June
2025
Vol. 20 – N. 1

SOCIETAS
HERPETOLOGICA
ITALICA

ISSN 1827-9635

Acta Herpetologica

FIRENZE
UNIVERSITY
PRESS

Acta Herpetologica

Acta Herpetologica è la rivista ufficiale della *Societas Herpetologica Italica* (S.H.I.), un'associazione scientifica che promuove la ricerca erpetologica di base e applicata, la divulgazione delle conoscenze e la protezione degli Anfibi e Rettili e dei loro habitat.

Acta Herpetologica is the official journal of the *Societas Herpetologica Italica* (S.H.I.), a scientific association that promotes basic, applied, and conservation researches on Amphibians and Reptiles.

Sito ufficiale S.H.I. (Official S.H.I. website): http://www-9.unipv.it/webshi

Modalità di associazione
Le nuove domande di associazione sono esaminate periodicamente dal Consiglio Direttivo; solo successivamente i nuovi soci riceveranno la comunicazione di accettazione con le modalità per regolarizzare l'iscrizione (ulteriori informazioni sul sito: http://www.unipv.it/webshi). La quota annuale di iscrizione alla S.H.I. è di € 35,00. I soci sono invitati a versare la quota di iscrizione sul conto corrente postale n. 62198205 intestato a: SHI *Societas Herpetologica Italica*. In alternativa è possibile effettuare un bonifico bancario sul Conto Corrente Postale: n. conto 62198205 intestatario: SHI *Societas Herpetologica Italica* IBAN: IT95Y0837433220000013351889.

Membership
The S.H.I. Council will examine periodically new applications to S.H.I.: if accepted, new Members will receive confirmation and payment information (for more information contact the official website: http://www.unipv.it/webshi). Annual membership fee is € 35.00 (Euro). Payments are made on the postal account of SHI *Societas Herpetologica Italica* no. 62198205, or by bank transfer on postal account no. 13/351889 at *Banca di Credito Cooperativo di Barlassina* IBAN: IT95Y0837433220000013351889 to *Societas Herpetologica Italica.*

Versione on-line: http://www.fupress.com/ah

SOCIETAS
HERPETOLOGICA
ITALICA

Acta Herpetologica

Vol. 20, n. 1 - June 2025

Firenze University Press

Acta Herpetologica 20(1): 3-16, 2025
DOI: 10.36253/a_h-17227

Genetically informed range extension for *Kurixalus inexpectatus* (Anura: Rhacophoridae) in Fujian, with ecological niche modeling to guide further searches

Dallin B. Kohler[1], Siti N. Othman[2,*], Zhenqi Wang[3], Yiyao Zhu[4], Yiyun Wu[5], Ming-Feng Chuang[6,7], Amaël Borzée[2,8,*]

[1] *Laboratory of Animal Behaviour and Conservation, College of Ecology and the Environment, Nanjing Forestry University, Nanjing 210037, China*
[2] *Laboratory of Animal Behaviour and Conservation, College of Life Sciences, Nanjing Forestry University, Nanjing 210037, China*
[3] *College of Life Sciences, Nanjing Forestry University, Nanjing 210037, China*
[4] *Yunxiao County Forestry Bureau, Zhangzhou 363300, China*
[5] *Fujian Forestry Prospect and Design Institute, Fuzhou 350001, China*
[6] *Department of Life Sciences, National Chung Hsing University, Taichung, 402202, Taiwan*
[7] *Global Change Biology Research Center, National Chung Hsing University, Taichung, 402202, Taiwan*
[8] *Department of Zoology and General Biology, Faculty of Life Sciences, Fergana State University, Fergana, 150100, Uzbekistan*
**Corresponding authors.* Email: dy.othman@gmail.com, amaelborzee@gmail.com

Submitted on: 2025, 27th January; revised on: 2025, 9th March; accepted on: 2025, 5th May
Editor: Andrea Gazzola

Abstract. Undocumented and unnamed biodiversity is difficult to conserve effectively. We encountered several *Kurixalus* (Anura: Rhacophoridae) individuals of an unknown species identity in Fujian, China, a province with no previous records of *Kurixalus*. This genus of frogs, of which many new species have been described in recent years, has been the subject of much historical taxonomic confusion, largely due to conserved morphology. We sequenced two mitochondrial and one nuclear genes (1748 total bp) for three individuals from Fujian and reconstructed their phylogeny including all known lineages of *Kurixalus* from mainland China, and other East Asia lineages for which data were available. Both Bayesian Inference and Maximum Likelihood trees consistently showed that the individuals from Fujian were most closely related to *K. inexpectatus*, which previously was only known from northern Zhejiang, over 830 km north of this new location. Our results help clarify the identity of the unknown *Kurixalus* in Fujian and the distribution of *Kurixalus*, particularly regarding the distribution of *K. hainanus*, which has previously been confused with other members of the *K. odontotarsus* species complex. Additionally, we created ecological niche models using Maxent for the clade including both *K. inexpectatus* and its sister species *K. idiootocus* to guide future survey efforts.

Keywords. *Kurixalus*, Maxent, cryptic species, tree frog.

INTRODUCTION

The genus *Kurixalus* Ye, Fei, and Dubois, 1999, sometimes referred to as the frilled swamp tree frogs, consists of 23 currently recognized species, with ten of these described since 2014 (Frost, 2024a). These small, arboreal frogs of the family Rhacophoridae are found mainly in Southeast Asia and have relatively similar morphologies, which has led to significant taxonomic confusion within the genus (Yu et al., 2017a; Lv et al., 2018; Nguyen et al., 2020). *Kurixalus* has also been the subject of multiple studies of biogeography and patterns of his-

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

www.fupress.com/ah

torical dispersal between islands and the Asian mainland (Yu et al., 2020; Mo et al., 2023), although some reported colonization timelines dating to the Miocene are incompatible with the estimated timing of geological formation of landmasses in the Pliocene (Lv et al., 2018; Ali, 2020).

