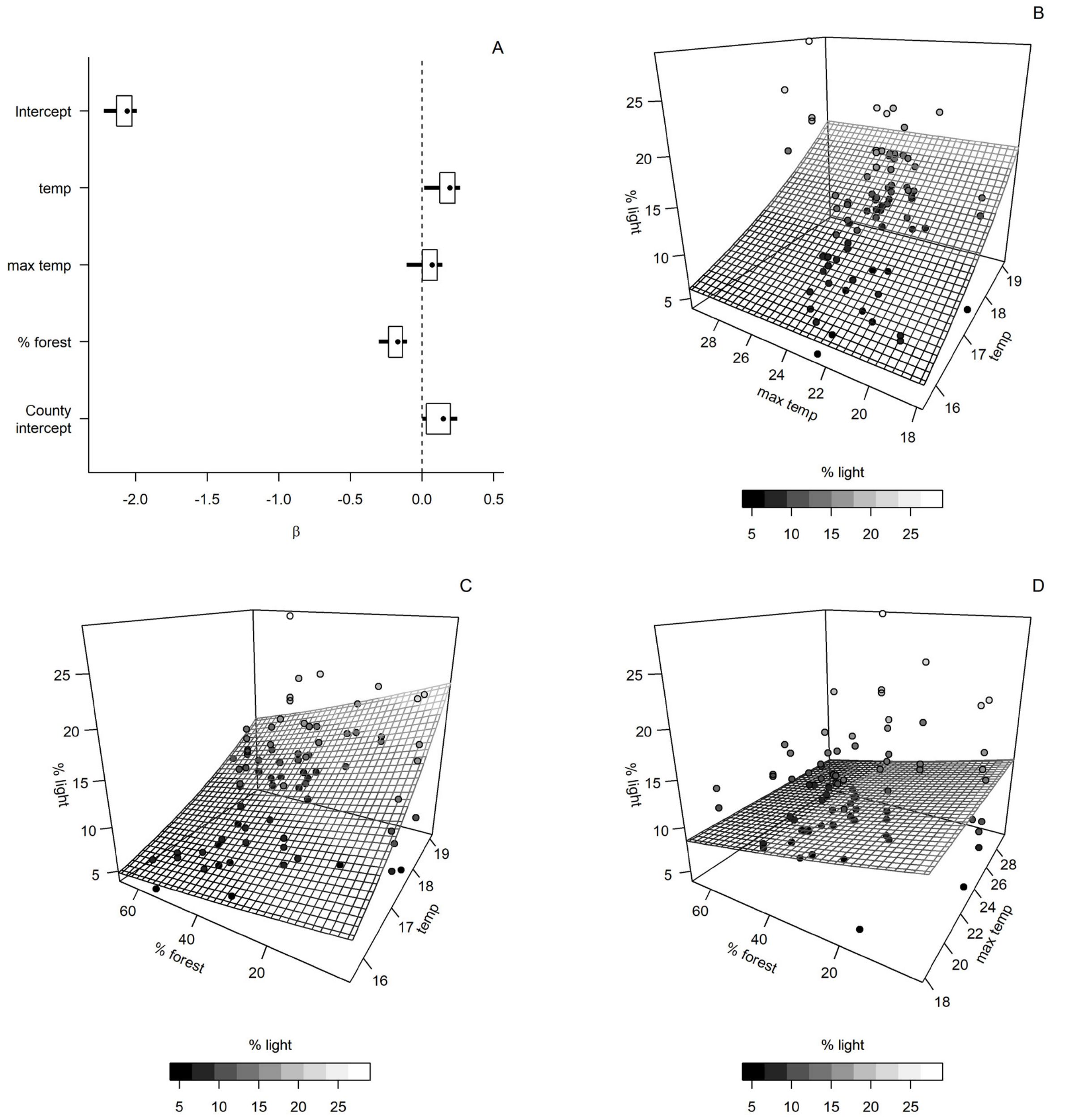

Figure 5. Relationship between proportion of light area on dorsal scales of *Lampropeltis nigra* (% light), average temperature (temp), average maximum temperature (max temp) and forest cover (% forest). Plot **A** displays coefficient estimates with central tendency (median) indicated by the filled circle, the 50% CIs represented by the box and the whiskers represent the 95% CIs. Plots **B**–**D** display model fit (grid plane)with observed data between combinations of explanatory variables. The grid plane and points are shaded in relation to their % light values.

We used logit linear mixed models with a beta distribution to relate *L. nigra* scale % light to temp, max temp, soil val, and % forest (fixed variables) with county as a categorical random variable to account for repeated sampling (multiple snakes) from within the same county (Supplementary material Table 2). For modeling, we transformed % light to its proportional form to meet assumptions of beta distribution (back-transformed for plotting). We developed six apriori models for comparison (Table 1). Model 1 included only the intercept as an ecological baseline to which hypothesised models could be compared. If model 1 received the most support, we considered our results to be inconclusive. Models 2 and 3 related % light to temp and max temp (model 2) and % light to temp and max temp and % forest (model 3). If models 2 or/and 3 received the most support, we considered colouration in *L. nigra* to be adapted for thermoregulation. Models 4 and 5 related % light to soil val (model 4) and % light to soil val, % forest, and the interaction of soil val and % forest (model 5). If models 4 or/and 5 received the most support, we considered colouration in *L. nigra* to be adapted for crypsis. Model 6 included % forest only for comparison. If model 6 received the most support, we considered our results inconclusive.

Fixed variables were scaled and centred before model fitting. We estimated model coefficients using the No-U-Turn Markov chain Monte Carlo algorithm

Table 1. Model number, model variables and pertinent hypothesis used to explain the percentage area of light colouration in dorsal scales (% light) of *Lampropeltis nigra*

Model No.	Variables*	Hypothesis
1	Intercept only	Inconclusive
2	temp + max temp	Thermoregulation
3	temp + max temp + % forest	Thermoregulation
4	soil val	Crypsis
5	soil val × % forest	Crypsis
6	% forest	Inconclusive

*All models included county as a random variable

with non-informative ("flat") priors in the brms R package (Bürkner, 2017; 2018). Posterior sampling was set to 2,000 warmup samples and 8,000 post-warmup samples thinned to 4,000. All models converged and we inspected model fit using posterior predictive checks - there was no indication of a violation of model assumptions (Supplementary material Figs. 3–8), so we continued with model comparisons. We used the leave-one-out cross validation information criterion (LOOIC) and corresponding model weights to compare models (Vehtari et al., 2017; Yao et al., 2018). The LOOIC is a Bayesian analogue to the more familiar Akaike information criterion (AIC) in that it provides an assessment of the quality of model fit relative to other candidate models, with the best competing model having the lowest LOOIC score (Arnold, 2010; Vehtari et al., 2017). Model weights based on LOOIC represent the relative likelihood of a given model base on the expected log predictive density (elpd; Yao et al., 2018). We also calculated total (fixed and random variables) and fixed Bayesian R^2 values to further characterise and compare models (Gelman et al., 2019). For the model with the lowest LOOIC and highest model weight, we plotted parameter estimates and their 50% and 95% credible intervals. All analyses and plots were produced in program R (R Core Team, 2021).

RESULTS

The % light of *L. nigra* dorsal scales averaged 12.24 (SD = 5.05), with a minimum value of 3.81 and a maximum value of 29.04 (Supplementary material Table 2). The temp from within counties in Mississippi where these snakes were collected was 17.71 ˚C (SD = 0.99 ˚C), with a minimum value of 15.56 ˚C and a maximum value of 19.17 ˚C. The max temp from within counties in Mississippi where these snakes were collected was 23.75 ˚C (SD = 2.16 ˚C), with a minimum value of 17.78 ˚C and a maximum value of 29.78 ˚C. The soil val measured for counties where snakes were collected averaged 3.82 (SD = 0.28), with a minimum value of 3.20 and a maximum value of 4.40. The % forest from within counties in Mississippi where these snakes were collected averaged 36.65 (SD = 17.81), with a minimum value of 0.09 and a maximum value of 66.84.

Table 2. Model number and fit metrics in relation to the percentage area of light colouration in dorsal scales (% light) of *Lampropeltis nigra*. Note: LOOIC = leave-one-out cross validation information criterion; elpd = expected log predictive density; Δ elpd = change in elpd in relation to best fit model; weight = weight of support for a model among candidate models; Bayesian R^2: fixed and total = relative fit of model to data for fixed variables and total (fixed and random variables).

