June

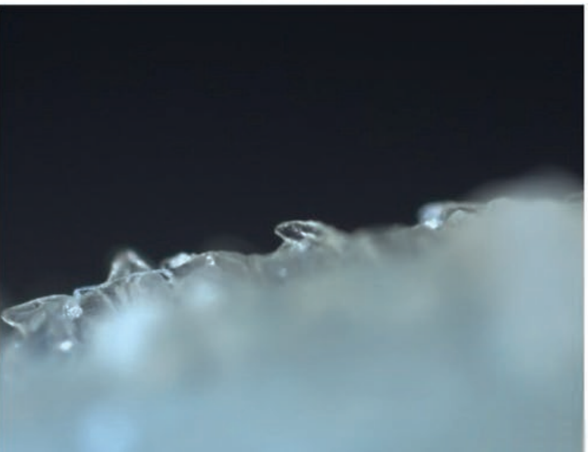
2024

Vol. 19 – N. 1

SOCIETAS HERPETOLOGICA ITALICA

Acta Herpetologica

ISSN 1827-9635



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SOCIETAS
HERPETOLOGICA
ITALICA

Acta Herpetologica

Vol. 19, n. 1 - June 2024

Firenze University Press

Acta Herpetologica 19(1): 3-12, 2024
DOI: 10.36253/a_h-14145

Hemipenial morphology does not provide insight on mating barriers between the two main lineages of *Hierophis viridiflavus* (Lacépède, 1789)

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Submitted on: 2023, 28th December; revised on: 2024, 9th January; accepted on: 2024, 16th January
Editor: Andrea Villa

Abstract. Copulatory organs are a key trait in reproductive compatibility and sexual isolation. The role of male genitalia in boosting mating success is well known and is often the outcome of behavioural and biological constraints, although no clear and common interpretation about their evolution appears broadly applicable. In snakes, hemipenial morphology has often been described under the perspective of sexual selection, taking into consideration both behavioural and morphological traits of both sexes. We investigated hemipenial morphology and ornamentation in the two subspecies of *Hierophis viridiflavus*, a male-male combating colubrid, and compared it to the sister species *H. gemonensis*, to assess intraspecific variation in size of genitalia and ornamentation richness. The male intromitted organ of this species is unilobed and bulbous, with rich ornamentations consisting of basal spines and apical calyculations. We detected no statistically significant difference in hemipenial size, basal spine count, and spine length between the two subspecies, suggesting that no copulatory barrier is present between the two clades. Although hemipenial morphology and anatomy do not seem suitable to assess intraspecific variability as shown in this study, they are often highly variable at the family or genus level, suggesting that the evolution of male genitalia is linked to phylogenetic relationships and that hemipenial divergence might be correlated to lineage splitting despite not being necessarily the main cause. Future studies should be aimed at investigating hemipenial morphology and anatomy across species under the perspective of comparative phylogeny and reproductive behaviours to address thoroughly the constraints of hemipenial evolution and development.

Keywords. *Hierophis viridiflavus*, mating barriers, hemipenes, anatomy, morphology.

INTRODUCTION

The anatomy and morphology of copulatory organs have been of great interest for herpetologists in the last century especially concerning snakes (Cadle, 2011; Folwell et al., 2022). As a matter of fact, hemipenes are postulated to play a major role in mating success, being supposedly species-specific (Cope, 1895; Keogh, 1999), and thus with marked implications in terms of reproductive biology and behaviour (Tokarz, 1988; King et al., 2009; Klaczko et al., 2017). Reptilian hemipenes show highly variable morphological traits, in terms of the gross shape of the organ itself (unilobed, bilobed) as well as of its external ornamentations, which can consist of rigid spines (spread across the organ or aggregated in a specific region, i.e., basal region or the apex; Fig. 1) or soft tissue folds, or otherwise can be completely absent (Zaher et al., 1999; Andonov et al., 2017).

ISSN 1827-9635 (print)
ISSN 1827-9643 (online)

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The extent of hemipenial morphological variability can remarkably vary among different families (Cadle, 2011; Andonov et al., 2017), but also at lower taxonomic levels (Inger and Marx, 1962; Branch, 1986; Zaher, 1999; Zuffi, 2002; Bernardo et al., 2012; Klaczko et al., 2014; Myers and McDowell, 2014). From this perspective, investigating the mechanisms that drive the evolution of specific features in copulatory organs can be of great interest to address phylogenetic relationships and species splitting over time as copulatory organs are some of the most rapidly evolving traits in squamates (Brennan and Prum, 2015; Klaczko et al., 2015, 2017; Folwell et al., 2022). Hypotheses have been proposed for the development of male genitalia, first of which the "lock-and-key", formerly postulated by Dufour (1844), states that male genitalia evolve to be complementary to those of females with noticeable species-specificity. Alternatively, the pleiotropy hypothesis for male genitalia differentiation has been partly supported, hypothesizing that they evolve through selective pleiotropic effects on other traits (Mayr, 1963; Edwards, 1993; Arnqvist and Thornhill, 1998; Hosken and Stockley, 2004). This hypothesis appears still unsuitable to be applied broadly as a common rule, because it assumes that the set of genes coding for general morphology codes also for genital morphological variation, which should not be selected against, implying tight genetic correlation between genital and general morphology (Arnqvist and Thornhill, 1998). These hypotheses have been revised broadly (Shapiro and Porter, 1989; Sota and Kubota Soto et al., 2013; Brennan and Prum, 2015) in an evolutionary perspective highlighting the role of genital morphology as a barrier against hybridisation, favouring coevolution between male and female genitalia (House et al., 2020; Greenwood et al., 2022).

Ophidian hemipenes have been studied vastly in the last decades (Zaher, 1999; Myers and McDowell, 2014) under the functional perspective as they are related to copulation duration as well: indeed, in New World natricines more complex and ornate hemipenes (as in *Thamnophis radix*) are associated to more prolonged copulations compared to the congeneric *T. sirtalis*, characterised by simple subcylindrical hemipenes with little ornamentation (King et al., 2009). From this perspective, the occurrence of abundant ornamentations on hemipenial surfaces of both apical and body part of the organ appears to be relevant in terms of how efficiently males remain attached to females (Friesen et al., 2014), significantly affecting the duration of copulation and thus male fitness. Additionally, Rivas et al. (2007) showed that male coiling during copulation in species that undergo mating balls (*Eunectes murinus* in this case) can impede other males from mating with the female and, in these cases, more conspicuously ornamented hemipenes (i.e., more abundant or large ornamentations) should favour copulation and operate synergically with behaviour. On the other hand, snakes that exhibit male-male combat behaviours are subject to sexual selection prior to copulation. Therefore, hemipenis morphology should not be selected to evolve more complex structures such as calyces, spines, and hooks. However, as shown by Andonov et al. (2017), hemipenial morphology does not always correlate with behavioural strategies, so the scenario of the evolution of male genitalia is a complex task to untangle.

The green whip snake *Hierophis viridiflavus* (Lacépède, 1789) is one of the most widespread species in Mediterranean Europe as it occurs from Northern Spain across France and throughout Italy to Northern Balkans (Sillero et al., 2014). From the phylogenetic point of view, this species has been object of debate and Mezzasalma et al. (2015), according to both molecular and morphological differences, have split the two subspecies *H. v. viridiflavus* and *H. v. carbonarius* (Western and Eastern clade respectively) and elevated them to the rank of species. Recently, Speybroeck et al. (2020) have proposed to pool them together as a unique species; however, the debate is still open. As a matter of fact, the phylogenetic relationships between the two lineages are still unclear and recent research on the genetic basis of its colour polymorphism (mtDNA and nDNA; Senczuk et al., 2021) has suggested that these two lineages might undergo asymmetrical gene flow from the Western into the Eastern clade, even though no decisive evidence has been gathered. Morphological variability in dentition, pholidosis, and hemipenes has also been used to characterise the phylogeny of the *Hierophis* genus by Schätti (1987, 1988), discriminating the members of this genus with respect to sister groups (i.e., *Spalerosophis, Eirenis, Platyceps* genera); however, interspecific variability patterns within genus are still unexplored (Schätti and Monsch, 2004; Utiger and Schätti, 2004). With this respect, nevertheless, Schätti and Vanni (1986) have investigated morphological traits of the target species, among which hemipenes too, but no difference among populations was found by the authors; however, hemipenial morphology was not the key subject for investigation and no in-depth study of morphology and ornamentations was performed.

In this scenario, the role of copulatory organs can be useful to cast light on the reproductive aspects of species/subspecies delimitations as marked differences in hemipenial morphology and ornamentation might impede interbreeding driving divergence between lineages; on the other hand, similar hemipenes would not act as a barrier to hybridization, favouring gene flow and inter-lineage mating (King, 1989; Sota and Kubota, 1998; Greenwood et al., 2022). For such reasons we investigated hemipenial morphology to address potential morphofunctional advantages of hemipenial structures, such as increased copulation efficiency and duration according to differences in size, shape, and ornamentation, in the scenario of intraspecific lineage diversification. Additionally, we compared the gross morphology of *H. viridiflavus* as a whole, to that of the sister species *H. gemonensis* to check the extent, if any, of morphological variability of hemipenes at the genus level.

MATERIALS AND METHODS

Gross morphology

For morphological description we referred to the categories in Dowling and Savage (1960) and Andonov et al. (2017). We collected the left hemipenis from both fresh and museum collection specimens, preserved in ~75% alcohol. For sample preparation from alcohol-preserved specimens, we slightly modified the method in Andonov et al. (2017) and Zuffi (2002): for hemipenis filling we preferred liquid paraffin over petroleum jelly (used by Pesantes, 1994; Myers and Cadle, 2003; Zaher and Prudente, 2003) because it is easier to use as it does not need to be kept in liquid state and, being less viscous, it is quicker and simpler to inject through syringe. We propose to use this technique in case the injection of petroleum jelly appears to be difficult. Firstly, the organs were soaked in 2% KOH after extraction for 30 minutes to 6 hours according to its size and duration of preservation. After this period, hemipenes were everted manually using tweezers, and subsequently filled with liquid paraffin. Hence, we sealed hemipenes at the base using a thin string. Fresh samples were processed using the same procedure as for alcohol-preserved specimens, except they were soaked in water rather than KOH solution for tissue softening before eversion. Lastly, all samples were stored in ~75% alcohol for permanent preservation. Each hemipenis was photographed on both sulcate and asulcate surfaces using a high-resolution reflex camera (NIKON D7100) by placing it on a black surface under two light sources on opposite sides to minimize shadows. We used the "magic wand" tool built in Photoshop CS3 (version 10.0) to eliminate any remaining shadow from the pictures to produce a clean photo of each object.

We prepared and analysed 10 left hemipenes for each currently accepted subspecies (20 in total, two road-killed and 18 alcohol preserved museum specimens; Table 1, for details). We also prepared a single left hemipenis of *H. gemonensis* for outgroup comparison.

Table 1. List of the specimens of *Hierophis viridiflavus* with the relative source of collection, clade, and morphometric measures. NHMPv Natural History Museum of Pavia; NHMPi: Natural History Museum of Pisa; NHMMi: Natural History Museum of Milan; Spines = number of basal spines; Length = hemipenial length; SVL = snout-to-vent length; Spine1-5 = length of five randomly selected basal spines.

Specimen	Source	Clade	Spines	Length (mm)	SVL (mm)	Spine 1 (mm)	Spine 2 (mm)	Spine 3 (mm)	Spine 4 (mm)	Spine 5 (mm)
LEC006 - 22	NHMPv	E	61	34.80	745	3.29	3.09	3.45	3.22	2.79
LEC008 - 22		E	51	30.87	875	3.64	3.53	2.79	3.14	2.56
CUN001 - 22		W	51	28.65	910	2.10	2.74	2.97	2.29	2.91
AGR001 - 22		E	46	25.98	796	3.73	2.65	2.83	2.12	3.40
LEC010 - 22		E	56	29.98	840	3.14	4.17	3.38	4.60	3.37
ARE001 - 22		W	63	28.17	950	1.99	2.63	3.38	2.15	1.72
ALE001 - 22		W	64	20.86	640	4.44	3.06	4.57	4.26	1.95
TOR006 - 22		W	52	20.45	897	2.24	1.83	2.70	2.10	1.74
1184	NHMPi	W	49	31.44	1053	4.00	3.75	2.34	4.32	3.04
1193		E	60	32.33	912	3.81	3.65	4.12	3.74	3.27
1213		E	60	36.62	910	3.22	2.25	2.36	2.45	3.11
1203		E	54	30.74	875	2.71	3.85	3.65	3.70	3.11
1206		E	39	25.06	880	4.67	4.10	2.92	3.11	2.66
1199		E	46	29.05	790	3.75	3.38	4.75	2.77	3.56
1191		W	52	31.04	972	3.42	5.37	3.84	3.67	4.01
GRO001 - 22	NHMMi	W	57	29.10	860	3.55	3.52	2.32	3.43	2.37
TOS001 - 22		W	51	30.85	740	2.64	3.68	4.29	2.50	2.54
TOS002 - 22		W	46	31.46	890	3.62	4.29	3.38	3.45	1.99
FIR001 - 22	field	W	56	22.96	715	3.04	2.66	3.22	2.15	2.76
GLP001 - 22		E	43	27.81	860	2.46	3.51	3.60	3.10	3.29

Quantitative analyses

To thoroughly describe any variation in hemipenial morphology and anatomy between the two main lineages of *H. viridiflavus* (Western and Eastern clades, hereinafter W and E respectively), we also recorded quantitative data concerning hemipenial size, number of basal spines, spine length, and snout-to-vent length (SVL) of each specimen. The count of the total number of spines was repeated three times to minimize counting error. From each hemipenis, we randomly extracted five spines from different parts of the basal region. Prior to performing any analysis, all predictors (hemipenial size, spine number, SVL, and clade) were tested for intercorrelation via Pearson's correlation test. No correlation was detected between spine count and hemipenial length (r = 0.13, P = 0.57), as well as between SVL and both hemipenial length (r = 0.37, P = 0.11) and spine count (r = -0.21, P = 0.38). Therefore, we performed a Linear Mixed-Effects (LME) model to test whether spine length depended on species clade when controlling for body size (SVL), hemipenial size, and number of spines. All those variables were implemented in the model as fixed effects. We also considered the two-way interactions between clade and both spine length and count to test for potential different effects in each group. Additionally, specimen entered the model as a random-intercept effect to account for inter-individual variability patterns that were unexplained by morphometry or lineage assignment. The model was performed with the *lme4* package (Bates et al., 2015); model visualisation was performed with the package *visreg* (Breheny and Burchett, 2017) and 95% confidence intervals were estimated with the package *bootpredictlme4* (Duursma, 2022). All analyses were performed on R 4.2.1 (R Core Team, 2022).

RESULTS

Gross morphology

The hemipenis of *Hierophis viridiflavus* is unilobed, bulbous, non-capitate (Fig. 1A-B). The basal region lacks ornamentation and does not show tissue swelling of any kind. The body part is characterised by numerous spines

Fig. 1. Hemipenes of *Hierophis viridiflavus* collected from two roadkilled specimens, respectively from the Western (A) and Eastern (B) clades, and hemipenis of *H. gemonensis* (C). The basal region (a) lacks ornamentations, whereas the body part (b) is completely covered in spines and the apical part (c) is markedly ornamented with calyculations (folds of tissue). Both the Western and Eastern clades show unilobed bulbous hemipenes and no difference in ornamentation and gross morphology. The sulcus spermaticus (white arrows) is unique and undivided from the base to the apex.

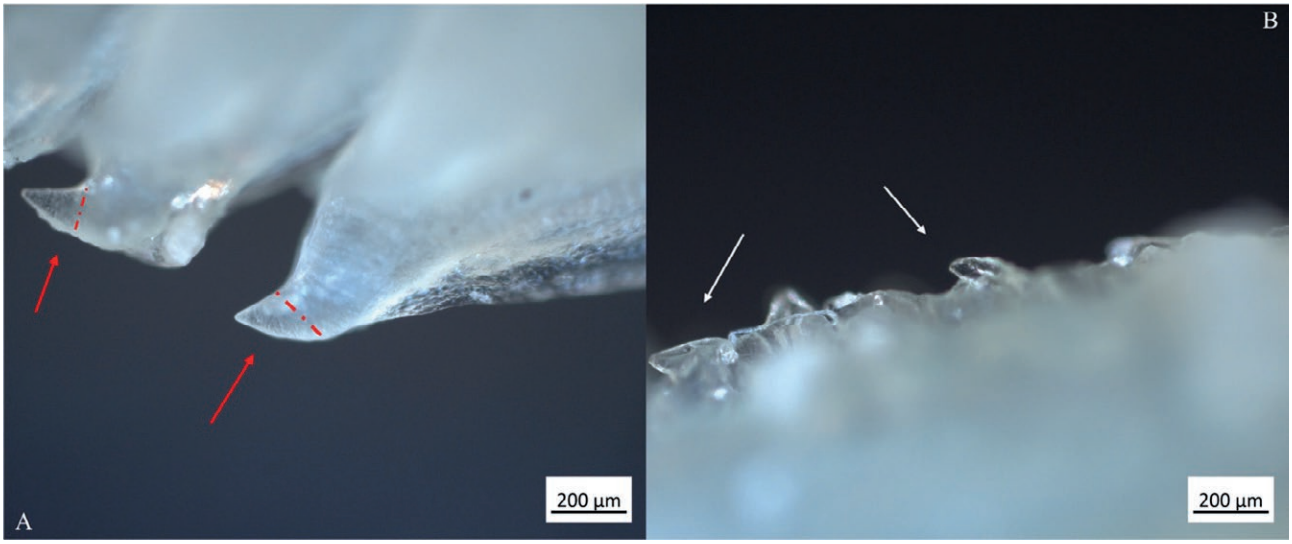

Fig. 2. High-resolution images of hemipenial spines (A, red arrows) and calyculations (B, white arrows). Picture 2A shows how spines, located across the body part of the organ, are embedded in soft tissue folds up to their terminal region (dotted red line).

(Fig. 2A), while the apical part is rounded and highly calyculate on both sulcate and asulcate surfaces (Fig. 2B). Calyculations form a reticulate pattern of more or less regular geometric shapes (hexagons, pentagons). The ridges of the calyces show small flounces of soft tissue (details in Fig. 2B). The apex is flat and ornamented with calyculations and hosts the termination of the *sulcus spermaticus*, which is oblique and undivided from the basal region to the apex (Fig. 1A). No evident morphological differences were detectable between the two clades (Fig. 1).

Similarly, the hemipenis of *H. gemonensis* is unilobed and non-capitate. However, upon inspection, its general shape is more elongate and less bulbous (Fig. 1C). The basal region lacks ornamentations and does not show any swelling. The body part is covered in large spines and the apical part is markedly calyculate on both surfaces (sulcate and asulcate); such calyculations form a reticulate pattern very much like that of the sister species. The ridges of the calyces consist of flounces of soft tissue. Also, the apex is flat and ornamented with calyculations and hosts the termination of the *sulcus spermaticus*, which in this case is less oblique and straighter than in *H. viridiflavus*, undivided from the basal region to the apex.

Quantitative analyses

Hemipenes length was on average 27.9 ± 3.3 mm when considering the whole sample of 20 whip snakes and, respectively, 27.5 ± 4.3 mm and 28.3 ± 3.0 mm for W and E clade specimens separately. The LME model run to investigate the effects of SVL, hemipenial length, spine count, and clade on the length of spines neither showed any significant effect of SVL, hemipenial length, and spine count nor any difference in spine length between the two clades. Similarly, all two-ways interactions between predictors were not significantly correlated with the response variable (Table 2). On the other hand, a marked variability of spine length at the individual level was found (LR-χ^2 = 9.760, df = 1, P = 0.0018; Fig. 3), which was not explained either by clade or morphometry, so that 18.7% of the total variance is explained by the random effect.

Table 2. Analysis of variance with Satterthwaite's method of the fixed effects and interactions implemented in the Linear Mixed-Effects model on the length of hemipenial spines in *Hierophis viridiflavus*. No significant effect was detected for any predictor nor interaction.

Fixed effect	F	df	P
SVL	0.112	1,12	0.42
clade	0.279	1,12	0.71
hemipenial length	0.075	1,12	0.30
spine count on hemipenis	0.633	1,12	0.55
SVL × clade	0.349	1,12	0.55
hemipenial length × clade	2.793	1,12	0.09
clade × spine count	0.447	1,12	0.50

DISCUSSION

Divergence in copulatory organs has been regarded as a barrier to interbreeding between closely related lin-

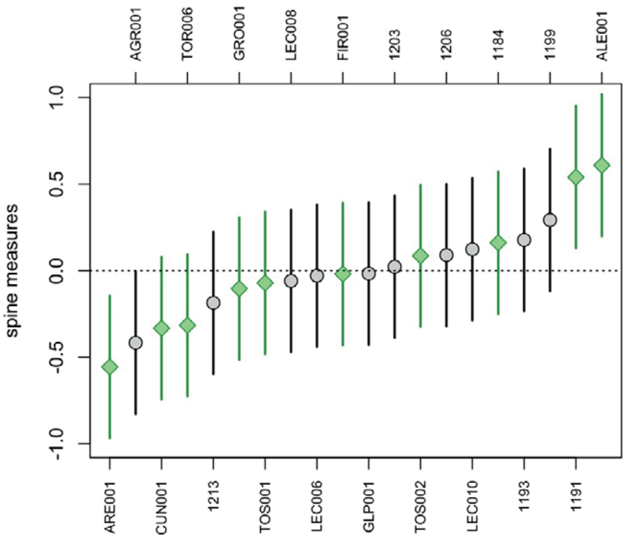

Fig. 3. Random-intercept effects on the length of hemipenial spines for each specimen, colour and symbol coded by clade (Eastern clade in grey circles; Western clade in green rhombi). Symbols correspond to each specimen's estimate ± SE retrieved from the LME model.

eages. So, we have investigated hemipenial morphology variations between the two major clades of the green whip snake *Hierophis viridiflavus*. This species is a Mediterranean colubrid that can be highly variable in phenotype (Vanni and Zuffi, 2011; Meier et al., 2023; Storniolo et al., 2023). It has been object of debate in the last decade as to whether its two major lineages should be considered as separate species according to gross morphology, karyotype, and mtDNA markers divergence (Nagy et al., 2004; Mezzasalma et al., 2015), while no investigation in reproductive barriers has ever been performed.

In our study we show that the hemipenis of *H. viridiflavus* is markedly ornamented in both subspecies, with noticeable morphological differentiation between the apical part (completely covered in tissue folds - calyculations) and the body region (covered in rigid spines). Furthermore, upon investigation, we have found that the general hemipenial morphology of our target species is markedly similar to that of *H. gemonensis*, especially in terms of ornamentations, with the body part covered in spines and the apical part in calyculations of soft tissue folds. Minor differences, however, were found, so that the organ itself is not bulbous and more elongate in the Balkan whip snake with respect to *H. viridiflavus* and the *sulcus spermaticus* is not oblique but straighter from the base to the apex.

Concerning the quantitative investigations for *H. viridiflavus*, no difference in snake size (SVL), hemipenial length, ornamentations, and length of spines was detected between the two major clades, suggesting the lack of copulatory barriers between the two lineages. On the other hand, we found a significant random effect at the individual level, indicating that spines are more variable within individual rather than between individuals or subspecies.

The evolution of male genitalia was firstly supposed to be driven by complementarity to those of females (Dufour, 1844), defined as "lock-and-key" mechanism, as a way of natural selection to minimise hybridisation (Shapiro and Porter, 1989; Brennan and Prum, 2015). With this respect, however, some key assumptions must be met, such as that, in sympatry, character displacement in sister species should occurr as the outcome of reproductive isolation due to natural selection (Eberhard, 2010; Simmons, 2014; Brennan and Prum, 2015; Ng et al., 2017), which is not always true. Alternatively, pleiotropy has been suggested to be driving genital coevolution, so that modifications in genitalia are supposed to be mainly neutral and are inherited alongside positively selected mutations on genes that code for the general morphology of the organism (Mayr, 1963; Edwards, 1993; Arnqvist and Thornhill, 1998; Hosken and Stockley, 2004). This hypothesis has not been supported broadly due to limited evidence except only few cases of insects (Arnqvist and Thornhill, 1998; Arnqvist and Danielsson, 1999), where pleiotropic effects on both general and genital morphology were found. Concerning snakes as well, the evolutionary mechanisms driving hemipenial diversification appear still to be unclear. On one hand, phylogenetic approaches can be potent to describe hemipenial morphological patterns at higher phylogenetic levels (Keogh, 1999; Zaher, 1999; Schargel and Castoe, 2003). At lower levels instead, as in Andonov et al. (2017), sexual selection and behavioural strategies in mating appear to be more suitable to explain different adaptations in hemipenial morphology. For species in which males do not actively compete with each other to mate with partners (e.g., natricines), rendering male size less crucial, hemipenial adaptations in ornamentations can significantly affect duration and efficiency of copulation (Perry-Richardson et al., 1990; Madsen and Shine, 1993; Luiselli, 1996; King et al., 2009) as a response to the ability of larger females to disengage the hemipenis from the vagina (as in some boids and colubrids; Joy and Crews, 1985; Perry-Richardson et al., 1990; Rivas et al., 2007). Differently, concerning species that undergo male-male competitive strategies such as combats, the role of hemipenial morphology is harder to define because morphological adaptations, especially in colubrids, are extremely variable even when mating strategies match. For instance, the hemipenial morphology of *Malpolon insignitus* is relatively simple to address (Andonov et al., 2017), as it lacks any form of lobation and ornamentations, which is consistent with the assumption that combating species do not require marked morphological adaptations to enhance copulation efficiency. Contrastingly, other male-male combating colubrids such as *Chironius* and *Zamenis* (Edgar and Bird, 2006; Klaczko et al., 2014), show highly ornamented hemipenes, more similar to those of natricines (Rossman and Eberle, 1977; Ota and Iwanaga, 1997; King et al., 2009), supposedly to boost duration of copulatory events. The green whip snake is commonly known for engaging prolonged sessions of male-male combats during the mating season (Capula et al., 1995, 1997); hence, according to the sexual selection hypothesis that highlights the role of ornamentations when males do not actively compete one another for the mates, hemipenial morphology should not be markedly developed (Perry-Richardson et al., 1990; King et al., 2009; Andonov et al., 2017). However, the differentiation and complexity we recorded is in contrast with this statement and suggests that hemipenial adaptations cannot be thoroughly explained under the perspective of sexual selection (Andonov et al., 2017; Klaczko et al., 2014). Alternatively, phylogenetic relationships, despite not being suitable to address hemipenial morphology at lower levels (species or subspecies), seem to be a good predictor of variability when considering closely related groups at the family level, such as *Hierophis* in the present study and its sister group *Dolichophis* from other studies (Andonov et al., 2017). Nevertheless, how and whether marginal populations interbreed along the contact zone between the two subspecies (Mezzasalma et al., 2015) is still untangled, making behavioural, reproductive and genetic studies an open and intriguing field of investigation to address the putative evolutionary history of *Hierophis viridiflavus*. With this respect, it needs to be pointed out that, in order to have a broad and accurate investigation of this matter, also female genitalia should be taken into consideration.

In conclusion, our study shows that variability in hemipenial morphology is not potent enough to discriminate populations at the subspecies level, as both major clades of *H. viridiflavus* show almost identical hemipenial features, in accordance to partial evidence collected by Schätti and Vanni (1986). Even by comparing hemipenial morphology between species, the extent of the diversification is limited: the hemipenes of *H. viridiflavus* and *H. gemonensis* are very similar to one another, which leads to potentially intriguing perspectives. If, on one hand, the similarity in copulatory organs at the subspecies level is consistent with morphological and molecular evidence (Meier et al., 2023; Storniolo et al., 2023), and suggests marked gene flow events between the two lineages, on the other hand the extent, if any, of gene flown between the two species is unknown but cannot be excluded, at least according to anatomy. With this respect, hemipenial morphology does not seem to be potentially impeding copulation. If these taxa are indeed reproductively isolated, alternative mechanisms other than genital compatibility must be at play to keep the two entities distinct, such as behavioural strategies (e.g., chemical communication as in Fornasiero et al., 2007) or selection against hybrids (Servedio et al., 2004). In accordance with past research, hemipenial features of both species are not consistent with their mating strategy, suggesting that hemipenial morphology and its evolution are more challenging then expected to investigate under the perspective of common hypotheses. Therefore, we believe that future research on hemipenial anatomy should address this matter under different approaches, such as comparative phylogeny and behavioural ecology with the aim to investigate the evolutionary and biological constraints of hemipenial evolution and development along with a thorough examination of female genitalia as well.

ACKNOWLEDGEMENTS

The authors would like to thank the Natural History Museum of the University of Pisa, the Natural History Museum of Milan, and the Natural History Museum of the University of Pavia, and their respective curators, especially Edoardo Razzetti of the NHM of the University of Pavia, for granting access to alcohol-preserved specimens and for allowing hemipenes extraction for the analyses.

We would also thank three anonymous reviewers for their interest in our research and the very useful comments that significantly improved the form of this work.

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Acta Herpetologica 19(1): 13-27, 2024
DOI: 10.36253/a_h-15271

The tale of the black viper: distribution and bioclimatic niche modelling of melanistic *Vipera aspis* in Italy

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Submitted on: 2023, 15th November; revised on: 2024, 7th March; accepted on: 2024, 3rd May
Editor: Stefano Scali

Abstract. For decades, the evolutionary role of melanism in reptiles has been highly debated. According to the thermal melanism hypothesis, melanistic phenotypes should provide thermal advantages, thus positively impacting various biological aspects of these individuals. Nevertheless, these benefits seem to be countered by environmental constraints and predatory pressure. Here, we mapped for the first time the distribution of the melanistic phenotypes in the highly polymorphic asp viper (*Vipera aspis*). We focused our research effort on the Italian peninsula, where this species reaches its highest level of taxonomic diversity with three currently described subspecies. Furthermore, we investigated via bioclimatic niche modelling, the influence of a wide array of bioclimatic variables on the distribution of melanism in Italian asp vipers. In general, our results seem to support the implications of the thermal melanism hypothesis, highlighting the central influence of mean annual temperature and elevation on the geographic distribution of melanistic *V. aspis*. At the finest scale, our analyses have highlighted a distinction in bioclimatic niches among the three assessed subspecies. However, further fine-scale investigations are needed in order to exclude the potential influence of latitude and elevation on the observed the intersubspecific bioclimatic niche segregation pattern.