Several recently described species of *Kurixalus* are known from only the type locality or very small ranges (Yu et al., 2018; Hou et al., 2021; Zeng et al., 2021; Guo et al., 2022; Messenger et al., 2022), but additional surveys may reveal new localities, as was the case with *K. lenquanensis* (Yu et al., 2017b; Pang et al., 2024). During field surveys at night in February 2024 in Yunxiao County (Fujian, China), we encountered multiple individuals of the genus *Kurixalus* of uncertain species identity (Fig. 1). The frogs were calling from underneath vegetation in a montane wetland at an elevation of around 800 meters (23.9189° N, 117.2022° E). As far as we are aware, there have been no published records for the genus *Kurixalus* in Fujian, with the closest records on the Chinese mainland being in Guangdong and belonging to *K. hainanus* (Yu et al., 2017a). Given the presence of paired dark patches on the belly, we hypothesized the unknown *Kurixalus* were most likely related to *K. idiootocus* or *K. inexpectatus* rather than *K. hainanus* (Zeng et al., 2021; Messenger et al., 2022). The *K. idiootocus* species complex has been in flux recently, with five mainland species having been described as sister to the island endemic *K. idiootocus*, namely *K. lenquanensis* (Yu et al., 2017b), *K. raoi* (Zeng et al., 2021), *K. silvaenaias* (Hou et al., 2021), *K. qionglaiensis* (Guo et al., 2022), and *K. inexpectatus* (Messenger et al., 2022). Of these, *K. silvaenaias* and *K. qionglaiensis* have been recognized to be the same species and the synonymy of *K. inexpectatus* with *K. idiootocus* has been proposed as well (Lyu et al., 2024). Additionally, the distribution and identity of members of the *K. odontotarsus* species complex in Southeast Asia is also in need of further clarification. Several likely species-level lineages have been identified (Yu et al., 2017a) but only some of these have been formally described, such as *K. yangi* (Yu et al., 2018). Within southeastern China, specifically Guangxi, Guangdong, and Hainan, the names *K. bisacculus*, *K. hainanus*, *K. odontotarsus*, and *K. verrucosus* have been used, but they likely correspond to a single lineage within the *K. odontotarsus* species complex (Yu et al., 2017a; Lv et al., 2018; Mo et al., 2023).

We conducted molecular phylogenetic analyses to assess the identity of the unidentified frogs and clarify the distribution of *Kurixalus* frogs in eastern Asia, with an emphasis on mainland China. Preliminary molecular barcoding indicated that the specimens from Fujian were most closely related to *K. inexpectatus*, though the only previously known locality of *K. inexpectatus* is in northern Zhejiang, 830 km further north. We also used ecological niche modeling with the known points for *K. inexpectatus* and its sister species, *K. idiootocus*, to predict the areas that may harbor additional undiscovered populations.

MATERIALS AND METHODS

Laboratory work

Upon encounter, three *Kurixalus* individuals from Fujian were collected and buccal swabs were taken to obtain DNA. We extracted genomic DNA from the buccal swabs of the three *Kurixalus* individuals sampled in Fujian and three individuals of *K. idiootocus* from Taiwan, as well as DNA from thigh muscle of four specimens of *K. inexpectatus* from Zhejiang (Table S1) using a Qiagen DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol. For the *Kurixalus* individuals from Fujian and three *K. idiootocus* from Taiwan, we sequenced one nuclear and two mitochondrial fragments. For the nuclear gene, we sequenced a 476 bp long fragment of Tyrosine exon-1 (*TYR*) using the primer pair L2976 (5'-TGC TGG GCR TCT CTC CAR TCC CA-3') H2977 (5'-AGG TCC TCY TRA GGA AGG AAT G-3'; Bossuyt and Milinkovitch, 2000). For the mtDNA, we sequenced an 827 bp fragment from a section of the partial 12S rRNA, complete tRNA-Valine, and partial 16S rRNA (*12S-Val-16S*) genes using the primer pair F0001 (5'-AGA TAC CCC ACT ATG CCT ACC C-3') R1169 (5'-GTG GCT GCT TTT AGG CCC ACT-3'; Wilkinson, Drewes, and Tatum, 2002). We also sequenced a 554 bp long fragment of the cytochrome oxidase subunit I (*COI*) gene using the primer pairs COI-CO1 (5'-TYT CWA CWA AYC AYA AAG AYA TTG G-3') COI-CO3 (5'-ACY TCY GGR TGA CCA AAR AAY CA-3') and Chmf4 (5'-TYT CWA CWA AYC AYA AAG AYA TCG G-3') Chmr4 (5'-ACY TCR GGR TGR CCR AAR AAT CA-3'; Che et al., 2012). For the *K. inexpectatus* from Zhejiang, which already had two gene fragments sequenced (Messenger et al., 2022), we sequenced *COI* using the ad hoc-designed primers CO1KuF (5'-CCT GGG CCG GAA TGA TCG-3') CO1KuR (5'-TTG ATA AAG AAC TGG GTC CCC-3'), as these samples failed to amplify with the COI primers mentioned above. We amplified all fragments using polymerase chain reactions (PCR) in a total volume of 25 µl, which contained 12.5 µl of 2× Hieff PCR Master Mix (without dye), 1 µl of a 10 µM solution of each primer, 2 µl of DNA sample at a concentration of 10 ng/µl (within the recommended range), and 8.5 µl ddH2O. We carried out amplification using an Arhat 96 thermal cycler (Shanghai, China).