Model	LOOIC	elpd	Δ elpd	weight	Bayesian R^2: fixed (total)
1	-278.0	139.0	-6.6	0.00	0.00 (0.55)
2	-286.7	143.3	-2.3	0.16	0.28 (0.53)
3	**-291.3**	**145.6**	**0.0**	**0.84**	**0.37 (0.55)**
4	-279.1	139.6	-6.1	0.00	0.08 (0.56)
5	-277.6	138.8	-6.9	0.00	0.14 (0.56)
6	-279.1	139.5	-6.1	0.00	0.07 (0.55)

The best competing model included temp and max temp and % forest (model 3) as fixed variables and county as a random variable (Table 2). There was a positive relationship between % light and temp (β = 0.23, 95% CIs = 0.13, 0.34) and % light and max temp (β = 0.11, 95% CIs = -0.00, 0.21), and a negative relationship between % light and % forest (β = -0.14, 95% CIs = -0.23, -0.04; Fig. 5). The total Bayesian R^2 value for the model was 0.55 while the fixed Bayesian R^2 value was 0.37. The second-best competing model included temp (β = 0.22, 95% CIs = 0.10, 0.33) and max temp (β = 0.10, 95% CIs = -0.01, 0.22), and had total and fixed Bayesian R^2 of 0.53 and 0.28 respectively.

DISCUSSION

Characteristics of ectotherms, like colouration, are highly influential on body temperature leading to selection of individuals which most efficiently thermoregulate in the local environment (i.e. the thermoregulation hypothesis; Huey & Kingsolver, 1989; Gross et al., 2004). Previous studies of snake colouration support these claims, with darker colouration (i.e. melanistic individuals) being more common in lower average temperature environments (Gibson & Falls, 1979; Andrén & Nilson, 1981; Seigel & Ford, 1987). Here we provide even greater support to this claim by showing a clear relationship between *L. nigra* colouration and temperature and how this relationship is modified by, or with respect to, forest cover. Conversely, we did not find support among our candidate models that colouration was related to soil value (i.e. the crypsis hypothesis).

The thermoregulation hypothesis posits that populations adapt to local thermal conditions but also predicts general large-scale patterns due to variation in thermal conditions. This has been observed repeatedly in other taxa including butterflies, flies, amphibians and other reptiles, most notably darker individuals are more common in higher latitudes (Clusella-Trullas et al., 2007; Alho et al., 2010). In our study, average temperature (temp) was strongly correlated with latitude (r = -0.96, 95% CIs = -0.94, -0.98), supporting prior observations. Particularly, that lighter coloured *L. nigra* are expected to be found at lower latitudes, in more southerly Mississippi.

Temperature patterns in Mississippi are expected to continue to change, as is the case in most parts of the world (Fill et al., 2019; Sharma et al., 2022). As climates change, species that have adapted to localised conditions through adaptive colouration related to thermoregulation may experience detriments to physiology, depending on their ability to adjust to short and long-term changes. If, as shown in the present study, *L. nigra* and other squamates exhibit colouration linked to thermal conditions, they may experience thermally induced behavioural modifications, as squamates cannot readily adapt to rapidly changing ambient conditions (Bennett, 1990). If the ambient temperatures experienced are outside that of a species' thermal optima then behavioural adaptations may result wherein some individuals with specific colouration experience thermal advantages (Tanaka, 2007). Some of these advantages may be more pronounced during various times when thermal benefits or constraints are more pronounced (Tanaka, 2007). These changes could also induce proximate and ultimate pressures on these species affecting their distribution and other population metrics as well as the elements of the communities they inhabit (Shine & Harlow, 1993; DeGregorio et al., 2015; Tully et al., 2020).

It is expected that changes in land cover have occurred within our study area during the period in which specimens used in our analysis were collected. However, during the time frame of this study it is unlikely that these changes in land cover occurred uniformly over large geographic areas, allowing process of drift and dispersal to occur whereas organisms adapted to local conditions could still access similar conditions through dispersal. However, the coupling of climate change and anthropogenic land use may push species such as *L. nigra* into situations where drivers that affect these ectotherms are supplanted by other factors that affect the adaptability of these organisms over much shorter time frames. The capacity for species like *L. nigra* to adapt to changing environmental conditions will also depend on future land use and community changes, including those conditioned on historical land use.

Understanding how climate can influence the distribution of species is key to understanding current and expected ecosystem change (Montoya & Raffaelli, 2010). Soil is a major storage reservoir for heat within an ecosystem, absorbing energy during the day and releasing heat to the surface at night (Onwuka & Mang, 2018). Energy discharged by soil can vary daily as well as seasonally and changes in soil temperature can affect plant and invertebrate diversity within systems where warming occurs (Robinson et al., 2018). How organisms adapt to the landscape or spatial synchrony and cross-correlations between organisms and the environment is growing in interest as climate change has promulgated in a variety of phenological and other mismatches, typically at large spatial scales (Koenig, 1999). Conversely, neutral drift and dispersal limitations among organisms typically occur at intermediate to fine scales (Hubbell, 2001).

Further research on thermoregulation via colouration in *L. nigra* could help the conservation community better understand if physiology and behaviour of *L. nigra* have coadapted with colouration (Clusella-Trullas at al., 2007). Such studies could include measures of temperatures internal to each snake, relative to ambient conditions. Additional application of multigenomics can also help shed light on the processes of colouration in this and other ectotherms as well as their genetic adaptability. Knowledge gained through these studies can better inform our understanding of colouration in ectotherms and perhaps provide insight to the conservation of species through anticipated climate change, and help biologists brainstorm creative solutions in protecting this and similar species.

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DATA ACCESSIBILITY

Data and R scripts used in the analysis of this study are available in the online supplemental material at the journal website.

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Endemic amphibians of the Cerrado and Caatinga: species richness, geographic range and conservation

Gisele do Lago Santana[1], Davi Lima Pantoja[2], Pedro Peloso[1,3], Geovania Figueiredo da Silva[4], Maiume Silva da Silva[1] & Gleomar Fabiano Maschio[1*]

[1]Programa de Pós-graduação em Zoologia, Instituto de Ciências Biológicas, Universidade Federal do Pará, Belém, Pará, Brazil
[2]Departamento de Biologia - CCN, Universidade Federal do Piauí, Teresina, Piauí, Brazil
[3]California State University, Cal Poly Humboldt, Department of Biological Sciences, Arcata, CA, USA
[4]Colégio Técnico de Floriano, Universidade Federal do Piauí, Floriano, Piauí, Brazil

The Cerrado and the Caatinga have few formally protected areas and encompass a high diversity of amphibians, which is still relatively unknown. In this study we present the list of amphibian species endemic to the Cerrado or Caatinga and their geographic range, highlighting current conservation status. We obtained the species list from the compilation of scientific publications (up to July 2022). We obtained the occurrence records from the databases SpeciesLink, GBIF, Portal da Biodiversidade and literature. We classified the species in the categories of threat according to the Brazilian list of threatened species and the global list of threatened species. We compiled 2,659 occurrences of amphibians, 1,335 from the SpeciesLink platform, 414 from GBIF, 371 from the Biodiversity Portal and 539 obtained from the literature. We recorded 100 endemic species, 82 from the Cerrado and 18 from the Caatinga. The Cerrado has three species Vulnerable (VU) (*Boana buriti*, *Bokermannohyla napolii* and *Scinax pinimus*), one Critically Endangered (CR) (*Proceratophrys moratoi*), 14 species Data Deficient (DD), 46 in the Least Concern (LC) category and 18 Not Evaluated (NE). The Caatinga has three species CR (*Adelophryne maranguapensis*, *Proceratophrys ararype*, *Rhinella casconi*), three DD, five LC and seven NE. Threatened, DD and NE species have few records within the protected areas of Cerrado and Caatinga. We consider that the Cerrado and Caatinga present a rich diversity of endemic amphibians, which have a geographic range relatively reduced, especially the threatened ones, DD and NE. Distribution and taxonomy data are essential, because the lack can hinder the assessment of conservation status, since threatened species, DD, NE, including LC, may be undervalued and at risk.

Keywords: amphibia, distribution, endemism, species list, species protection

INTRODUCTION

Amphibians are among the most diverse vertebrate groups, with 8,483 known species (Amphibiaweb, 2022). About 1,188 occur in Brazil, making it the richest country in amphibian species (Segalla et al., 2021). However, amphibians are globally threatened with extinction. The Neotropical region concentrates the largest number of threatened amphibians (Amphibiaweb, 2022), especially because of the destruction of native habitats, pollution, introduction of exotic species (Duellman & Trueb, 1994), climate change (Ficetola & Maiorano, 2016) and diseases (Fisher & Garner, 2020).