Keywords. Bioclimatic model, habitat suitability, MaxEnt, Mediterranean, melanism, snake, *Vipera aspis.*

INTRODUCTION

In snakes, polychromatism is a widespread phenomenon (Wolf and Werner, 1994; Pizzatto and Dubey, 2012; Ruane et al., 2018; de Avila et al., 2019; Regnet et al., 2022) and its related functions have recently been investigated (Allen et al., 2013; Cox and Davis Rabosky, 2013). It is generally believed that dorsal colouration in snakes may have various functions (Allen et al., 2013) such as anti-predatory purpose, expressed as both camouflage

ISSN 1827-9635 (print)
ISSN 1827-9643 (online)

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and aposematism (Madsen, 1987; Santos et al., 2018; Cyriac and Kodandaramaiah, 2019), advantage in thermoregulation (Monney et al., 1995; Bittner et al., 2002; Clusella-Trullas et al., 2007b), and role in intraspecific communication and interactions (Shine, 1993; Brooks et al., 2022).

Intraspecific polymorphism is generally considered an evolutionary advantage, linked to a greater range of adaptive possibilities towards perturbative events, such as climate or habitat variations (Forsman et al., 2008; Pizzatto and Dubey, 2012; Forsman, 2016). Within intraspecific polychromatism, less frequent forms may occur compared to the standard chromatic range of a given species, which can be indicated as chromatic anomalies.

Among chromatic anomalies in wild snakes, melanism is certainly amongst the most well-known and widespread (e.g., Andrén and Nilson, 1981, Bittner et al., 2002; Lorioux et al., 2008; Castella et al., 2013; Benito et al., 2022; Kalogiannis, 2021; Bruni et al., 2022). Melanism is the condition whereby individuals are characterised by darker pigmentation either as intraspecific polymorphism or as consistent variation between closely related taxa (Majerus, 1988; True, 2003; Clusella-Trullas et al., 2007b; Pernetta and Reading, 2009). Melanism can occur not only as an anomalous condition but also as a common form in some taxa (e.g., in *Hierophis viridiflavus carbonarius*; see Storniolo et al., 2023).

In reptiles, the darkening of the skin colour is mainly determined by a surplus in the production (or dispersion) of pigment by melanophores and, although environmental factors can contribute to physiological (e.g., in lizards) and ontogenetic colour variations, the condition is genetically determined (Sherbrooke and Frost, 1989; Clusella-Trullas et al., 2007b). Although various hypotheses have been formulated, the factors that determine the expression and maintenance of melanism in some individuals or taxa remain to be better understood (Clusella-Trullas et al., 2007b; San-Jose and Roulin, 2018). In ectothermic animals, melanism is considered an advantageous condition with regards to thermoregulation, in fact dark colours reflect less light, absorbing a greater amount of energy. According to the thermal melanism hypothesis, in areas with low temperatures, melanistic individuals are advantaged because they warm-up faster and maintain optimal body temperatures more easily (Jong, et al., 1996; Clusella-Trullas et al., 2007b; Martínez-Freiría et al., 2020). Furthermore, melanistic phenotypes appear to be less susceptible to the damaging effects of UV rays (Fu et al. 2022). Nonetheless, the extent of this phenomenon and the molecular mechanisms underlying it in ectotherms are still debated (see Chang and Zheng, 2003; Cox et al. 2013; Reguera et al., 2014; Jin et al. 2020; Senczuk et al. 2021). Moreover, they can achieve greater body size, wider activity cycle, better body condition and a higher fitness than their clearest conspecifics (Huey and Kingsolver, 1989; Luiselli, 1992; Capula and Luiselli, 1994; Monney et al., 1996; Tanaka, 2009). On the other hand, melanism also has disadvantages such as lower camouflage which is reflected in a higher rate of predation, lower efficiency as predators and other stress-related problems (Gibson and Falls, 1979; Andrén and Nilson, 1981; Pérez-Tris et al., 2004; San-Jose et al., 2008).

The asp viper *Vipera aspis* (Linnaeus, 1758) is a polytypic and highly polymorphic species for which four subspecies are currently recognised: *Vipera aspis aspis* (Linnaeus, 1758), *Vipera aspis francisciredi* Laurenti, 1768, *Vipera aspis hugyi* Schinz, 1833 and *Vipera aspis zinnikeri* Kramer, 1958 (Speybroeck et al., 2016; Di Nicola et al., 2021; Sindaco and Razzetti, 2021).

Vipera a. aspis is widespread in a territory that includes the Italian, French and Swiss western Alps and a large part of France with the exclusion of the northern areas and those around the Pyrenees (Zuffi, 2002; Ursenbacher et al., 2006). Its dorsal pattern is usually formed by two transverse series of dorsal dark bars of variable thickness, which can extend up to the sides, in which a further series of rounded spots can run (De Smedt, 2006). The ground colour can be grey, reddish, brown or yellowish. A separate discussion can be made on the highly distinctive *V. a. aspis* alpine populations (formerly known as ssp. *atra*, Meisner, 1820; see Ursembacher et al., 2006; Golay et al., 2008; Barbanera et al., 2009 for the synonymy of the two subspecies). These present a highly variable dorsal pattern (Mebert et al., 2011; Dubey et al., 2015), which can be made up of transverse bars, a marked dorsal zig-zag line or as a series of large rounded blotches more or less fused together or totally absent in the patternless individuals (Mebert et al., 2011; Tessa, 2016) (Fig. S1A).

Vipera a. francisciredi is widespread from central Italy to the central-eastern Italian Alps, including Elba Island, with some populations beyond the borders in Switzerland and Slovenia (Kumar, 2009; Grano et al., 2017; Di Nicola et al., 2021). *Vipera a. francisciredi* is characterized by a dorsal pattern similar to *V. a. aspis*, mainly consisting of four transverse series of dark bars offset from each other, two on the back and two on the sides (Bruno, 1985; De Smedt, 2006). The ground colour can be brown, grey, reddish, or yellowish (Fig. S1B).

Vipera a. hugyi is endemic to southern Italy, being distributed in central and southern Campania, Apulia and Basilicata (excluding its northernmost portions), Calabria, Sicily and on Montecristo Island, where it was introduced in historical times (Masseti and Zuffi, 2011; Di Nicola et al., 2021). *Vipera a. hugyi* is a highly distinctive subspecies, characterized by a pattern consisting of a vertebral series of rounded blotches more or less in contact with each other and often surrounded by a dark margin (Zuffi et al., 2011). On the flanks there are small dark circles, offset from the dorsal blotches. The ground colour ranges from light grey to brown and patternless individuals are known (Di Nicola and Faraone, 2020) (Fig. S1C).

Vipera a. zinnikeri Kramer, 1958 – the only subspecies absent in our study area (i.e., Italy) –, has a geographic range that includes the Pyrenees and the contiguous territories of France and Spain (Ursenbacher et al., 2006). This subspecies usually has a dorsal pattern characterized by a marked zig-zag band of variable thickness, which may expand to a broad vertebral band (Geniez, 2015).

In the asp viper, melanism is a fairly recurrent mutation, which appears with different frequency in the four subspecies. In *V. a. aspis*, melanism appears very frequent in the Alpine populations (Bruno, 1976; Broenniman et al., 2014; Castella et al., 2013; Muri et al., 2015) but rare elsewhere (De Smedt, 2006). The melanistic specimen records for *V. a. zinnikeri* are not infrequent (Pottier, 2001; Rivera et al., 2001; Baena and Oliveras, 2015; García-Roa and Carbonell, 2020), as well as for *V. a. francisciredi,* for which they are located exclusively in the regions of central Italy (Bruno, 1976; Di Nicola et al., 2019, 2021; Borgianni and Paolino, 2020). As for *V. a. hugyi,* melanism has been found in only a few cases, located in the Calabria region (Brodmann, 1987; Di Nicola and Meier, 2013).

In any case, scientific literature on the presence and distribution of melanistic *V. aspis* in Italy is scarce, and limited to localized individuals or populations (see Bruno, 1976; Monney et al., 1996; Di Nicola and Meier, 2013; Borgianni and Paolino, 2020). Since Italy is a key territory for *V. aspis*, hosting the highest intraspecific diversity for the taxon, the present research aimed to fill such a lack of information by collecting all possible records of melanistic *V. aspis* and using them to produce both a real and potential distribution for these individuals.

Accordingly, the main objectives of the present study were: (1) to describe the current knowledge on the geographical distribution of melanism among the Italian populations of *V. aspis*, mapping the occurrence of the three subspecies present in the study area; (2) to assess and analyse the altitudinal distribution of melanistic individuals and compare the results obtained for each of the three subspecies; (3) to test the relationship between the onset of melanism and some climatic parameters by implementing distribution models and assessing their link with the detected geographic ranges and subspecific identity. Based on the literature available on the asp viper (Castella et al., 2013; Broennimann et al., 2014; Muri et al., 2015; Martínez-Freiría et al., 2020), we expect to detect a relationship between the onset of melanism and bioclimatic and altitudinal variables in the study area, and to record a similar pattern in the three subspecies.

MATERIALS AND METHODS

Eligibility criteria for melanistic individuals

For the creation of the dataset of melanistic individuals, privately collected data and third-party records (see next section) were used.

In snakes, melanism is a highly variable condition and its expression can vary in intensity, also depending on the part of the body affected (Lorioux et al., 2008; Bruni et al., 2022). Without the availability of photographs taken in a targeted and standardized way, a trait that was verifiable in all the different photos was needed to define the melanistic individuals and evaluate their suitability for the dataset. For this purpose, the overall colouration of dorsal and cephalic scales was chosen, with the following two forms considered eligible:

- Highly dark individuals (tending towards black), with the background colouring presenting very dark reddish-brown tones, with respect to which the dark pattern is only scarcely visible and not very distinguishable (or becomes so only in some light conditions such as under direct sunlight) (Fig. 1A, B);
- Individuals with a uniformly black dorsal colouration – which eventually leaves space only for tiny and sporadic lighter spots –, in which the dorsal pattern is not visible (Fig. 1C).

A distinction between uniformly black individuals and highly dark individuals was not made because the different lighting of the photos would not have guaranteed sufficient precision in this type of evaluation (i.e., a highly dark individual underexposed may appear completely black).

On the other hand, individuals with dark grey or anthracite dorsal background colour and sufficiently distinguishable dark pattern (Fig. S2A, B) were not included in the dataset, as well as individuals in which the dark pattern is anomalously expanded but a lighter background colour is still present and clearly distinguishable (Fig. S2C). The latter was excluded because, with the photographic material available, it would not have been possible to quantify the expansion of the dark pattern and therefore establish a threshold beyond which individuals would no longer be considered eligible.

Finally, individuals in pre-shedding condition were excluded as this is a life phase that is not always suitable for evaluating colouration.

Fig. 1. Examples of melanistic *Vipera aspis* individuals from Italy. A) Highly dark male *V. a. aspis* from Piedmont; B) Highly dark female *V. a. francisciredi* from Lazio; C) Uniformly black female *V. a. hugyi* from Calabria. Photo credit: Matteo R. Di Nicola.

Distribution

Information on the distribution of melanistic *Vipera aspis* in Italy was obtained from observations carried out by three of the authors (MRDN, NB and LL) during herpetological surveys or hiking carried out from July 2011 to August 2022 (32% of the whole dataset), and combined with data received from collaborators and citizen science, updated until May 2023 (68% of the whole dataset). Moreover, a review of both scientific and popular bibliographic sources was undertaken, along with interactions with expert herpetologists and museum curators. The goal was to enhance understanding of the actual distribution of melanistic individuals in Italy, bridging any gaps between the sample and the documented real distribution. No bibliographic or museum records outside the regions already covered by the authors' dataset have been recorded. Furthermore, the few records deemed ineligible due to imprecise coordinates or data unreliability were located in regions already included in the dataset.

Each snake found by the authors was identified on morphological and geographical basis (see Di Nicola, 2019; Di Nicola et al., 2021, 2022); the date, time, coordinates, and altitude were recorded, and a photo of the dorsal pattern was taken. As for the records from third parties, the data was obtained through direct requests to collaborators or surveyors following consultation of citizen science platforms (i.e., Inaturalist.it, Ornitho.it and Observation.org) and social networks such as Flickr.com, and the Facebook group "Identificazione Anfibi e Rettili" (https://www.facebook.com/groups/283231695476830), administered by MRDN. Only the records provided with coordinates with an accuracy of at least 200 m and accompanied by a photo with sufficient resolution were considered. The altitudes of third-party records were obtained using the Google Earth Software (ver. 9.194.0.0).

The records were used to compile the Italian distribution map of melanistic *V. aspis* individuals, following the subspecies spatial division indicated by Barbanera et al. (2009), and an ETRS89 / ETRS-LAEA 10×10 km grid covering the Italian territory. The altitudinal values of melanistic individuals of *V. aspis* individuals were compared with the relative species' values in Italy. The latter were obtained from the dataset provided by the Global Biodiversity Information Facility network, selecting only the directly observed (non-museum) records, equipped with photos, with a coordinate accuracy of at least 180 m (GBIF.org, 2023). Upon extraction of the altitudinal values for all individuals, normality and homoscedasticity of the data were tested using Shapiro-Wilk and Levene's test, respectively. As the normality assumption was not met, statistical differences were compared using a Wilcoxon rank sum test. All analyses were performed in R software version 4.3.1 (R Core Team, 2023).

Bioclimatic niche models

The bioclimatic niche occupied by melanistic individuals of *V. aspis* was modelled using the correlative presence-background software MaxEnt 3.4.1. (Phillips et al., 2017). Only occurrence records with precise coordinates (n = 83) were used for this purpose. A first model was computed using all occurrence records and a mask of the known areal of the species in Italy as background. Then, the records were divided among *V. aspis aspis* (n = 36), *V. aspis francisciredi* (n = 36), and *V. aspis hugyi* (n = 11). Despite the low number of occurrences, these records should constitute a sufficient sample size to produce accurate predictions of species suitability, especially given the restricted extent of the study area (van Proosdij et al., 2015; Støa et al., 2019). Nonetheless, care was taken in order to minimize model overfitting by checking the data for spatial autocorrelation, avoid multicollinearity between predictor variables, and reducing models' complexity (Boria et al., 2014). As each subspecies occupies a distinct geographic region within the Italian peninsula (Barbanera et al., 2009), three additional models were computed, each using a customized background mask representing the area available to the relative subspecies. Finally, in order to test the effect of background selection on model performance and predictions, minimum bounding polygons were drawn from each subspecies occurrence records and used to draw background points for the relative models. The predictor variables used for modelling were downloaded from the WorldClim 2.1. database (Fick and Hijmans, 2017) at a 30 arc-sec spatial resolution. These contained 19 bioclimatic layers (bio1-19), elevation, and monthly solar radiation (srad01-12). Slope aspect (exposition) was derived from elevation. As aspect is a circular rather than continuous variable, it was split into four classes: North (0°-45° and 315°-360°), East (45°-135°), South (135°-225°) and West (225°-315°). Additionally, a land cover raster was obtained from the Global Land Cover National Mapping Organizations (GLCNMO v3, available at https://globalmaps.github.io/glcnmo.html, accessed on 20 August 2023). Values for all 34 layers were extracted at each presence location, and pairwise Pearson's correlation coefficients were calculated. In order to minimize multicollinearity among predictors, a single variable was retained from each correlation group ($|r| > 0.75$). The selected variable was the one deemed most ecologically relevant for the distribution of the species. As high positive correlation was observed among solar radiation values during the period of activity of the species (May-October), an average was calculated (srad05-10) and used as predictor in the models. Upon selection, the variables used for modelling were: mean annual temperature (bio1), mean diurnal temperature range (bio2), annual precipitation (bio12), precipitation of the coldest quarter (bio19), average solar radiation of the month of April (srad04), average solar radiation of the period May-October (srad05-10), land cover (lc) and slope exposition (exp). As melanistic individuals of *V. aspis* are often linked with high altitude environments, we also tested the effect of using elevation (elev), in place of mean annual temperature, to model suitability, given the high negative correlation ($r < -0.9$) between the two. Accordingly, four model combinations were tested for *V. aspis* and for each subspecies: i) only climatic variables (n = 6); ii) climatic variables plus land cover (n = 7); iii) climatic variables plus land cover and slope exposition (n = 8); and iv) climatic variables plus land cover and slope exposition, but using elevation instead of bio1 (n = 8). For each model combination, 10 bootstrap replicates were computed in MaxEnt (default settings, logistic output), each randomly selecting 70% of records for model training and 30% for testing. Permutation importance was used to estimate the relative contribution of each predictor variable to the models. Model performance was evaluated based on the area under the receiver operating curve (AUC), a measure of discrimination between presence and background points (Fielding and Bell, 1997). Additionally, omission rates (OR) were inspected to evaluate model overfitting. These express the proportion of records predicted to fall outside the area defined as suitable by the model, based on various theoretical thresholds. For the purpose of this study, the 10th percentile training presence omission rate (OR10) was adopted (Boria et al., 2014). The relative OR10 logistic threshold was also used to set the minimum suitability threshold to MaxEnt continuous suitability outputs. As the output maps for different model combinations did not differ greatly from each other, an average model was calculated for *V. aspis* and each of its subspecies, applying the average OR10 threshold for minimal suitability. These ensemble models were used for final representation. All analyses were performed in R 4.3.1 (R Core Team, 2023) and QGIS 3.14 (QGIS Development Team, 2020).

Principal component analysis

A principal component analysis (PCA) was computed in order to explore potential differences between the bioclimatic niches occupied by melanistic individuals of *Vipera aspis aspis, V. a. francisciredi*, and *V. a. hugyi*. To do so, the presence and background points used to compute the MaxEnt models were plotted across the two first principal component dimensions, showing their variation along the continuous predictor variables used for modelling (see the "Bioclimatic Niche Modeling" section). All analyses were performed in R 4.3.1 using the packages '*FactoMineR*' (Lê et al., 2008), '*factoextra*' (Kassambara and Mundt, 2020) and '*ggplot2*' (Wickham, 2016).

RESULTS

Distribution

A total of 124 eligible observations of melanistic *Vipera aspis* were collected (40 through personal surveys, 84 through collaborators and citizen science), carried out between 1984 and 2023, of which 90% (n = 112) were from 2010 onwards (Table S1). The altitudinal range of the records covered from 515 to 2212 meters above sea level (mean value = 1533 m a.s.l.; Fig. S3 A).

At the subspecific level, 50 observations of melanistic *V. a. aspis* (five from Aosta Valley and 45 from Piedmont; altitudinal range = 954-2212 m a.s.l., mean value = 1630 m a.s.l.); 58 observations of melanistic *V. a. francisciredi* (35 from Abruzzo, 19 from Lazio, one from Marche, two from Molise and one from Umbria; altitudinal range = 515-2200 m a.s.l., mean value = 1505 m a.s.l.); 16 observations of melanistic *V. a. hugyi* (four from Basilicata, 10 from Calabria and two from Campania; altitudinal range = 645-1783 m a.s.l., mean value = 1333 m a.s.l.) were collected (Fig. 2; Fig. S3A-C).

The 124 observations fall into 57 ETRS89 / ETRS-LAEA 10×10 km squares (Fig. S4); 24 squares concern *V. a. aspis*, 22 *V. a. francisciredi* and 11 *V. a. hugyi*. There was a significant difference (Wilcoxon's W = 13979, p-value < 0.001) between the altitudinal range of melanistic individuals (n = 124; this study) and that of *V. aspis* individuals from the whole species (n = 451; GBIF.org, 2023). On average, melanistic individuals were encountered at a higher altitude (Mean = 1533 m a.s.l.) and showed a more restricted distributional range (Min = 515 m; Max = 2212 m; St. Dev. = 323 m) compared to other individuals (Mean = 976 m a.s.l.; Min = 0 m; Max = 2816 m; St. Dev. = 665 m) (Fig. S5).

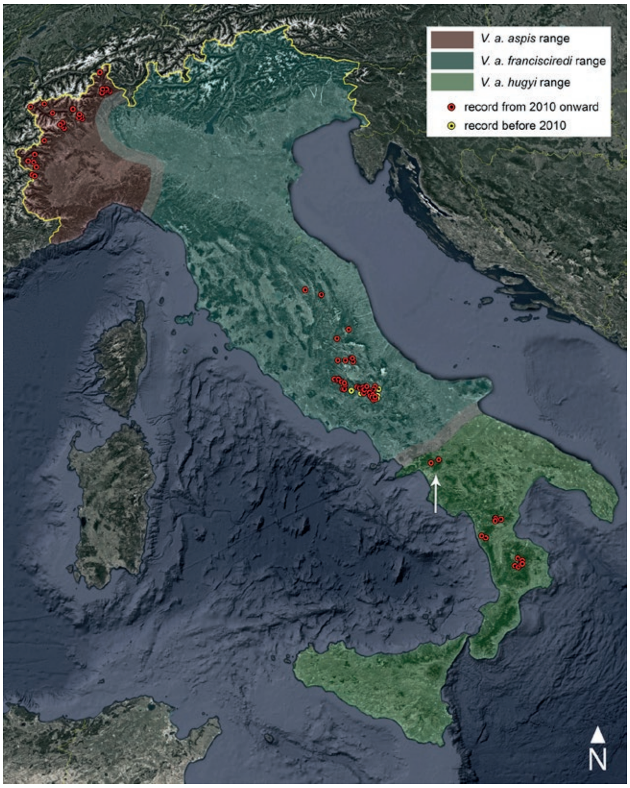

Fig. 2. Map of Italy with the 124 verified observations of melanistic *Vipera aspis* (the positioning of each point is slightly approximated for conservation reasons). The approximative subspecies range is in agreement with Barbanera et al. (2009). Two records indicated by a white arrow come from an area (i.e., Monti Picentini, Campania) of possible intergradation between the subspecies *hugyi* and *francisciredi*, where non-melanistic individuals recurrently have dorsal patterns that can be defined as intermediate between those of the two taxa (MRDN personal obs.). Map credits: Google Earth. Data SIO, NOAA, U.S. Navy, NGA, GEBCO. Image Landsat / Copernicus (modified).

Bioclimatic niche models

All models computed showed robust statistical performances (see Table S1). Mean annual temperature (bio1) was the most influential predictor for all models (42.9-84.1% contribution). Given the high correlation between bio1 and elevation ($r < -0.9$), the latter was the most important predictor for models that did not contain bio1 (24.8-85.1% contribution). The models for *Vipera aspis* were also influenced by the mean solar radiation of the month of April (srad04; 3.5-9.5%) and precipitation of the coldest quarter (bio19; 1.7-6.6%), whereas other variables showed low overall contribution (<5%). The same pattern was observed for model combinations computed for *V. a. aspis* in NE Italy, with srad04 (12.2-16.8%) followed by bio19 (4.2-13.3%). Contrarily, models for *V. a. francisciredi* in Central Italy were mostly influenced by the mean solar radiation of the period May-October (srad05-10; 24.2-32.3%) and mean annual precipitation (bio12; 4.3-11.6%) rather than srad04 (0.5-1.3%) or other variables. Finally, model combinations computed for *V. a. hugyi* in Southern Italy showed low overall contribution of solar radiation (0-4.0%) and were instead most influenced by mean diurnal temperature range (bio2; 17.2- 56.7%) and bio19 (4.9-27.1%). These models also showed highest contribution of land cover (3.2-7.2%) and slope exposition (3.7-4.1%) compared to the previous models.

Areas predicted as suitable for melanistic individuals of *V. aspis* were in agreement across the four model combinations and also between the ensemble model and the independent models computed

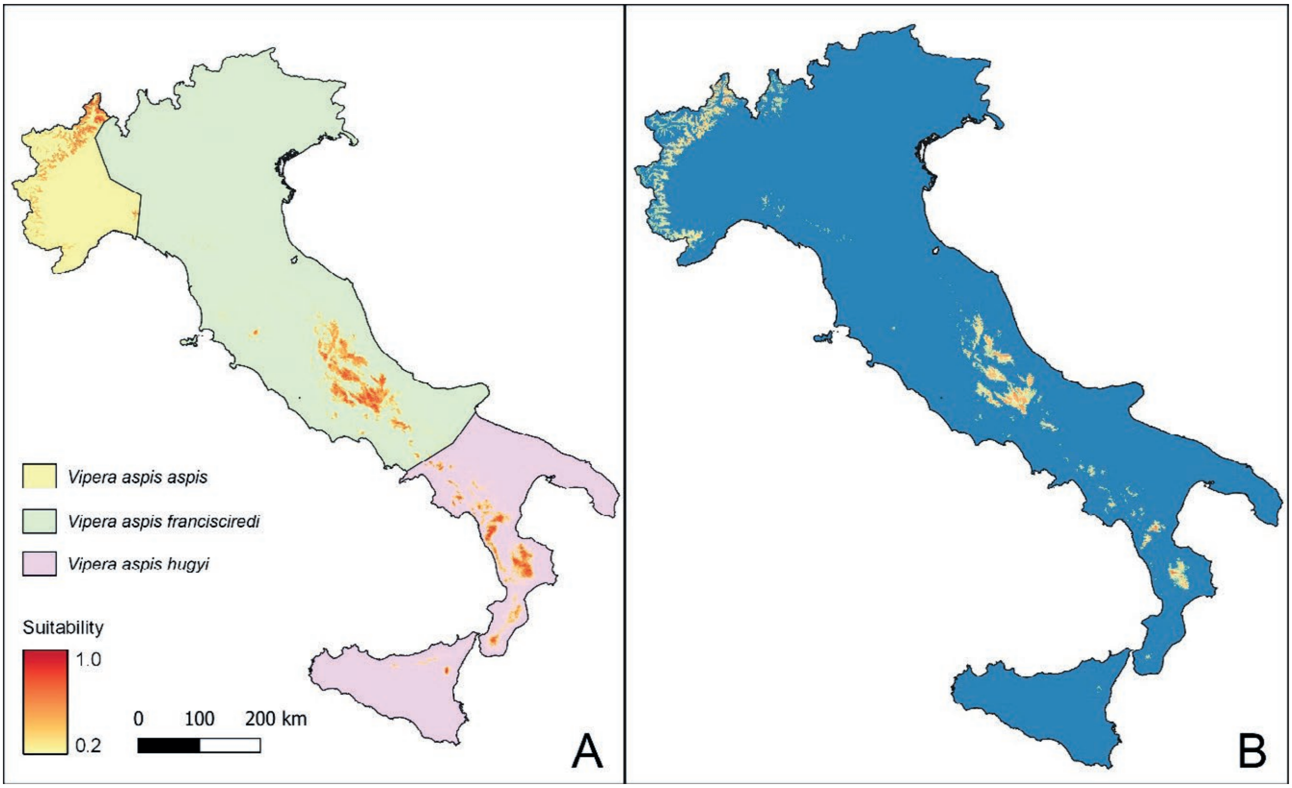

Fig. 3. Predicted bioclimatic suitability for melanistic individuals of *Vipera aspis* in Italy. A) Suitability models computed independently for subspecies *V. a. aspis, V. a. francisciredi* and *V. a. hugyi* within their relative geographic ranges (according to Barbanera et al., 2009). B) Ensemble model computed using all occurrence records together and the complete Italian range as background. The outputs shown represent the average of four model combinations, each replicated 10 times (see materials and methods for details). Warmer colours indicate higher suitability, the background falls below the minimum suitability threshold for each model (see Table S2).

for each subspecies (Fig. 3A, B). The average model shows areas predicted as suitable for the species by all model combinations, using the relative OR10 as threshold for minimum suitability for each subspecies (Table S2). The model predicts high suitability across three main areas, respectively in Northern, Central and Southern Italy: in Northern Italy (involving Piedmont and secondarily Val d'Aosta), the western sector of the central Alps (Lepontine and Pennine Alps), and, with less involvement, the eastern portions of the Graian and Cottian Alps in the Western Alps; in Central Italy (mainly involving Abruzzo, but also Marche, Umbria, Lazio, Molise and Campania), the central Apennines, where the Abruzzo Apennines are mainly involved, including in the North the southern portion of the Umbria-Marche Apennines and, to the south, the northern portion of the Southern Apennines (specifically, the Samnite Apennines); in Southern Italy (involving Calabria and a small part of Southern Basilicata), the Southern Apennines involving the Lucanian Apennines and the Calabrian Apennines.

It is important to highlight that the models presented in Fig. 3 are produced using few occurrence records localized over a large available background. As such, they depict a conservative projection of bioclimatic suitability, intended to highlight areas sharing very similar environmental conditions to those where melanistic *V. aspis* individuals were recorded. Vice versa, restricting the modelling background to areas directly surrounding the presence records produced significantly larger predictions of bioclimatic suitability, which include the Northern Apennines and Eastern Alps (Fig. S6). However, projections based on restricted backgrounds are strongly influenced by environmental variables and features being limited to the range encountered during training. The treatment of variables outside their training ranges is likely to have a strong effect on predicted suitability, so predictions in those areas should be treated with strong caution (Elith et al., 2011. For these reasons, we focused our discussion on the most conservative models for the purposes of this study.

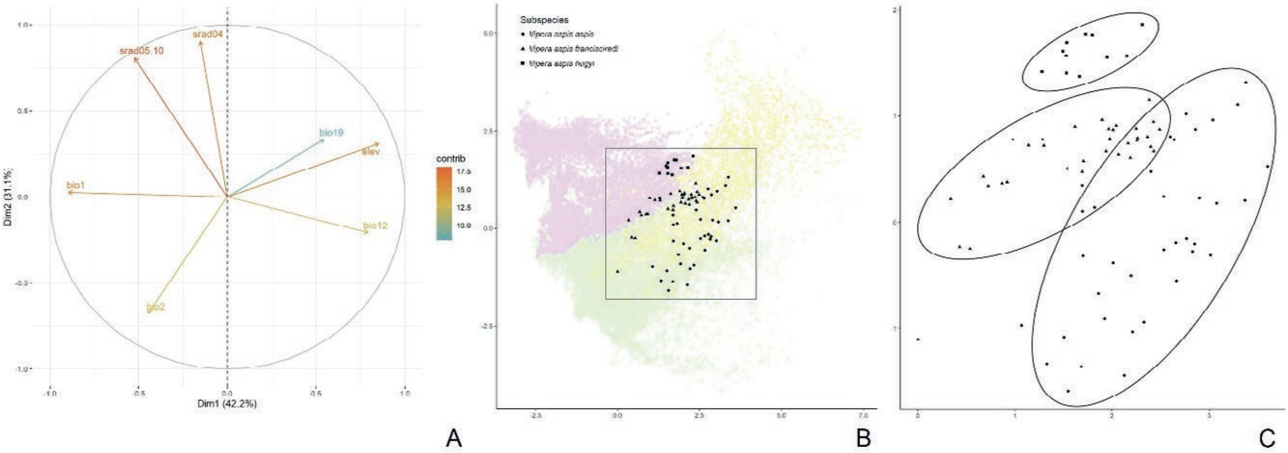

Fig. 4. Principal component analysis of melanistic individuals of *Vipera aspis* in Italy. A) Bioclimatic variables used for the analysis, defined by their contribution to the first two dimensions (warmer colours indicate higher contribution). B) Distribution of the background (coloured points) and occurrence records (black points) represented in bidimensional environmental space, divided by subspecies (different colours and shapes). C) Focus on the inset of the panel B, showing only the occurrence records (ellipses indicate the 95% confidence interval).