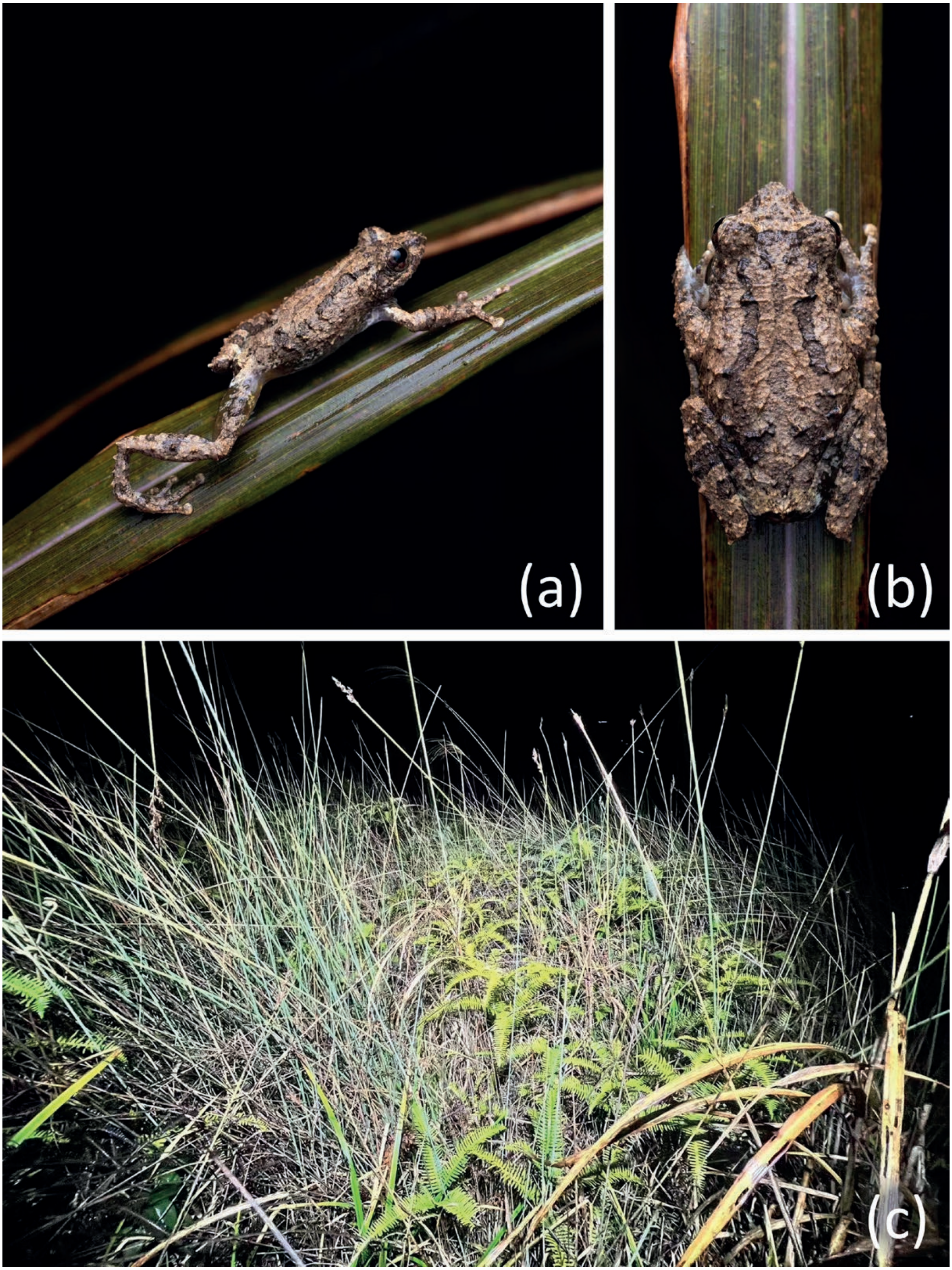

Fig. 1. *Kurixalus inexpectatus* from Fujian. Lateral (a) and dorsal (b) views of the *Kurixalus* found in southern Fujian. These individuals were found in a montane wetland composed mainly of the plant species *Lepidosperma chinense*, *Dicranopteris pedate*, and *Miscanthus floridulus* (c). Photos by Zhenqi Wang.

Thermal profiles for PCR were as follows: initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 54 °C for *TYR*, 55 °C for *12S-Val-16S*, and 46 °C for *COI* for 1 minute, and extension at 72 °C for 1 minute. The cycles were followed by a 10-minute final extension at 72 °C. PCR amplifications and double reads sequencing for all samples were performed by Sangon Biotech Co., Ltd. (Shanghai, China) and Tsingke Biotech Co., Ltd. (Beijing, China). The *Kurixalus* individuals from Fujian were released after initial DNA barcoding indicated they were not an undescribed species and no morphological measurements were taken.

Molecular analyses

To complement our sequences and reconstruct alignments for phylogeny, we downloaded homologous sequences for *Kurixalus* species from GenBank (www.ncbi.nlm.nih.gov/genbank). We added a sequence of *Theloderma albopunctatum* (Rhacophoridae) as the outgroup. To identify our unknown samples using phylogenetic analyses, the sequences in our alignments covered all identified clades of *Kurixalus* found in mainland China (Fig. 2, Table S1), according to the literature (Wilkinson, Drewes, and Tatum, 2002; Frost et al., 2006; Li et al., 2008, 2013; Nguyen, Matsui, and Duc, 2014; Wu et al., 2016; Yu et al., 2017a, 2020; Hou et al., 2021; Zeng et al., 2021; Messenger et al., 2022; Luo et al., 2023; Lyu et al., 2024; Xu et al., 2024). We trimmed and aligned our sequences using Muscle v. 5.1 (Edgar, 2004) in Geneious Prime 2023.2.1 (Kearse et al. 2012; www.geneious.com). We constructed three different sequence alignment datasets, namely: (1) a 1748 bp long concatenation of three gene fragments (802 bp of partial *12S-Val-16S*, 553 bp of *COI*, and 393 bp of *TYR*), including four individual *K. inexpectatus*, three *K. idiootocus*, and the three *Kurixalus* sampled in Fujian as the ingroup taxa, and two outgroups (*K.* cf. *bisacculus* and *T. albopunctatum*); (2) a 553 bp long *COI* fragment composed of 91 *Kurixalus* individuals and one *T. albopunctatum*, which had the greatest taxonomic coverage of our datasets; and (3) an 802 bp long fragment of *12S-Val-16S* composed of 43 *Kurixalus* individuals and one *T. albopunctatum*, which had a balance of taxonomic coverage and fragment length.

We used Partition Finder v. 2.1.1 (Guindon et al., 2010; Lanfear et al., 2012, 2016) to determine the best partitioning of the defined subsets, considering a fixed model for non-coding fragments and one for every codon position with respect to the coding fragments. We selected the models based on corrected Akaike information criterion (AICc) values. All models were treated as priors and used for further phylogenetic analyses, which were conducted using Bayesian inference (BI) implemented in MrBayes v. 3.2.4 (Ronquist et al., 2012) and maximum-likelihood (ML) implemented in IQ-Tree (iqtree.cibiv.univie.ac.at; Chernomor et al. 2016; Trifinopoulos et al. 2016; Hoang et al. 2018). For the BI analysis for each dataset, four independent runs were conducted, each of which was performed with MCMC algorithm for 10 million generations. We sampled every 1,000 generations, and discarded the first 25% of samples as burn-in. To ensure the convergence of all runs, we ensured that the analyses reached split frequencies below 0.005. For the ML analysis, we generated consensus trees using 1,000 bootstrap replicates.

Ecological niche models

Ecological niche modeling, most commonly implemented using Maxent (Phillips et al., 2017), relies on occurrence and environmental data to predict suitable areas for species (Ananjeva et al., 2015; Hou et al., 2023). Such models can be used to guide field surveys for uncommon species (Rhoden et al., 2017; Sarker et al., 2019; Entiauspe-Neto et al., 2024) and aid in conservation planning (Kidov and Litvinchuk, 2021; Shin et al., 2021). We used Maxent within R v. 4.2.1 to predict what other areas in eastern Asia may harbor undiscovered populations of *Kurixalus* related to those we found in Fujian (Phillips et al., 2017; Rhoden et al., 2017; R Core Team, 2022). As barcoding suggested, the individuals in Fujian were most closely related to *K. inexpectatus*, we used occurrence records of *K. inexpectatus* (Messenger et al., 2022; Li, 2023). However, since three localities is insufficient for accurate species distribution modeling (van Proosdij et al., 2016), we also opted to include an initial 29,251 records from GBIF for *K. idiootocus* (GBIF.org, 2024b), its sister species (Messenger et al., 2022). We removed the duplicates and thinned the datapoints at a distance of 1 km using the thinData function of the SDMtune package (Vignali et al., 2020), resulting in 1,585 total occurrence points for model training.