The South American dry diagonal of structurally open vegetation includes the Cerrado, Chaco and Seasonally Dry Tropical Forests. These biomes experience strong seasonal droughts, contain a significant number of endemic species and high taxon diversity, but all are highly threatened (Werneck, 2011; Fonseca et al., 2017; Medeiros et al., 2022). The Cerrado is the largest and most threatened tropical savanna (Myers et al., 2000) and has a great diversity of amphibians with more than 209 species (Valdujo et al., 2012). Such diversity is under intense threat, mainly from agricultural activities, in addition to the intense use of toxic agrochemicals, the construction of hydroelectric dams, and from frequent fires (Ribeiro et al., 2020). The intense degradation of the Cerrado places this biome in second place in the Brazilian deforestation ranking, with around 409,000 hectares lost by 2018 (MapBiomas, 2019).

The Caatinga, considered the largest extension of the Seasonally Dry Tropical Forests in South America (Silva et al., 2017), is the fourth most deforested biome in Brazil, with about 12,200 hectares of extension lost by 2018 (MapBiomas, 2019). Moreover, Caatinga has suffered from a gradual increase in desertification, caused by human activities with the additional influence of the dry climate (Souza & Oyama, 2011). The species richness of amphibians in the Caatinga is considered high, with 98 species recorded, however this biome is still poorly studied (Garda et al., 2017).

Correspondence: Gleomar Fabiano Maschio (gleomarmaschio@gmail.com)

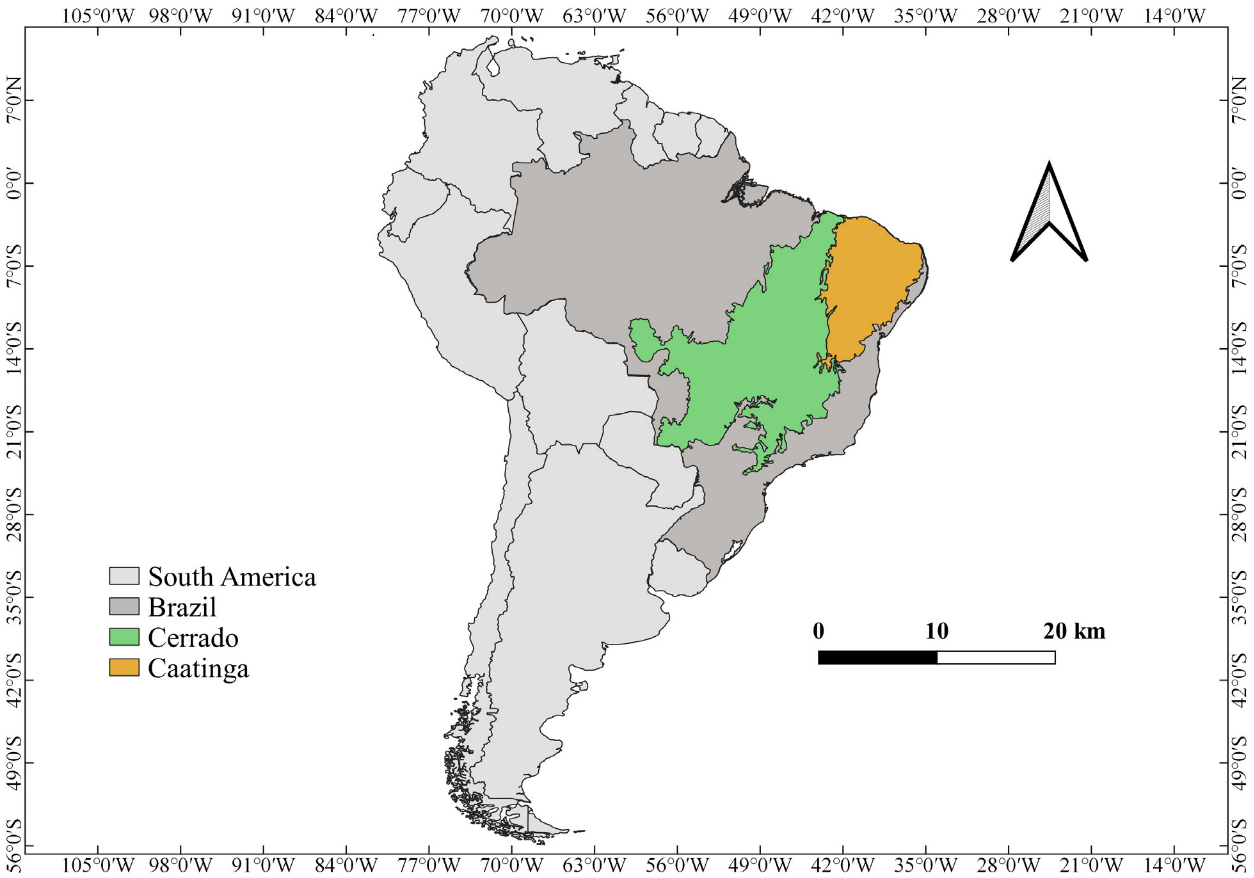

Figure 1. Location of the Cerrado and Caatinga biomes in South America. Shapefile provided by the Brazilian Institute of Geography and Statistics (IBGE, 2020).

Endemic species of each biome and those with restricted geographic distribution are among the most affected by habitat loss (Silvano et al., 2016). Habitat loss can reduce the size of the geographic range of endemic species (Mayani-Parás et al., 2019) since the low dispersal capacity and physiological restrictions (Smith & Green, 2005) may present limitations to locomotion between habitats. In this sense, endemism and the species geographic range serve as essential predictors for assessing the risks of extinction of the species (Purvis et al., 2000).

Faced with the risk of species extinctions in open vegetation biomes in South America, the allocation of areas for environmental protection becomes even more important for the conservation of biodiversity (Rodrigues et al., 2004). This is most evident when it was discovered that about 24% of amphibian species worldwide are not located within protected areas (Nori et al., 2015). In Brazil, both the Cerrado and the Caatinga have few protected areas, corresponding to 8.6% and 7.7% of their total area, respectively (Vieira et al., 2019). By 2020, the National Biodiversity Commission established that at least 17% of the total area of every biome in the world should be protected (CONABIO, 2013).

Knowledge of the geographic range of each species individually allows us to understand the organisation of these species on a regional scale (Valdujo et al., 2012) and support conservation proposals (Keil & Hawkins, 2009). Thus, here we have gathered and discussed the updated list of amphibian species endemic to the Cerrado or Caatinga, their geographic range, and their current conservation. We were guided by the following questions considering the endemism of amphibian species in the Cerrado or Caatinga biomes: 1) what is the available knowledge regarding the geographic range and conservation of the species; 2) what is the current number of known species and what are the advances and contrasts since the last compilations; 3) do endemic threatened species occur within protected areas; 4) do digital databases provide substantial information regarding the occurrence of endemic species?

MATERIALS & METHODS

Study area

The species listed in this study are endemic to the Brazilian biomes called Cerrado and Caatinga, defined and delimited by the Brazilian Institute of Geography and Statistics (Instituto Brasileiro de Geografia e Estatística - IBGE) (IBGE, 2020) (Fig. 1).

The Cerrado is considered the largest savanna area and the second-largest biome in South America, occupying an area of 1,983,017 km^2 (IBGE, 2019). The

Cerrado has boundaries with the biomes in South America Caatinga, Amazon Forest, Atlantic Forest, Pantanal and the Pampa. The prevailing climate in the Cerrado is of the Aw type, according to the Köppen classification, which is markedly seasonal, with dry winters and annual rainfall ranging from 1,300 to 2,300 mm (Alvares et al., 2013).

The Cerrado has a great diversity of endemic species, most listed as Threatened, one of the reasons why it is considered a hotspot of global biodiversity (Myers et al., 2000). This biome has been affected by intense habitat degradation caused mainly by suppressing native vegetation for use in agricultural expansion (MapBiomas, 2022; Zalles et al., 2019). Of the total area of the biome, about 44% is destined for farming activities, in which pasture is the activity that occupies most of the biome, followed by monocultures of soybean, sugarcane and rice (MapBiomas, 2022). Due to its high flammability, the Cerrado also suffers from frequent fires (Oliveira et al., 2021).

The Caatinga is an exclusively Brazilian biome, covering about 913,000 km², and is primarily located in Brazil's north-east region (Silva et al., 2017). The Caatinga has extensive flat surfaces with altitudes ranging from 300 to 500 m, relatively high temperatures ranging from 26 to 30 °C, and annual rainfall ranging from 300 to 1,200 mm (Prates & Navas, 2009; Silva et al., 2017). The vegetation is dominated by small, thorny trees with twisted trunks and by many succulent and cactus plants, which respond efficiently to the minimum levels of precipitation in this biome (Queiroz et al., 2017).