Principal component analysis

The results of the principal component analysis (PCA) are shown in Fig. 4. The first two PCA dimensions explain 42.2% (Dim1) and 31.1% (Dim2) of the variation observed in the data. Mean annual temperature (bio1), elevation (elev), and annual precipitation (bio12) contribute mostly to the variation along the first dimension, whereas solar radiation (srad04 and srad05-10) and diurnal temperature range (bio2) influence most of the variation along the second dimension. Precipitation of the coldest quarter (bio19) shows little contribution. The distribution of the background points used for the MaxEnt models shows a clear distinction in the bioclimatic environments available to the three subspecies of *Vipera aspis* in Italy (Fig. 4A). Nonetheless, the presence records of melanistic individuals used for the analyses occur within similar bioclimatic conditions, shared by the background environments. On a finer scale, a distinction is present between the bioclimatic niche occupied by melanistic individuals of the three subspecies, mostly along the second dimension of the PCA (Fig. 4B). However, due to the contribution of solar radiation and diurnal temperature range to Dim2, this difference could be attributed to the latitudinal gradient rather than a specialization towards different niches.

DISCUSSION

Vipera aspis can be considered as one of the most geographically widespread snakes in Italy (Zuffi, 2006), but despite that, melanistic individuals of this species appear to be characterised by a very limited and fragmented distribution (Bruno et al., 1985; Grano et al., 2017).

For elusive and not always easily contactable animals such as vipers, there is always the risk of underestimation in sampling. Nevertheless, considering the multiple channels exploited for data acquisition and the quantity of *V. aspis* records examined from each area in Italy, we believe that the mapping of melanistic individuals here presented does not deviate significantly from the actual distribution of these snakes.

According to our distributional data, melanistic individuals of *V. aspis* seems to be restricted to alpine and temperate bioclimatic regions along the Alps and the southern Apennines, with no evidence of melanistic individuals within the Mediterranean bioclimatic region (Attorre et al., 2007) (Fig. 2). These results are in strong agreement with the outputs of our bioclimatic niche modelling analyses (Fig. 3A, B). However, the overall species model highlighted a few areas of modest suitability that currently lack formal records of melanistic individuals of *V. aspis*. In particular, low but still noticeable suitability was found in proximity of Mount Etna, a small portion of the Ligurian Apennines and within a restricted area of the southern part of the Tuscan-Emilian Apennines (Fig. 3B). These areas are highly explored from a herpetological point of view (e.g., Zuffi, 1984; Bassini et al., 1991; Turrisi and Vaccaro, 2001, 2004), and so far, no melanistic individuals of *V. aspis* have been formally found. Even if this phenomenon may be the product of research biases, correlated with the cryptic nature of the taxon, mechanisms such as predation pressure, genetic isolation, and biographic pattern may explain the lack of melanistic phenotypes of *V. aspis* within the suitable areas highlighted by our models. These potential explanations become even more relevant when considering the broader suitability zones provided by the less conservative model shown in Fig. S6.

In particular, populations of *V. a. hugyi* from Sicily and Southern Apulia represent an old clade which diverged from the rest of the subspecies around 1.5-1.2 Mya and poorly contributed to the expansion of the taxon in the southern part of the Italian peninsula during the Pleistocene (Barbanera et al., 2009). On the other hand, populations of *V. a. hugyi* from the rest of its geographic range were subject to more complex patterns of gene flow which involved introgression with *V. a. francisciredi* (Barbanera et al., 2009). The melanistic phenotype in the latter populations could have independently arisen via random mutation or, it may be the product of historic introgression with *V. a. francisciredi* as found in other species (McRobie et al., 2019; Storniolo et al., 2023).

Therefore, the absence of melanistic individuals of *V. a. hugyi* in the suitable areas highlighted by the model may be the consequence of the lack of the phenotype among the highly divergent and genetically isolated populations of southern Apulia and Sicily.

Moreover, modest suitability was highlighted in the proximity of the Lombardy Prealps, part of the Lepontine Alps and a small sector of the Rhaetian Alps in north-western Lombardy (Fig. 3B). Interestingly, anomalously dark grey individuals, characterised by the presence of visible dorsal pattern and high concentration of melanin (*sensu* Fănaru et al., 2022), have been observed in the highlighted areas (MRDN and AVP, pers. comm.) (Fig. S2). This geographic region is likely to represent a transition zone between *V. a. aspis* and *V. a. francisciredi* (Ursenbacher et al., 2006), but formal genetic studies from this area are currently lacking (Barbanera et al., 2009), thus individuals from north-western Lombardy are formally ascribed to the subspecies *francisciredi* (Ursenbacher et al., 2006; Di Nicola et al., 2021). As the melanistic phenotype is very common in the alpine populations of the nominal subspecies (Mebert et al., 2011), the presence of partially melanistic individuals of *V. a. francisciredi* in the area may be the result of genetic introgression between the two subspecies. Another possibility involves the sudden increase of random mutations and interactions between genes correlated with pigment regulation in the focal populations, such as the pro-opiomelanocortin gene (POMC) (Ducrest et al., 2014). It is possible to speculate that partial melanism may have been then maintained at low frequency within these populations via weak selection due to environmental-related advantages (Martínez-Freiría et al., 2020).

In this regards, our bioclimatic niche modelling analyses highlighted a general correlation between the distributional pattern of melanistic *V. aspis* and various bioclimatic variables (Table S2). Specifically, the strong influence of mean annual temperature and elevation seems to support the implications of the thermal melanism hypothesis (Watt, 1968; Clusella-Trullas et al., 2007b). According to the latter, melanistic individuals should be in fact favoured in cooler environments thanks to their ability to thermoregulate in sub-optimal thermal conditions. The adaptive role of melanisms in reptiles, and in particular in snakes, has been highly debated. While some studies have highlighted physiological advantages correlated with melanism (Luiselli, 1993; Bittner et al., 2002; Clusella-Trullas et al., 2007a; Tanaka, 2009; Moreno Azócar et al., 2020; Hantak et al., 2022), others failed to find any related evidence of selection (San-Jose et al., 2008; Matthews et al., 2016; Bury et al., 2020, 2022; Fănaru et al., 2022). Within European vipers, the benefits associated with a melanistic pigmentation appear to be context-dependent with a high variability in terms of observed advantages across populations (Madsen and Stille, 1988; Luiselli, 1992; Monney et al., 1995, 1996; Strugariu and Zamfirescu, 2011; Broennimann et al., 2014; Lucchini et al., 2020). Our models indicated temperature and elevation as main predictors of suitability for melanistic *V. aspis* individuals in Italy. However, differences were observed among the three subspecies in their response to other predictor variables, which could be the consequence of bioclimatic niche specialization.

When annual average temperature was excluded in the models, altitude represented the most important predictor for the distribution of melanistic individuals of *V. aspis*, apart from *V. a. hugyi*. Melanistic individuals were found, on average, at significantly higher elevations than non-melanistic individuals (Fig. S5), nevertheless, both general and subspecies' altitudinal averages of melanistic *V. aspis* were found below the treeline within both the Alps and the Apennines (Bonanomi et al., 2020; André et al., 2023). This was further corroborated by the high ratio of melanistic individuals found within – or at the margins of – forested areas (authors, pers. comm.) and by the high suitability in forested areas highlighted by our models (Fig. S7). Similar results were shown by Muri and colleagues (2015), with substantial difference in microhabitat choice between melanistic and non-melanistic *V. aspis* individuals, with the former exploiting environments characterised by higher vegetation productivity. Exploitation of different microhabitats between non-melanistic and melanistic individuals is likely due to the ability of the latter to inhabit areas where their lower skin reflectance may represent an ecological advantage, such as forested areas characterised by low solar radiations (Borgianni and Paolino, 2020; Martínez-Freiría et al., 2020). Due to the less cryptic nature of melanistic individuals, inhabiting forested areas may reduce predator detection probability, especially in the case of avian taxa (Andrén and Nilson, 1981; Monney et al., 1996; Tanaka, 2009). On the other hand, phenotypes such as the blotched or the concolor ones seem to be more advantageous in open areas characterised by low vegetation coverage (Castella et al., 2013; Dubey et al., 2015; Tessa, 2016; Pizzigalli et al., 2020).

At a finer scale, melanistic individuals from the three analysed subspecies seems to be characterised by three quite distinct bioclimatic niches (Fig. 4). Surprisingly, the breadth of the nominal subspecies' bioclimatic niche resulted wider compared to the others despite a quite restricted geographic distribution (Zuffi, 2006; Di Nicola et al., 2021). This seems to showcase the ability of melanistic *V. a. aspis* to adapt to a wide variety of heterogenous bioclimatic conditions (Brodmann et al., 1987). On the other extreme, melanism in *V. a. hugyi* appears to be correlated with a very narrow set of bioclimatic and environmental conditions. While the thermal melanism hypothesis implicates a positive correlation between skin reflectance and solar radiations (Clusella-Trullas et al., 2008), melanistic individuals of the latter subspecies seem to follow an opposite trend, with their bioclimatic niche being characterised by the highest levels of solar radiations among the investigated subspecies (Fig. 4). Melanistic pigmentation and relative low reflectance should provide a stronger protection against UV rays exposure compared to lighter pigmentation, thus shielding internal organs from UV-related damages (Clusella-Trullas et al., 2008; Goldenberg et al., 2021). According to the Gloder's rule, individuals characterised by high level of melanism should be favoured in warm and humid environments, due to better protection from solar radiations and pathogens (Delhey, 2017). Melanistic individuals of *V. a. hugyi* have been observed in forested areas, where levels of humidity are likely to be higher compared to other habitats inhabited by this subspecies (Brodmann, 1987; Di Nicola and Meier, 2013). Moreover, at low latitudes, solar radiations rather than air temperature may represent a better predictor for skin reflectance (Clusella-Trullas et al., 2008). Therefore, the observed correlation between the assessed bioclimatic variables and melanistic individuals of *V. a. hugyi* seems to agree, at least in part, with the implications of the Gloder's rule.

Nevertheless, it is critical to point out that this apparent bioclimatic niche partitioning among the assessed subspecies may be, at least in part, an artefact derived by changes in environmental conditions caused by latitudinal gradients rather than pure differences in bioclimatic niches (Andersen et al., 2022). A good amount of the variation in the PCA analysis is in fact primarily correlated with solar radiation and daily temperature variation, which are both factors highly affected by latitude. Thus, while melanistic individuals from the three subspecies may have similar general bioclimatic requirements (Fig. 4), the observed niche partitioning could be the results of differences in the available bioclimatic conditions at different latitudes.

In the same way, some of the observed similarities among the relative bioclimatic niches may be caused by the homogenizing effect of altitude on bioclimatic conditions (Körner, 2007). Therefore, due to the complex interplay between the above-described factors, our results at the subspecies level must be taken with caution. Further studies aimed at characterising the microhabitats of melanistic individuals of *V. aspis ssp.* are thus needed to test for our observed differences in inter-subspecific niche partitioning.

In conclusion, this study reported, for the first time, an accurate geographic distribution of melanistic individuals of *Vipera aspis* in Italy. Moreover, on a broad scale, our bioclimatic niche modelling results for melanistic *V. aspis* seems to agree with the insights provided by the thermal melanism hypothesis. On the other hand, finer scale analyses of bioclimatic niche partitioning between the three subspecies of *V. aspis* portraited a discrete level of differentiation, although confounding factors may overestimate the real extent of bioclimatic niche differentiation.

ACKNOWLEDGEMENTS

The authors would like to thank all the people who have kindly provided us with their finding data and photos of melanistic *Vipera aspis* from Italy: Angela Tomei, Angelina Iannarelli, Bruno Di Cecco, Carlo Zanetta, Chiara Santini, Daniel Li Veli, Daniele Seglie, Domenico Belcastro, Emanuele Lotti, Ezio Giuliano, Fabrizio Bernardi, Fabrizio Moglia, Federica Gaydou, Francesco Di Toro, Francesco Raffaele, Gaetano De Persiis, Giandomenico La Barbera, Gianluca Congi, Gianni Liguori, Giulia Tessa, Luciano Caporale, Maria Felicia Coppola, Mario Marconi, Maurizio Caniglia, Maurizio D'Amico, Maurizio Lo Fiego, Mauro Saltarelli, Michela Mastrella, Mimmo Ippolito, Mirko Galuppi, Pasquale Buonpane, Raffaele Ferretti, Sergio De Santis, Tiziano Fioriti and Valter Rossotti. Author's records were carried out in field surveys authorized by the following permits: MATTM reg. 0012442, 13.06.2017; ISPRA Prot. 25913, 26.5.2017; Parco Nazionale d'Abruzzo, Lazio e Molise Prot. N.

0005299/2017, 26/06/2017; MATTM reg. 0083124, 16/10/2020; Prot. ISPRA 46387, 12/10/2020; MITE reg. 0024526, 28.2.2022; Prot. ISPRA 0009384/2022 del 23.02.2022; Parco Nazionale d'Abruzzo, Lazio e Molise Prot. N. 0004651/2022, 07/04/2022. AVP is supported by the Natural Environment Research Council grant number NE/S007164/1.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <http://www-9.unipv.it/webshi/appendix/index.html> manuscript number 15271.

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Acta Herpetologica 19(1): 29-39, 2024
DOI: 10.36253/a_h-15334

Effects of traffic noise on calling activity of *Aplastodiscus leucopygius* (Anura, Hylidae)

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Submitted on: 2023, 8th November; revised on: 2024, 13th January; accepted on: 2024, 18th January.
Editor: Ilaria Bernabò

Abstract. Advertisement calls are the main communication form of anurans, and other individuals can use it to evaluate several aspects of the calling individual. In this context, environmental disturbances, such as traffic noise, can potentially affect this recognition. Therefore, this study aims to evaluate the response of *Aplastodiscus leucopygius* to traffic noise in a fragment of Atlantic Forest within the city of São Paulo. The experimentation consisted of recording the calling individual previously, during and after an exposure to urban noise. After that, individuals were measured to evaluate the Scaled Mass Index (SMI), and individual and environmental temperatures were taken. Also, considering that individuals of this species present harmonic shifting, we tried to evaluate which factors (individual, acoustic, or environmental) are associated with this phenomenon. We observed that the individuals showed an increase in call activity after exposure to traffic noise, but none of the evaluated aspects here could explain the harmonic shifting in their calls. Considering that this increasing on call activity also means an increasing of individual's expense of energy, traffic noise is potentially harmful to the communication of *A. leucopygius* and, consequently, to its permanence in the site.

Keywords. Amphibian, Hylinae, anthropogenic noise, advertisement call, Atlantic Forest.

INTRODUCTION

Males of anurans use advertisement calls to attract females and segregate territories (Toledo et al., 2015). While these calls are emitted, other individuals use hearing to evaluate several aspects of the calling individual through call characteristics. These characteristics can be divided into two groups: spectral, such as dominant frequency, frequency bandwidth, and harmonics, and temporal, such as call rate, call duration, and interval between calls (Köhler et al., 2017). Spectral variables are less sensitive to environmental characteristics, such as temperature and precipitation, and are more related to intrinsic aspects of calling individual (Tonini et al., 2020; Maria et al., 2023), unlike temporal variables, which can be influenced by several aspects of environment surrounding the calling individual (Lingnau and Bastos, 2007; Both and Grant, 2012; Caorsi et al., 2017).

Since most anuran communication is performed through sound, some sound-related aspects must influ-

ISSN 1827-9635 (print)
ISSN 1827-9643 (online)

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ence this process. One of them is environmental noise, which can modify the call of individuals or even impair communication since it interferes with the auditory information transmitted to the receiver (Feng and Schul, 2007). Among these environmental noises, it is possible to distinguish two groups: natural noises, which are a consequence of the natural environment where each individual is inserted, such as rivers or wind (Lingnau and Bastos, 2007), and anthropogenic noises, which are human-produced and can promote an impact on natural populations. Among the effects of anthropogenic noises, such as traffic noise, it is possible to observe an increase in recognition time of males by females in the reproductive display (Bee and Swanson, 2007), decreased activity, which reduces the reproductive success (Kaiser et al., 2011), and the increase of the amplitude of call, which potentially results in waste of energy that could be used for reproduction (Gerhardt and Klump, 1988; Lima et al., 2022).

One of the characteristics of calls on several species is the presence of harmonics. They consist of frequencies that are separated in bands multiple of the lowest resulting from periodic patterns of oscillation (Köhler et al., 2017). Several anuran species present their calls consisting of observable harmonics, such as *Boana albomarginata* (Giasson and Haddad, 2006; Rebouças et al., 2020; Rebouças, 2021), *Eleutherodactylus iberia* (Estrada and Hedges, 1996) and those of *Aplastodiscus* genus (Zina and Haddad, 2006a,b). In this way, it was already observed that some species present the dominant frequency of their calls shifting between harmonics, such as *Boana albomarginata* (Rebouças et al., 2020) and *Aplastodiscus leucopygius* (Zina and Haddad, 2006b), but the possible causes of this phenomenon remain understudied.

Although anthropogenic noise can have harmful effects on anuran populations, its specific effects are highly variable (Zaffaroni-Caorsi et al., 2023). Some species modify their call activity, increasing the call rate and duration in noisy environments (Lima et al., 2022), while others present no effect on call activity (Cunnington and Fahrig, 2010), or even some species are reported to shift their call frequency (Parris et al., 2009). Thus, evaluating anthropogenic noise effects on anuran calling activity is necessary to predict consequences of communication disturbance in population or species level. In this study, we aimed to experimentally assess the impact of anthropogenic noise on the call activity of an isolated population of *Aplastodiscus leucopygius* in an urban forest fragment within the municipality of São Paulo, Brazil. Considering that this species commonly occurs in habitats far from anthropogenic noise sources, we evaluated if this noise could represent a factor that could impair this occupancy. Also, we evaluated which factors are able to predict the shifting of dominant frequency in the harmonics of calls. Here, we tested the hypothesis that individuals modify their calling activity structure as a consequence of anthropogenic noise. Specifically, we evaluated if the magnitude of this modification is related to (i) intrinsic aspects of individual calling, such as body condition, or (ii) temperature of the environment where each individual is inserted. Also, we evaluated if (iii) harmonic shifting is more related to extrinsic than intrinsic aspects, as proposed by Zina and Haddad (2006b).

MATERIALS AND METHODS

Sampling site and species

Individuals of *Aplastodiscus leucopygius* were captured in the Parque Estadual das Fontes do Ipiranga (PEFI), an urban Atlantic Forest fragment in the municipality of São Paulo, Brazil. The specific collecting site was between two boulevards, Avenida do Cursino and Avenida Miguel Stefano (23º38'21.55"S, 46º37'7.25"W), at a distance of 514 m and 891 m, respectively. We selected this specific place to minimise the influence of other anthropogenic noise in our experiment (Fig. 1).

Aplastodiscus leucopygius is a species of the Hylidae family, with occurrence in the Atlantic Forest in the states of Rio de Janeiro and São Paulo, Brazil (Frost, 2023). It breeds in small streams or ponds, calls in marginal vegetation above the water body, and lays eggs in subterranean nests constructed by males (Zina and Haddad, 2006a). Males of this species present three call types: territorial, multi-note call, and advertisement call, which is the most

Fig. 1. Sampling site of *Aplastodiscus leucopygius* in the Estadual das Fontes do Ipiranga, municipality of São Paulo (photo by Victor Fávaro).

common (Haddad and Sawaya, 2000; Zina and Haddad, 2006b). Advertisement calls are described as composed by four visible harmonics, with the dominant frequency in the first or third harmonic.

Experiment

We conducted this study from November 2020 to April 2021, between 19:00 and 22:30, in the natural calling site of individuals. Our experiment consisted of recording the call of 20 males of *A. leucopygius* during three uninterrupted minutes while they were exposed to three consecutive trials of one minute each: (i) pre-playback, when each individual was recorded with no influence of noise (control trial); (ii) playback, when each individual was recorded during the emission of traffic noise by a speaker; and (iii) post-playback, when each individual was recorded after speaker turned off. Thus, each individual was exposed sequentially to a pre-playback, playback and post-playback trial. Both male's calls and traffic noise recordings were made with a YOGA 9600 unidirectional microphone and a Tascam DR-40 digital recorder. The traffic noise sound for the playback trial was recorded in the Avenida Miguel Stefano during the rush hour, for one minute. In all playback trials, individuals were exposed to the same traffic recording (Supplementary Materials, Fig. S1). During the recording of calls, the microphone was placed at a distance of 1 m from the focused individual, and during the playback trial, the speaker was placed at the same distance in a parallel position to the microphone, in a position of ~45º of the individual, to reduce the interference of sound emission into the recording (Fig. 2). The recordings were made at a sampling rate of 44.1 kHz and with 16 bits of resolution. For the playback trial, we used a JBL Extreme speaker because of its relatively good frequency response (Fig. S2) and Bluetooth connection, which allowed us to perform the experiment in the natural environment of individuals. The noise was emitted through its connection to a cell phone. We kept the traffic noise emission as it was recorded, which implies some variation of levels, which ranged from -55 until -85.4 dBFS (scale C), measured with a digital decibel meter Instrutherm DEC-500 during all recording periods. Thus, we used a decibel meter to calibrate the sound pressure of the speaker to the same levels at 1 m distance (Fig. 3). Although inserted between two avenues, the noise generated by them does not reach the collecting site (see Lima et al., 2022). We avoided performing the experiments on rainy or windy days to reduce the further influence of other noises that were not the playback, and to record individuals close to each other to ensure that individuals would be exposed to only the specified time of noise.

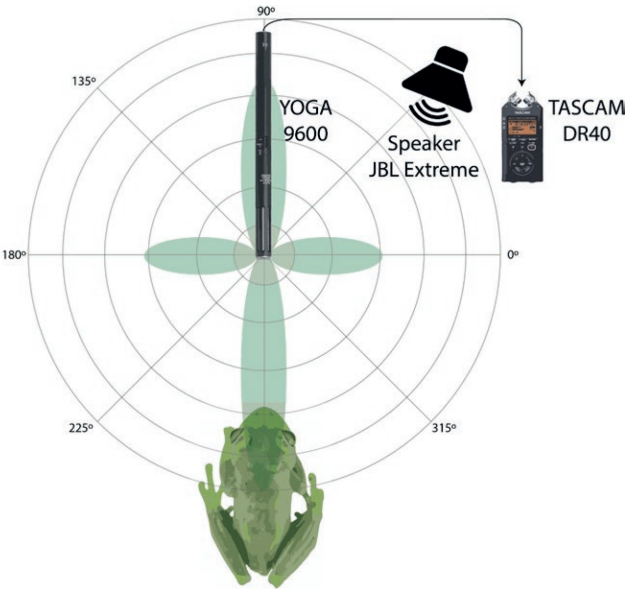

Fig. 2. Experimental design, with the location of the speaker according to the recording range of the microphone, with the aim to reduce the influence of traffic noise exposed to the recorded individual on posterior analysis (individual not in scale).

After the experiment, we captured the individuals and measured their snout-vent length (SVL) with a digital calliper (to the nearest 0.01 mm), and their weight with a digital scale (to the nearest 0.1 g). These measurements were used to calculate the Scaled Mass Index (SMI) of individuals (Peig and Green, 2009). This is a measurement based on the population parameters used as an indicator of energy reserves of an animal (Peig and Green, 2009). At the collecting site, we evaluated the air temperature with a mercury thermometer (to the nearest 0.1 ºC) and the body temperature of the focal individual, with an infrared thermometer (to the nearest 0.1 ºC). To avoid performing the experiment twice with the same individual and consequently avoiding pseudo replications, each individual was marked with Visible Implant Elastomer, applied subcutaneously in the ventral part of the thigh (Nauwelaerts et al., 2000), and recordings of recaptured individuals were discarded.

Call Analysis

We analysed all calls in Raven Pro 1.6 (K. Lisa Yang Center for Conservation Bioacoustics, 2019) with the following settings: Hann window type with size of 512 samples, 3 dB filter bandwidth of 2.7 kHz, time grip with an overlap of 50%, hop size of 256 samples, DFT size of 512 samples and spectral resolution of 1.88 kHz. Spectrograms were visualised with contrast of 75% and bright of 60%. Recordings in all trials were deposited according to previous recommendations (Dena et al., 2018, 2020) in Fonoteca Neotropical Jaques Veilliard (FNJV 58961 - 59020).

We used four spectral and three temporal variables in call analysis. As spectral, we used the dominant frequency through the function 'peak frequency'; minimum and maximum frequency, obtained through the function 'frequency at 5%' and 'frequency at 95%', respectively; and the bandwidth, which was the difference between the minimum and maximum frequencies. We used this latter function to avoid the inclusion of frequency measurements that were not related to individual calls (see Köhler et al., 2017). As temporal variables, we used the interval between calls, the number of calls in the recorded minute, and the duration of the call.

Statistical Analyses

To verify multicollinearity between variables in all models, we performed an initial model and used the variation inflation factor (VIF) through the "vif" function of the "*car*" package (Fox and Weisberg, 2019). We checked the performance of each model with the package "*performance*" (Lüdecke et al., 2020) (Fig. S3-S9). We considered an indicator of multicollinearity when the variables reached a VIF higher than 10 (Quinn and Keough, 2002). We used a Generalised Linear Mixed Models analysis (GLMM) to evaluate if the trial (pre-playback, playback, and post-playback) influenced each of the measured variables of calls. We excluded the minimum frequency and frequency bandwidth of analysis during the playback trial since traffic noise overlapped these measurements. Considering that we have several measurements of the same individual in each trial, we used "individual" as a random factor and Gaussian family with identity link, for analysis with dominant, minimum, and maximum frequencies, frequency bandwidth, call duration and the interval between calls as response variables. To evaluate the influence of trial on the number of calls, we used a GLMM with a Poisson family and logit link. Additionally, we ran a GLM, with Gaussian distribution and "identity" link, using the residuals of those models, which showed the influence of trial on a specific call variable as a response, and SMI, individual temperature and air temperature as predictors to evaluate which factor influenced in the response of individuals to traffic noise. Also, to evaluate which factor is better predicting the harmonic shifting in calls (Zina and Haddad, 2006b), we also used a GLMM with harmonic of dominant frequency (coded as 0 for the first harmonic and 1 for the third) as the response variable and, as the predictor, the trial (only used pre- and post-playback trials, since playback could give a false estimative of first harmonic due to experimental noise), temporal variables (call duration, interval between calls), minimum and maximum frequencies, individual variables (SMI and body temperature), and habitat variables (air temperature and number of surrounding individuals calling). We used a binomial distribution with logit link and individual as random factor.

To determine the effect of each factor on the response variable, we used the analysis of variance with the type II Wald chi-square test through the "Anova" function of the "*car*" package (Fox and Weisberg, 2019). All analyses were performed in R 4.2.1 (R Core Team, 2022) with a confidence interval of 95 %, parameters of all models are available in supplementary material, and information in tables were provided according the best practices to allow transparency and reproducibility with the package "*report*" (Makowski et al., 2023).

RESULTS

Calls of *Aplastodiscus leucopygius* consisted of a single-pulsed note with most energy concentrated in three harmonics (Fig. 3). We observed that among spectral parameters of call, individuals of *A. leucopygius* showed a reduction in the dominant frequency during playback trial which was not observed for maximum frequency. However, in relation to temporal parameters, during playback trial calls were less frequent and more spaced (Table 1, Fig. 4).

None of our variables presented a VIF higher than 10, so we considered all in our analysis (minimum frequency: 1.83; maximum frequency: 7.67; dominant frequency: 1.37; bandwidth: 8.81; interval between calls: 2.21; number of calls: 2.64; call duration: 1.26). Our analyses showed that the complete trial (pre-playback, playback and post-playback) presented a significant influence on dominant frequency ($\chi^2 = 10.28$, $P = 0.006$), call duration ($\chi^2 = 7.17$, $P = 0.03$), interval between calls ($\chi^2 = 43.47$, $P < 0.001$) and number of calls ($\chi^2 = 494.87$, $P < 0.001$), but presented no influence on maximum frequency ($\chi^2 = 1.36$, $P = 0.51$), minimum frequency ($\chi^2 = 0.19$, $P = 0.66$), and bandwidth ($\chi^2 = 0.44$, $P = 0.51$) (Table 2, Figs. S3-S9). Specifically, during the playback trial, individuals showed a reduction in call duration, call rate, and dominant frequency and an increase in the interval between calls. Additionally, during the post-playback trial, the call rate increased compared to the two previous trials (Table 2).

Individuals presented a weight of 4.09 ± 0.47 g (3.1-4.9 g), SVL of 38.56 ± 2.03 mm (32.9-42 mm) and SMI

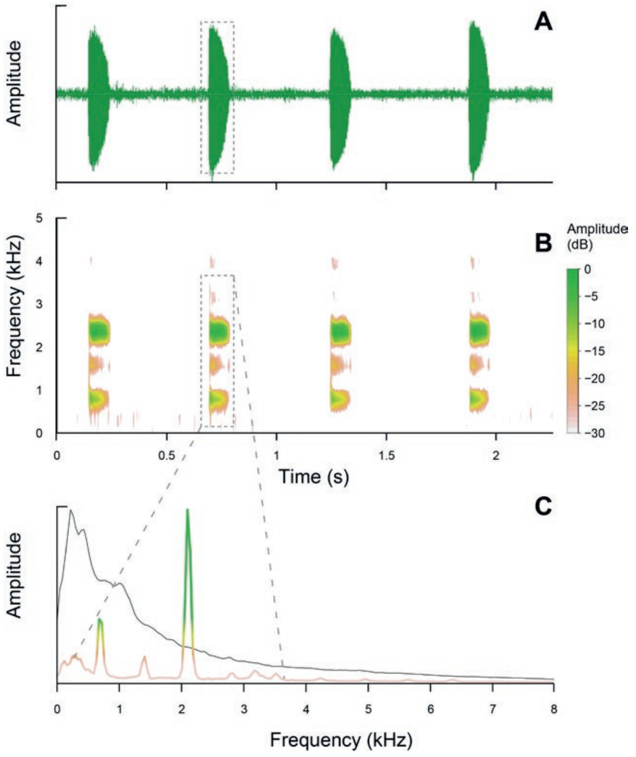

Fig. 3. Call of *Aplastodiscus leucopygius*: Oscillogram (A), Spectrogram (B) and frequency spectrum of the call in relation to the noise of boulevard (grey) (C).