We defined the area for model construction as a 50 km buffer around the occurrence records (Hijmans, 2024). We started with the standard 19 bioclimatic variables as climate data (Fick and Hijmans, 2017) and supplemented them with additional layers for elevation, slope, and tree cover (Farr et al., 2007; Zanaga et al., 2021) via the geodata package v.0.6.2 (Hijmans et al., 2024), all at a resolution of 30 arc seconds (approx. 1 km^2). We used the built-in GRASS plug-in in QGIS v.3.32.2 (QGIS.org, 2023) to calculate Pearson correlation coefficients for our environmental variables within

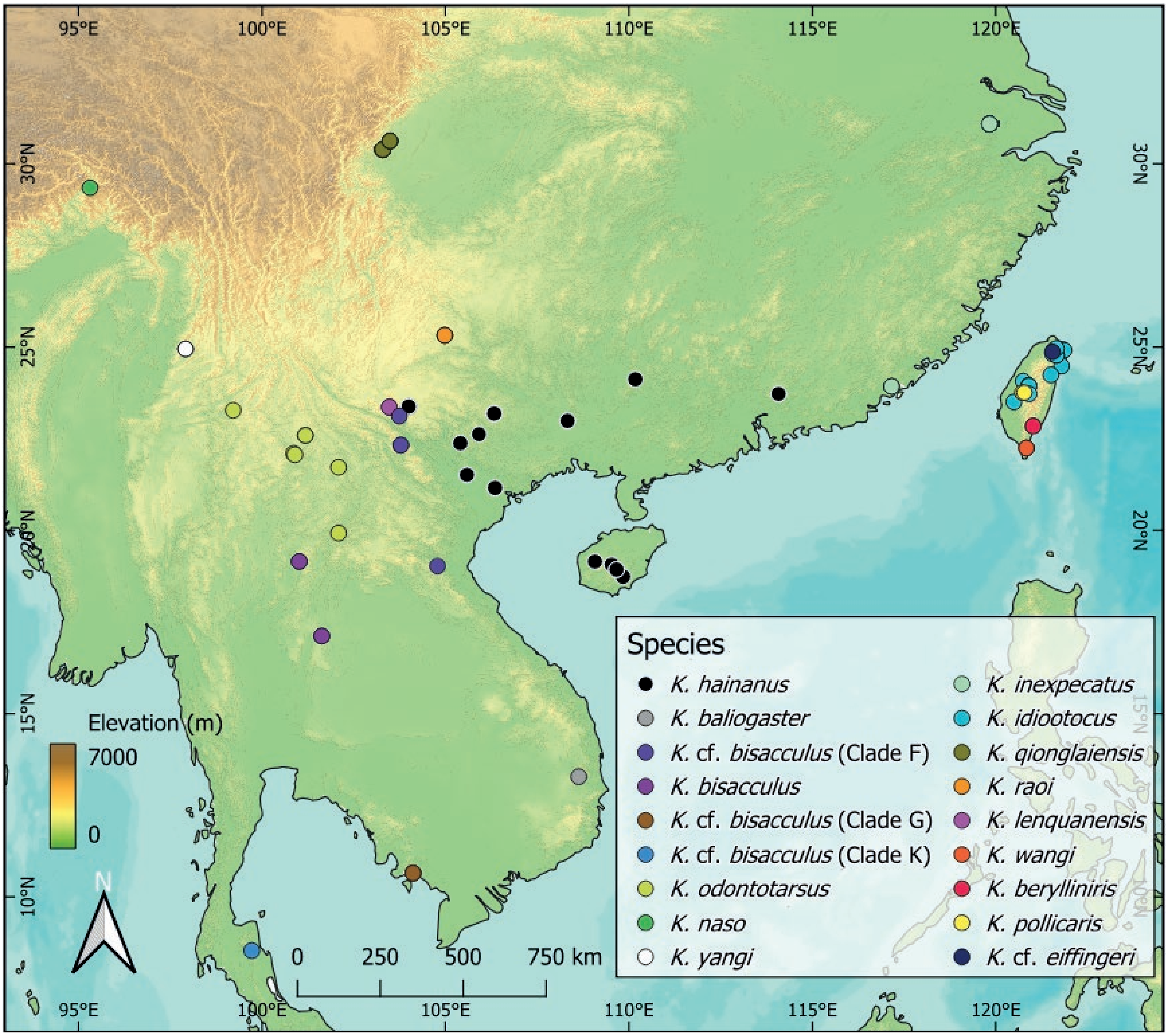

Fig. 2. Sampling locations and distribution of *Kurixalus* in Eastern Asia. Species names given following Frost (2024) for described species and Yu et al. (2017a) for clades that remain undescribed. Sampling included all known lineages of *Kurixalus* in mainland Southern China; coverage for the genus in Indochina is not comprehensive. Base map from World Terrain Base by Esri (https://server.arcgisonline.com/ArcGIS/rest/services/World_Terrain_Base/MapServer) and elevation layer from Fick and Hijmans (2017). Colors of species dots correspond to phylogenetic trees.

the study area and identify highly correlated (r > |0.8|) variables (Elith et al., 2011) to remove, though this step is arguably unnecessary (Feng et al., 2019). The following variables were used for modeling: Bio 1 (annual mean temperature), Bio 2 (mean diurnal range), Bio 3 (isothermality), Bio 5 (maximum temperature of warmest month), Bio 12 (annual precipitation), Bio 13 (precipitation of wettest month) and Bio 17 (precipitation of driest quarter), as well as elevation, slope, and tree cover. A total of 10,000 background points were selected from the study area, with selection weighted using a bias raster file generated from 539,243 records of anurans from GBIF within 115.8° to 125.5° latitude and 19.9° to 35.1° longitude (GBIF.org, 2024a) to reduce spatial bias in the occurrence data set (Kramer-Schadt et al., 2013; Zhu and Qiao, 2016).

Candidate Maxent models were generated using ENMeval (Kass et al., 2021) using 30 combinations of feature classes (“L”, “LQ”, “H”, “LQH”, “LQHP”, “LQHPT”) and regularization multipliers (1-5), with data partitioned using 'checkerboard2'. We selected the model with the lowest AICc, which balances fit and complexity (Warren and Seifert, 2011), as the best model and projected to southeastern Asia. To evaluate model accuracy, we calculated the Boyce index and area under the receiver operating characteristic curve (AUC; Breiner et al., 2015; Liu et al., 2024).