The Caatinga is recognised as one of the world's richest dry forests, with 3,150 species of vascular plants, 276 species of ants, 386 fish, 98 amphibians, 191 reptiles, 548 birds and 183 mammals (Silva et al., 2017). The Caatinga is one of the least scientifically explored Brazilian biomes, meaning thousands of new species are yet to be described (Tabarelli et al., 2018). About 35% of the Caatinga is already destined for agricultural activities, including pasture, sugarcane and soybean plantations (MapBiomas, 2022). In recent decades, the Caatinga suffered an intense process of desertification, which can have serious consequences, for example, in the hydrological cycle in the semi-arid region of north-eastern Brazil (Souza & Oyama, 2011).

Data collection and analysis

We produced a list of amphibian species endemic to the Cerrado and Caatinga biomes from the compilation of works by Valdujo et al. (2012) and Azevedo et al. (2016) for the Cerrado, and Garda et al. (2017) for the Caatinga. We also compiled records from descriptions of species published later, up to July 2022. We consider as endemic the species that occurs exclusively within the limits of the Cerrado or Caatinga biomes, including adjacent transition areas. We followed the taxonomic updates adopted by Segalla et al. (2021).

To compile the new species descriptions, considering the period from 2013 to 2022 for the Cerrado, and from 2018 to 2022 for the Caatinga, we consulted national and international scientific articles, available in the Google Scholar and Scopus databases. Internet searches were guided by the keywords 'amphibians', 'endemic amphibians', 'Cerrado and Caatinga' and by the specific name of the species.

We compiled species occurrence records by searching the SpeciesLink (SpeciesLink, 2022), Global Biodiversity Information Facility (GBIF) (GBIF, 2022), and Biodiversity Portal platforms (SISBIO, 2022). Additionally, we searched for publications with occurrence records of endemic species. For this, we consulted scientific articles, distribution notes, masters' dissertations and doctoral theses. The search included national and international documents on the geographic distribution of endemic species, available in Google Scholar and Scopus databases, and scientific journals such as Herpetological Review, Biota Neotropica, Check List, Herpetology Notes, Zootaxa and South American Journal of Herpetology.

We checked the current geographic range of the species compiled through the Biodiversity Portal of the Chico Mendes Institute for Biodiversity Conservation (Portuguese: Instituto Chico Mendes de Conservação da Biodiversidade, ICMBio) (SISBIO, 2022) (Disponívelem: https://biodiversidade.icmbio.gov.br/portal/), the International Union for Conservation of Nature (IUCN) (IUCN, 2022) and Frost (2022).

To avoid taxonomic uncertainties regarding the identification of species in the different databases and thus have reliable records, we considered those that presented the name of the collector or identifier and the available collection in which the specimen was deposited. We considered uncertain those records that did not have such information. Furthermore, we did not consider records of species with uncertain identifications, such as those listed as aff., cf., gr. or sp. We consulted expert Adrian Garda to clarify uncertainties about the taxonomy of Caatinga species.

We classified the species in the categories Least Concern (LC), Data Deficient (DD), Near Threatened (NT), Vulnerable (VU), Endangered (EN), Critically Endangered (CR) and Not Evaluated (NE) according to the Brazilian list of threatened species (MMA, 2022), the Red Book of Brazilian Fauna Threatened with Extinction (2018), and to complement, the global list of threatened species (IUCN, 2022). In order to verify the occurrence of endemic species within the Brazilian, municipal, state and federal protected areas (PAs), and of Integral Protection and Sustainable Use of the Cerrado and Caatinga, we overlapped the occurrence records of the species with the referred PAs. We extracted the file with the PAs in shapefile format, made available by the Ministry of the Environment (MMA, 2020). We used the QGIS 3.6.2 software to overlay the occurrence records with the PAs and verify if the species are within these protected areas (QGIS, 2020). We considered only the records identified within the limits of the PAs. We grouped species by conservation status to ascertain the distribution of occurrence records through a histogram, removing, in this case, duplicate records.

Table 1. List of amphibian species endemic to the Cerrado and Caatinga biomes: CE=Cerrado; CA=Caatinga; CS=Conservation status; N=Number of records with no duplicates; REF=Source; LC=Least Concern; DD=Data Deficient; CR=Critically Endangered; EN=Endangered; VU=Vulnerable; NE= Not Evaluated; 1=Garda et al. (2017); 2=Azevedo et al. (2016); 3=Valdujo et al. (2012); * Species compiled after the studies by Garda et al. (2017), Azevedo et al. (2016) and Valdujo et al. (2012), and added in this study.

AMPHIBIA	**CA**	**CE**	**CS**	**N**	**REF**
ANURA					
Aromobatidae					
Allobates goianus (Bokermann, 1975)		■	DD	9	3
Brachycephalidae					
Ischnocnema penaxavantinho Giaretta, Toffoli & Oliveira, 2007		■	DD	11	3
Bufonidae					
Rhinella casconi Roberto, Brito & Thomé, 2014	■		CR	1	1
Rhinella cerradensis Maciel, Brandão, Campos & Sebben, 2007		■	LC	13	3
Rhinella inopina Vaz-Silva, Valdujo & Pombal, 2012		■	LC	8	3
Rhinella veredas (Brandão, Maciel & Sebben, 2007)		■	LC	10	3
Ceratophryidae					
Ceratophrys joazeirensis Mercadal de Barrio, 1986	■		LC	6	1
Craugastoridae					
Oreobates antrum Vaz-Silva, Maciel, Andrade & Amaro, 2018		■	NE	1	*
Oreobates remotus Teixeira, Amaro, Recoder, Sena & Rodrigues, 2012		■	LC	3	3
Pristimantis dundeei (Heyer & Muñoz, 1999)		■	LC	18	3
Pristimantis relictus Roberto, Loebmann, Lyra, Haddad & Ávila, 2022	■		NE	12	*
Pristimantis rupicola Taucce, Nascimento, Trevisan, Leite, Santana, Haddad & Napoli, 2020	■		NE	13	*
Pristimantis ventrigranulosus Maciel, Vaz-Silva, Oliveira & Padial, 2012		■	LC	2	3
Dendrobatidae					
Ameerega berohoka Vaz-Silva & Maciel, 2011		■	LC	3	3
Ameerega braccata (Steindachner, 1864)		■	LC	1	3
Eleutherodactylidae					
Adelophryne baturitensis Hoogmoed, Borges & Cascon, 1994	■		LC	1	1
Adelophryne maranguapensis Hoogmoed, Borges & Cascon, 1994	■		CR	2	1
Hylidae					
Aplastodiscus heterophonicus Pinheiro, Pezzuti, Berneck, Lyra, Lima & Leite, 2021		■	NE	5	*
Aplastodiscus lutzorum Berneck, Giaretta, Brandão, Cruz & Haddad, 2017		■	NE	4	*
Boana botumirim (Caramaschi, Cruz & Nascimento, 2009)		■	LC	14	3
Boana buriti (Caramaschi & Cruz, 1999)		■	VU	2	3
Boana caiapo Pinheiro, Cintra, Valdujo, Silva, Martins, Silva & Garcia, 2018		■	NE	18	*
Boana cipoensis (B. Lutz, 1968)		■	LC	22	3
Boana ericae (Caramaschi & Cruz, 2000)		■	LC	3	3
Boana goiana (B. Lutz, 1968)		■	LC	21	3
Boana jaguariaivensis (Caramaschi, Cruz & Segalla, 2010)		■	LC	6	3
Boana paranaiba (Carvalho, Giaretta & Facure, 2010)		■	LC	18	*
Bokermannohyla alvarengai (Bokermann, 1956)		■	LC	3	3
Bokermannohyla diamantina Napoli & Juncá, 2006	■		DD	1	1
Bokermannohyla flavopicta Leite, Pezzuti & Garcia, 2012	■		NE	2	1
Bokermannohyla juiju Faivovich, Lugli, Lourenço & Haddad, 2009	■		DD	1	1
Bokermannohyla nanuzae (Bokermann & Sazima, 1973)		■	LC	26	3
Bokermannohyla napolii Carvalho, Giaretta & Magrini, 2012		■	VU	2	2
Bokermannohyla pseudopseudis (Miranda-Ribeiro, 1937)		■	LC	7	3
Bokermannohyla ravida (Caramaschi, Napoli & Bernardes, 2001)		■	DD	3	3
Bokermannohyla sapiranga Brandão, Magalhães, Garda, Campos, Sebben & Maciel, 2012		■	NE	16	3
Bokermannohyla saxicola (Bokermann, 1964)		■	LC	5	3
Bokermannohyla sazimai (Cardoso & Andrade, 1982)		■	LC	10	3
Corythomantis botoque Marques, Haddad & Garda, 2021		■	NE	8	*
Dendropsophus araguaya (Napoli & Caramaschi, 1998)		■	LC	5	3
Dendropsophus cerradensis (Napoli & Caramaschi, 1998)		■	DD	4	3
Pseudis tocantins Caramaschi & Cruz, 1998		■	LC	13	3
Scinax cabralensis Drummond, Baêta & Pires, 2007		■	DD	7	3