Table 1. Summary statistics of *Aplastodiscus leucopygius* call during pre-playback, playback and pos-playback trials.

	Pre-playback	Playback	Post-playback
Minimum Frequency (Hz)	740.1 ± 95.83 (598.7 - 1097.8)	-	736.4 ± 85.57 (580.9 - 1015.9)
Maximum Frequency (Hz)	2551 ± 265.37 (2212 - 3201)	2535 ± 213.1 (2248 - 3159)	2558 ± 292.4 (2205 - 3328)
Dominant Frequency (Hz)	1830.5 ± 686.56 (750 - 2449.2)	1629.5 ± 632.7 (703.1 - 2374.2)	1832.3 ± 704.74 (750 - 2437.5)
Bandwidth (Hz)	1811 ± 281.67 (1254 - 2451)	-	1821 ± 300.09 (1333 - 2578)
Call duration (s)	0.098 ± 0.008 (0.081 - 0.109)	0.093 ± 0.11 (0.067 - 0.107)	0.097 ± 0.008 (0.082 - 0.109)
Interval between calls (s)	0.709 ± 0.28 (0.4 - 1.67)	2086 ± 1.53 (0.64 - 7.16)	0.604 ± 0.183 (0.384 - 1.079)
Number of calls	79 ± 2.39 (33 - 121)	33.25 ± 18.14 (3 - 80)	89.35 ± 21.73 (51 - 126)

of 4.11 ± 0.52 (3.37-5.21). Body temperature had an average of 20.77 ± 1.18 °C (18.4-22.6 ºC), and air temperature had an average of 22.68 ± 0.98 °C (20-24 ºC). None of these variables were excluded based on their VIF (SMI: 1.22, body temperature: 1.36, air temperature: 1.46). None of these variables showed any influence on the response of individuals to traffic noise (Table 3).

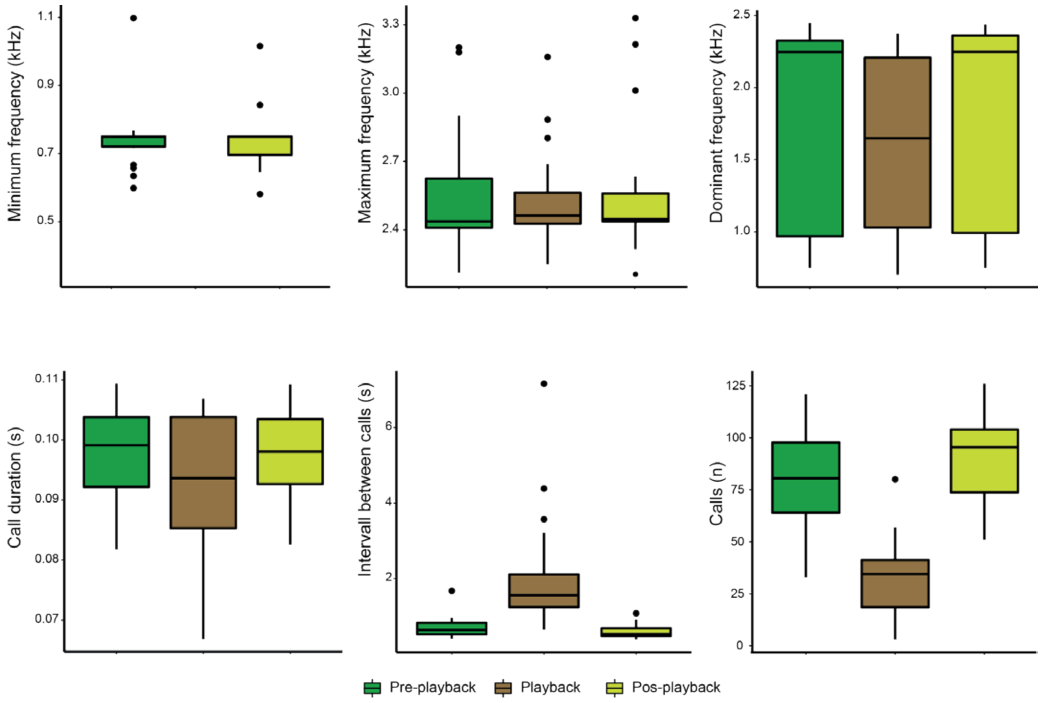

Fig. 4. Spectral and temporal variables of the call of *Aplastodiscus leucopygius* in the three trials: pre-playback (green), playback (brown) and post-playback (yellow).

Table 2. Coefficients of the Generalised Linear Mixed Effects model considering the influence of each trial (pre-playback, playback, and post-playback) on each call parameter as response variables (t or z values are corresponding to Gaussian and Poisson families, respectively).

Response	Parameter	Coefficient	95% CI	t/z	P	Effects	Group	Std. Coef.	Fit
Call Duration	Intercept	0.1	0.09, 0.1	44.39	< 0.001	fixed		-0.32	
	exp [POS]	4.29E-03	0, 0.01	2.22	0.031	fixed		0.45	
	exp [PRE]	4.66E-03	0, 0.01	2.41	0.019	fixed		0.49	
		7.10E-03				random	individual		
		6.12E-03				random	residual		
	AICc								-368.44
	R2 (conditional)								0.59
	R2 (marginal)								0.05
	Sigma								0.006
Minimum frequency	Intercept	740.12	698.92, 781.32	36.43	< 0.001	fixed		0.02	
	exp [POS]	3.71	-21.10, 13.67	-0.43	0.67	fixed		-0.04	
		86.71				random	individual		
		27.11				random	residual		
	AICc								432.02
	R2 (conditional)								0.91
	R2 (marginal)								4.28e-04
	Sigma								27.11
Maximum frequency	Intercept	2534.98	2418.89, 2651.07	43.76	< 0.001	fixed		-0.05	
	exp [POS]	22.83	-17.44, 63.1	1.14	0.261	fixed		0.09	
	exp [PRE]	15.97	-24.3, 56.24	0.79	0.43	fixed		0.06	
		251.15				random	individual		
		63.54				random	residual		
	AICc								728.65
	R2 (conditional)								0.94
	R2 (marginal)								0.001
	Sigma								63.54
Bandwidth	Intercept	1810.83	1678.85, 1942.81	27.83	< 0.001	fixed		-0.02	
	exp [POS]	10.58	-21.78, 42.93	0.66	0.51	fixed		0.04	
		286.62				random	individual		
		50.45				random	residual		
	AICc								500.45
	R2 (conditional)								0.97
	R2 (marginal)								3.39e-04
	Sigma								50.45
Dominant frequency	Intercept	1629.53	1326.89, 1932.17	10.79	< 0.001	fixed		-0.2	
	exp [POS]	202.81	57.1, 348.53	2.79	0.007	fixed		0.3	
	exp [PRE]	200.97	55.25, 346.69	2.76	0.008	fixed		0.3	
		635.01				random	individual		
		229.93				random	residual		
	AICc								862.05
	R2 (conditional)								0.89
	R2 (marginal)								0.02
	Sigma								229.93

(Continued)

Table 2. (Continued).

Response	Parameter	Coefficient	95% CI	t/z	P	Effects	Group	Std. Coef.	Fit
Interval between calls	Intercept	2.09	1.68, 2.49	10.31	< 0.001	fixed		0.85	
	exp [POS]	-1.48	-1.98, -0.98	-5.91	< 0.001	fixed		-1.32	
	exp [PRE]	-1.38	-1.88, -0.87	-5.49	< 0.001	fixed		-1.23	
		0.44				random	individual		
		0.79				random	residual		
	AICc								167.67
	R2 (conditional)								0.51
	R2 (marginal)								0.36
	Sigma								0.79
Number of calls	Intercept	3.46	3.3, 3.62	43.19	< 0.001	fixed		3.46	
	exp [POS]	0.99	0.9, 1.08	21.79	< 0.001	fixed		0.99	
	exp [PRE]	0.87	0.77, 0.96	18.74	< 0.001	fixed		0.87	
		0.31				random	individual		
	AICc								532.75
	R2 (conditional)								0.95
	R2 (marginal)								0.63
	Sigma								1

Finally, our GLMM analysis showed that neither call, individual aspects nor environmental variables explained the harmonic shift between the first and the third harmonic (Table 4).

DISCUSSION

In our analyses, we observed that urban traffic noise had a significant influence on several aspects of *Aplastodiscus leucopygius* calls, even when it was not present anymore. Also, we observed that neither the body nor environmental aspects measured are related to these responses, which probably means that all individuals are subjected to this modification, independently of their body condition or temperature. We observed, during the playback trial, an influence of noise on almost all aspects of the call, except for the maximum frequency. All temporal variables showed a significant influence of playback trial, with calls becoming shorter, less frequent, and with a larger interval between them. It is consistent with most anuran species, since a recent study showed that 49% of anuran species decrease their call rate during exposure to a noise (Zaffaroni-Caorsi et al., 2022). These changes in the call pattern can directly imply communication with females. Similar results were also observed for *Scinax nasicus* (Leon et al., 2019)*, Hyla arborea* (Lukanov and Naumov, 2019), *Rana clamitans*, *R. pipiens*, *H. versicolor* (Cunnington and Fahrig, 2010), and *Pseudacris crucifer* (Hanna et al., 2014), with calls presenting less duration in noisy environments than in silent ones. In *Bokermannohyla hylax*, a species from the same subfamily of *A. leucopygius*, when in noisy environments, males present longer, more frequent and less spaced calls (Lima et al., 2022), similar to the one observed for *Dendropsophus triangulum* (Kaiser and Hammers, 2009). It reveals that the effect of anthropogenic noise on anuran call is not the same for all species (Zaffaroni-Caorsi et al., 2022), but that they probably tend to modify temporal aspects of the call, with only some exceptions (e.g., Parris et al., 2009; Grenat et al., 2019).

In the post-playback trial, individuals presented a significant increase in the number of calls when compared to the playback trial, but the estimate of our models showed slight increase in the number of calls also in relation to the pre-playback trial. Consequently, in the post-playback trial, individuals emitted calls at shorter intervals. It probably means that traffic noise stimulates individuals to increase the call rate, i.e., spend more energy on calling activity, even when the noise stimulus is no longer present. Similar results were observed for *Hyperolius pickersgilli,* a native species from South Africa, which presents an increase of 18% in call rate after anthropogenic noise stimulus, in this case, aeroplane noise (Kruger and Du Preez, 2016). Calling is one of the most energetic spending activities of anurans (Ryan, 1988; Grafe and Thein, 2001; Wells and Schwartz, 2007), with metabolic rates rising up to tenfold over the resting metabolism (Wells and Schwartz, 2007). Consequently, the increasing calling activity after anthropogenic noise

Table 3. Coefficients of Generalised Linear Models between residuals of models which showed a significant influence of traffic noise, as response, and Scaled Mass Index (SMI), body temperature and air temperature as predictive variables.

Model	Parameter	Coefficient	95% CI	t/z	*P*	Fit
Call Duration	Intercept	0.01	-0.02, 0.05	0.71	0.48	
	SMI	-1.77E-04	0,0	-0.14	0.89	
	Air temperature	-6.35E-04	0,0	-0.68	0.5	
	Body temperature	7.91E-05	0,0	0.11	0.91	
	AICc					-453.67
	R2 (conditional)					-452.56
	R2 (marginal)					-443.2
	Sigma					5.26E-03
Dominant frequency	Intercept	-174.72	-1531.99, 1182.56	-0.25	0.8	
	SMI	7.03	-99.98, 85.92	-0.15	0.88	
	Air temperature	4.51	-62.05, 71.08	0.13	0.89	
	Body temperature	4.89	-47.13, 56.91	0.18	0.85	
	AICc					806.49
	R2 (conditional)					807.6
	R2 (marginal)					816.96
	Sigma					191.14
Interval between calls	Intercept	1.18	-4.01, 6.38	0.45	0.65	
	SMI	-0.06	-0.42, 0.3	-0.33	0.74	
	Air temperature	-0.02	-0.27, 0.24	-0.14	0.89	
	Body temperature	-0.03	-0.22, 0.17	-0.25	0.8	
	AICc					138.72
	R2 (conditional)					139.83
	R2 (marginal)					149.19
	Sigma					0.73
Number of calls	Intercept	-0.84	-9.93, 8.26	-0.18	0.86	
	SMI	-0.18	-0.8, 0.45	-0.55	0.58	
	Air temperature	4.77E-03	-0.44, 0.45	0.02	0.98	
	Body temperature	0.07	-0.28, 0.41	0.37	0.71	
	AICc					205.85
	R2 (conditional)					206.96
	R2 (marginal)					216.32
	Sigma					1.28

stimulus can induce individuals to spend more energy, and consequently impair some other activities which also demand great amounts of energy, such as reproduction. In an experiment with *Hyla chrysoscelis,* evaluating the time response of females to mating calls, it was observed that in silent environments, females tend to respond faster to the call of males than in noisy environments, which means that anthropogenic noise masks the mating call emitted by males in a chorus (Bee and Swanson, 2007).

Furthermore, a study in Belize showed that anthropogenic noise promoted a decrease in the number of males present in choruses and the duration of the chorus during the night, and considering that females join the reproduction site lately than males, which could substantially reduce reproductive success in these species (Kaiser et al., 2011). Unlike *B. hylax* (Lima et al., 2022), *A. leucopygius* only breeds at sites far from the boulevard in the PEFI (Lisboa et al., 2021). Therefore, individuals are probably not used to the levels of anthropogenic noise of

Table 4. Coefficients of Generalised Linear Mixed Effects Models analysis (GLMM) using harmonic (first or third) as response variable and trial, temporal variables (call duration and interval between calls), spectral variables (minimum and maximum frequencies), individual variables (SMI and body temperature) and habitat variables (air temperature and number of individuals calling) as predictors.

Parameter	Coefficient	95% CI	z	*P*	Effects	Group	Std. Coef.	Fit
Intercept	232.33	-5.62E+06, 5.62E+06	8.11E-05	> 0.99	fixed		235.29	
exp [POS]	-207.19	-6.62E+06, 6.61E+06	-6.14E-05	> 0.99	fixed		-211.76	
exp [PRE]	-106.09	-6.68E+06, 6.68E+06	-3.11E-05	> 0.99	fixed		-105.02	
Call duration	-0.55	-2.32E+06, 2.32E+06	-4.64E-07	> 0.99	fixed		-0.03	
Interval between calls	2.76	-2.19E+06, 2.19E+06	2.47E-06	> 0.99	fixed		0.26	
Minimum frequency	-4.93	-2.40E+06, 2.40E+06	-4.02E-06	> 0.99	fixed		-2.95	
Maximum frequency	2.38	-2.46E+06, 2.46E+06	1.90E-06	> 0.99	fixed		4.49	
SMI	10.75	-2.92E+06, 2.92E+06	7.23E-06	> 0.99	fixed		10.69	
Body temperature	-15.03	-2.71E+06, 2.71E+06	-1.09E-05	> 0.99	fixed		-15.4	
Dominant frequency	266.95	-2.27E+06, 2.27E+06	2.30E-04	> 0.99	fixed		269.6	
Air temperature	8.6	-2.83E+06, 2.83E+06	5.94E-06	> 0.99	fixed		10.35	
n individuals	-52.25	-2.51E+06, 2.51E+06	-4.08E-05	> 0.99	fixed		-58.75	
	0.01				random	individual		
AICc								26.09
R2 (conditional)								1
R2 (marginal)								1
Sigma								1
Log loss								2.22E-16

the playback trial. However, it highlights that, as previously observed in other species (Bee and Swanson, 2007; Leon et al., 2019; Lukanov and Naumov, 2019), this type of noise can be harmful to individuals of *A. leucopygius* and consequently could be a factor that explains the non-occurrence of this species close to anthropogenic noise sources. Finally, we did not test for other noise sources, such as white noise or waterfall noise, to verify if the results observed here are specifically related to anthropogenic noise (e.g., white noise or traffic noise) or to any sort of noise those individuals are not used to (e.g., waterfall noise). However, considering that individuals of *A. leucopygius* typically occur in very silent habitats (Zina and Haddad, 2006a), probably both noise sources (anthropogenic and natural) could present an influence on their call parameters, and further studies are still necessary to evaluate this aspect.

We observed that individuals of *A. leucopygius* have the dominant frequency in the third of the three visible call harmonics. However, it also presented the dominant frequency in the first harmonic in several calls. It was consistent with observation for other species of the same genus, such as *A. albosignatus* (Moser et al., 2022), for other species of a different genus but in the same family, such as *Boana albomarginata* (Rebouças et al., 2020) and *B. punctata* (Brunetti et al., 2015), and for other species from a different family, such as *Thoropa lutzi* (Nunes-de-Almeida et al., 2016). However, we observed that none of the examined variables were able to explain this phenomenon. Although Zina and Haddad (2006b) reported that individuals of *A. leucopygius* present dominant in the first harmonic when calling in antiphony and dominant frequency in the third harmonic when calling alone, we did not evaluate the number of individuals calling in the habitat in this study. This aspect requires further studies explicitly designed to observe this harmonic shifting, especially in an experimental approach.

This study demonstrated that individuals of *A. leucopygius* present calling activity influenced by anthropogenic noise, with a reduction of calling activity during the exposure to noise and a significant increase after that. Also, we observed that the harmonic shifting observed in this species is not related to traffic noise, nor to individual and environmental aspects. These results reinforce that further studies are still needed and that anthropogenic noise, generated by human activities in the city surrounding the habitat of this species (Lisboa et al., 2021), represents a potentially harmful influence on this population.

ACKNOWLEDGMENTS

We thank to Natália Raga Catai, Iago Vinícius de Sá Fortes Junqueira, Ana Maria Macagnan, Matheus de Moraes dos Santos, Acácio Paiva and Karin Saito by their help in fieldwork. We also thank to Diogo Borges Provete by its help in analysis, to Instituto de Botânica by its authorisation for this research and access to PEFI, and the former Fundação Parque Zoológico de São Paulo by its research funding. This study was carried out with the license of SISBIO (#52015-3) and SisGen (#A7FA4F8). This work was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior under Grant #001, and São Paulo Research Foundation under Grant #2022/09659-4.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <http://www-9.unipv.it/webshi/appendix/index.html> manuscript number 15334.

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Acta Herpetologica 19(1): 41-55, 2024
DOI: 10.36253/a_h-15046

Autecology of the gecko *Phyllopezus periosus* (Squamata: Phyllodactylidae) inhabiting rock cliffs of a waterfall canyon from the semi-arid region of northeastern Brazil

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Submitted on: 2023, 31st August; revised on: 2023, 5th December; accepted on: 2024, 23rd January.
Editor: Mattia Falaschi

Abstract. *Phyllopezus periosus* is the largest phyllodactilid in Brazil, with a relictual distribution in the Caatinga Domain. Herein, we investigated the autecology of *P. periosus*, describing activity patterns, microhabitat use (spatial niche dimension), foraging mode, body temperature, reproduction, diet (trophic niche dimension), and temperature. Also, we tested the influence of seasonality and ontogeny on these biological aspects. Geckos (n = 116, 54 females, 33 males, and 29 juveniles) were sampled from October 2013 to September 2014. *Phyllopezus periosus* has an unimodal activity pattern, with peak activity in the early hours of the night. Males are more robust than females, and forelimbs in females are longer than in males. The body temperatures of this thermoconformer gecko are higher in the dry season. The reproductive season begins in the early dry season, with a fixed clutch of two eggs. Although we did not find evidence of more than one clutch per year, the studied population of *P. periosus* reproduces for an extended period throughout the dry season, as highlighted by the presence of juveniles all year. *Phyllopezus periosus* is saxicolous and presents a generalist diet composed mainly of arthropods, also including vertebrates such as frogs and lizards.

Keywords. Caatinga, daily activity, diet, foraging mode, microhabitat use, reproduction, sexual size dimorphism.

INTRODUCTION

The life history of lizards has been strongly shaped by the environments occupied during their evolutionary history. The interaction between individuals of a species and habitat dynamics stands as a pivotal subject investigated through autecological investigations (Walter and Hengeveld, 2014). These studies provide valuable insights into adaptations, behaviours, and survival strategies of a species in its ecological context (e.g., Alcantara et al., 2018; Barden and Shine, 1994; Ribeiro et al., 2015; Sousa and Ávila, 2015). Ecological factors such as temperature, food availability, and microhabitat heterogeneity are some of the main factors that determine the distribution of lizards in a specific place (Gonçalves-Sousa et al., 2023; Gonçalves-Sousa et al., 2022; Sinervo et al., 2010; Wang et al., 2016).

Studying the various dimensions of the ecological niche of a species is necessary to understand its role within the ecosystem (Pianka, 1973; Pianka et al., 2017). These dimensions can encompass spatial aspects (such as habitat and microhabitat use), trophic interactions (dietary preferences), reproductive behaviors (breeding season, nesting sites, mating rituals, and parental care), behavioral patterns (foraging strategies and territoriality), physiological characteristics (ecological tolerances and

ISSN 1827-9635 (print)
ISSN 1827-9643 (online)

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requirements regarding physical and chemical factors), as well as temporal factors (diurnal or nocturnal activity, seasonal rhythms, or migrations) (Hutchinson, 1957; Pianka, 1973; Pianka et al., 2017). Understanding the ecological niche breadth of species plays a crucial role in the formulation of conservation strategies, as it allows us to evaluate how environmental changes, such as climate change or natural land conversion, could impact species survival (Pianka et al., 2017).

In the present study, we investigated the autecology of *Phyllopezus periosus*. This gecko is a member of the Phyllodactylidae, a family encompassing 163 species and 10 genera extant in the New World, Northern Africa, Europe, and the Middle East (Gamble et al., 2008; Uetz et al., 2022). The genus *Phyllopezus* comprises eight large-bodied species, crepuscular and nocturnal, oviparous, saxicolous, and arboreal. These species are found in open vegetation in South America (Cacciali et al., 2018; Gamble et al., 2012) and six species are known to occur in Brazil: *P. diamantino, P. lutzae, P. periosus, P. pollicaris, P. przewalskii,* and *P. selmae.*

Phyllopezus periosus is the largest Brazilian phyllodactilid, inhabiting rocky environments in the semiarid region of northeastern Brazil, with a relictual distribution in the Caatinga morphoclimatic domain (Andrade et al., 2013; Franzini et al., 2019; Neta et al., 2014). Recently, *P. periosus* biological data have become available, regarding activity period, demography, foraging, microhabitat use, and morphometry (Freitas et al., 2015; Palmeira et al., 2021; Ragner et al., 2014). Data on communal nests and body temperatures throughout the rainy season is also available (Lima et al., 2011; Passos et al., 2013). However, information about the annual fluctuation or influence of seasonality on the ecology of *P. periosus* is still lacking.

Our primary aim was to investigate the autecology of *P. periosus* inhabiting the rocky cliffs of the Missão Velha waterfall canyon, located in the semiarid region of Brazil. Specifically, we investigated activity patterns, microhabitat use, foraging mode, body temperature, reproduction, diet, and temperature and estimated the spatial and trophic niche breadth dimensions. Each of these aspects contributes valuable insights to our understanding of how this species interacts with its habitat and ultimately plays its ecological role.

MATERIAL AND METHODS

Study site

Our study was conducted on the rocky cliffs of the Missão Velha waterfall canyon (7°13'S; 39°08'W), Missão Velha municipality, Ceará state, Brazil (Fig. 1A). The waterfall is around 12 meters high, and it is accompanied by other river erosion features, such as a canyon (Fig. 1B; Mochiutti et al., 2012). The vegetation is dense, forming a riparian forest contrasting with the vegetation of adjacent shrubby Caatinga, which mainly is characterized by deciduous and hypoxerophytic formations.

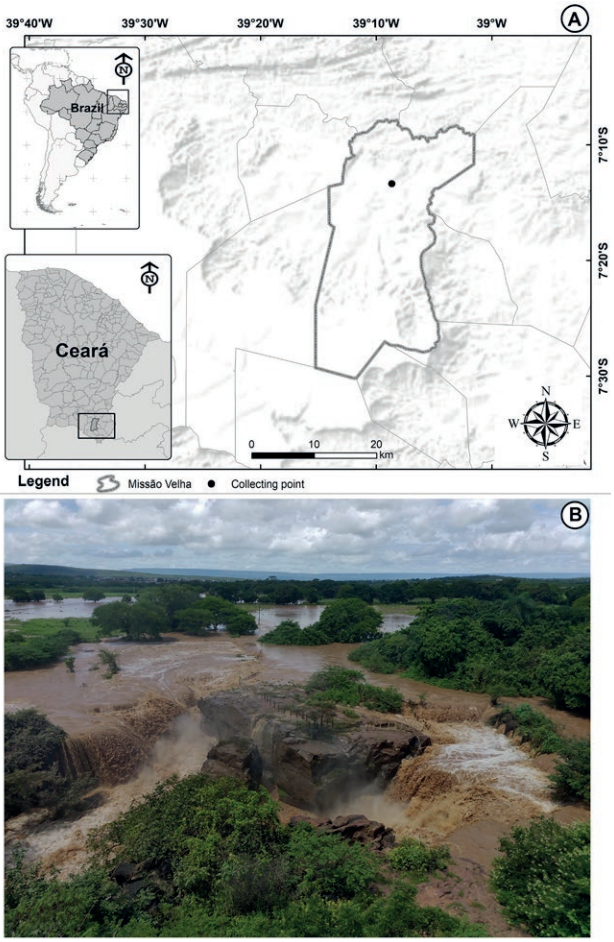

Fig. 1. Sampling area of *Phyllopezus periosus*. A: Map showing the location of Missão Velha waterfall, Missão Velha municipality, Northeast Brazil. B: Missão Velha waterfall in the rainy season.

The studied area is located in the semi-arid region of northeastern Brazil. There are two distinct seasons in this region: one long dry and one short rainy season. The rainy season is condensed from January to April, with annual rainfall of 987 mm and average temperature ranging from 24°C to 26°C (IPECE, 2017).

Activity

We conducted field expeditions from October 2013 to September 2014, one night per month. Two collectors

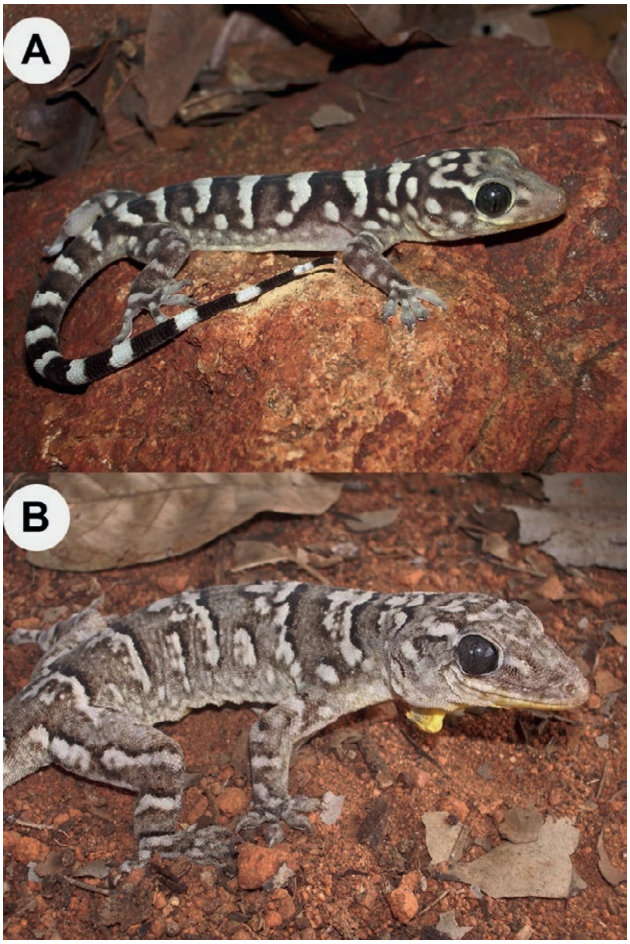

Fig. 2. *Phyllopezus periosus* of rocky cliffs of the Missão Velha waterfall. A: juvenile; B: adult individual.

performed active searches from 18:00 to 23:30, searching for *P. periosus* (Fig. 2) in all microhabitats available in the waterfall canyon, including the riparian forest. The total sampling effort was 11 hours per day (5 hours and 30 minutes per collector), totaling 132 hours of sampling. For each individual sighted, we recorded the time of sighting and the microhabitat used. We used a thermo-hygrometer (Instrutherm HT-300; precision ± 0.8 °C temperature and ± 3.5% relative humidity) to measure the temperature and humidity every hour (Fig. 3).

Microhabitat use

To evaluate the spatial niche of each species, we recorded the microhabitat used by each gecko according to the following categories: tree canopy and trunk, leaf litter, rock, and open ground. To measure the spatial niche breadth (Bs) of each species, we calculated the inverse of the diversity index of Simpson (1949), which values may range from 1 (exclusive use of a single category of microhabitat) to 5 (equal use of all microhabitat categories recorded). We calculated microhabitat overlap between sexes, and between adults and juveniles in Ecosim software v7.0 (Gotelli and Entsminger, 2004) using the overlap index of Pianka (1973), with values of overlap ranging from 0 (without overlap) to 1 (total overlap). We also estimated the height of the microhabitats used. As this population inhabits the walls of the Missão Velha waterfall canyon and the ground of the canyon is very uneven, we assumed the ground (0 m) to be the same as the water level and based our height estimates on this.

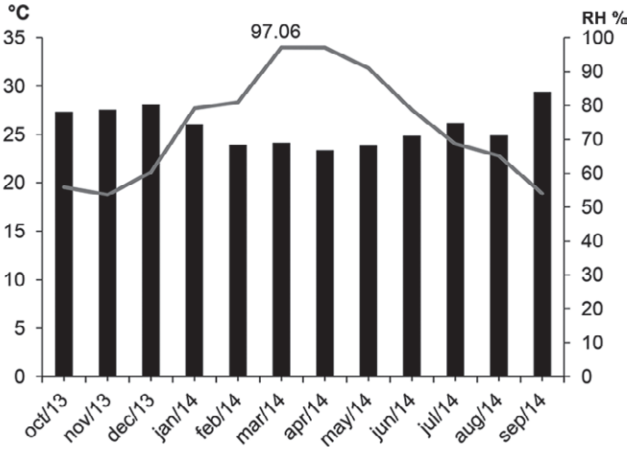

Fig. 3. Annual fluctuation of temperature (black bars) and relative humidity (gray line) in the area of the Missão Velha waterfall. Value above gray line represent the highest humidity record.