RESULTS

Molecular analyses

Though the BI and ML trees produced some inconsistencies, in all reconstructed trees the three individuals from Fujian were consistently grouped within a clade of *K. idiootocus* and *K. inexpectatus* (Figs. 3-5). Specifically, phylogenetic trees based on the three concatenated gene fragments (Fig. 3) and independent *12S-Val-16S* (Fig. 4) datasets indicated the Fujian individuals formed a monophyletic group with *K. inexpectatus* (Bayesian posterior probability (BPP) = 1.00 and 1.00; ML bootstrap: 97% and 83%, respectively). The tree reconstructed from independent *COI* fragments (Fig. 5) showed an unclear resolution of this group, forming polytomies for the Fujian samples, *K. idiootocus*, and *K. inexpectatus*. According to the BI trees, the group of *K. idiootocus*, *K. inexpectatus*, *K. raoi*, *K. lenquanensis*, and *K. qionglaiensis* formed a monophyletic group (BPP = 1.00) which was sister to a clade of island endemics consisting of *K. wangi*, *K. berylliniris*, *K. pollicaris*, and *K.* cf. *eiffingeri* (BPP = 1.00). The taxonomy for species names following Frost (2024a) as of July 2024, with unnamed clades following naming from Yu et al. (2017a). In addition, the remaining *Kurixalus* samples within the *K. odontotarsus* species group formed a strongly supported clade (BPP = 1.00). Samples from Guangxi, Guangdong, Hainan, and northern Vietnam clustered within the *K. odontotarsus* species complex but distinct from both *K. bisacculus* sensu stricto and *K. odontotarsus* sensu stricto. Other undescribed clades (i.e. *K.* cf. *bisacculus* Clades F, G, and K) previously identified in the *K. odontotarsus* species complex (Yu et al., 2017a) clustered together, but showed unclear resolution between each other.

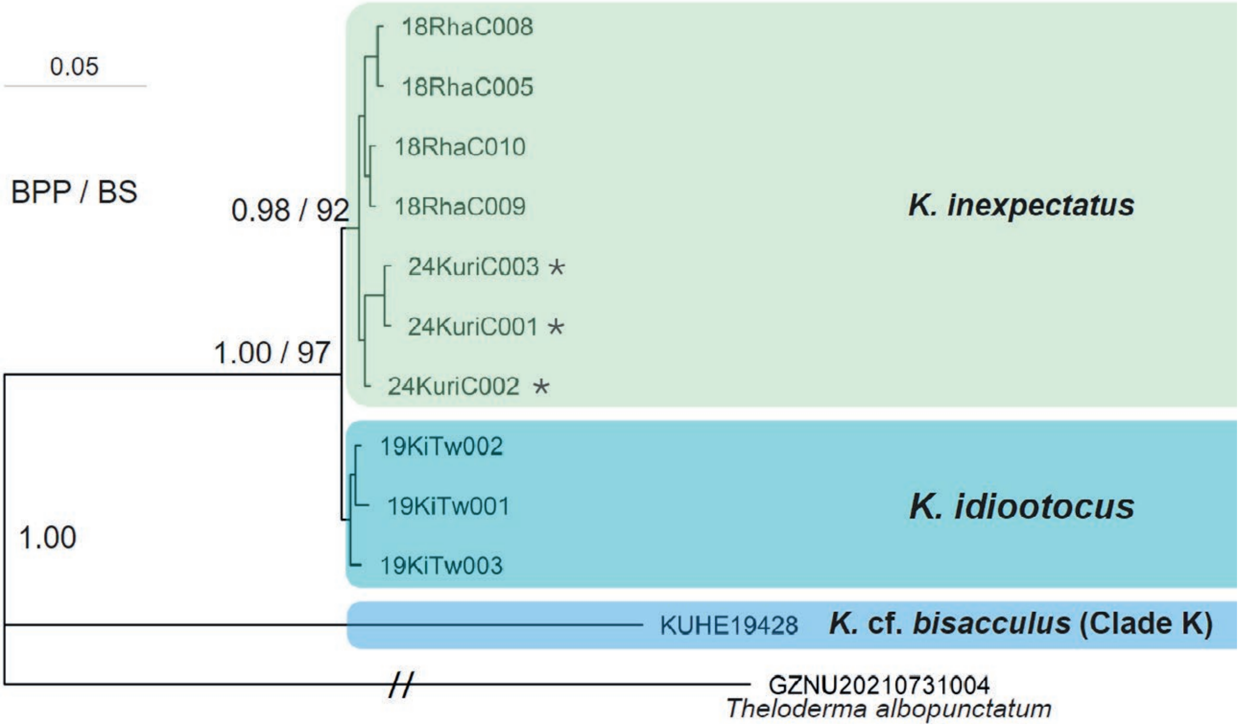

Fig. 3. Phylogenetic tree of *Kurixalus* based on concatenated genetic sequences. Bayesian Inference tree from 1748 bp concatenation of three gene fragments (802 bp of *12S-Val-16S*, 553 bp of *COI*, and 393 bp of *TYR*) with no missing data from the focal clade including *K. inexpectatus* and *K. idiootocus*, with one sample of *Theloderma albopunctatum* and one of *K.* cf *bisacculus* Clade K as designated by Yu et al. (2017a). Here, *K. inexpectatus* and the Fujian *Kurixalus* samples (24KuriC001-3; marked with asterisks) formed a monophyletic group sister to *K. idiootocus*. Species clade colors correspond to those in the sampling presented in Fig. 2.