AMPHIBIA	CA	CE	CS	N	REF
Scinax canastrensis (Cardoso & Haddad, 1982)		■	LC	27	3
Scinax centralis Pombal & Bastos, 1996		■	LC	11	3
Scinax curicica Pugliesse, Pombal & Sazima, 2004		■	LC	56	3
Scinax goya (Andrade, Santos, Rocha, Pombal & Vaz-Silva, 2018)		■	NE	1	*
Scinax machadoi (Bokermann & Sazima, 1973)		■	LC	39	3
Scinax maracaya (Cardoso & Sazima, 1980)		■	LC	13	3
Scinax montivagus Juncá, Napoli, Nunes, Mercês & Abreu, 2015	■		NE	4	1
Scinax pinimus (Bokermann & Sazima, 1973)		■	VU	2	3
Scinax pombali Lourenço, Carvalho, Baêta, Pezzuti & Leite, 2013		■	NE	1	2
Scinax rogerioi Pugliesi, Baêta & Pombal, 2009		■	LC	4	3
Scinax rupestris Araujo-Vieira, Brandão & Faria, 2015		■	NE	1	*
Scinax skaios Pombal, Carvalho, Canelas & Bastos, 2010		■	LC	2	3
Scinax tigrinus Nunes, Carvalho & Pereira, 2010		■	LC	4	3
Trachycephalus mambaiensis Cintra, Silva, Silva, Garcia & Zaher, 2009		■	DD	9	3
Hylodidae					
Crossodactylus trachystomus (Reinhardt & Lütken, 1862)		■	LC	6	3
Hylodes otavioi Sazima & Bokermann, 1983		■	DD	5	3
Leptodactylidae					
Adenomera saci Carvalho & Giaretta, 2013		■	LC	8	2
Leptodactylus avivoca Carvalho, Seger, Magalhães, Lourenço & Haddad, 2021		■	NE	5	*
Leptodactylus camaquara Sazima & Bokermann, 1978		■	LC	31	3
Leptodactylus kilombo Alves da Silva, Magalhães, Thomassen, Leite, Garda, Brandão, Haddad, Giaretta & Carvalho, 2020		■	NE	4	*
Leptodactylus oreomantis Carvalho, Leite & Pezzuti, 2013	■		LC	9	1
Leptodactylus payaya Magalhães, Lyra, Carvalho, Baldo, Brusquetti, Burella, Colli, Gehara, Giaretta, Haddad, Langone, López, Napoli, Santana, de Sá & Garda 2020	■		NE	18	*
Leptodactylus sertanejo Giaretta & Costa, 2007		■	LC	23	3
Leptodactylus tapiti Sazima & Bokermann, 1978		■	DD	11	3
Physalaemus claptoni Leal, Leite, Costa, Nascimento, Lourenço & Garcia, 2020		■	NE	1	*
Physalaemus deimaticus Sazima & Caramaschi, 1988		■	DD	9	3
Physalaemus evangelistai Bokermann, 1967		■	LC	18	3
Pseudopaludicola coracoralinae Andrade, Haga, Lyra, Carvalho, Haddad, Giaretta & Toledo, 2020		■	NE	2	*
Pseudopaludicola jazmynmcdonaldae Andrade, Silva, Koroiva, Fadel & Santana, 2019		■	NE	2	*
Pseudopaludicola matuta Andrade, Haga, Lyra, Carvalho, Haddad, Giaretta & Toledo, 2018		■	NE	3	*
Pseudopaludicola mineira Lobo, 1994		■	LC	22	3
Pseudopaludicola murundu Toledo, Siqueira, Duarte, Veiga-Menoncello, Recco-Pimentel & Haddad, 2010		■	DD	31	3
Microhylidae					
Chiasmocleis centralis Bokermann, 1952		■	DD	5	3
Odontophrynidae					
Odontophrynus monachus Caramaschi & Napoli, 2012		■	LC	2	3
Proceratophrys ararype Mângia, Koroiva, Nunes, Roberto, Ávila, Sant'Anna, Santana & Garda, 2018	■		CR	4	*
Proceratophrys bagnoi Brandão, Caramaschi, Vaz-Silva & Campos, 2013		■	DD	2	2
Proceratophrys branti Brandão, Caramaschi, Vaz-Silva & Campos, 2013		■	LC	17	2
Proceratophrys carranca Godinho, Moura, Lacerda & Feio, 2013		■	DD	2	2
Proceratophrys cururu Eterovick & Sazima, 1998		■	LC	9	3
Proceratophrys dibernardoi Brandão, Caramaschi, Vaz-Silva & Campos, 2013		■	LC	9	2
Proceratophrys goyana (Miranda-Ribeiro, 1937)		■	LC	34	3
Proceratophrys huntingtoni Ávila, Pansonato & Strüssmann, 2012		■	NE	6	2
Proceratophrys minuta Napoli, Cruz, Abreu & Del-Grande, 2011	■		LC	3	1
Proceratophrys moratoi (Jim & Caramaschi 1980)		■	CR	19	3
Proceratophrys redacta Teixeira, Amaro, Recoder, Vechio & Rodrigues, 2012	■		LC	2	1
Proceratophrys rotundipalpebra Martins & Giaretta, 2013		■	LC	4	2
Proceratophrys salvatori (Caramaschi, 1996)		■	LC	16	3
Proceratophrys velhochico Mângia, Magalhães, Leite, Cavalheri & Garda, 2022	■		NE	2	*
Proceratophrys vielliardi Martins &Giaretta, 2011		■	LC	4	3

AMPHIBIA	CA	CE	CS	N	REF
Phyllomedusidae					
Phasmahyla jandaia (Bokermann & Sazima, 1978)		■	LC	4	3
Pithecopus araguaius Haga, Andrade, Bruschi, Recco-Pimentel & Giaretta, 2017		■	NE	5	*
Pithecopus centralis (Bokermann, 1965)		■	DD	2	3
Pithecopus gonzagai Andrade, Haga, Ferreira, Recco-Pimentel, Toledo & Bruschi, 2020	■		NE	8	*
Pithecopus megacephalus (Miranda-Ribeiro, 1926)		■	LC	4	3
Pithecopus oreades (Brandão, 2002)		■	LC	8	3
GYMNOPHIONA					
Typhlonectidae					
Chthonerpeton arii Cascon & Lima-Verde, 1994	■		DD	3	1
Chthonerpeton tremembe Maciel, Leite, Silva-Leite, Leite & Cascon, 2015		■	NE	1	*

RESULTS

We recorded 100 species of endemic amphibians: 82 from the Cerrado (81 frogs and one caecilian) and 18 from the Caatinga (17 frogs and one caecilian) (Table 1). For the Cerrado, the most represented families were Hylidae (36 species), Leptodactylidae (14) and Odontophrynidae (12). The least representative families were Aromobatidae, Brachycephalidae, Microhylidae and Typhlonectidae, with only one species each. For the Caatinga, the most representative families were Hylidae and Odontophrynidae with four species, and Craugastoridae, Eleutherodactylidae and Leptodactylidae with two species each, while the least represented were Bufonidae, Ceratophryidae, Phyllomedusidae and Typhlonectidae with one species each.

Thirty-seven species in Cerrado and two in Caatinga are no longer considered endemic, because their range is now known to occur elsewhere (Table S1). We consider *Pristimantis* sp., cited as endemic by Garda et al. (2017), because its taxonomy has recently been clarified, now known as *Pristimantis rupicola* (Taucce et al., 2020; consultation with specialist Adrian Garda). In addition, we compile 16 new species endemics to the list for the Cerrado, compiled considering the period from 2013 to 2022 (Anura: *Aplastodiscus heterophonicus*, *Aplastodiscus lutzorum*, *Boana caiapo*, *Boana paranaiba*, *Corythomantis botoque*, *Leptodactylus avivoca*, *Leptodactylus kilombo*, *Oreobates antrum*, *Physalaemus claptoni*, *Pithecopus araguaius*, *Pseudopaludicola coracoralinae*, *Pseudopaludicola jazmynmcdonaldae*, *Pseudopaludicola matuta*, *Scinax goya*, *Scinax rupestris*; Gymnophiona: *Chthonerpeton tremembe*) and six species for the Caatinga, compiled considering the period from 2018 to 2022 (Anura: *Leptodactylus payaya*, *Pithecopus gonzagai*, *Pristimantis relictus*, *Pristimantis rupicola*, *Proceratophrys ararype*, *Proceratophrys velhochico*) (Table 1). We clarify that the species added in this study were already known by science but can be considered new if compared with the records obtained in Garda et al. (2017) for the Caatinga, and Valdujo et al. (2012) and Azevedo et al. (2016) for the Cerrado.