Foraging

We decreased the brightness of the flashlight after finding each gecko to avoid behavioral disturbances and observed them from a distance of about 5 m (± 1 m). Geckos were observed for up to 12 minutes.

We ended observations before 10 minutes when the lizard showed signs of disturbance or vanished from view. Observations shorter than 90 seconds were removed. After registering the foraging behavior, we hand-collected the geckos whenever possible. For each individual observed, we recorded the total time of observation, time spent moving, number of movements, the approximate distance covered, number of prey capture attempts, and number of successful prey captures. We then calculated the proportion of time spent moving (PTM) and the number of moves per minute (MPM) (Cooper Jr et al., 2001).

Body size

In laboratory conditions, we measured the body mass of each individual using a digital scale (standard deviation ± 0.01), euthanize with a lethal dose of 2% lidocaine hydrochloride (following Resolution #714 of the Conselho Federal de Medicina Veterinária, 2002), and used a digital caliper (± 0.01mm) to measure the following variables: snout-vent length (SVL), tail length (TLE), body width (BWD), body height (BHT), head width (HWD), head height (HHT), head length (HLE), rostrum labial commissure (RLC), forelimb length (FRL), hind limb length (HDL), and original length of the tail (TLO - from the cloaca to the beginning of the regenerated part in individuals who have autotomized the tail at least once in their lifetime). Then, we fixed the euthanized lizards with 10% formalin, preserved them with 70% ethanol, and deposited them at the Coleção Herpetológica of the Universidade Regional do Cariri.

To remove the effect of SVL on the other variables in the morphometric analysis, we used SVL and the residuals of linear regressions between the SVL and each of the other morphometric variables. This method was appropriated because it removes the tendency for individuals with larger body sizes (SVL) to have proportionately larger additional body parts than individuals with smaller bodies. Using the "car" R-package (Fox and Weisberg, 2019), we performed a multivariate analysis of variance (MANOVA) using the "Manova" function to test whether there was sexual or ontogenetic dimorphism in body shape, and a one-way ANOVA with the "Anova" function to assess differences in each variable independently. We also used the "t.test" function from the stats R package (R Core Team, 2019) to conduct t-tests for independent samples, aiming to evaluate sexual differences in body mass.

Reproduction

The sex of each specimen was determined by dissection and direct examination of gonads. We considered it as reproductively mature when males had enlarged testis and convoluted epididymis, and females had vitellogenic follicles or oviductal eggs. Sexually immature males and females were generically called "juveniles". We estimated the size of sexual maturation by the smallest sexually mature specimen of each sex. For females, we categorize the stage of development of follicles in pre-vitellogenic (small and whitish follicle clusters, when only pre-vitellogenic is present, or intercalated to vitellogenic follicles), vitellogenic (larger and yellow follicles, varying in size for each studied species), and eggs (when it presents the formation of the shell). Then we established the clutch size by counting the eggs present in the oviduct or vitellogenic follicles. When females presented eggs, vitellogenic follicles, or corpus luteum in the ovaries, we considered them to be in reproductive activity.

We recorded the length and width of testis in males, and the length and width of vitellogenic follicles and eggs in females to estimate the volume using the ellipsoid formula. We used SVL and the residuals of testis volumes (as described in the Body Size section) to check whether SVL and testis volume are correlated through the nonparametric Spearman correlation ("cor.test" function, method "spearman") in R. Then, we made a scatter plot with the volume and residual volume of the testis to determine the reproductive season of males, and to verify whether the reproductive season of males corresponds to the reproductive season of females throughout the year. When males presented positive values of volume testis, we considered them to be in reproductive activity.

Trophic niche

We removed the stomach content of all collected geckos and analyzed them under a stereomicroscope to identify prey items to the lowest taxonomic level possible (usually Order, except Formicidae). We measured the length and width of intact prey items with digital calipers (± 0.01 mm). Assuming a roughly equal width and depth of each prey item, we estimated its volume using the ellipsoid formula: $V = \frac{4}{3}\pi\left(\frac{l}{2}\right)\left(\frac{w}{2}\right)^2$, where V = volume, l = length, and w = width. Afterwards, we calculated numeric and volumetric percentages of each prey category. From these percentages, we obtained numeric and volumetric niche breadths using the inverse of the Simpson diversity index. Then, we used the Pianka niche overlap index using the volume of each prey category to evaluate trophic niche overlap between sexes, and between juveniles and adults. We assessed whether there were sexual and/or ontogenetic differences in prey diversity, average abundance, and average volume of items per stomach using the non-parametric Mann-Whitney test U ("wilcox.test" function) in R.

To determine the relative contribution of each prey category, we calculated the index of relative importance (IRI) for each species using the formula (Powell et al., 1990): $I = \frac{F\% + N\% + V\%}{3}$, where F%, N%, and V% are the percentages of frequency, number, and volume, respectively.

We used the Sorensen similarity index to evaluate similarities in diet between females, males, and juveniles. To test for sexual and ontogenetic differences in the diet, we conducted nonparametric similarity multivariate analyses (ANOSIM), using the Bray-Curtis similarity coefficient and 9999 permutations. Then we performed a discriminant analysis (SIMPER) to determine which prey categories were responsible for sexual and ontogenetic dissimilarities in diets. The ANOSIM and SIMPER analyses were executed in the software PAST 3.0 (Hammer et al., 2001). To assess the seasonal difference in the number or volume found per stomach, we used the Mann-Whitney U test in R. Finally, we used the non-parametric Spearman correlation to verify whether there was a correlation between the gecko cloacal temperature (Tc) and the total number or volume of prey ingested per stomach.

Temperature

We measured cloacal temperature (Tc), using a thermal sensor (Instrutherm S-02k, accuracy ± 1 °C) coupled to a digital thermo-hygrometer of fast reading (Instrutherm HT-300), in a maximum of 15 seconds after gecko capture. We also assessed the temperature of the substrate (Tsub), air (Tair; 2-3 cm above the substrate), and relative humidity close to the substrate (~10 cm) at the time of capture.

We used one-way analysis of variance (ANOVA) to check whether there were significant seasonal differences between Tc, Tsub, and Tair, and between Tsub and Tair of the dry and rainy seasons. We performed a multiple linear regression to verify the effects of environmental variables on body temperature. We verified whether there was a relationship between SVL and Tc through a non-parametric Spearman correlation. We used ANOVA to check if there were sexual and ontogenetic differences in Tc.

RESULTS

Activity

We recorded the daily activity of 224 individuals of *P. periosus*. The first active gecko was observed at 18:28, with activity peaking between 19:00 and 20:59, gradually declining until 22:59. After 23:00, only four individuals were sighted (Fig. 4B).

Microhabitat use

Phyllopezus periosus was found on rocks 194 times out of 224 observations (Fig. 4C), showing that it has specialized preferences for this habitat type (Bs = 1.309). We classified 99 of the 194 individuals found on rocks, categorizing them as follows: 60 individuals were found inhabiting vertical surfaces of rocks (60.6%), 22 in crevices (22.2%), 10 on slabs (10.1%), and 7 on diagonal surfaces (7.1%). We estimated the height of microhabitats occupied by 192 individuals: we found 10 individuals on the ground, 11 between 4-5 m, four between 6-7 m, and one at 10 m high (tree canopy). Other 166 individuals were at 1.38 ± 0.96 m (Fig. 4D).

Foraging

We recorded the foraging behavior of 41 individuals of *P. periosus*: 31 individuals were on rocks, eight on tree trunks, one a on tree canopy, and one on a leaf litter. The total observation time was 347.2 min. There were 154 moves (MPM = 0.444) and the total time the individuals spent moving was 2.19 min (PTM = 0.0063). The average PTM was 0.0084 ± 0.0086 and the average MPM was 0.5855 ± 0.0014. We detected 47 attempts of prey capture (30.52% of total moves), with 26 successful tries (55.32%; Table 1). We were able to collect 20 of the 41 individuals observed (10 females, five males, and five juveniles), with 15 having tails at an advanced stage of regeneration. We did not find *P. periosus* with autotomized and non-regenerated tail in foraging activity or exposed to sighting in their microhabitats.

Body size

We collected 116 specimens of *P. periosus* (54 females, 33 males, and 29 juveniles; Fig. 4A). Males of *P. periosus* are larger than females (Tables 2, 3), but considering the variables individually, females have longer forearms than males, which is the only variable with a significant sexual difference (Table 3). There is no sexual difference in body mass (t = -1.33; P = 0.187).

Reproduction

We found 17 females in breeding activity: five females had eggs in their ovaries about to be laid (two eggs per female), five had vitellogenic follicles (always two), and seven had corpus luteum in the ovary. One female had only one egg in its ovary, but there was evidence of recent oviposition. Although we did not find evidence of more than one clutch per year, such as the presence of eggs and vitellogenic follicles at the same time, the studied population of *P. periosus* reproduces for an extended period throughout the dry season, as highlighted by the presence of juveniles all year (except in October and February). Nevertheless, smaller juvenile lizards (SVL < 60 mm) were only found from November to January (four on average), and in March (one specimen). The reproductive period of females appears to

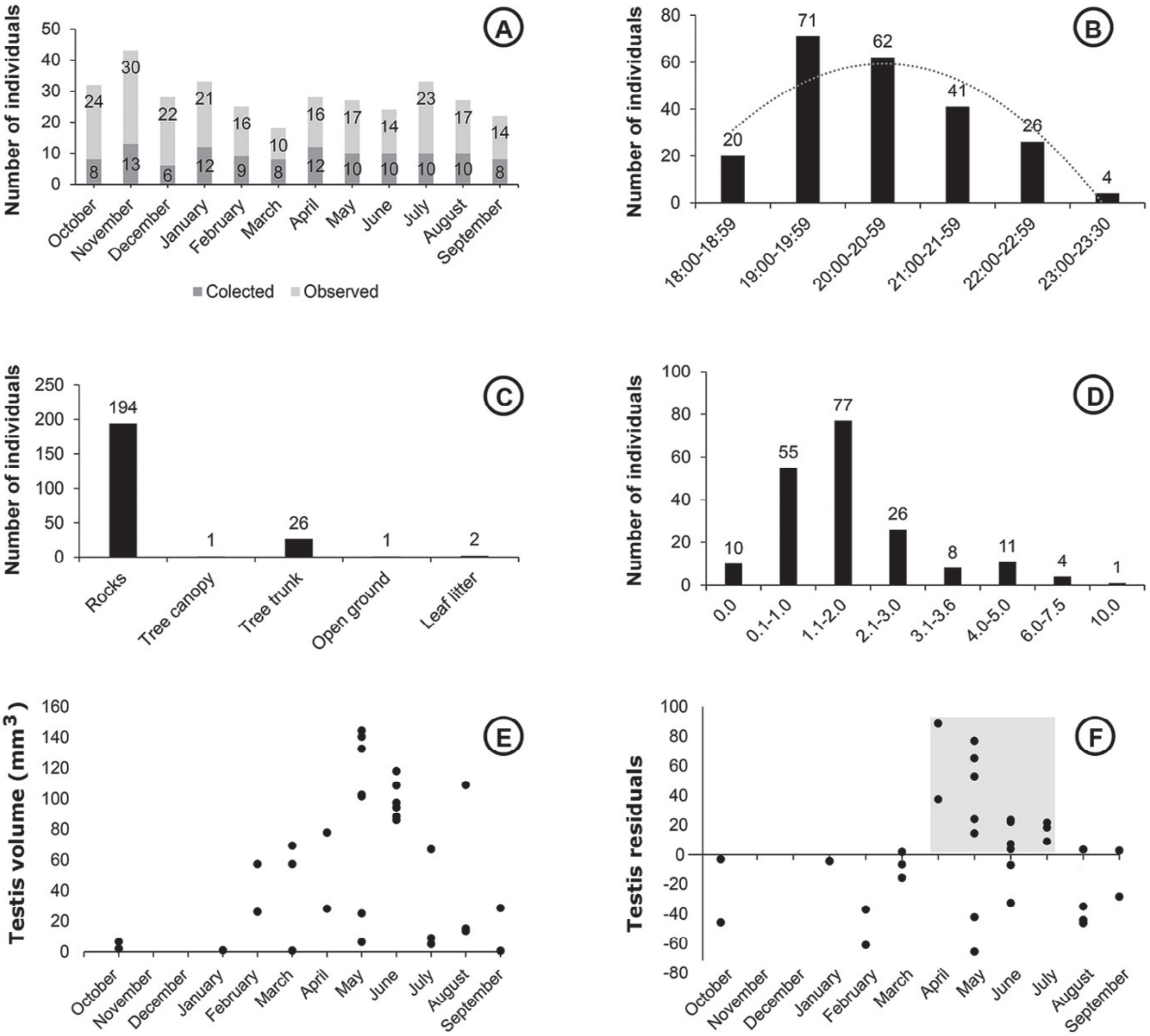

Fig. 4. Ecological data of *P. periosus* from Missão Velha waterfall, Brazilian semiarid region. A: number of individuals observed (light grey bar) and collected (dark grey bar); B: daily activity pattern; C: used microhabitats; D: height categories (in meters) where individuals were first sighted; E: volume (mm^3) of testis; F: residuals of linear regressions between the SVL and the volume of testis (gray square highlights individuals with enlarged testis).

have started in May and lasted until November, peaking in July (two females had eggs and two had vitellogenic follicles). The SVL of the smallest sexually mature female was 79.19 mm.

Throughout the year, we collected 17 reproductively active males. The male reproductive period began and ended earlier than that of females, lasting from March to September (Fig. 4–E, F). The volume of the testis is positively correlated with SVL (Spearman = 0.611; P = 0.002). The smallest sexually mature male had an SVL of 84.77 mm.

In November, we found five clutches of *P. periosus*; three of them were in communal nests (Fig. 5). The first and second communal nests had six and four eggs, respectively. They were 15 cm away from each other, one near the edge and the other at the bottom of the same horizontal crevice, which was around 2 cm larger at a height of 180 cm (Fig. 5A). We classified these clutches as separate nests because the eggs of *P. periosus* adhere to each other and to the substrate, establishing a clutch of pooled eggs. The third nest had 10 eggs of *P. periosus* and

Table 1. Foraging of *P. periosus*. Tobs = Observation time in minutes; PTM = Proportion of time moving; MPM = movements per minute; ADT= average distance traveled during the observed time (in cm); PCS = Prey capture success (successful captures/total attempts).

P. periosus	n	Tobs	PTM	MPM	ADT	PCS
Total	41	347.2	0.0063	0.444	42.39	55.32% (26/47)
Females	10	63.54	0.0105	0.704	59.8	42.86% (3/7)
Males	5	49.45	0.0014	0.121	13.33	100% (1/1)
Juveniles	5	51.00	0.0022	0.569	63	73.33% (11/15)
Not collected	21					

Table 2. Body mass (g) and morphometric variables (mm) of *P. periosus* (mean ± standard deviation) on rocky cliffs of the Missão Velha waterfall, Northeastern Brazil. SVL = Snout-vent length; TLE = tail length; BWD = body width; BHT = body height; HWD = head width; HHT = head height; HLE = head length; RLC = rostrum labial commissure; FRL = forelimb length; HDL = hind limb length.

	Females	Males	Juveniles
Mass	31.74 ± 11.23	36.26 ± 11.61	6.57 ± 4.96
SVL	103.82 ± 10.49	106.43 ± 10.61	61.84 ± 14.31
TLE	98.39 ± 25.49	96.19 ± 28.47	66.67 ± 19.52
BWD	21.85 ± 4.03	22.33 ± 4.06	14.41 ± 15.71
BHT	14.58 ± 2.56	15.43 ± 2.36	7.71 ± 1.95
HWD	20.19 ± 2.04	21.06 ± 2.25	12.84 ± 2.73
HHT	12.48 ± 1.58	13.05 ± 2.03	10.02 ± 12.37
HLE	28.25 ± 2.39	28.9 ± 2.35	18.67 ± 3.35
RLC	17.47 ± 1.65	18.22 ± 2.09	11.47 ± 2.4
FRL	32.57 ± 3.32	32.34 ± 3.00	19.60 ± 4.64
HDL	45.56 ± 4.73	46.40 ± 4.74	27.62 ± 7.03

two of *P. pollicaris*, found in a vertical crevice of approximately 40 cm in height and 15 cm in width (Fig. 5B). The eggs of *P. pollicaris* have harder shells, do not adhere to each other, and were piled over *P. periosus* eggs. The three *P. periosus* eggs at the bottom of this clutch had many cracks in the shells. We found deteriorated eggshells 30 cm away from that communal nest, revealing that this nest site had previously been used for oviposition.

Diet

Among the 116 geckos collected, 93 had stomach contents (43 females, 23 males, and 27 juveniles), whereas the remaining had empty stomachs (11 females, 10 males, and 2 juveniles). We identified 24 prey categories, with an average diversity of prey per stomach of 1.59 ± 0.74 (1.72

Table 3. Results of discriminant analysis between *Phyllopezus periosus* males and females and between adults and juveniles on rocky cliffs of the Missão Velha waterfall, Northeastern Brazil. SVL = Snout-vent length; TLE = tail length; BWD = body width; BHT = body height; HWD = head width; HHT = head height; HLE = head length; RLC = rostrum labial commissure; FRL = forelimb length; HDL = hind limb length. F = Fisher; * = p-values < 0.05.

	Males x Females		Adults x Juveniles	
	F	*p*-value	F	*p*-value
Body shape	2.867	0.006*	0.426	0.903
SVL	1.260	0.265	-	-
BWD	0.223	0.638	0.107	0.744
BHT	1.140	0.288	0.433	0.512
HWD	3.640	0.060	0.154	0.696
HHT	0.863	0.355	0.052	0.820
HLE	0.279	0.599	0.510	0.477
RLC	2.400	0.125	0.007	0.932
FRL	5.910	0.017*	1.280	0.260
HDL	0.007	0.934	0.784	0.378

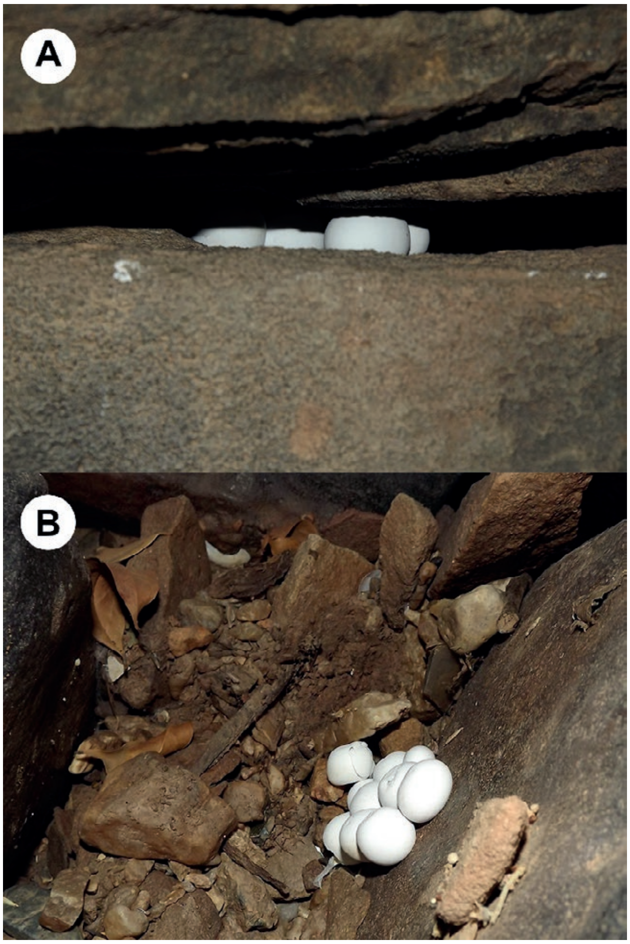

Fig. 5. Communal nests of *P. periosus* in horizontal rocky crevice (A), and of both *P. periosus* and *P. pollicaris* in diagonal crevice (B).

Table 4. Diet composition of *P. periosus* on rocky cliffs of the Missão Velha waterfall, Northeastern Brazil. F = frequency; N = number; V = volume; I = relative importance index.

Prey item	F	F%	N	N%	V	V%	I
Invertebrates							
Advanced digestion	6	4.11	6	2.182	6313.45	7.475	4.589
Araneae	9	6.16	11	4.000	4872.39	5.769	5.311
Blattodea							
Imago	6	4.11	7	2.545	1500.19	1.776	2.810
Eggs	3	2.05	3	1.091	64.86	0.077	1.074
Coleoptera	32	21.9	57	20.727	5257.46	6.225	16.29
Diplopoda	3	2.05	3	1.091	730.85	0.865	1.337
Diptera	3	2.05	3	1.091	792.36	0.938	1.361
Formicidae	6	4.11	6	2.182	192.43	0.228	2.173
Gastropoda	2	1.37	2	0.727	257.38	0.305	0.801
Hemiptera	8	5.48	9	3.273	5104.69	6.044	4.932
Hymenoptera	4	2.74	5	1.818	307.48	0.364	1.641
Insect larvae	1	0.68	3	1.091	3631.78	4.300	2.025
Isoptera	14	9.59	106	38.545	9414.89	11.147	19.76
Lepidoptera							
Imago	5	3.42	6	2.182	781.01	0.925	2.177
Larvae	1	0.68	1	0.364	882.10	1.044	0.698
Neuroptera	2	1.37	2	0.727	34.70	0.041	0.713
Orthoptera	25	17.1	29	10.545	29874.96	35.371	21.01
Pseudoscorpione	1	0.68	1	0.364	26.85	0.032	0.360
Scolopendomorpha	3	2.05	3	1.091	331.98	0.393	1.180
Scorpione	1	0.68	1	0.364	3300.76	3.908	1.652
Vertebrates							
Anura	2	1.37	2	0.727	1326.38	1.570	1.223
Lizard shed skin	2	1.37	2	0.727	1295.34	1.534	1.210
Lizard	5	3.42	5	1.818	7049.52	8.346	4.530
Vertebrate egg	2	1.37	2	0.727	1118.35	1.324	1.140
Total	146	100	275	100	84462.12	100	100
Niche breadth			4.79		6.03		
Empty stomachs	23	19.82					

± 0.77 for females, 1.52 ± 0.73 for males, and 1.44 ± 0.70 for juveniles). There was no significant difference in prey diversity per stomach between males and females (U = 411.5; P = 0.267) or between adults and juveniles (U = 746.5; P = 0.223).

The average number of items per stomach was 2.97 ± 4.11 (3.26 ± 4.40 in females, 3.09 ± 5.01 in males, and 2.41 ± 2.59 in juveniles), and the average volume (in mm^3) of prey per stomach was 908.34 ± 1215.48 (985.93 ± 1117.75 in females, 1367.06 ± 1714.94 in males, and 394.79 ± 500.20 in juveniles). There were no significant differences in the abundance (U = 411.5; P = 0.267) or volume (U = 438; P = 0.451) of items found in the stomachs for the two sexes. Although there was no ontogenetic difference in the abundance of items found in the stomachs (U = 838; P = 0.657), adults consumed larger prey than juveniles (U = 601; P = 0.014).

The three most important categories in the diet composition of *P. periosus* were the same for both sexes and ontogeny, although they were ranked in reverse order of importance. The categories Isoptera (21.95%), Orthoptera (17.91%), and Coleoptera (15.01%) were the most important items for females; Orthoptera (28.64%), Isoptera (21.44%), and Coleoptera (11.49%) for males; and Coleoptera (22.81%), Orthoptera (15.37%), and Isoptera (12.86%) for juveniles. Coleoptera and Orthoptera were the most frequent categories for females (18.84% and 15.94%, respectively), males (17.65% and 23.53%), and juveniles (28.89% and 13.33%). In number, Isoptera (40.29%), Coleoptera (20.86%), and Orthoptera (9.35%) were the most consumed categories by females; Isoptera (48.61%), Orthoptera (13.89%), and Coleoptera (9.72%) by males; and Coleoptera (32.31%), Isoptera (23.08%), and Orthoptera (9.23%) by juveniles. Volumetrically, the most important categories were Orthoptera (28.44%), Isoptera (15.40%), and lizards (11.55%) for females; Orthoptera (48.52%), insect larvae (11.51%), and Scorpiones (10.46%) for males; and Orthoptera (23.55%), lizards (20.24%), and Araneae (19.41%) for juveniles.

The numerical and volumetric trophic niche breadth of the population was 4.79 and 6.04, respectively (Table 4). Adult males had the smallest numerical ($B_N = 3.630$) and volumetric ($B_V = 3.719$) niche breadths. Juveniles had a greater numerical trophic niche width ($B_N = 5.552$) than females ($B_N = 4.541$). Contrarily, adult females had a greater volumetric niche breadth ($B_V = 7.282$) than juveniles ($B_V = 4.479$).

When the proportions of prey categories were compared, there were no sexual (ANOSIM, R = 0.296; P = 0.2035) or ontogenetic differences (ANOSIM, R = 0.259; P = 0.195), although SIMPER revealed a dissimilarity of 48.86% between males and females and 45.34% between adults and juveniles. The categories Isoptera (18.71%), Orthoptera (15.88%), and Coleoptera (7.81%) contributed most to the difference between males and females, while Isoptera (15.05%), Coleoptera (15.21%), and Orthoptera (13%) best explained the difference between adults and juveniles. Furthermore, there was no significant difference in the number (U = 882.00; P = 0.076) or volume (U = 832.00; P = 0.110) of prey items consumed by *P. periosus* during the dry and rainy seasons.

Phyllopezus periosus also ingested frogs and lizards. An adult female specimen ate a frog (Leptodactylidae), and a juvenile ingested an anuran that could not be identified due to the high degree of digestion. One adult female ate the congener *P. pollicaris*, and one juvenile consumed a lizard (Gekkonidae), also not identified due to the degree of digestion. Furthermore, evidence of cannibalism was found, with two adult females and one juvenile that ingested smaller individuals of their own species.

Body temperature was correlated to prey volume (Spearman = -0.228; P = 0.038), but not to prey abundance (Spearman = -0.1192; P = 0.283) found in the stomachs.

Temperature

Average Tc (27.85 ± 1.91 °C; n = 102) was higher than Tsub (26.23 ± 2.11 °C), and Tair (25.49 ± 2.09 °C). Both Tsub and Tair have an influence on Tc ($R^2 = 0.618$; $F_{2,99} = 79.99$; P <0.001), but the β coefficient suggests that Tsub ($\beta_{sub} = 0.88$) exerts more influence than Tair ($\beta_{air} = -0.097$) on Tc.

There were seasonal differences in Tc (F = 27.29; P = 0.0001; Dry season Tc = 28.54 ± 1.78 °C; Rainy season Tc = 26.73 ± 1.56 °C). Both Tsub and Tair were significantly higher (Tsub: F = 13.85, P = 0.0003; Tair: F = 12.83; P = 0.0005) during the dry season (Tsub = 26.81 ± 2.33 °C; Tair = 26.04 ± 2.31 °C) than in the rainy season (Tsub = 25.36 ± 1.24 °C; Tair = 24.64 ± 1.27 °C). In contrast to the dry season (F = 3,44; P = 0.0658), Tsub and Tair were significantly different in the rainy season (F = 6.13; P = 0.0155).

There was no significant difference in Tc between males (Tc = 27.57 ± 1.78 °C) and females (28.08 ± 2.07 °C; F = 1.15; P = 0.287), or between adults (27.9 ± 1.98 °C) and juveniles (27.69 ± 1.72 °C; F = 0.22; P = 0.638).

There was no relationship between SVL and Tc (Spearman = -0.0931; P = 0.352), nor between Tc of individuals with a tail in the process of regeneration (27.95 ± 1.96 °C; F = 0.34; P = 0.5599) and those who had never been autotomized (27.72 ± 1.75 °C; F = 0.34; P = 0.5599).

DISCUSSION

Activity

The daily cycle of *P. periosus* is unimodal, peaking between 19:00 and 21:00, and effectively ending around 23:00. Other populations of this gecko showed a time and unimodal activity patterns similar to ours, although a few individuals were also found until close to dawn (Passos et al., 2013; Ragner et al., 2014). *Phyllopezus periosus* had a restricted distribution, occurring almost exclusively on the rocky cliffs of the canyon formed downstream of the Missão Velha waterfall. During the rainy season, the water level rose around 2 m (personal observation, JGGS and HFO), which could cause dispersion out of the canyon. However, although we looked extensively in the Caatinga vegetation adjacent to the canyon in both seasons for *P. periosus*, we were unable to find it there.

Phyllopezus periosus coexists with the congener *P. pollicaris* in the canyon of the Missão Velha waterfall, but with a substantially higher population density than *P. pollicaris* (personal observation). *Phyllopezus periosus* preys on *P. pollicaris* and has the advantage of competing for space due to its larger body size. Other potential predators of geckos in the Missão Velha waterfall include the snakes *Oxyhropus trigeminus*, *Micrurus bonita*, and *Pseudoboa nigra*, and the owls *Glaucidium brasilianum* and *Megascops choliba* (personal observation, JGGS and HFO). The competitive and predatory pressure exerted by *P. periosus* and these other predators contributes to the low abundance of *P. pollicaris* in the canyon region. In contrast, we found *P. pollicaris* in Caatinga vegetation around the waterfall canyon, where *P. periosus* was not found.

Microhabitat use

In the present study, *P. periosus* was highly specialized in the use of rocky microhabitats. Rocky substrates favor thermoregulatory activities, which have an impact on all other dimensions of the niche in lizards (Faria and Araujo, 2004; Huey, 1982). A good rocky spot, for example, can reduce the time it takes for the lizard to reach its ideal body temperature, lowering the risk of predation (Vitt et al., 1996). Several studies have reported similar specialized usage of microhabitats (Freitas et al., 2015; Gonçalves-Sousa et al., 2023; Palmeira et al., 2021; Passos et al., 2013; Ragner et al., 2014), except in the type locality, where Rodrigues (1986) found *P. periosus* in nine microhabitat categories. However, this author does not present data on the frequency of use in any of them.