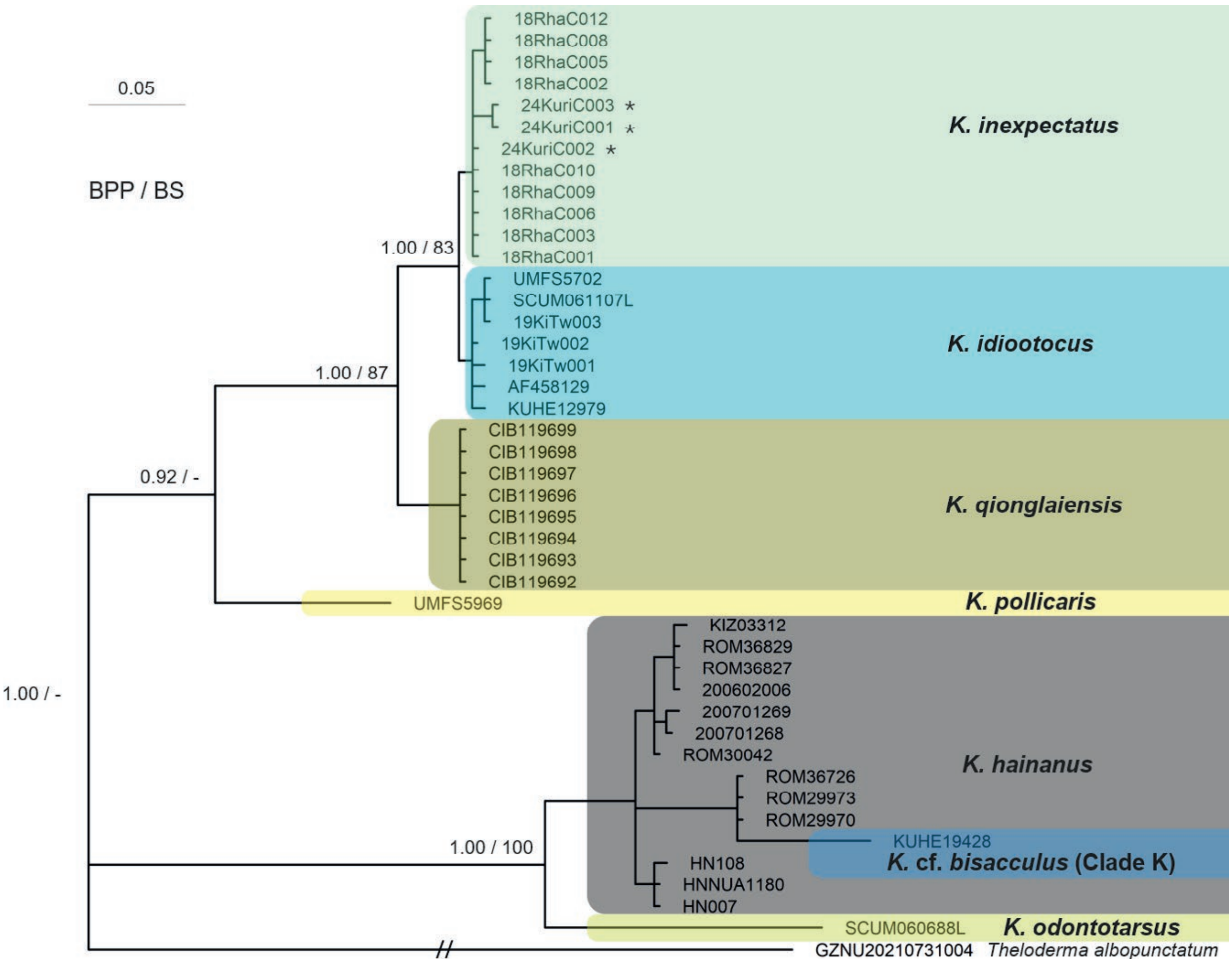

Fig. 4. Phylogenetic tree of *Kurixalus* based on the *12S-Val-16S* gene fragment. Bayesian Inference tree based on 809 bp alignment of *12S-Val-16S* from 43 *Kurixalus* individuals and *Theloderma albopunctatum* as the outgroup. The three *Kurixalus* from Fujian are marked with asterisks. Bayesian posterior probabilities are given, as are Maximum Likelihood bootstrap values exceeding 70%. Species clade colors correspond to the sampling map in Fig. 2.

Ecological niche models

Out of 30 candidate models, the species distribution model for the combined clade of *K. inexpectatus* and *K. idiootocus* (Fig. 6) with the lowest AICc used LQHPT feature classes and a regularization multiplier of one. The AUC value was moderately high (0.80), while the Boyce index value was very high (0.98). The highly suitable habitat predicted in Taiwan matched the known distribution of *K. idiootocus* well, and the model also predicted broad areas of potentially suitable habitat across mainland Southern China and Vietnam. Additional highly suitable areas on the Asian mainland were in northern Vietnam, southern Guangxi, central Sichuan, and along the coast of Guangdong.

DISCUSSION

Our previously unknown samples from Fujian clustered with *K. inexpectatus*, which represents a range extension of over 830 km for the species. A previous analysis based on only mtDNA proposed that *K. inexpectatus* be synonymized with *K. idiootocus* (Lyu et al., 2024). We acknowledge that the morphological differences between the two species are minor, and that vocalizations within the genus can vary by context and deserve to be studied in further detail (Zhu et al., 2017; Deng et al., 2024; Lyu et al., 2024). Our concatenated tree of *12S-Val-16S*, *COI*, and *TYR* showed *K. idiootocus* to be definitively monophyletic, not paraphyletic with respect to *K. inexpectatus* as previously suggested (Lyu et al., 2024), with high BPP