We gathered 2,659 occurrences of amphibians, of which 2,544 were from the Cerrado and 115 from the Caatinga (1,335 from the SpeciesLink, 414 from GBIF, 371 from the Biodiversity Portal and 539 records from the literature).

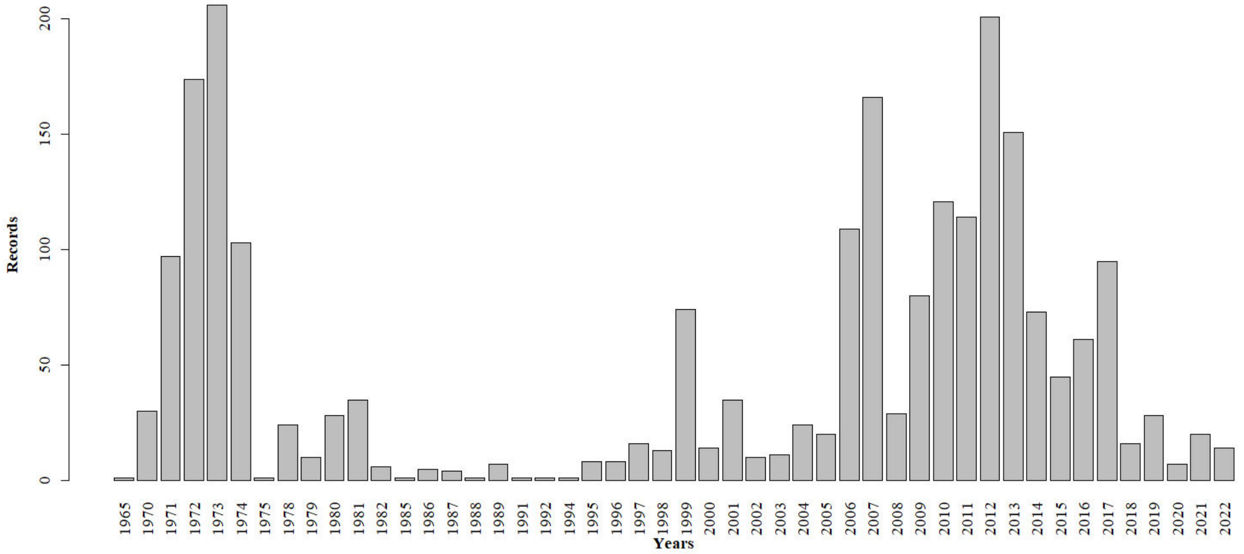

Figure 2. Number of occurrence records obtained on platforms and in the literature with the corresponding years

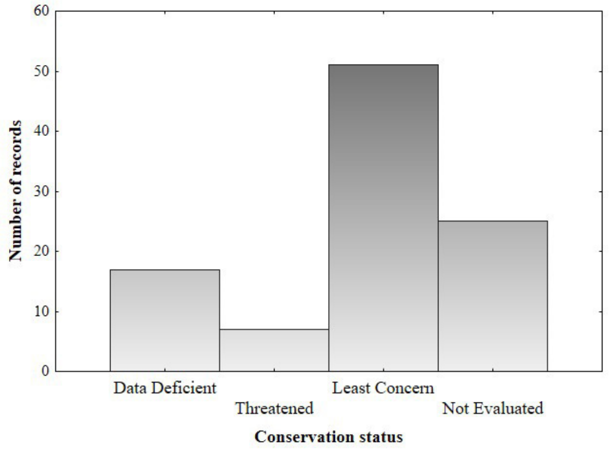

Figure 3. Distribution of the number of occurrence records for the threatened species, LC, DD and NE of Cerrado and Caatinga

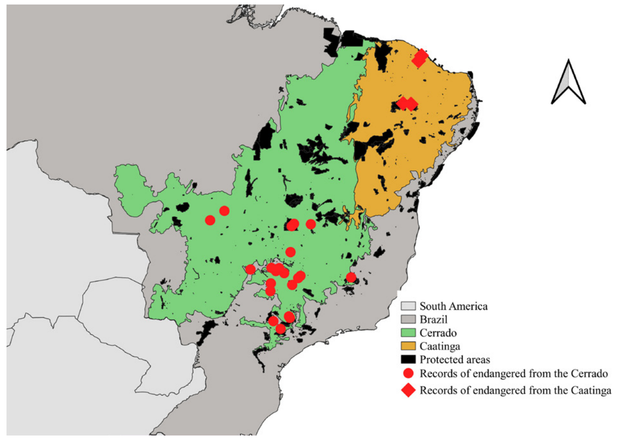

Figure 4. Overlap of the Protected Areas (PAs) with the occurrences of endangered species from the Cerrado and Caatinga

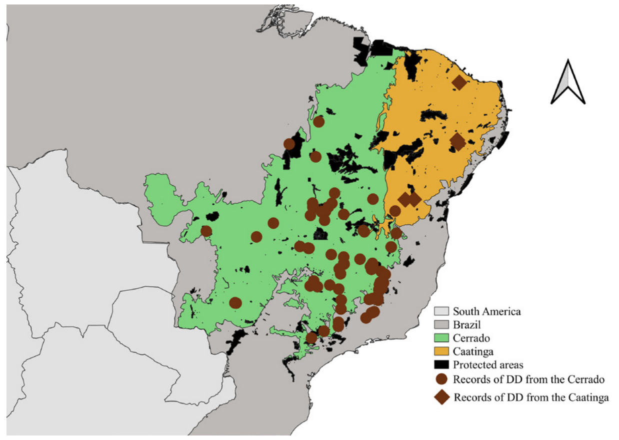

Figure 5. Overlap of the Protected Areas (PAs) with the occurrences of the DD species of the Cerrado and Caatinga

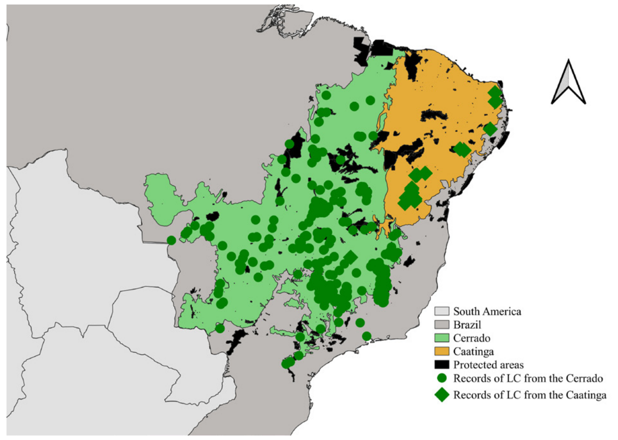

Figure 6. Overlap of the Protected Areas (PAs) with the occurrences of the LC species from Cerrado and Caatinga

However, when removing the duplicate occurrences, we obtained a total of only 897 unique occurrences: 805 for Cerrado and 92 for Caatinga (Table 1).

Considering the accuracy of the occurrence records obtained from the databases, GBIF presented 415 secure records with the presence of the name of the collector/identifier and the available collection and 937 uncertain records, due to the absence of this information; SpeciesLink presented 1,335 secure records, all with the collector/identifier name and collection, and 3,012 uncertain records; and the Biodiversity Portal had 371 secure records and 668 uncertain ones. The species with the highest number of uncertain data were *Bokermannohyla saxicola* (75), *Bokermannohyla alvarengai* (46), *Proceratophrys goyana* (38), *Proceratophrys cururu* (34), *Pseudis tocantins* (33), *Pithecopus megacephalus* (30), *Crossodactylus trachystomus* (26), *Pithecopus gonzagai* (24), *Ameerega braccata* and *Adelophryne baturitensis* (17), *Phasmahyla jandaia* (15), *Ceratophrys joazeirensis* and *Bokermannohyla pseudopseudis* (14), *Bokermannohyla sazimai* (13), *Rhinella veredas*, *Leptodactylus camaquara*, *Pithecopus centralis*, *Pithecopus oreades* and *Adelophryne maranguapensis* (9), *Ameerega berohoka* (8), *Bokermannohyla nanuzae* (7), *Leptodactylus sertanejo* and *Physalaemus evangelistai* (6), *Rhinella cerradensis*, *Bokermannohyla diamantina* and *Boana buriti* (4).

The records covered the period from 1965 to 2022, and records from previous periods were not considered due to not presenting collector or identifier data and the collection. The highest number of records are from 1972 (174), 1973 (206) and 2012 (201), while the years 1965, 1975, 1985, 1988, 1991, 1992 and 1994 had the lowest number of records (one each) (Fig. 2). A large number of records (1,577) were not considered in the analyses due to not having the year.