Foraging

Phyllopezus periosus is a typical sit-and-wait forager (present study; Palmeira et al., 2021). This kind of forager usually has a PTM lower than 0.10 (Perry, 1995), indicating that they spend most of their time waiting for prey to approach. Sit-and-wait foragers generally feed on large active foraging prey that requires little movement to capture (Huey and Pianka, 1981; Perry and Pianka, 1997). The fact that *P. periosus* eats highly mobile prey (crickets, beetles, and termites) confirms its status as a sit-and-wait forager.

Data on foraging in Gekkota species is limited to only 1% of the species in this order (Palmeira et al., 2021). The great majority of these species are sit-and-wait foragers (e.g., Bauer, 2007; Cooper Jr. et al., 2001; Cummings et al., 2021; Palmeira et al., 2021; Whiting et al., 1999) that generally return to their original site after a capture attempt (e.g., Alcantara et al., 2018; Vitt, 1995), while *P. periosus* remains in the exact location where the prey was located, regardless of whether the capture was successful. This strategy divergence could be due to better prey availability in the humid environment around Missão Velha waterfall during most of the year, as suggested by the average PTM found by us (0.0084), which is considerably lower than that found in the population studied by Palmeira et al. (2021) in an area of Caatinga (1.7). On the other hand, because *P. periosus* has a larger body mass than other geckos, this variation may be compensatory, reducing energy expenditure with movements.

We did not find *P. periosus* with an autotomized and non-regenerated tail in foraging activity or exposed to sighting. This finding suggests that after autotomy, *P. periosus* adopts suboptimal foraging behaviors, such as foraging near refugia (mostly rock crevices) or reducing foraging in situations where predation is high risk (see Bels et al., 2019), which occurs as a result of reduced mobility and the loss of the autotomy possibility as a defense strategy. In lizards, tail loss through autotomy has a significant impact on their capacity to flee, jump, and climb, which has a direct impact on their foraging ability (Kuo et al., 2019).

Body size

Although females have longer forelimbs, males of *P. periosus* are larger than females, as in other gecko species (e.g., Johnston and Bouskila, 2007; Massetti et al., 2017; Nieva et al., 2013; Saenz and Conner, 1996). In lizards, sexual dimorphism is usually associated with larger limbs, a larger head, and a longer torso, which can improve locomotor performance and provide benefits in territorial disputes, ensuring the best site for foraging and female selection (Husak and Fox, 2008; Olsson et al., 2002; Van Damme et al., 2008).

The main cost of reproduction in lizards is locomotor performance loss caused by egg formation (Kuo et al., 2019). Females may have evolved this differential allometric growth in the forelimb to aid its support and mobility in vertical microhabitats, such as the rocky cliffs of the Missão Velha waterfall canyon, during the period of egg production, when their body mass greatly increases. Nevertheless, additional research is needed to further understand how this sexual difference in forelimb size affects locomotion and substrate adhesion.

Reproduction

The reproductive period of males begins and ends earlier than that of females in *P. periosus.* This suggests a potential reproductive strategy wherein the females of *P. periosus* can retain sperm from previous mating encounters to fertilize their eggs at a later time, maximizing their reproductive success. This behavior has been observed in a wide range of taxa, from insects to mammals (Cunningham and Birkhead, 1998). The reproductive period of *P. periosus* started at the end of the rainy season, and the smallest specimens were found at the beginning of the rainy season. This strategy ensures that neonates will find enough food to survive in localities where prey availability fluctuates throughout the year, especially in those from arid or semiarid environments (McCluney and Sabo, 2009; Pinheiro et al., 2002; Vasconcellos et al., 2010).

This gecko has fixed two eggs as the clutch size, and communal nests in diagonal and horizontal rock crevices are rather common (Present study; Lima et al., 2011). Lizards from several families, including all seven from the Gekkota infraorder, share a fixed clutch size reproductive method (Meiri et al., 2020; Mesquita et al., 2016). Other geckos from the semiarid Caatinga, such as *Gymnodactylus geckoides, Hemidactylus mabouia, Lygodactylus klugei, and P. pollicaris*, typically reproduce continuously throughout the year (Vitt, 1986; Vitt, 1992). In highly seasonal environments, continuous reproduction throughout the year ensures that the persistence of the population is not jeopardized by unfavorable periods. Seasonality is a strong limiting factor that influences the partial or total halt of the reproductive activity of lizards in climatically atypical years (Castro-Franco and Bustos-Zagal, 2011; Paulissen, 1999; Vitt and Breitenbach, 1993). This is probably what happens to most lizards in the Caatinga in months of greater climatic severity. Temperature and humidity, on the other hand, do not appear to be limiting factors for *P. periosus* in the Missão Velha waterfall because the availability of water and relatively high humidity for most of the year do not result in significant fluctuations in food resources. In fact, the water level of the waterfall appears to be the limiting factor for *P. periosus* activities, as it rises and reduces favorable foraging and oviposition sites after the first rains, which could explain why we did not find females carrying eggs or vitellogenic follicles during the rainy season.

Diet

The diet of *P. periosus* is mainly composed of arthropods, but it also includes anurans, lizards, vertebrate eggs, and lizards shed skin. Geckos from the semi-arid region of northeastern Brazil usually have an opportunistic feeding habit and exhibit plasticity in response to food availability (Alcantara et al., 2019; Gonçalves-Sousa et al., 2023; Sousa et al., 2017). The most important categories in the diet of the population studied were crickets, termites, and beetles. In other populations of *P. periosus*, spiders (41.5% of total prey consumed) were followed by beetles (25.9%) in Cuité, Paraíba state (Gonçalves-Sousa et al., 2023), and beetles (52.1%) in the Ecological Station of Seridó, Rio Grande do Norte state (Palmeira et al., 2021). Furthermore, because of its abundance, Coleoptera is an essential food item for other geckos from Caatinga, including *Hemidactylus brasilianus* (26.1%), *Lygodactylus klugei* (24.3%), and *P. pollicaris* (24.8%) (Gonçalves-Sousa et al., 2019). Saurophagy reinforces that the diet of *P. periosus* is not only generalist but also opportunistic, with sympatric lizard interactions occasionally leading to predation attempts. *Phyllopezus periosus* feeds on the lizards *P. pollicaris*, *Hemidactylus brasilianus,* and *Tropidurus semitaeniatus* in addition to its own species (Present study; Palmeira et al., 2021).

Rodrigues (1986) suggested that when food is scarce due to drought, adult *P. periosus* may diverge from optimal foraging (MacArthur and Pianka, 1966) and feed on small animals only enough to keep themselves alive. Food, on the other hand, is not a scarce resource for this population. The relatively high humidity and presence of water in the lower part of the waterfall most of the year favor a lower seasonal fluctuation of arthropods, which is supported by the presence of both large (mainly crickets) and small (primarily termites and small beetles) prey in the diet of *P. periosus*, as well as the lack of significant food variation between dry and rainy seasons. Moreover, the studied population showed a lower frequency of empty stomachs (19.83%) than other Neotropical geckos (22.4%) or the overall average for nocturnal lizards (24.1%) (Huey and Pianka, 1981). Finally, we found a positive correlation between body temperature and the volume of prey consumed by *P. periosus*. Increased Tc improves digestion by accelerating metabolism and lowering digestion time (Secor, 2009). This mechanism improves the metabolic efficiency of lizards, giving them more time to perform other daily activities or even to feed more in the same night.

Temperature

Phyllopezus geckos are thermoconformers, showing a body temperature that fluctuates in accordance with environmental temperature. The body temperature of these geckos appears to be plastic and unrelated to SVL. For example, the average body temperature of *P. periosus* in Missão Velha waterfall (Tc = 27.5 °C; maximum SVL = 122.5 mm) seasonally fluctuated, being higher in the dry season (Tc = 28.54 °C); yet, Tc was lower than that of another *P. periosus* population in the rainy season (Tc = 29.4 °C; Passos et al., 2013). The average Tc of *P. pollicaris* (maximum SVL = 77.9 mm) ranged from 24.4 °C in a Cerrado vegetation area to 30.8 °C in a Caatinga vegetation area (Ferreira et al., 2014; Recoder et al., 2012). Contrarily, other phyllodactylids, such as *Gymnodactylus geckoides* (maximum SVL = 48 mm) from Brazilian Caatinga, *Phyllodactylus bordai* (69 mm) and *Phyllodactylus tuberculosis* (59.8 mm) from the Mexican semiarid, and *Phyllodactylus xanti* (69 mm) from the Mexican island of San Esteban, were classified as active thermoregulators because their body temperature was around 2 °C higher than the temperature of the substrate in which they inhabited (Lara-Resendiz et al., 2013a; Lara-Resendiz et al., 2013b; Vitt, 1995).

In summary, *P. periosus* is a typical sit-and-wait forager with an unimodal activity pattern, thermoconformer, sexually dimorphic, highly specialized in microhabitat use, but highly generalist and opportunistic in the use of food resources, and has a long reproductive period, beginning early in the dry season.

ACKNOWLEDGMENTS

All data were collected following ethical guidelines provided by the American Society of Ichthyologists and Herpetologists (ASIH), The Herpetologists' League (HL), the Society for the Study of Amphibians and Reptiles (SSAR), Resolution #714 of the Conselho Federal de Medicina Veterinária, and the geckos were collected under permission from the Instituto Chico Mendes de Conservação da Biodiversidade (license number 29613-1). We thank the Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico for a postdoc fellowship granted to JGGS (FUNCAP/CNPq FC3-0198-00136.01.00/22; 150110/2023-5), and to Conselho Nacional de Desenvolvimento Científico (CNPq) for a research fellowship granted to RWA (303622/2015-6; 305988/2018-2; 307722/2021-0).

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Acta Herpetologica 19(1): 57-68, 2024
DOI: 10.36253/a_h-13529

Diet patterns of water green frogs (*Pelophylax esculentus* complex) in mixed population systems in Serbia

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Submitted on: 2024, 19th January; revised on: 2024, 3rd March; accepted on: 2024, 3rd April
Editor: Enrico Lunghi

Abstract. Population systems in which all three taxa of the *Pelophylax esculentus* complex coexist are rare and in Serbia can only be found along the Danube. Although several studies describe the diet in populations with only one taxon, there is a lack of data from mixed populations. We studied the diet in mixed populations of edible frogs at three sites for three years. We collected gut contents from 221 adult frogs using the stomach flushing method and identified 1,477 prey items. The diet consisted mainly of insects (88%). For all three taxa, the most frequently consumed prey groups were Hymenoptera (28%), Coleoptera (18%), Lepidoptera (17%) and Diptera (11%). Larger prey (in terms of length and volume) was mainly consumed by *P. ridibundus*, followed by *P. esculentus*. The smallest prey were mainly consumed by *P. lessonae*. However, *P. ridibundus* consumed fewer prey items than *P. lessonae*. No significant difference was found between the taxa in the staple diet, while there were differences in the less abundant prey categories, especially between *P. ridibundus* and *P. lessonae*. The narrowest trophic niche width was observed in *P. lessonae*, followed by *P. esculentus*, and the widest in *P. ridibundus*. Our results suggest that the diet of the three taxa is diverse and consists of a large number of invertebrate groups. However, the diet was locality-specific, with the dominance of different prey groups in different localities depending on habitat characteristics. These results confirm our expectation that the frogs of the *Pelophylax esculentus* complex follow an opportunistic foraging strategy, as predicted by optimal foraging theory.

Keywords. European water frogs, feeding habits, diet composition, prey diversity, Danube, Serbia.

INTRODUCTION

Frogs have an important position in the trophic network by regulating the populations of invertebrates and other groups of organisms on which they feed (Hocking and Babbitt, 2014). Larval stages feed on algae and detritus and, as primary consumers, influence the ecosystem structure and function by altering algal communities, patterns of primary production, and organic matter dynamics in a variety of freshwater habitats (Kupferberg, 1997; Flecker et al., 1999). Within trophic webs, adult frogs occupy an intermediate position and are involved in aquatic and terrestrial food webs as both prey and predator of various taxa due to their complex life history (McCoy et al., 2009). Frogs are reported as prey for various vertebrates and invertebrates, but also as predators of invertebrates and some vertebrate species (e.g., small lizards, snakes, birds, mammals, and other anurans) (Toledo et al., 2007).

Green frogs are semi-aquatic ambush ("sit and wait") predators (Moore and Biewener, 2015) that feed both during the day and at night (Cogălniceanu et al., 2000). The success of this strategy depends on factors such as prey density, prey mobility, and energy requirements of the predator (Moore and Biewener, 2015). According to the requirements of optimal foraging theory, animals

ISSN 1827-9635 (print)
ISSN 1827-9643 (online)

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with such a strategy must be generalists across the spectrum and opportunists in prey selection to maximize energy intake per unit of time (Pianka, 2000; Glaudas et al., 2019).

Information about dietary habits and trophic interactions are the key determinants of the structure and dynamics of ecological niches in coexisting species (Lunghi et al., 2022). They are necessary for basic understanding of the life history of anurans and the fluctuation patterns of their populations (Anderson et al., 1999), for identifying environmental conditions and habitat changes (Batista et al., 2011), and for designing and implementing management and conservation strategies for species that require protection (Stuart et al., 2004). Although some species among anurans are considered specialists (Simon and Toft, 1991), frogs are mainly considered generalists and opportunists (e.g., ÇiÇek and Mermer, 2007; Almeida-Gomes et al., 2007). In generalist species, aspects such as phylogeny, foraging mode, prey availability and abundance, and morphological constraints to capture and ingest a particular type of prey, may be related to resource partitioning among species (Lima, 1998; Vignoli et al., 2009). In addition, community niche micro-differentiation of both frog taxa and prey may be influenced by anthropogenic activities that can affect ecological interactions (Albrech and Gotelli, 2001).

Serbia is populated by all three taxa of the *Pelophylax esculentus* complex: *Pelophylax ridibundus* (*P. rid*), *Pelophylax lessonae* (*P. les*), and their hybrid *Pelophylax esculentus* (*P. esc*). Throughout its range, mixed populations in which all three taxa of the *P. esculentus* complex coexist in the same locality are generally rare ("REL population systems", named after the initial letters of the taxa that compose them) (Suriadna et al., 2020). In Serbia, these population systems are detected in a few areas along the Danube (Krizmanić and Ivanović, 2010). We believe that REL population systems provide the opportunity to study all three taxa living in syntopy and sharing the same resources. Although they are likely to have similar ecological needs, they should have measurable differences in resource use that reduce the possibility and/or extent of competition between taxa and thus promote coexistence (Costa-Pereira et al., 2019). Before the clarification of the taxonomic status of green frogs (Berger, 1973) and the advent of molecular identification techniques, field identification of taxa in this complex was difficult (see Breka et al., 2020). We note that most trophic studies treated green frogs as a single unit without considering possible dietary differentiation within the complex (e.g., Lőw and Török 1998; Cogălniceanu et al., 2000). Trophic studies on green frogs have mostly been reported for populations with a single taxon (e.g., Šimić et al., 1992; Mollov, 2008; Sas et al, 2009), while only a few have reported on diet in mixed populations where at least one parental species coexists with the hybrid taxon - LE or RE population systems (Sas et al., 2007; Ferenti et al., 2009), and a few on diet in REL systems (Popović et al., 1992; Paunović et al., 2010; Karaica et al., 2016). More recently, field studies on green frogs have been conducted on population systems based on inter-taxon differences, especially when considering implications for effective conservation and management of altered aquatic and wetland habitats (Jośko and Pabijan, 2020).

The aim of the present study was to (1) provide the necessary data for an overview of the diet composition of the *P. esculentus* complex in Serbia in REL population systems; (2) determine the dietary pattern and niche breadth for each taxon at three sites with different habitat quality and anthropogenic pressure; (3) determine whether there are niche shifts in the composition of the staple diet and/or dietary partitioning between three taxa in syntopic population systems of green frogs.

MATERIALS AND METHODS

Study area

Frogs were collected in three consecutive years (2016-2018) at three sites in the South Banat district, Serbia (Fig. 1). The sampling sites were selected according to the confirmed presence of the REL system (Krizmanić and Ivanović, 2010) and represent typical habitats for water frogs, but with different levels of preserved natural features and anthropogenic activities and pressures: 1) Stevanove ravnice (STR) (44°49'57.8"N 21°18'33.1"E) is an alluvial plain of the Danube within the special nature reserve "Deliblatska peščara" (Deliblato Sands). Here, underground waters form semi-permanent pools along the sandy plains. During high water or floods, these pools merge into larger water bodies that are connected to the Danube. The pools are overgrown with helophytes, which form extensive reed belts. The area is traditionally used as pastureland, but has retained its characteristic structure and composition of a mosaic landscape characterised by an alternation of steppe and hygrophyte vegetation depending on the microtopography and water level. The frogs were collected in the wet meadows between the ponds. 2) The Danube-Tisa-Danube Canal (DTD) (44°51'14.4"N 21°18'17.8"E) is an artificial, man-made structure in which natural aquatic vegetation only grows in a very narrow belt along the shoreline. Frogs have been collected along the canal in the macrophyte beds and the canal embankment. The embankment is a popular picnic, recreation, camping, and fishing spot for locals

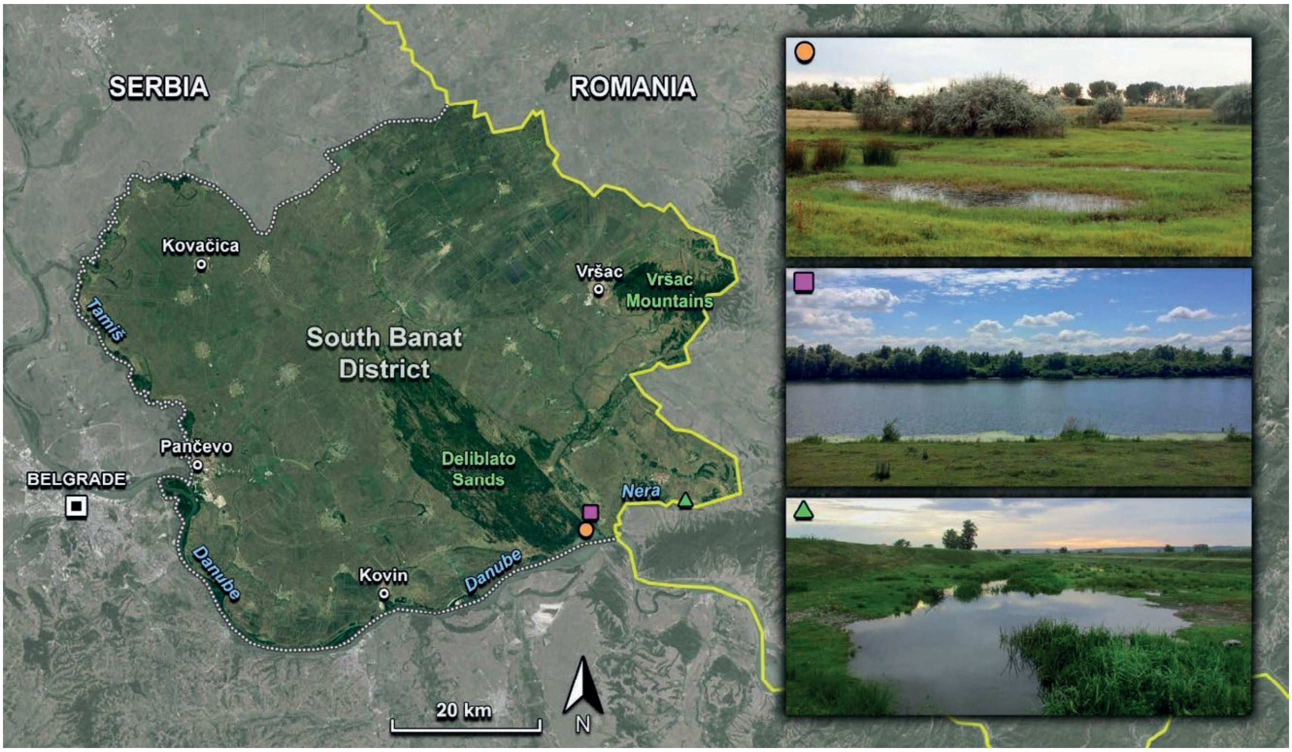

Fig. 1. Map of the South Banat district (dotted border) in northern Serbia showing three sites where green frogs (*P. esculentus* complex) were sampled: STR (orange circle), DTD (pink square) and JRG (green triangle).

and tourists with planned management activities. 3) Jaruga (JR) is a canal on the edge of the outstanding natural landscape area "Karaš-Nera" near the village of Kusić (44°52'30.8"N 21°28'16.0"E). It was built to regulate the flooding of the Nera River and to ameliorate the surrounding agricultural land, but it has lost its main purpose and is no longer used. The canal is surrounded on one side by a mosaic of agricultural fields and orchards and on the other by floodplain vegetation and abandoned gravel pits. When the Nera is at low water, which is the case for several months of the year, the canal has a low water flow. This allows the formation of dense macrophyte beds, which consist mainly of underwater plants. Frogs were collected within the canal where access was possible and in the floodplain meadows along the canal.

Data collection

Frogs were sampled at night after the breeding season from May to October using dip nets and then transported to a field laboratory for further processing. Frogs were identified to taxon level according to Breka et al., (2020). Individuals with a snout-vent length < 55 mm (*P. ridibundus*) or < 45 mm (*P. esculentus, P. lessonae*) were considered juveniles and not included in further analyses (Mikulíček et al., 2014).

The prey samples were extracted from the stomach contents using the stomach flushing method (Solé and Rödder, 2010), which allows to obtain residuals of the last foraging activity without sacrificing the individual. Due to its ability to digest food very quickly, the stomach of each frog was flushed immediately (within one hour) after capture to minimise the time between frog capture and prey extraction. Stomach contents were stored individually and preserved in vials containing 70% ethanol. Individual prey items were identified to the lowest taxonomic level based on their digestive status. After stomach flushing, all frogs were released near their capture site and checked to ensure they behaved normally.

The identification of prey taxa was carried out at the Institute of Zoology, University of Belgrade - Faculty of Biology, Serbia, using published key references (Nilsson, 1996; Nartshuk, 2003; Horsák et al., 2013; Brock, 2017; Araneae, version 03.2022) and adopting the current nomenclature (Fauna Europaea version 2.4, 2011). The length and width of intact prey were measured using a digital calliper, while the lengths of incomplete prey were estimated using known proportional ratios of body parts (Chinery, 1993). Prey volume was estimated from the volume of a prolate spheroid (Quiroga et al., 2009). We considered stomach contents as a random sample of food selected by an individual over an unspecified period of time prior to capture.

Data analysis

All identified prey were categorised into 17 broader categories ("prey categories"). They were assigned to categories defined by their ecomorphological characteristics to maximise clarity of biological interpretation and variation in diet composition (Vignoli et al., 2009).

To identify differences in diet composition between taxa and localities, we used a non-parametric similarity analysis (ANOSIM using Euclidean distance with N = 9,999 permutations) with pairwise comparisons based on a step-down sequential Bonferroni procedure. Variations in diet were compared between taxa and localities using a repeated measure permutational multivariate analysis of variance (PERMANOVA, with N = 9,999 permutations of the Euclidean distance matrix). SIMPER analysis was used to identify the specific prey group contributing the most to the observed differences and the possible existence of niche shifts. All tests were performed using the software programme PAST (Paleontological Statistics Software Programme, Ver. 4.13 Hammer et al., 2001).

For further analysis, an explanatory matrix was created in which each frog was assigned to a specific combination of locality and taxon (loc_tax): STR*Esc*, STR*Rid*, STR*Les*, DTD*Esc*, DTD*Rid*, DTD*Les*, JRG*Esc*, JRG*Rid* and JRG*Les*.

The evenness and richness of prey categories were estimated using the Simpson index (D') and Simpson dominance index (D), which are used in studies of niche differentiation as indices of specialised measures of niche width and can provide insights into details of trophic ecology (Krebs, 2014). All indices are given according to the notation in Hammer et al., (2001). The analysis was performed in the diversity module of PAST, with the options "unbiased" and "use ACE for S" selected. The ACE richness estimator corrects for the rare occurrence of food items and, in our case, the effects of prey sample size (see: Gotelli and Colwell 2011, Hammer et al., 2001). The standardised Levin's Index (B_A), as proposed by Hurlbert (1978), was used to calculate niche width values for each loc_tax combination using Ecological Methodology software, version 7.4 (Kenney and Krebs, 2003).

The available data were further analysed using multivariate statistical analysis. Following the suggestion of Leps and Smilauer (2003), we chose linear redundancy analysis (RDA), a multivariate direct gradient analysis (ter Braak and Prentice, 1988). It can be seen as a constrained form of PCA in which the ordination of the variables of interest is constrained by linear combinations of external explanatory variables. This allows us to assess how much of the variation in the structure of a multivariate data set (e.g., species composition, in our case diet composition) is explained (i.e., predicted) *a priori* by one or more independent external variables (Legendre and Legendre, 1998). We chose this procedure because we opted for a method that allows explicit prediction of food composition determination rather than exploratory post hoc interpretation. The resulting RDA ordination plot is presented as a triplot with the following symbology: 1) prey categories are shown as arrows (17 elements); 2) the three frog taxa and three sampling sites are shown as symbols (six in total); and 3) additionally, the centroids of the individual frogs sampled at each site are shown (nine in total). In this representation, the distance between symbols approximates the average dissimilarity of prey composition as measured by their Euclidean distances. These distances can be considered proportional to their trophic overlap. The scaling of loadings and scores (prey categories and frog taxa, localities, and individual frogs in our case) focused on standardised prey category scores and a scaling that preserves the relative distances between cases. The prey category arrows point in the direction of the steepest increase in prey category abundance in the samples. The length of a diet category arrow together with the angle with respect to an axis also indicates the relative contribution of that category to the axes shown in the triplot as well as the dominance and contribution of each diet category to the extracted gradients. The angle between the arrows indicates the approximate correlation between the dietary categories: The approximate correlation is positive when the angle is sharp and negative when the angle is more than 90 degrees. The projected orthogonal distance of the loc_tax symbols on the arrows of the dietary categories indicates the relative importance of this category in this sample. Ordination analysis was performed with CANOCO 5.15 (ter Braak and Smilauer, 2002). The significance of the two canonical axes was tested using a permutation test. Additional post-hoc tests (t-test with XLSTAT, ver. 3.1, 2021) were performed for the taxa and site assessment scores to confirm the trends observed by visual interpretation of the resulting ordinations. For all analyses, $P < 0.05$ was set as the significance level.

RESULTS

We captured 317 adult green frogs and obtained diet samples from 221 frogs. The breakdown of samples by location and frog taxa is given in Table 1. Empty stom-

Table 1. Breakdown of the total number of frogs caught by taxon and location (abbreviations are given in the text).

Locality/Taxon	*P. rid*	*P. les*	*P. esc*	Total
STR	46	11	129	186
DTD	11	9	53	73
JRG	6	9	43	58
Total	63	29	225	317

achs were present in 78 frogs (24.6%) and were excluded from further analysis. The taxon with the highest percentage of empty stomachs was *P. ridibundus* (30%), while this percentage was slightly lower in the other two taxa (18%). The site with the highest percentage of empty stomachs was STR (31%), while DTD and JRG had a lower percentage of empty stomachs (18% and 14%, respectively). Plant material (small leaves, seeds, lentils) was identified in 9 stomachs (3%) and was not included in further analysis as we considered it as unintended prey items. A further 9 stomachs (all *P.esc.*) contained unidentifiable prey items. In total, 1477 prey items were identified. The frogs' diet consisted mainly of insects (88%), while the remaining 12% consisted of other small invertebrates – spiders, crustaceans, molluscs (snails), and vertebrates. Insect larvae made up 20% of the total prey. In all three frog taxa, the most frequently observed prey groups were typical ground-dwelling terrestrial invertebrates, e.g., ants, ground beetles, weevils, caterpillars, and spiders (50%), followed by aerial groups (e.g., midges, flying ants and leafhoppers, 36%) and aquatic prey (6.5%).