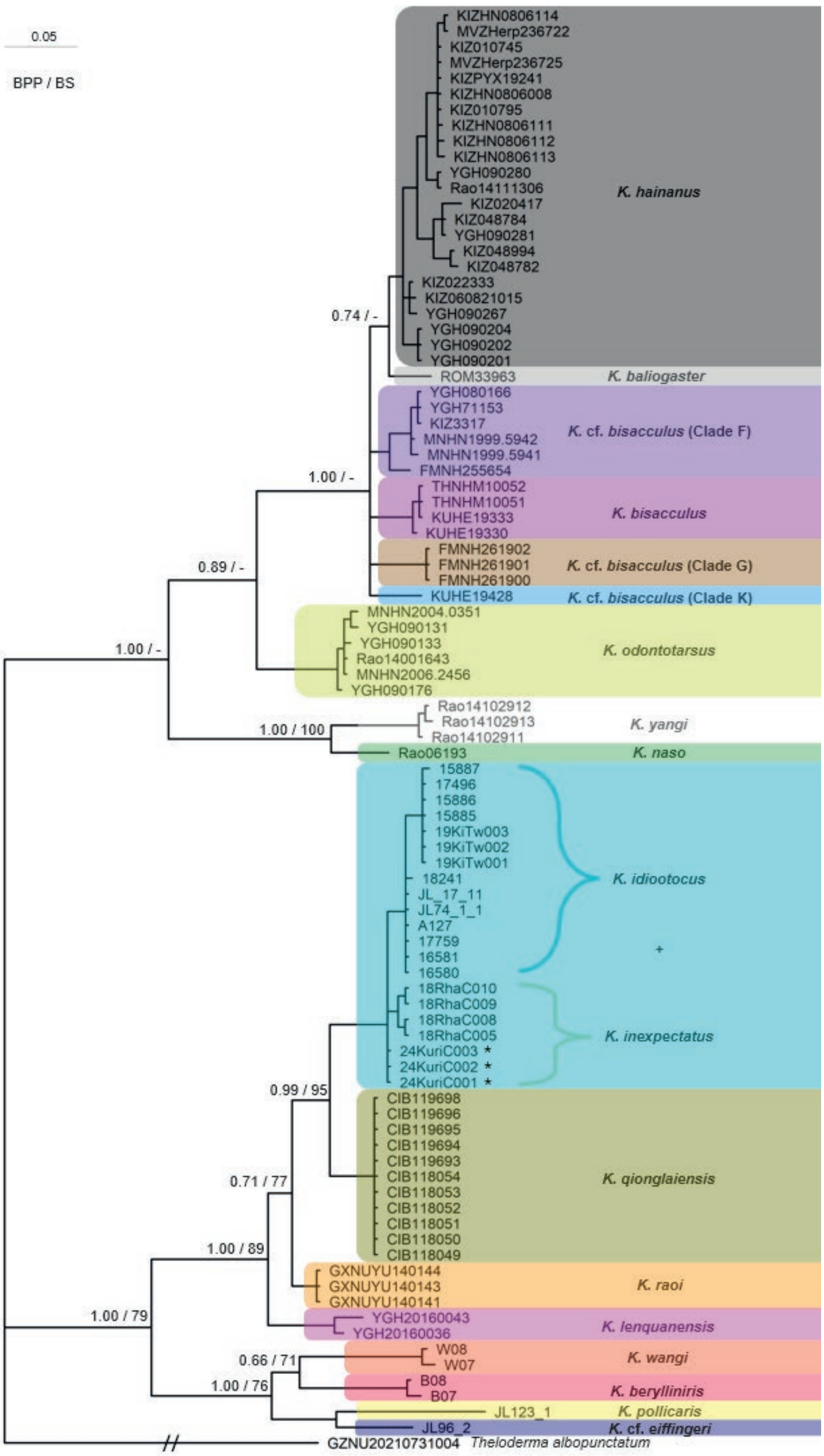

Fig. 5. Phylogenetic tree of *Kurixalus* based on the *COI* gene fragment. Bayesian Inference tree based on a 553 bp *COI* alignment from 91 *Kurixalus* individuals, with *Theloderma albopunctatum* as the outgroup. This phylogenetic tree had the broadest taxonomic coverage of our datasets, but did not resolve the relationships within the clade containing the Fujian *Kurixalus* samples (24KuriC001-3; marked with asterisks), *K. inexpectatus*, and *K. idiootocus*. Bayesian posterior probabilities are given, as are Maximum Likelihood bootstrap values exceeding 70%. Several lineages within the *K. odontotarsus* species complex in Southeast Asia remain unnamed, with clades G, K and F following the naming scheme of Yu et al. (2017a). Species clade colors correspond to those in the sampling map presented in Fig. 2.

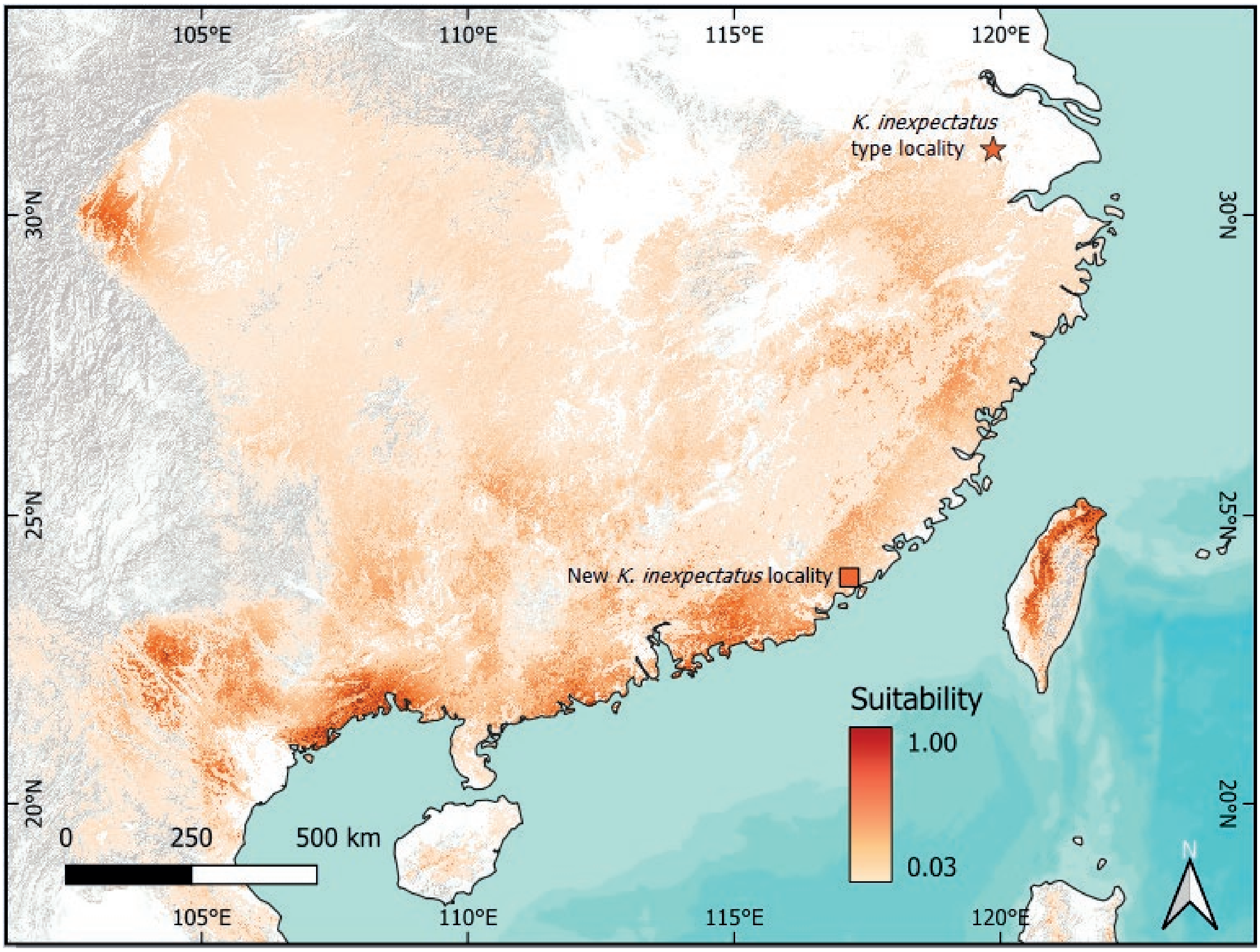

Fig. 6. Model projection for *K. inexpectatus* and *K. idiootocus*. The Maxent output from our best model for the clade of *K. inexpectatus* and *K. idiootocus*, with increasingly dark orange indicating better areas of predicted climatic suitability. Currently, *K. inexpectatus* is known only from the two marked areas, while *K. idiootocus* is found throughout much of Taiwan Island. Basemap from World Terrain Base by Esri (https://server.arcgisonline.com/ArcGIS/rest/services/World_Terrain_Base/MapServer).