Regarding the conservation status, the Cerrado has three species classified nationally as VU (*Boana buriti*, *Bokermannohyla napolii* and *Scinax pinimus*) and one globally as CR (*Proceratophrys moratoi*), whereas 14 are considered DD, 46 are LC, and 18 are NE (Table 1). The Caatinga has three species classified as CR (*Adelophryne maranguapensis*, *Proceratophrys ararype*, *Rhinella casconi*), and three listed as DD status, five as LC and seven are NE (Table 1). In general, the species are not well known and have unique records of occurrences, ranging from one to 50 records, with threatened and DD species having fewer records than the others (Table 1, Fig. 3).

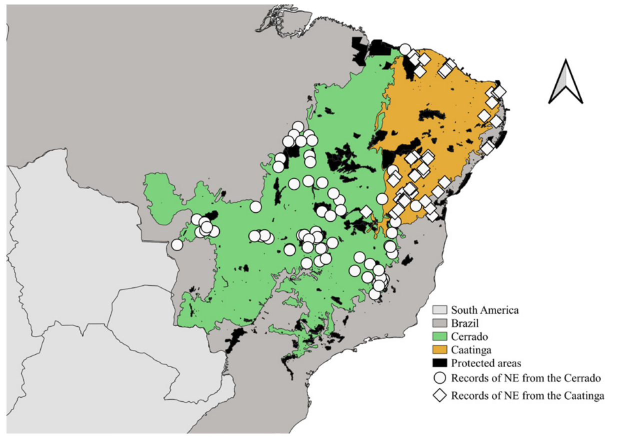

Figure 7. Overlap of the Protected Areas (PAs) with the occurrences of the NE species of Cerrado and Caatinga

We recorded 72 protected areas that included species occurrence (12 for the Caatinga and 61 for the Cerrado). The protected areas of the Cerrado that had the most records of occurrence of endemic amphibians were: Morro da Pedreira Environmental Protection Area (72), João Leite Environmental Protection Area (31), Pouso Alto Environmental Protection Area (27), Environmental Protection Area Environmental Águas Vertentes (21) and Chapada dos Veadeiros National Park (15). For the Caatinga they were the Chapada Diamantina National Park (10), the Serra do Barbado Environmental Protection Area (5), and the Chapada do Araripe Environmental Protection Area (4).

Verifying the presence of endemic amphibian species within the protected areas of the Cerrado and Caatinga, we identified 81 species that occur within the protected areas (68 for the Cerrado and 13 for the Caatinga), with 19 species not being recorded in these areas (14 for the Cerrado and five for the Caatinga) (Table S2). We did not identify occurrence records of the endangered species *B. napolii*.

In general, the species had few occurrence records within the protected areas, in which the threatened species had from one to four records (Fig. 4), the DD from one to eight records (Fig. 5), the LC from one to 15 (Fig. 6) and NE species from one to three records (Fig. 7).

DISCUSSION

The Caatinga is a unique biome that has suffered great anthropic pressure. This region is known to receive less attention in relation to research, requiring more conservation strategies directed at it and its biodiversity (Lessa et al., 2019). In addition, the Caatinga has a relatively unknown richness of endemic species (about 18 species), requiring more sampling and studies, especially in poorly accessed areas, for a complete understanding of its biodiversity (Albuquerque et al., 2012).

The Cerrado is considered the richest savanna in terms of biodiversity, which has been intensely threatened by increasing human pressures (Silva & Bates, 2002), and has an enormous richness of endemic amphibian species (about 82 species). It is essential to carry out more studies in the Cerrado, in order to better understand its biodiversity and propose conservation strategies in the face of anthropic pressures. It is known that human pressures will probably advance in future scenarios, which will eventually drastically reduce its native vegetation (Resende et al., 2019), harming the entirety of its biodiversity with the imminent loss of their natural habitats.

Geographic occurrence data allowed us to better understand the geographic area of amphibian species endemic to the Cerrado or Caatinga, and the real diversity of these species. It is important to know the size of the species geographic range and their changes over time, because besides being an ecological and evolutionary characteristic of species, it can be a predictor of extinction risk (Gaston & Fuller, 2009). Biodiversity records present in digital databases (e.g. GBIF) greatly facilitate access to information and are essential because they are based on verifiable specimens and therefore have ballast in scientific collections (Nelson & Ellis, 2018). These data can be used for purposes of basic research in ecology, applied ecology in conservation, scientific outreach, and integrated studies (Smith & Blagoderov, 2012). However, care must be taken with data quality, avoiding uncertainty and bias (Jin & Yang, 2020).

The endemic species from the Cerrado and Caatinga, especially the threatened ones, DD and NE, have few occurrence records and reduced geographical range. According to Smith & Green (2005), the geographical range of species is determined by several factors, such as geographic and ecomorphological restrictions and low dispersal capacity. In this sense, we assume that, for most species, this smaller geographical range may be related to a lower local abundance, which could explain the low number of records, in addition to the ecomorphological characteristics of the different species (Gaston, 1990).

The Cerrado and the Caatinga have few species classified as threatened (four and three, respectively), but have many species considered DD and NE. The species previously considered threatened in the Cerrado, *Allobates goianus*, *A. brunneus* and *Proceratophrys moratoi* (classified as EN, CR, and EN, respectively) were removed from the Brazilian list of threatened species updated in 2022 (MMA, 2022). However, in the IUCN global assessment *A. goianus* is classified as DD and *P. moratoi* CR. The Cerrado species *Boana buriti*, *Bokermannohyla napolii* and *Scinax pinimus* previously classified as LC, NE, and DD respectively are now considered threatened, classified as VU (MMA, 2022). The Caatinga species *Proceratophrys arарype* and *Rhinella casconi* previously classified as NE are now classified as CR along with *Adelophryne maranguapensis* (MMA, 2022). These updated results highlight the essential importance of knowing more about the species geographic ranges and the environmental quality in order to better assess the species conservation status, because species previously assessed as DD, NE, and even LC may be underestimated and at risk.

Doubtful taxonomy and poor knowledge on geographic distribution affect the assessment of the species conservation status, as in the case of *A. brunneus*, which was considered endemic and threatened from the Cerrado (Lima et al., 2009), but due to the similarity with others, such as *Allobates magnussoni*, there are many dubious records in the literature, for example, for the Brazilian Amazon, Bolivia and Colombia (Lima et al., 2014).

The DD species needs more attention for their conservation and strategies that consider their diversity, their abundance, geographic reach, and the conservation status of the environments in which they live. Studies suggest that up to 63% of DD species are at high risk of extinction and are neglected due to the lack of information about them (Howard & Bickford, 2014). Therefore, it is vital to better understand the taxonomy of species and their geographic range, whether LC, DD, threatened or NE, in order to assess their actual conservation status and propose effective conservation measures.

The protected areas of the Cerrado and Caatinga play an essential role in protecting the species. The Cerrado has about 8.6% of its extension formally protected and the Caatinga has about 7.7%, which is still low compared to other biomes like the Amazon (27.8%) and the Atlantic Forest (10.1%) (Vieira et al., 2019). Moreover, all these percentages are considerably below conservation targets proposed by the National Biodiversity Commission (CONABIO, 2013).

We emphasise the importance of amphibian occurrence records to know their biodiversity, their geographic range, their conservation status and thus propose effective measures for their protection. In this sense, we suggest that the records on endemic amphibians be increasingly included in digital databases to make them accessible. However, it is necessary that these inserted data are refined, containing all the necessary information about the record of the specimen such as the year of registration, location, collector, among others.

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DATA ACCESSIBILITY

The data supporting the supplementary material (Table S1) can be accessed at https://osf.io/4w8yx/?view_only=4bf4cbbec17c413eb961ef992f7ab76d. The data supporting the supplementary material (Table S2) can be accessed at https://osf.io/8snue/?view_only=bb69383d4260446f9d8013d7ab43526d.

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SHORT NOTE

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First natural history observations of the Critically Endangered Wattle-necked softshell turtle *Palea steindachneri* in its native range

Pham Van Thong[1*], Ha Van Ngoan[1], Vi Van Dinh[1], Nguyen Huu Trung[1], Luong Van Nam[1], Lo Tien Bieu[1], Pham Thi Ha Nguyen[1], Pham Minh Phuong[1], Nguyen Van Thai[1], Tran Xuan Cuong[2], Hoang Anh Tuan[3] & Russell J. Gray[1]

[1]Save Vietnam's Wildlife, Cuc Phuong commune, Nho Quan district, 430000, Ninh Binh province, Vietnam

[2]Pu Mat National Park, Con Cuong town, Con Cuong district, 460000, Nghe An province, Vietnam

[3]Quang Trung commune, Thong Nhat district, Dong Nai province, Vietnam

The Wattle-necked softshell turtle *Palea steindachneri* is poorly understood in its natural habitat, despite being highly productive in farms and commonly observed in the turtle trade. Field observations conducted in Vietnam's Pu Mat National Park represent the inaugural instance of direct, in-the-wild natural history documentation for the species within its native habitat. In contrast, prior research relied on interview data gathered from local communities. The species was found in slow-moving water in a sandy stream, consistent with previous information from community interview data. The limited range and its frequent appearance in the food and traditional medicine trade have led to the species' classification as Critically Endangered by the IUCN Red List.