The prey categories were as follows: Gastropoda (Gas.), Clitellata (Cli.), Arachnida (Ara.), Myriapoda (Myr.), Malacostraca (Mal.), Ephemeroptera (Eph.), Odonata (Odo.), Orthoptera (Ort.), Hemiptera (Hem.), Hymenoptera (Hym.), Coleoptera (Col.), Coleoptera larvae(Col_l), Lepidoptera (Lep.), Lepidoptera larvae(Lep_l), Diptera (Dip.), Diptera larvae(Dip_l) and Vertebrata (Ver.). The diet of all three frog taxa was dominated by insect orders Hymenoptera (28%), Coleoptera (18%), Lepidoptera (17%) and Diptera (11%). Other groups were represented with proportions below 10%. Rank-abundance curves of the 17 selected prey categories are shown in Fig. 2. Prey categories that were represented with a frequency of more than 10% in the whole sample were considered as staple diet prey - Hymenoptera, Coleoptera and Lepidoptera larvae. Those represented with a frequency of 5-10% were considered common prey - Diptera, Arachnida, Hemiptera. Rare and accessory prey groups were represented by the eleven remaining

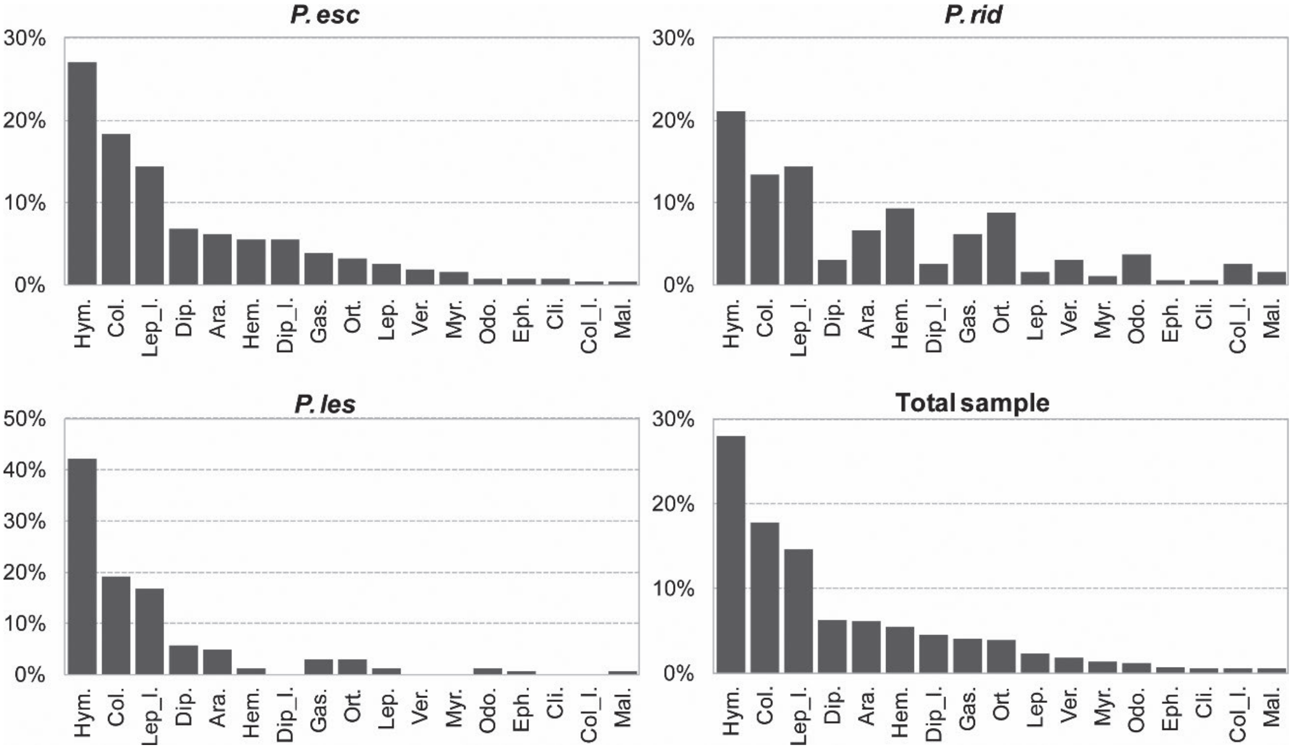

Fig. 2. Rank abundance curve for 17 selected prey categories (abbreviations are given in the text) for three taxa of green frogs. The order of the food categories for the individual species corresponds to their order in the total sample.

categories (less than 5%), e.g., snails, springtails, annelids and centipedes, and vertebrates (small fish, froglets, smaller voles). In *P. lessonae*, most prey items belonged to the dominant prey groups – Hymenoptera, Coleoptera and Lepidoptera larvae, while rare food items were present in small proportions. In contrast to *P. lessonae*, all 17 categories of prey were present in *P. ridibundus*. In addition to the predominant prey categories, *P. ridibundus* also consumed prey from other diet categories, e.g., Hemiptera, Gastropoda, Orthoptera, Odonata and Coleoptera larvae. The hybrid taxon also consumed all 17 prey categories, and as the largest number of frogs analysed were identified as *P. esculentus*, this influenced the pattern of the overall rank abundance curve. Rare prey categories were present in the diet of the hybrid taxon, but in lower proportions than in *P. ridibundus*.

The rank abundance curves for all three sites are shown in Fig. 3. The site STR was the only one where all 17 prey categories were present. Compared to the rank abundance curves of the total sample, there are some important differences: the most dominant prey categories were different, while some prey categories that are considered staple diet were hardly present (e.g. Lep_l. in the locality STR, < 2%). At the JRG and DTD sites, rare and accessory prey categories were represented by less than 2%. We note that a slightly larger proportion of Orthoptera and Lepidoptera were present in the DTD locality (11%) and Vertebrata in the STR locality (3%) in relation to the total sample.

Overall, individuals from the *P. esculentus* complex consumed 6.75 ± 0.58 (mean ± SE) prey items with an average length of 10.39 ± 0.23 mm and a volume of 830.86 ± 62.76 mm^3. The largest average prey length and volume were found in *P. ridibundus* (12.24 ± 0.65 mm and 1515.58 ± 257.1 mm^3, respectively) and the smallest in *P. lessonae* (9.07 ± 0.58 mm and 533.31 ± 91.62 mm^3, respectively). However, *P. ridibundus* consumed fewer individual prey items (4.97 ± 5.81), while *P. lessonae* consumed smaller prey items per stomach in greater numbers (7.43 ± 11.14). This is consistent with the body size pattern of these species. The breakdown of basic statistics and prey measurements in the total sample by taxon is shown in Table 2.

ANOSIM identified a weak but significant difference in diet between localities (R = 0.1, P < 0.01), but not between taxa (R = -0.03, P = 0.72) (Fig. 4). Subsequent pairwise comparisons showed that each locality differed significantly from all others (Table S1). In addition, SIMPER analysis showed that the overall dissimilarity between localities was 85.74% and 3 of the total 17 prey categories (Coleoptera, Hymenoptera and Lepidoptera larvae) yielded more than 50.65% of cumulative dissimilarity (Table S2). PERMANOVA analysis supported these results, finding a significant effect of locality (F = 58.44, P < 0.01) but not between taxa (F = 0.68, P = 0.65) and the interaction between these two factors (F = 0.94, P = 0.45).

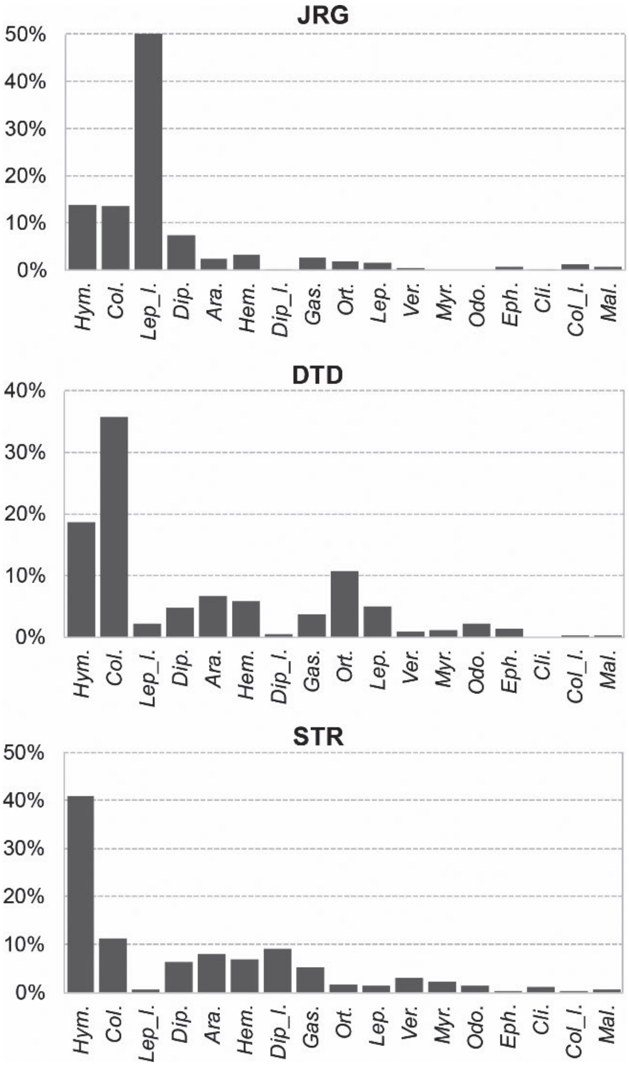

Fig. 3. Rank abundance curve for three localities (abbreviations are given in the text).

The estimates of prey diversity and niche width for the nine loc_taxa groups are shown in Table 3. Among the sites, the highest number of prey categories is documented at site STR (S = 17), followed by DTD (S = 16), while the site with the lowest number of prey categories is JRG (S = 15). In general, the lowest diversity of frog prey was found in frogs at site JRG, which was accompanied by correspondingly higher values of dominance.

In terms of trophic niche width, the widest Levins-standardised niche width for all sites was found in *P. ridibundus* (B_A = 0.489), while the narrowest niche width

Table 2. Basic statistics of green frogs and their stomach contents by taxon

	P. rid (*n* = 63)	*P. les* (*n* = 29)	*P. esc* (*n* = 225)
Average body size of frogs (mm)	75.8 ± 1.32	69.06 ± 1.48	72.62 ± 0.71
Average number of consumed prey items per stomach	4.97 ± 5.81	7.43 ± 11.14	6.95 ± 8.70
Average length of the consumed prey items (mm)	12.24 ± 0.65	9.07 ± 0.58	10.26 ± 0.26
Average volume of the consumed prey items (mm^3)	1515.58 ± 257.1	533.31 ± 91.62	757.27 ± 68.1

Table 3. Summary of frogs' diet diversity indices and niche breadth

Loc_tax	STR*Rid*	DTD*Rid*	JRG*Rid*	STR*Les*	DTD*Les*	JRG*Les*	STR*Esc*	DTD*Esc*	JRG*Esc*
Number of prey categories (*S*)	16	11	9	7	11	8	17	14	15
Prey abundance in sample (*n*)	99	43	52	73	40	48	537	277	308
Simpson Index (*D*')	0.86	0.83	0.69	0.40	0.76	0.64	0.81	0.80	0.71
Simpson's dominance index (*D*)	0.15	0.17	0.31	0.60	0.24	0.36	0.19	0.20	0.29
Levins' standardized measure of trophic niche breadth (B_A)	0.3	0.37	0.14	0.04	0.2	0.11	0.28	0.25	0.15

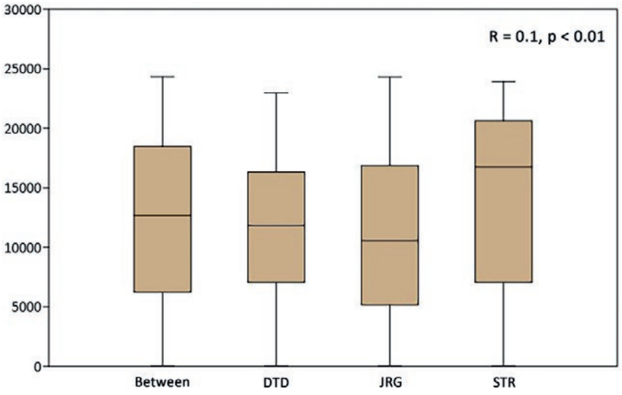

Fig. 4. Box whisker plot of the ANOSIM analysis comparing the diet of frogs at three different localities. Boxes indicates values from 25th (bottom) to 75th (top) percentile; horizontal black line indicates the median.

(B_A = 0.186) was found in the second parental species *P. lessonae* at all three sites. The trophic niche width values for the hybrid taxon *P. esculentus* were between the values of the parental species (B_A = 0.365). The observed differences in the standardised Levin's measure of niche width between loc_tax were not statistically significant.

The RDA triplot is shown in Fig. 5. The first RDA axis explains 4.16% of the variance in Euclidean distance, and the proportion increases to 5.38% when the second axis is added. About 50% of the unconstrained ordination is explained by the constrained axes, while the first two axes of the unconstrained ordination were able to extract 17% of the variance in individual frog diet composition.

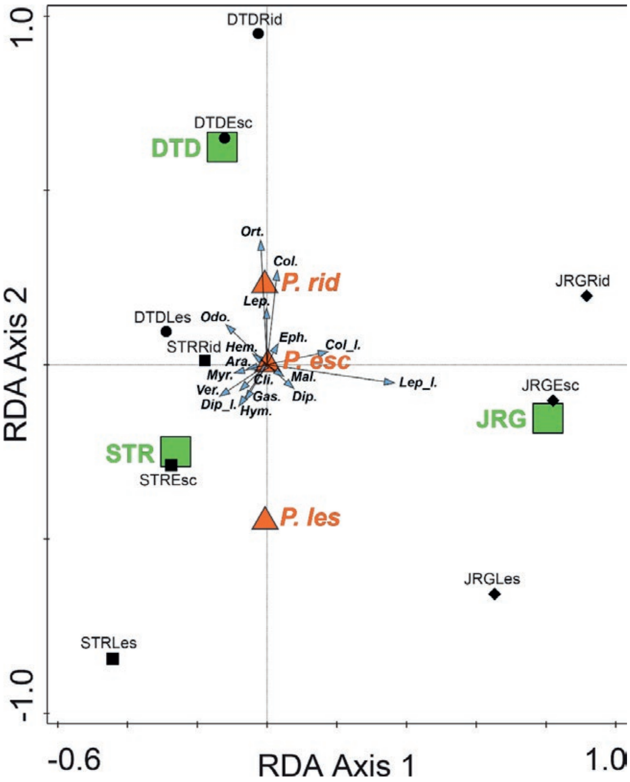

Fig. 5. Redundancy analysis (RDA) triplot of standardised taxon/locality scores derived from correlation matrices of 17 prey categories analysed (abbreviations as in text). The scaling preserves the distances between the loc_tax combinations, the angles between the vectors indicate the correlation, the length of the arrows the dominance.

As observed, frog taxa and sites are arranged such that the first RDA axis separates the JRG site from DTD and STR (two-tailed t-test of ordination values: $t_{219} = 73.25$, $P < 0.01$). The second RDA axis separates two parental taxa, with *P. ridibundus* being positive and *P. lessonae* being negative (two-tailed t-test of ordination scores: $t_{57} = -6.31$, $P < 0.01$). The hybrid taxon lies approximately in the middle of the ordination. Moreover, the second axis separates the DTD locality from the other two localities (two-tailed t-test of ordination values: $t_{219} = 26.37$, $P < 0.01$). The RDA with the two extracted gradients was statistically significant ($F = 3.4$, $P < 0.01$).

DISCUSSION

The frogs of the *P. esculentus* complex were found to feed on different invertebrates, especially ground-dwelling arthropods and aerial insect groups. Aquatic prey made up only a small proportion of the total prey, suggesting that the green frogs feed mainly on land or wait for terrestrial prey to come close to the water ("sit and wait" strategy). According to our data, Hymenoptera, Coleoptera, and Lepidoptera predominate in the diet of all three frog taxa of the complex. A comparison of our results with the only published data on the diet of the green frog in a REL system from Serbia, reported by Paunović et al., (2010), showed a similar dietary spectrum, albeit with differences in the proportions of prey. In their study, the dominant prey groups were Coleoptera, Hymenoptera, and Gastropoda (each with a frequency of > 30%). In our study Gastropoda were more abundant in the diet only in the STR locality (5%), a marshy locality with similar characteristics to the locality in Paunović et al., (2010). Insect larvae were less present in Paunović et al., (2010) (11%) than in our study (20%). In both studies, no vertebrate prey was observed in *P. lessonae*, while in the other two species the proportion of vertebrate prey was low (1 – 3%). Our results also agree with those of Karaica et al., (2016) from REL population systems in the north-western part of Croatia, where the predominant prey groups were Coleoptera, Diptera, and Hymenoptera. In both studies, a higher proportion of aquatic prey was observed in *P. ridibundus* than in the other two taxa. In contrast to our results, no vertebrate prey was observed, and insect larvae were present in a much lower proportion (1%). The diet composition of the frogs of the *P. esculentus* complex from Serbia was similar to that observed in populations of *P. ridibundus* in Turkey (ÇiÇek and Mermer, 2006; ÇiÇek and Mermer, 2007), Bulgaria (Mollov, 2006; Mollov et al., 2010) and Russia (Ruchin and Ryzhov, 2002), and to that in a mixed LE population system in Romania (Sas et al., 2007).

According to studies on the diet of green frogs, arthropods accounted for 90.1-97.3% of the total prey in different population systems (Cogâlniceanu et al., 2000; Ruchin and Ryzhov, 2002; ÇiÇek and Mermer, 2007; Rakojević et al., 2022), which is consistent with the results of our study (92.4%). Most previous studies show that green frogs feed predominantly on terrestrial prey (e.g. ÇiÇek and Mermer, 2007; Karaica et al., 2016; Rakojević et al., 2022), which is consistent with our results (93.46%), with the largest proportion of aquatic prey found in *P. ridibundus* (15.46%) and the smallest in *P. lessonae* (1.90%). However, according to Ruchin and Ryzhov (2002), who based their results on a single taxon population (*P. ridibundus*), the frogs consumed aquatic rather than terrestrial prey. This phenomenon is generally not confirmed in other studies. Adult forms of invertebrates dominated the diet of green frogs in our sample (79.83%), which is consistent with previous studies (e.g. ÇiÇek and Mermer, 2007; Paunović et al., 2010). This suggests that members of the *P. esculentus* complex generally seize more active prey, as adult invertebrates are generally more active than their larvae. All three taxa had an almost equal proportion of larvae in their diet (about 20%), although interestingly a high dominance of caterpillars (mainly moth larvae of the family Geometridae) was recorded in the JRG locality (as much as 51.57% of the total prey, while in other localities this percentage was much lower 10.44% in STR and only 3.9% in DTD). The abundance of caterpillars in the diet of green frogs is probably not the result of their preference, but the result of the abundance of caterpillars at the particular site, especially in samples collected in late summer/early autumn. Although this result is not the subject of this article, it could indicate a possible phenological effect specific to that site. However, this hypothesis needs to be further confirmed.

Vertebrates were "on the menu" of *P. esculentus* and *P. ridibundus* at all three sites, with the largest proportion found at STR. The most numerous vertebrate prey were newly-metamorphosed frogs from the *P. esculentus* complex (besides small fish and voles). The STR site with the highest percentage of cannibalism (3%) was also the site with the highest percentage of empty stomachs. Intraspecific predation has been previously documented in green frogs as a mechanism that enhances the survival of the individual under certain conditions such as drought, lower food availability, etc. (Crump, 1992; Çiçek and Mermer, 2007). Since in our study vertebrate prey was mainly present in autumn, the possibility of a phenological effect of prey preference cannot be completely excluded. However, we consider it more likely that an explanation for the observed phenomena is related to a relative prey deficit associated with season and/or location.

The diet of all three green frog taxa is dominated by the same prey categories, which means that there is no difference in the staple diet between the taxa. However, in the diet of the parental species there is a difference in terms of rare and accessory (marginal) prey, especially those that are present in the diet of *P. ridibundus*, while they are almost absent in the diet of *P. lessonae*. In the diet of the hybrid taxon, most of these prey categories were present, but in smaller or negligible proportions. These marginal prey categories are mostly represented by larger prey such as Odonata, Gastropoda and Orthoptera, and various species of vertebrates. This could be due to the fact that *P. ridibundus* frogs are larger and can therefore consume larger prey, whereas *P. lessonae* frogs fulfill their nutritional requirements with smaller prey, but in larger numbers (Löw and Török, 1998). This could also indicate a tendency of smaller green frog taxa to concentrate on fewer prey categories when foraging and would explain the dominance of only three prey categories in the diet of *P. lessonae*. Each of the dominant prey categories was most common in different locations. These differences in the dominance of prey at all three sites can be explained by differences in the type and configuration of the habitats. As the STR and JRG sites are floodplains under the strong influence of the surrounding rivers (Danube and Nera), the presence of hydrophilic and aerial categories was to be expected. The orchards surrounding the JRG site may also have had an influence on the high dominance of moth larvae. At the DTD site, a deforested artificial embankment planted with clover influenced the dominance of epigeobiont species.

The RDA showed a high variance in the diet of the individual frogs. This is not an unexpected result, as frogs are known to be opportunistic feeders, from which we conclude that there should be a large heterogeneity in diet between individual frogs. The RDA revealed a differentiation between the localities according to the dominance of certain prey categories. In the JRG locality, for example, Lepidoptera larvae had the largest contribution, in the DTD Coleoptera and Orthoptera stood out, while in the STR locality only Vertebrata and Hymenoptera had relevant loadings. A very high dominance of a single prey category (e.g. Lepidoptera larvae in JRG) indicates a lower diversity at this site, which is confirmed by the values of the diversity indices. At STR, on the other hand, the arrows of the prey categories were about the same length, which, together with the values of the diversity indices, indicates higher prey diversity. The "swarm" of arrows pointing to a sampling site is an indicator of the diversity of the food composition of that sample and consequently may indicate higher trophic production/availability at that site. According to the results of the multivariate analyses, the frog taxa are well separated both in ordination and at specific sites that differ in diet. For each locality, the position of each frog score corresponds to the position of the respective taxon on the second axis. The position of the centroids of the hybrid taxon was always close to the locality score, whereas the centroids of the parental species were always above (*P. ridibundus*) or below (*P. lessonae*) the locality score, reflecting their relative position on the secondary gradient. We note that the ordination of taxa on the secondary gradient corresponds to the ordination of the localities on the same gradient (P. ridibundus and the DTD locality have higher positive scores on the second axis, P. lessonae and the STR and JRG have negative scores on this axis). Furthermore, *P. lessonae* had the lowest values for prey diversity (as well as the highest dominance of certain prey categories) and Levins' standardised niche breadth. We conclude that *P. lessonae* has the narrowest trophic niche among the three taxa, which contradicts previous studies in mixed population systems where the hybrid taxon had the narrowest niche (Paunović et al., 2010; Karaica et al., 2016). Trophic diversity for the other two taxa was approximately the same at all three sites, with the lowest values at the JRG site and the highest at the STR site. The positions of their centroids on the RDA plot support this result. The direct gradient analysis presented in this study provides further insight into these patterns. Since the gradient extraction in RDA is hierarchical, we can interpret the two gradients as follows: 1) the first dominant gradient, predicting site position, is an environmental gradient related to habitat quality, defining the differential trophic availability of potential food components specific to a site through differences in secondary production; 2) the second, subdominant gradient predicting species position is a taxon-specific gradient separating parental species from hybrids. It probably follows the characteristics of their life form and differences, especially between parental species – different details of foraging strategy, size-related prey acquisition, etc.

The success of the "sit-and-wait" predation strategy depends, among other conditions (high mobility of prey and low energy requirements of the predator), on a fairly high prey density (Moore and Biewener, 2015). A high proportion of certain taxa in the diet could therefore also be due to the fact that green frogs, as "sit-and-wait" predators, compensate for the rarity or low specific mass of the prey taken by the number of individuals taken (Löw and Török, 1998). This would also suggest that prey items are acquired in proportion to their dominance in the immediate environment. We hypothesise that differences in the prey composition of all three taxa in the study area are influenced by differences in the local availability of prey groups, possibly resulting from differences in habitats and their secondary production. A review of the diet composition of the *P. esculentus* complex across its range revealed that some dominant taxonomic prey groups were consistent, but other prey categories varied in abundance. This suggests that the diet of frogs of the *P. esculentus* complex is determined by prey availability and habitat characteristics rather than active selection. Consequently, niche shifts among localities and variations in prey composition are to be expected, as green frogs from the *P. esculentus* complex can be characterised as non-selective predators that show a generalist feeding habit and consume prey according to their body size and other morphological features such as the mouth gap (Mollov, 2008).

In summary, the diet of all three taxa of the *P. esculentus* complex is diverse but regionally specific. There is no difference in the staple diet between the taxa of the complex, but in the less frequent and rare prey categories, especially in the parental species *P. ridibundus* and *P. lessonae*. Understanding feeding habits and trophic ecology is of particular importance for syntopic and marginal populations of the complex and for understanding the details of co-occurrence in human-modified landscapes. This is particularly important for *P. lessonae*, one of the parental species, as the southern Banat represents its southern geographical boundary. Although *P. lessonae* is classified as a Least Concern Species (LC) by the International Union for Conservation of Nature and populations are declining worldwide (Kuzmin et al., 2009), it can be considered threatened at the regional level as it is exposed to constant and increasing environmental threats throughout its range, which increase the risk of local extinction (Sjögren-Gulve, 1994; Vukov et al., 2015).

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (grant no. 451-03-47/2023-01/200178). Collection of adult frogs of *Pelophylax esculentus* complex from a natural population was approved by the Ministry of Environmental Protection of the Republic of Serbia (Permits No. 353-01-1170/2016-17 and 353-0l-370/2018-04). The authors would like to acknowledge the contribution of the Association for the Protection of the Danube and Sava Rivers in the Republic of Serbia for providing infrastructure and laboratory facilities during the field work.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <http://www-9.unipv.it/webshi/appendix/index.html> manuscript number 13529

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Acta Herpetologica 19(1): 69-73, 2024
DOI: 10.36253/a_h-15519

The lateral stripe – a reliable way for software assisted individual identification for *Hyla arborea*

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Submitted on: 2023, 12th December; revised on: 2024, 20th February; accepted on: 2024, 25th February
Editor: Enrico Lunghi

Abstract. Traditional mark-recapture methods for amphibians and reptiles involve the application of invasive physical markers, such as toe clipping. Photographic identification methods are non-invasive alternative that use natural colouration of individuals for inexpensive, reliable identification. The relatively small species of the Hylidae family are susceptible to toe clipping – so dorsal, ventral, or leg patterns have been used in studies on different hylid species. The present study aims to test the usefulness of the lateral stripe in the Common tree frog *Hyla arborea* in software-assisted image recognition, which would allow for a reliable and time-efficient individual identification. A total of 258 adult tree frogs from a pond near the village of Oshtava, SW Bulgaria, were captured by hand in twelve sessions throughout the spring (April-May) of 2022 and 2023. The right and left side of each frog was photographed and the animals were released at the site of capture. Images were loaded into Hotspotter – free software for image recognition that has been demonstrated to work very well for several amphibian species. Results revealed 108 recaptures of 46 individual tree frogs (including 11 tree frogs captured more than twice and three recaptures between years). Only 12% of all frogs had similar right and left lateral stripes, with the others displaying significant variations. This study highlights both the applicability of the lateral stripe for individual recognition in this species and the need for consistency in image processing.

Keywords. Colouration, Common tree frog, recognition, variation.

For several decades, individual identification of animals has been a crucial aspect of conservation biology and behavioural ecology. Studies focused on life-history traits and long-term monitoring require to repeatedly and reliably identify captured individuals. Traditional mark-recapture methods for amphibians and reptiles involve the application of physical markers with varying degrees of invasiveness – from paint marking to toe clipping (review in Ferner, 2007). For amphibians in particular, popular markings include toe clipping and subcutaneous injections/pit tags, with toe clipping being the most widespread for many decades because of its affordability. However, toe clipping does inflict temporary (i.e., newts regenerate toes) or permanent physical harm to the animal, and it is well-established that it could cause diminished survival and altered behaviour in some smaller species (e.g., Clarke, 1972; Guimaraesh et al., 2014). Alternatives to toe clipping have been implemented since the turn of the century, incl. visible implant elastomers (VIE) (Pittman et al., 2008; Antwis et al., 2014) and visible implant tags (Pittman et al., 2008). VIE provide reliable and rapid identification, but are expensive so their usefulness is often limited by their cost (Le Chevalier et al., 2017). During the past decade, there has been a growing number of studies investigating the potential of using the natural colouration pattern of various amphibian spe-

ISSN 1827-9635 (print)
ISSN 1827-9643 (online)

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cies in photographic identification methods (PIM), as the rapid advance in technology allows for inexpensive, reliable and non-invasive identification (Kenyon et al., 2009; Bendik et al., 2013; Elgue et al., 2014; Schoen et al., 2015; Sannolo et al., 2016; Kim et al., 2017; Renet et al., 2019; Lukanov, 2022). The relatively small species of the Hylidae family are susceptible to toe clipping, as it has a negative effect on their survival and capture probability, especially when multiple toes are removed (Waddle et al., 2008) – so dorsal, ventral or leg patterns have been used in PIM studies on some hylid species (Kim et al., 2017). While manual visual identification is very useful for smaller samples, it becomes unreliable and time-consuming for larger catalogues, and in recent years, there have been developed some specialized software packages aimed at image pattern recognition (Speed et al., 2007; Bolder at al., 2012; Crall et al., 2013; Matthé, 2018). As the usefulness of software-assisted image recognition for *Hyla* sp. has not been investigated, the present study aims to test whether photographs of the lateral stripe in the Common tree frog *Hyla arborea* (L., 1758) would allow for a reliable and time-efficient individual identification.

The Common tree frog is distributed from the Southern Balkans to North-Western Europe. In Bulgaria, it is present in the Struma river basin, whereas the Eastern tree frog *Hyla orientalis* (Bedriaga, 1890) occurs across the rest of the country (Dufresnes et al., 2015). The current study is part of an ongoing project on tree frog morphology and distribution in Bulgaria, which aims to compare populations across the country. The present study pond is located near the village of Oshtava in South-Western Bulgaria, with a surface area around 0.50 hectares and fluctuating water level (spring maximums and summer minimums, incl. desiccation). It is surrounded by grassy meadows and mixed forests, and water vegetation is dominated by reeds (*Phragmites* sp.). While the maximum depth is over 1m, there are shallow waters along the edges of the pond, and this combination provides excellent breeding conditions for many amphibian and reptile species: Agile frog *Rana dalmatina* Fitzinger, 1838, Balkan spadefoot toad *Pelobates balcanicus* Karaman, 1928, Common tree frog *Hyla arborea* (L., 1758), Green toad *Bufotes viridis* (Laurenti, 1768), Common toad *Bufo bufo* (L., 1758), Marsh frog *Pelophylax ridibundus* (Pallas, 1771), Buresch's crested newt *Triturus ivanbureschi* Arntzen & Wielstra, 2013, Grass snake *Natrix natrix* (L., 1758).

A total of 258 tree frogs (51 females, 207 males) were captured in 12 sessions in the period April-May of 2022 (141 frogs from six sessions) and 2023 (117 frogs from six sessions). In addition to this number, in each year, 10 of the captured tree frogs were randomly chosen and kept in individual containers until the end of the session; these frogs were photographed each night for the duration of the sessions and the images were used as control for the individual recognition tests. Tree frogs were collected at night (between 21:30h and 23:30h), by hand or by using a fishing hand-net, in both shallow (both male and female tree frogs swimming or sitting in the shallows) and in deeper water (male tree frogs calling from the reeds at up to 1m depth). Each frog was held in hand and photographed laterally from both sides to document the shape of its lateral stripe, after which it was released at the site of capture. We chose the lateral view, rather than the dorsal or the ventral, as the lateral stripe has been proven to be individually specific for other tree frog species (Kim et al., 2017), while colouration, in contrast, is known to be varying and unreliable (Bolger et al., 2012). For individual identification, we used the software Hotspotter v. 1.0 (Crall et al., 2013), which has been demonstrated to work very well for some amphibian species (e.g., Naumov and Lukanov, 2018; Patel and Das, 2020; Burgstaller at al., 2021; Lukanov, 2022). The region of interest (ROI) which is used for comparison between the images, was set as close around the frog body as possible to minimize external factors, but at the same time to allow all elements of the lateral stripe to be clearly visible (for a detailed description of image processing and analysis, see Crall et al., 2013; Naumov and Lukanov, 2018). Suggested matches by the program were always manually verified by a human observer.

Results revealed 108 recaptures of 46 individual tree frogs (26 in 2022 and 20 in 2023, or a recapture rate of approximately 42% and 41%, respectively), incl. 11 tree frogs captured more than twice (all males) and three recaptures between years (one female and two males). In order to assess the software match, the images from the control groups were compared to the ones taken in the field and were very consistent, with the verified matches always appearing as the first suggested matches. Unlike coloration, the shape of the lateral stripe remained constant (at least in the short-term, as evidenced by frogs from the control group) and allowed for easy individual identification (Fig. 1). As demonstrated for other species (Burgstaller et al., 2021), the type of camera did not matter and photos from different cameras performed equally (incl. smartphones, as we used Xiaomi Redmi Note 8 and Mi A2, as well as Huawei P Smart, with default settings and flashes turned on).

We compared image scores (values assigned by the program based on image similarity, see Crall et al., 2013) between First match (score of the first suggested match for verified matches), Last match (aggregate score for all verified matches after the first suggested match, when more

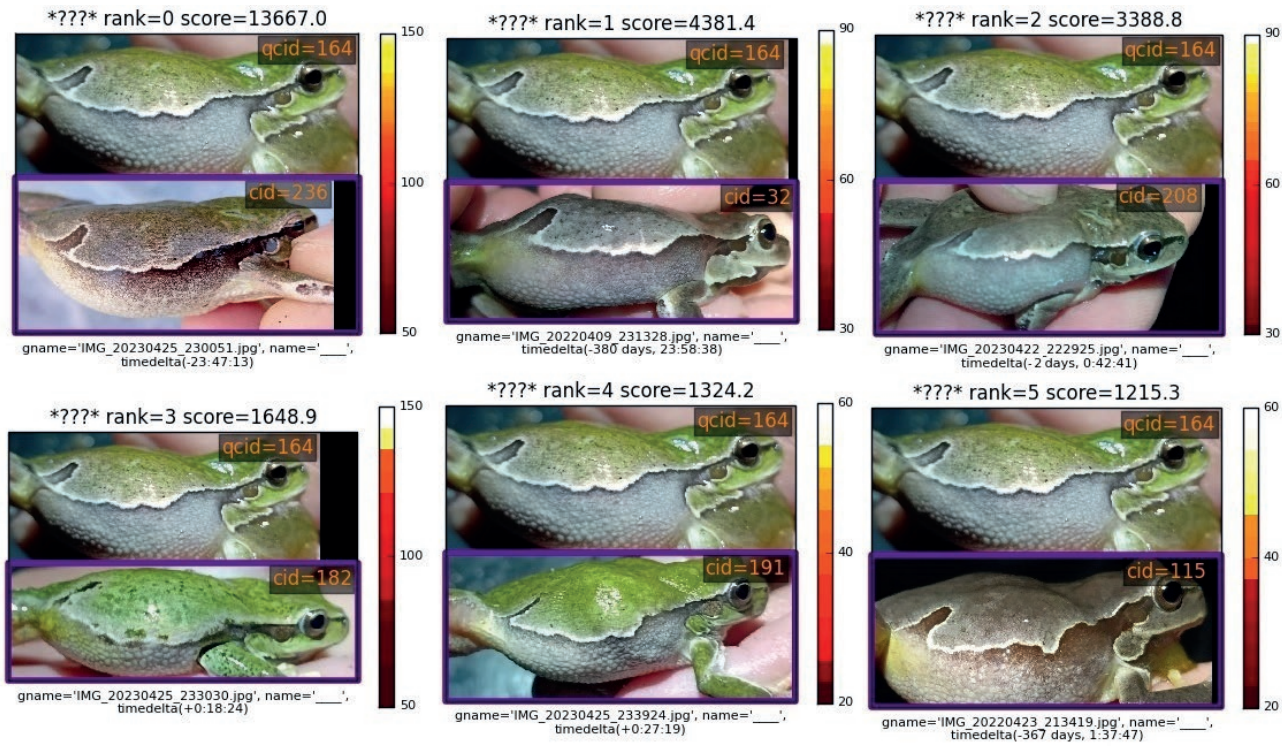

Fig. 1. Results from Hotspotter showing a male first captured on April 9th 2022 and then recaptured on 22nd, 24th and 25th April 2023. Scores for recaptures (first row) are 2-10 times higher than the other three suggestions (second row).

than one verified match was registered), and No match (score of the first suggested match for individuals without verified recapture). As data was not normally distributed, we used a Kruskal-Wallis and Multiple comparisons test in Statistica v. 7 (StatSoft, Inc. 2004), with level of statistical significance set at $P < 0.05$. The First match had an average similarity score of 13 735, with min-max range of 3590-52 541; these values were 3814 (1619-6482) for the Last match and 1314 (573-1805) for the No match, respectively. The Kruskal-Wallis test revealed significant differences between the three groups ($H_2 = 34.60$, $P < 0.001$), and Multiple Comparisons test confirmed differences between First match and No match ($P < 0.001$), Last match and No match ($P < 0.001$), but not between First match and Last match ($P = 0.075$). Although the average value for Last match was higher than the minimum of First match, there were no false matches between the first and the last suggested match. This is probably due to the fact that only 11 frogs were captured more than two times (i.e., in most cases there was no other match than the first), and of these, only five were captured four times (i.e., with three consecutive matches).

The scores assigned by the Hotspotter algorithm tend to vary depending on the species and the size of the image set, with small image sets producing higher scores (Crall et al., 2013). For this reason, match score values cannot be used as a sole indicator of a positive match, and manual verification is required. Nevertheless, there were no false positives (i.e., similarly high scores for different individuals) and a careful manual examination of all photos confirmed there were also no false negatives (i.e., low scores for images of the same individual, or identical individual image not suggested as a potential match by the program).

Results from comparisons between the right and left lateral stripes were virtually identical, i.e., all frogs that were identified as recaptures from images of the right side of their body, were also identified from images of the left side, and vice versa. This was reflected in the similarity scores, which were very similar (Mann Whitney U test: First match $U = 51.0$, $P = 0.732$; Last match $U = 19.0$, $P = 0.335$; No match $U = 32.0$, $P = 0.270$). However, there were often contrasting differences between the left and right lateral stripes in the same individual (Fig. 2A), and in only 12% of all frogs the right and left lateral stripes were similar (but still not identical) (Fig. 2B).

Our results lead to two conclusions: 1) the lateral stripe in the Common tree frog could be reliably used in PIM for both sexes, at least for short-term studies (the number of recaptures between years was very low and no definite conclusions could be made) and 2) a soft-

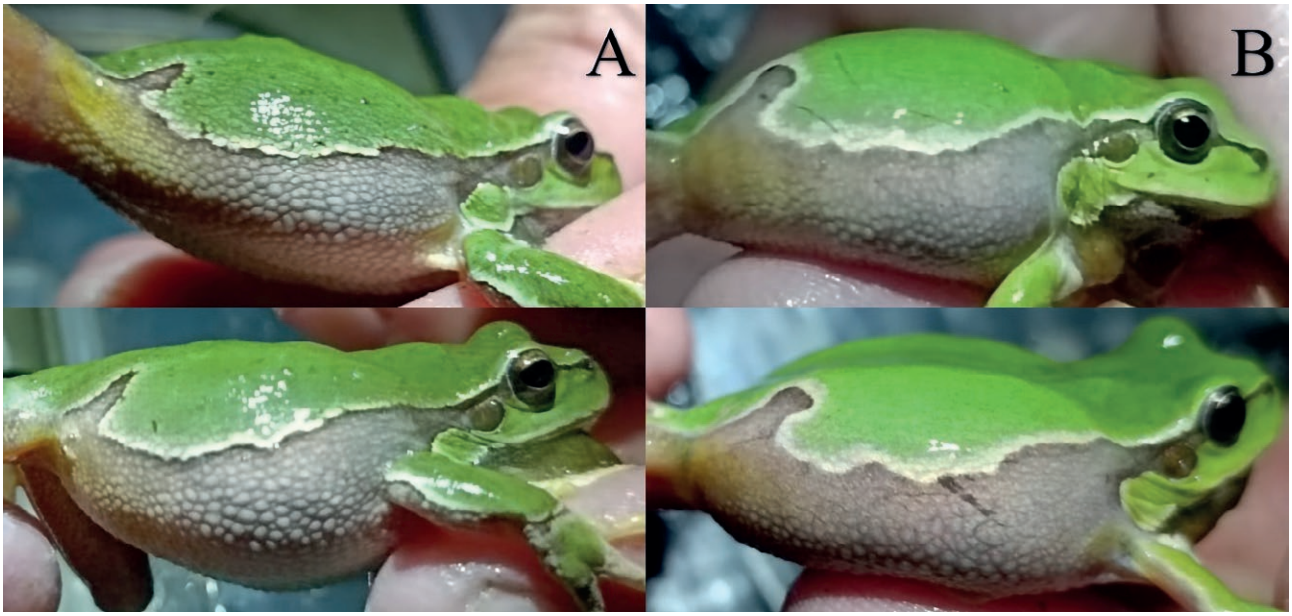

Fig. 2. A) Individual with very different right (top) and left (bottom) lateral stripes; B) Individual with similar right (top) and left (bottom) lateral stripes. Images of the left side are reversed to the right for easier comparison.

ware-assisted identification would provide time-efficient and reliable results. We still need to stress the necessity of manual verification, as relying solely on matching scores could lead to false positives, especially in smaller samples. Under certain conditions, some amphibians may change their colour pattern over long periods (see Naumov and Lukanov, 2018), and the applicability of this method for long-term population studies on *Hyla* species remains to be confirmed – but it is undoubtedly a very useful tool for non-invasive individual recognition. One way to prove the long-term usefulness of colour patterns in this frog genus is to match it with results from VIE, as this method has been demonstrated to work well for up to six years in the salamander *Hydromantes italicus* (Lunghi and Bruni, 2018). It also needs to be noted that Hotspotter seems to outperform other image recognition programs such as Wild-ID, which did not produce satisfactory results for the Japanese tree frog *Dryophytes japonicus* (Günther, 1859), which was photographed in a manner that was similar to ours - being held in hand and released immediately (Kim et al., 2017). This would confirm the observations of Naumov and Lukanov (2018) and Burgstaller et al. (2021), who conclude that Hotspotter performs consistently better than the other image recognition software they tested (for *Triturus dobrogicus* (Kiritzescu, 1903) and *Bufotes viridis*, respectively). Importantly, Burgstaller et al. (2021) point out that camera type has a negligible effect on the performance of all tested image recognition programs. It has to be noted that Hotspotter may prove unreliable for larger samples (over 900–1000 images), as in such cases it is known to crash and disrupt the workflow (S. Lukanov, pers. obs.; S. Burgstaller, pers. comm. 2022); however, no problems of this kind were reported by Dunbar et al. (2021), who used 2136 images of Hawksbill turtles *Eretmochelys imbricata* (L., 1766).

ACKNOWLEDGEMENTS

This study was supported by the National Science Fund of Bulgaria under Grant contract № KP-06-N31/11 from 11.12.2019. Handling of animals was carried out in accordance with Bulgarian Ministry of Environment and Water permit N° 861/13.01.2021. We thank Martin Marinov, Martin Tzvetkov, Irena Atanasova and Emiliya Vacheva for their help with the fieldwork in 2022.

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Acta Herpetologica 19(1): 75-80, 2024
DOI: 10.36253/a_h-14922

Fluorescence in amphibians and reptiles: new cases and insights

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Submitted on: 2023, 11th July; revised on: 2023, 17th October; accepted on: 2024, 21st February
Editor: Raoni Rebouças

Abstract. Fluorescence in amphibians and reptiles has emerged as a prominent study subject in recent years, with research focused on understanding its function and diversity. As the knowledge of fluorescence in vertebrates is still understudied, we surveyed amphibian and reptile species in montane and lowland Atlantic Forest sites to evaluate presence or absence of fluorescence. By randomly sampling species, we found evidence of fluorescence in amphibians of the genera *Scinax*, *Brachycephalus* and *Hylodes*, and reptiles of the genera *Bothrops*, *Enyalius* and *Hemidactylus*. Our findings increase the list of known species that may benefit from fluorescent patterns. Fluorescence was either ocular, dermal, or subdermal related to the skeleton or ossified dermal structures. Whether these species are able to see and interpret the different the patterns generated by fluorescent structures is yet to be discovered.

Keywords. Biofluorescence, coloration, herpetofauna, natural history, ontogeny, sexual dimorphism, terrestrial vertebrates, visual communication.

Biofluorescence was first described in the early 16th century through the study of medicinal herbs by a Spanish researcher (Lagorio et al., 2015). However, the first *in situ* observation was only reported in the 20th century for green-blue algae (Tswett, 1911). Cockayne (1924) published the first studies about biofluorescent animals. Since then, fluorescence has been the subject of investigation by numerous researchers. Among vertebrates, biofluorescence was primarily reported for marine species (Wucherer and Michiels, 2012; Sparks et al., 2014), while research on terrestrial tetrapods has only gained attention in recent years (Prötzel et al., 2021). For amphibians and reptiles, three main types of fluorescence are known. In reptiles, bone fluorescence stands out (Prötzel et al., 2018; Sloggett, 2018; Jeng, 2019; Top et al., 2020; Pinto et al., 2021; Maria et al., 2022), along with dermal fluorescence (Paul and Mendyk, 2021; Prötzel et al., 2021). In amphibians, in addition to dermal (Taboada et al., 2017a, b; Deschepper et al., 2018; Chaves-Acuña et al., 2020; Whitcher, 2020) and bone fluorescence (Goutte et al., 2019; Rebouças et al., 2019; Nunes et al., 2021), ocular fluorescence was reported recently (Deschepper et al., 2018; Alvarez et al., 2022).

Although the evolution of biofluorescence is still barely understood (Macel et al., 2020), some hypotheses have been proposed to explain its function. These include prey attraction (Haddock and Dunn, 2015; Paul and Mendyk, 2021), predator avoidance (Rebouças et al., 2019), camouflage (Sparks et al., 2014), visual communication (Goutte et al., 2019; Gray, 2019; Alvarez et al.,

ISSN 1827-9635 (print)
ISSN 1827-9643 (online)

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2022), visual recognition, mate choice, and sexual attraction (Hausmann et al., 2003; Prötzel et al., 2018).

In this study, we describe and illustrate fluorescence in four amphibian and four reptile species, and provide a list of amphibians that apparently did not display fluorescence when observed under UV light.

We used an UltraFire WF-5016 flashlight with a wavelength of 365 nm to test UV light fluorescence of several amphibians and reptiles. To maximize the chances of finding fluorescence, we caught and exposed the amphibians to light on all body sides, including the ocular region. When detecting the presence of UV fluorescence, we photographed the animal using a Nikon D7100 digital camera with a 100mm Sigma macro lens, with an aperture of f/5, ISO sensitivity of 3200, and a shutter speed of 1/200. After the tests, we recorded the presence or absence of fluorescence in a field spreadsheet and released the animals into the same location where we captured them. We categorized the fluorescence as dermal when detected on the surface of the animal's skin or in soft tissues, as bone fluorescence when reflected in areas such as the skull or vertebral column, and as ocular fluorescence when the fluorescence was displayed in the animal's eyes.

Individuals of *Brachycephalus nodoterga* were found in March 2022 in the Núcleo Santa Virgínia of the Parque Estadual da Serra do Mar, Natividade da Serra, São Paulo, Brazil. The specific location was known as "trilha do campinho" (23.866667°S, 45.568611°W, 855 m a.s.l.). Natividade da Serra is in mosaic-like Atlantic Forest vegetation, consisting of primary and secondary forests in different stages, bordered by eucalyptus plantations and pastures. The rainy season in this area occurs from October to March, while the drier season spans April to September. We searched opportunistically for other amphibians and reptiles from May to June 2023, during routine monitoring in the area of the NGO Projeto Dacnis (23.462947°S, 45.132943°W; 15-500 m a.s.l.). Projeto Dacnis encompasses a private reserve spanning 136 ha within the Atlantic Forest in Ubatuba, São Paulo, Brazil. The area is a swampy forest in low-lying areas and patches of primary and secondary dry forest on steep terrain. The climate is humid with rainfall incidence throughout the year. Finally, we also tested one individual of *Bokermannohyla alvarengai* in August 2023 in Monumento Natural Estadual Várzea do Lajeado e Serra do Raio, Serro, Minas Gerais, Brazil. The location is close to Caminho dos Escravos, in the district of São Gonçalo do Rio das Pedras (18.43019°S, 43.464654°W, 1165 m a.s.l.). The Serro region is predominantly covered by high-altitude savannah vegetation, with rocky and sandy fields and humid floodplains. There is also Atlantic Forest, with secondary forests, and areas deforested for agricultural use. The climate is characterized by two well-defined seasons, cold and dry winter, between April and September, and hot and humid summer, between October and March.

In total we tested 122 individuals of 25 amphibian and four reptile species (Table 1). Among the tested amphibians, five species displayed fluorescence: *Brachycephalus nodoterga* had dermal bones fluorescence on the dorsum (Fig. 1A–B); *Scinax argyreornatus* displayed dermal fluorescence on the dorsum, inguinal region, jaw, and upper part of the head (Fig. 1C–F); *Bokermannohyla alvarengai* presented dermal fluorescence on the entire dorsum, but in the blue spectrum (Fig. S1); *Hylodes phyllodes* and *H. asper* showed fluorescence only on their eyes (Fig. S2). Among reptiles, all four tested species displayed fluorescence. The lizard *Enyalius perditus* had fluorescence on the skull, with more evident reflections in males, both on the back and the lateral side of the head (Fig. 2A–F). A juvenile *Hemidactylus mabouia* gecko displayed fluorescence in both the skull and the vertebral column (Fig. 2G–I). The adult, photographed from a distance, showed fluorescence only on the upper part of the head and jaw. *Bothrops jararaca* and *B. jararacussu* displayed fluorescence only on the tail tip of juveniles (Fig. S3). From the three *B. jararaca* individuals (total length 28, 43, and 62 cm), the largest individual showed fluorescence only at a small portion of the tail tip.

Bone fluorescence in *Brachycephalus nodoterga* showed a distinct pattern from *B. ephippium*, *B. pitanga* and *B. rotenbergae* (Goutte et al., 2019; Nunes et al. 2021). This difference is due to the amount and distribution of dorsal ossified plates in these species (Goutte et al., 2019; Nunes et al. 2021). UV light fluorescence in a species of the genus *Scinax* and *Bokermannohyla* is here reported for the first time, despite fluorescence being recorded in other genera and species of hylid treefrogs (Taboada et al., 2017 a, b; Deschepper et al., 2018; Chaves-Acuña et al., 2020; Whitcher, 2020). Fluorescence in frogs could be related to intraspecific communication as a visual signal that complements acoustic signalling (Goutte et al., 2019; Gray, 2019) and can contribute to achromatic vision and the detection of other individuals in low-light environments (Lamb and Davis, 2020). Fluorescence in frogs of the genus *Hylodes* is also reported for the first time here. Furthermore, ocular fluorescence is reported only for four other anuran species in the genera *Boana* (Hylidae) and *Rana* (Ranidae) (Deschepper et al., 2018; Alvarez et al., 2022). Deschepper et al. (2018) suggest that fluorescent eyes are related to intraspecific recognition, whereas for Alvarez et al. (2022) this fluorescence type may be related to interspecific communication among sympatric species, thus avoiding predatory conflicts or disputes for food and territory.

Table 1. Amphibians and reptiles tested under UV light (wavelength 365 nm) and fluorescence type when present (bold).

Species	Fluorescence	Type	Nº ind. tested
Anura			
Brachycephalidae			
Brachycephalus nodoterga	**Yes**	**Bone**	**15**
Ischnocnema sp. (aff. *guentheri*)	No		6
Bufonidae			
Dendrophryniscus haddadi	No		4
Rhinella ornata	No		3
Craugastoridae			
Haddadus binotatus	No		3
Cycloramphidae			
Cycloramphus boraceiensis	No		9
Phyllomedusidae			
Phasmahyla sp. (aff. *cruzi*)	No		5
Pithecopus rohdei	No		3
Hylidae			
Boana albomarginata	No		3
Boana faber	No		2
Boana semilineata	No		2
Bokermannohyla alvarengai	**Yes**	**Dermal**	**1**
Bokermannohyla hylax	No		2
Itapotihyla langsdorffii	No		4
Scinax argyreornatus	**Yes**	**Dermal**	**9**
Scinax hayii	No		3
Scinax littoralis	No		5
Scinax perpusillus	No		3
Hylodidae			
Hylodes asper	**Yes**	**Ocular**	**6**
Hylodes phyllodes	**Yes**	**Ocular**	**5**
Hemiphractidae			
Fritziana mitus	No		2
Gastrotheca albolineata	No		1
Leptodactylidae			
Adenomera marmorata	No		5
Leptodactylus latrans	No		3
Physalaemus atlanticus	No		3
Squamata			
Leiosauridae			
Enyalius perditus	**Yes**	**Bone**	**6**
Gekkonidae			
Hemidactylus mabouia	**Yes**	**Bone**	**4**
Viperidae			
Bothrops jararaca	**Yes**	**Dermal**	**3**
Bothrops jararacussu	**Yes**	**Dermal**	**2**

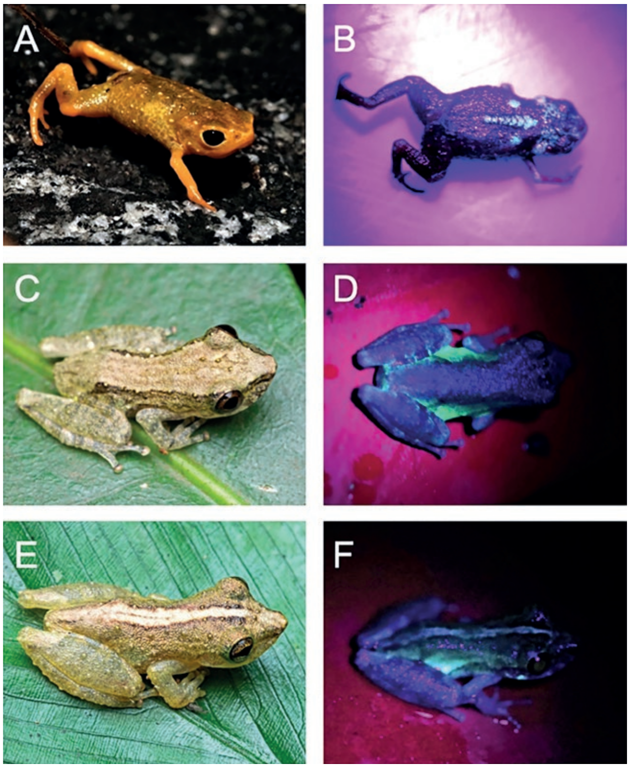

Fig. 1. Amphibian species with fluorescence: *Brachycephalus nodoterga* photographed with flash (A) and UV light (B); *Scinax argyreornatus* photographed with flash (C) and under UV light (D); another *Scinax argyreornatus* with a dorsal stripe, photographed with flash (E) and under UV light (F).

Bone fluorescence for a species of the genus *Enyalius* is a novel information, similar to that reported by Prötzel et al. (2018) in chameleons of the genus *Caluma*, where males display more cranial fluorescence than females. In lizards, bone fluorescence has also been reported for the gekkonid genera *Chondrodactylus* (Sloggett, 2018), *Cyrtodactylus* (Jeng, 2019; Top et al., 2020), *Kolekanos* (Pinto et al., 2021), and *Hemidactylus* (Maria et al., 2022). Maria et al. (2022) reported fluorescence on the head and mandible of *Hemidactylus platyurus*, as well as a more pronounced fluorescence in juvenile individuals, similarly to what we observed for *H. mabouia*. Bone fluorescence in lizards was suggested to play a role in interspecific visual communication, serving as a secondary visual communication system that does not compromise their camouflage, as well as for attracting sexual partners (Prötzel et al., 2018; Top et al., 2020).

Our record of tail tip fluorescence in two pit viper species of the genus *Bothrops* is a novel information, although Paul and Mendyk (2021) already reported tail tip fluorescence in eight pit viper genera known or suspected to display tail luring to attract prey. Juveniles of both *B. jararaca* and *B. jararacussu* feed predominantly on frogs and use caudal luring to attract and catch this prey type (Sazima, 1991, 1992; Hartmann et al., 2003; Sazima, 2006). As frogs have UV light sensitivity (Thomas et al., 2022), this sense may be used to detect prey, and

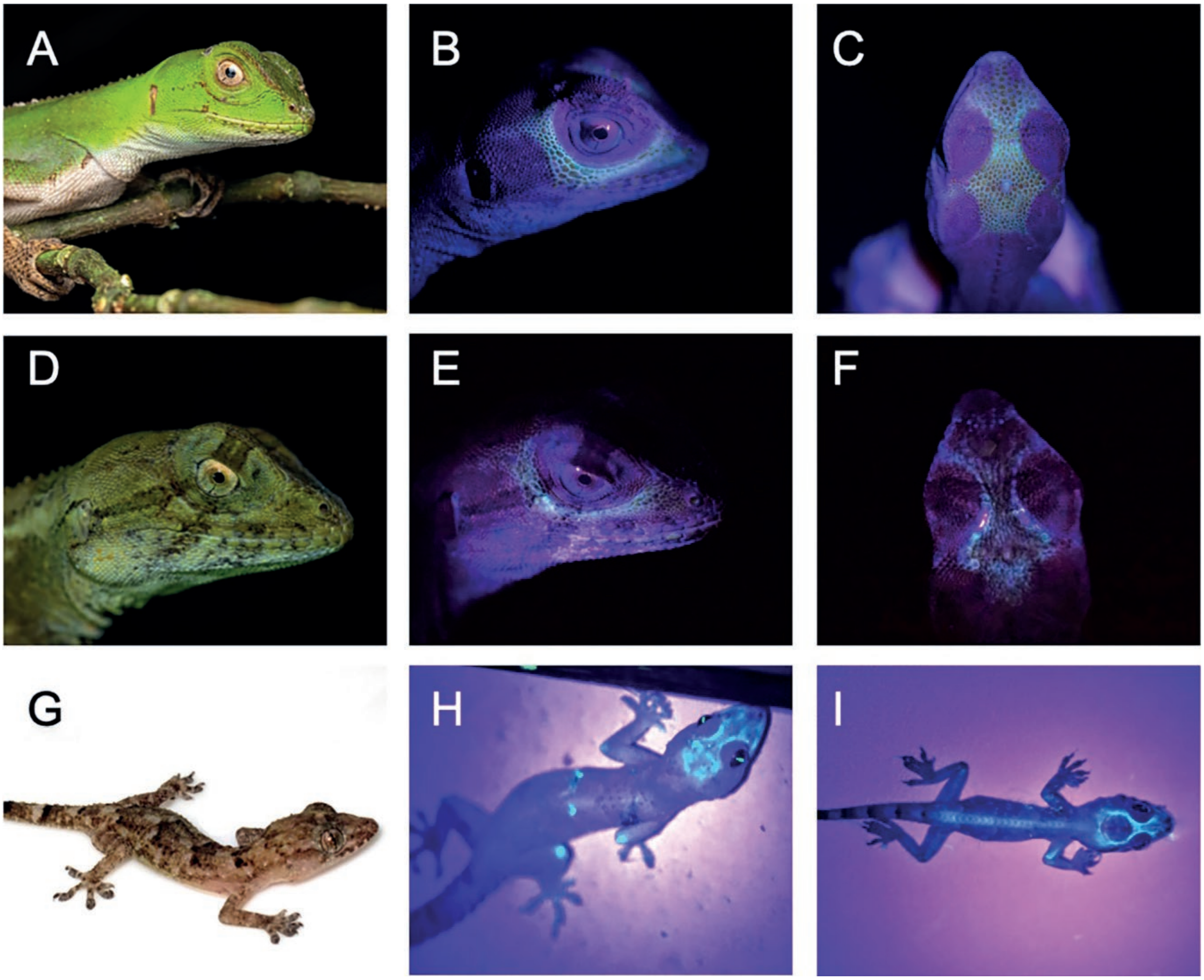

Fig. 2. Lizard species with fluorescence: Male *Enyalius perditus* photographed with flash (A) and under UV light (B-C); Female *Enyalius perditus* photographed with flash (D) and under UV light (E-F); *Hemidactylus mabouia* photographed with flash (G) and, under UV light, an adult (H) and a juvenile (I).

the fluorescence of the two snakes' tail tips could play an important role: attracting frogs at night (Sazima, 1991; Sazima and Haddad, 1992).

The noticeable decrease in tail tip fluorescence of the largest *B. jararaca* individual (not adult yet, see Sazima, 1992) is likely related to ontogenetic diet changes. Adult individuals ambush or actively hunt rodents and small mammals, and no longer display caudal luring (Sazima, 1991; Hartmann et al., 2003). Fluorescence decrease on the tail tip of large individuals was already reported in other vipers, also related to ontogenetic diet changes (Paul and Mendyk, 2021).

We failed to detect UV fluorescence in an additional 20 species tested. However, we do not exclude the possibility of fluorescence in those species. We illuminated them with a wavelength of 365 nm, and suggest experiments with longer wavelengths, as some species may only display fluorescence when exposed to lights of 400–415 nm (Whitcher, 2020).

As we showed, fluorescence in anurans and reptiles may be widespread (Deschepper et al., 2018), especially when considering that studies on fluorescence in terrestrial tetrapods have only begun to increase in recent years (Prötzel et al., 2021). Therefore, testing other species in different localities may reveal fluorescence of numerous other species, as well as provide insights into the ecological and evolutionary relevance of such coloration patterns.

ACKNOWLEDGMENTS

We would like to thank Elsie Laura Rotenberg for reviewing the text. The National Council of Scientific and Technological Development provided fellowships (CNPq #302834/2020-6; #300992/79-ZO) and São Paulo Research Foundation provided a grant (FAPESP #2022/11096-8) to LFT.. Permits to capture and handle animals were provided by Biodiversity Authorization and Information System – SISBIO Number: 27745-24.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <http://www-9.unipv.it/webshi/appendix/index.html> manuscript number 14922

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Finito di stampare da
Logo s.r.l. – Borgoricco (PD) – Italia

Cover: *Hierophis viridiflavus*, melanic male (photo by Edoardo Razzetti); isolated hemipenis (photo by Federico Storniolo) and ornamentation particulars (photo by Mattia Gilio).

Università degli Studi di Firenze
Firenze University Press
via Cittadella 7, 50144 Firenze, Italy
http://www.fupress.com/
E-mail: journals@fupress.com

Periodicità: semestrale
ISSN 1827-9643 (online)
ISSN 1827-9635 (print)
Registrata al n. 5450 del 3.11.2005
del Tribunale di Firenze

ACTA HERPETOLOGICA

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June **2024** Vol. **19** – N. **1**