Keywords: Natural history, observation, *Palea steindachneri,* Vietnam turtle, Wattle-necked softshell turtle

The natural history and ecology of the Wattle-necked softshell turtle *Palea steindachneri* remains poorly understood throughout its entire range (Le Duc et al., 2020a). While the species is native to Vietnam, Laos and China, all prior data regarding its natural history have stemmed from local communities, lacking verification through field observations (Fong et al., 2021; Le Duc et al., 2020a; Markus, 2011; Zuklin et al., 2021; Ducotterd et al., 2022). Notably, there have been no confirmed field observations in its native range to date. However, there have been recorded observations of the species in the wild in Hawaii, USA, which lies outside its native range (Radford, 2011). The majority of knowledge on the species has been obtained through farming, turtle trade, genetics, diseases in captivity, and consumption (Ernst & Bogadek, 2005; Gaillard et al., 2017; Han et al., 2017; Le Duc et al., 2020a; Tang et al., 1997; Tong et al., 2009). The species is highly productive in farms, which has led to extensive farming in China and Vietnam, and it is commonly observed in the turtle trade but is not easily seen in the wild (Haitao et al., 2008; Le Duc et al., 2020a). While Le Duc et al. (2020a) claimed the species is carnivores, Marchetti & Engstrom (2016) believe it is omnivorous. Respondents from local communities report that the species mostly inhabits slow moving water bodies in sandy streams and rarely enters rivers or large water bodies such as lakes or reservoirs (Le Duc et al., 2020a; 2020b). Interviews with farmers who farm *P. steindachneri* in Vietnam revealed that the species can grow up to 45 kg in weight or 450 mm in carapace length, recorded in captivity, and they lay up to 28 eggs per clutch, intermittently between the months of March and July, and can produce 4–5 clutches per year (Le Duc et al., 2020a; Pham, pers. com.). In captivity, the species hibernates from November to February while it does not do so in Hawaii (Le Duc et al., 2020a; Radford, 2011). Due to its limited range and frequent appearance in the food and traditional medicine trade, the species was recently reassessed as Critically Endangered by the IUCN (Fong et al., 2021). In this study, we present the first natural history observations of the species from its native range through field data collected by Save Vietnam's Wildlife (SVW)'s anti-poaching team (a local NGO in Vietnam that focuses on the protection and rehabilitation of wildlife in the country) and a survey conducted using aquatic traps in Pu Mat National Park (PMNP) in the central Annamite mountain range of Vietnam.

Starting from 2018, the alliance between SVW's 16 anti-poaching teams and PMNP's 74 rangers has been conducting patrols across the entire PMNP while employing SMART (Spatial Monitoring and Reporting Tool) to monitor and record forest violations and wildlife observations. During one of these patrols on 22 September 2020, VVD, one of the authors of this paper, observed a juvenile *P. steindachneri* (Fig. 1a) near a sand bank of Chat stream at an elevation of 203 m a.s.l. Chat stream is one of the main streams in PMNP. The water body was estimated to be 1000 cm in length, 300 cm in width, and 200 cm in depth at the time he captured the individual (Fig. 1c). The co-ordinates of the discovery have not been disclosed for the safety of the animals.

Correspondence: Pham Van Thong (phamthong.86@gmail.com)

Figure 1. *Palea steindachneri* found by chance in the forest stream by VVD **A.** and by trapping **B**. Habitat where VVD captured the juvenile *P. steindachneri* **C.** and location where subadult one was trapped **D**.

The second observation occurred serendipitously during a survey conducted using 20 funnel aquatic traps (sized at 40 x 80cm). This survey was originally undertaken to locate Big-headed turtles *Platysternon megacephalum* and determine suitable release sites for the 120 rescued individuals currently housed at the PMNP rescue centre (SVW, unpub. data). The traps were set from 22–28 April 2023, with a total of 1321.87 trapping hours and were set in the afternoon along a ~ 5 km stream length. The locations of the traps were changed every morning. The trapping hours were calculated using the formula H = RT - ST (where H represents hours, RT is the trap removal time, and ST is the trap setting up time). All 20 traps were set for an average of 9 hours per day before being moved to other locations. Chicken intestines were used as bait. While no *P. megacephalum* were found, a sub-adult male *P. steindachneri* (weighing 2330 g, carapace length (CL) 27.5 cm, carapace width (CW) 23.6 cm) (Fig. 1b) and an adult female Four-eyed turtle *Sacalia quadriocellata* (weighing 253 g, CL 12.8 cm, CW 11.4 cm) were found in the traps. The *P. steindachneri* was caught on 27 April 2023 at an elevation of 283 m a.s.l. The water body where the *P. steindachneri* was trapped was an estimated 170 cm wide, 300 cm long, and 110 cm deep with a submerged sandbank (Fig. 1d). The trapping effort for the *P. steindachneri* was 0.000757 individual/hour/trap (see Pham et al., 2018 for further detail on trapping method). All turtles were unharmed and released to the same location after basic measurements were taken using a tape measure for carapace length and carapace width and photos were taken.

Palea steindachneri is known for its high productivity in captivity, with females capable of laying a substantial number of eggs (Le duc et al., 2020a; Pham, pers. com.). However, despite this high reproductive potential, the species seems to exhibit a low survival rate, resulting in low population density even in areas where hunting threats are absent. This was exemplified by Radford's (2011) study, which conducted trapping efforts for 24 days but only managed to capture three *P. steindachneri* individuals.

Throughout the trapping period, the team also captured several species of fish and crab by chance, these were tentatively identified by relevant regional experts based on photos we provided. The captured species, included *Pterocryptis cochinchinensis*, *Tachysurus* sp., *Traccatichthys taeniatus*, *Bardodes semifasciolatus*, *Sarcocheilichthys nigripinnis*, *Onychostoma* sp., *Coreoperca* sp., *Hemibagrus* sp. and crabs (*Indochinamon* sp.) that could potentially serve as prey for *P. steindachneri*, as they coexist in the same stream. Further research on the diet of *P. steindachneri* should be conducted, as the available information on the species' diet only comes from captive specimens that are

primarily fed on fish (mainly *Hypophthalmichthys* ssp.) as their main food source, with earthworms and snails (*Achatina fulica*) provided as supplementary food (Le Duc et al., 2020a). However, studies conducted on the species in Kauai (USA), where *P. steindachneri* is non-native, have discovered a variety of items in the species' fecal matter, including rocks, hair, styrofoam, plastic, vegetation, seeds, insects, and introduced species of snails, prawns, and clams (Engstrom et al., 2009). This suggests that the species may have an omnivorous diet, but further research is needed to better understand its feeding habits in its native range.

In both our observations, *P. steindachneri* was found in a stream in a tropical evergreen forest with vines, ferns, grasses, forest banana and a complex forest structure with small to large trees on the sides of the stream. In both instances, the turtle was submerged in slow-moving water in the deeper and wider section of the stream. These findings are consistent with the information provided by local communities during interviews (Le Duc et al., 2020b), which stated that the species mostly lives in small water bodies in sandy streams and rarely enters a river or a large water body such as lakes or reservoirs. However, Ducotterd et al. (2022), based on interview information, argued that the species is also found in large river systems. Hence, our findings represent the first time this species has been recorded in the wild in its native range by scientists. The location where the species was found is about nearly 20 km away from the nearest human settlement. This location is considered as a safe zone of PMNP as a result of the effort of SVW and PMNP to stop poaching in the PMNP (SVW, unpub.data)

The discovery of a juvenile individual offers hope for the survival of the species in PMNP as it is indicative of a breeding population and relatively recent recruitment. Conducting surveys to gather information on the population size, distribution, and threats to *P. steindachneri* is crucial for conservation efforts in its native range. This information will help to develop and implement effective conservation strategies to protect the species and their habitats globally.

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Websites:
Lang, J., Chowfin, S. & Ross, J.P. (2019). *Gavialis gangeticus*. The IUCN Red List of Threatened Species 2019: e.T8966A149227430. Downloaded on 3 October 2019. http://dx.doi.org/10.2305/IUCN.UK.2019-1.RLTS.T8966A149227430.en.

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