(1.00) for the split between the two. Approaches utilizing more loci and longer fragments are more accurate for species delimitation (Blair and Bryson, 2017; Hofmann et al., 2019; Chan et al., 2022), and previous calls for synonymization with *K. idiootocus* were based on only one mitochondrial fragment from *K. inexpectatus* (Lyu et al., 2024). The divergence between *K. idiootocus* and *K. inexpectatus* shown in our concatenated tree is shallow though, and the single gene *COI* tree did not show two reciprocally monophyletic lineages in this clade yet did delineate other known lineages. An integrated taxonomic approach using both morphological measurements and genome-level molecular data would be helpful to clarify the species status of *K. inexpectatus*. At present, we consider *K. inexpectatus* to be a valid species, currently known from only northern Zhejiang and southern Fujian (Fig. 2).

Geographically, the closest mainland population to the *K. inexpectatus* found in Fujian is *K. hainanus* (Fig. 2), though these two congeners are not closely related. Populations of *K. hainanus* in Guangdong, Guangxi, and Hainan, and northeastern Vietnam have been previously referred to as *K. bisacculus* and *K. odontotarsus* (Yu et al., 2017a; Lv et al., 2018; Mo et al., 2023), but based on the *COI* tree these individuals form a monophyletic clade, distinct from both *K. bisacculus* sensu stricto, which is not found in China, and *K. odontotarsus* sensu stricto, whose presence in China is limited to Yunnan (Yu et al., 2017a). Additional undescribed lineages of the *K. odontotarsus* species complex exist in Yunnan and Southeast Asia (Yu et al., 2017a, 2020; Frost, 2024b), and significant further work remains to be done in the genus, such as regarding the status of *K. pollicaris* and *K.* cf. *eifffingeri*

(Dufresnes and Litvinchuk, 2022). Though it is unknown if *K. inexpectatus* and *K. hainanus* overlap in distribution, they can be easily distinguished by the presence (*K. inexpectatus* and *K. idiootocus*) or absence (*K. hainanus*) of paired symmetric dark blotches on the chest (Zhao et al., 2005; Zeng et al., 2021; Messenger et al., 2022).

The results of our modeling indicated broad areas of potentially suitable habitat throughout mainland Southern China for the combined clade of *K. inexpectatus* and *K. idiootocus* (Fig. 6). However, it is highly unlikely that *K. inexpectatus* occurs continuously from northern Zhejiang to southern Fujian, especially given the small range of *K. inexpectatus* and the other mainland species in the clade (*K. qionglaiensis*, *K. raoi*, and *K. lenquanensis*) and the impact of continued habitat degradation (Pan et al., 2019; Li et al., 2024; Pang et al., 2024). Nevertheless, the existence of additional undocumented populations is possible, particularly in the regions immediately surrounding the two known localities of *K. inexpectatus*, though surveys near the type locality of the species did not detect it (Kohler et al., 2024). The species has not been evaluated by the IUCN Red List of Threatened Species and estimates of population trends in both known populations of *K. inexpectatus* are lacking, however, the suitable habitat is decreasing at both localities because of bamboo plantation and the development of infrastructures for tourism (Messenger et al., 2022). Therefore, given the very low extent of occurrence of the species (c. 175 km^2) we recommend the species to be listed as endangered under the criteria B1 as the species is present at less than five location (B1a) and there is an observed continuing decline in the quality of the habitat (B1b(iii); IUCN Standards and Petitions Committee, 2024). While habitat loss is the main threat to the species, climate change, pollution are likely to also threaten the species (Luedtke et al., 2023). Interestingly, the highly suitable area forecasted by our model in central Sichuan is actually occupied by the next most closely related species, *K. qionglaiensis* (Hou et al., 2021; Guo et al., 2022). Similarly, the highly suitable area in southern Guangxi and northern Vietnam is occupied by another congener, *K. hainanus*. Based on our modeling results and the currently known range of *K. inexpectatus*, we anticipate two of the more promising areas to search for undiscovered populations to be: (1) near the northern coast of Guangdong; and (2) the northern Wuyi Mountains, Fujian, which is one of the recently identified hotspots of amphibian biodiversity in China (Xu et al., 2024). In addition to our model of broad habitat suitability, future search efforts should also take into account microhabitat and possible breeding conditions conducive to Rhacophorids (Lin and Kam, 2008; Madhushanka and Manamendra-Arachchi, 2021). We are optimistic that additional populations of *Kurixalus* in mainland China can be discovered with additional searching, as has been the case for other Maxent-guided field efforts (Rhoden, Peterman, and Taylor, 2017; Sarker et al., 2019).

ACKNOWLEDGEMENTS

This project was funded by the Research Fund for International Scientists (RFIS) from the National Natural Science Foundation of China (NSFC; W2432021) and the Foreign Youth Talent Program of the Ministry of Science and Technology of the People's Republic of China (QN2023014004L) awarded to AB. No permits were required for sampling on the mainland, and *Kurixalus idiootocus* samples were collected under the permit 103-16. This research received the approval from the IACUC Ethics Committee of Nanjing Forestry University, number 2024013.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <http://www9.unipv.it/webshi/appendix> Manuscript number 17227.

REFERENCES

Ali, J.R. (2020): Geological data indicate that the interpretation for the age-calibrated phylogeny for the *Kurixalus*-genus frogs of South, South-east and East Asia (Lv et al., 2018) needs to be rethought. Mol. Phylogenet. Evol. **145**: 106053.

Ananjeva, N.B., Golynsky, E.E., Lin, S.-M., Orlov, N.L., Tseng, H.-Y. (2015): Modeling habitat suitability to predict the potential distribution of the Kelung cat snake *Boiga kraepelini* Steineger, 1902. Russ. J. Herpetol. **22**: 197-205.

Blair, C., Bryson, R.W. (2017): Cryptic diversity and discordance in single-locus species delimitation methods within horned lizards (Phrynosomatidae: *Phrynosoma*). Mol. Ecol. Resour. **17**: 1168-1182.

Bossuyt, F., Milinkovitch, M.C. (2000): Convergent adaptive radiations in Madagascan and Asian ranid frogs reveal covariation between larval and adult traits. Proc. Natl. Acad. Sci. **97**: 6585-6590.

Breiner, F.T., Guisan, A., Bergamini, A., Nobis, M.P. (2015): Overcoming limitations of modelling rare species by using ensembles of small models. Methods Ecol. Evol. **6**: 1210-1218.

Chan, K.O., Hertwig, S.T., Neokleous, D.N., Flury, J.M., Brown, R.M. (2022): Widely used, short 16S rRNA mitochondrial gene fragments yield poor and erratic results in phylogenetic estimation and species delimitation of amphibians. BMC Ecol. Evol. **22**: 37.

Che, J., Chen, H., Yang, J., Jin, J., Jiang, K., Yuan, Z., Murphy, R.W., Zhang, Y. (2012): Universal COI primers for DNA barcoding amphibians. Mol. Ecol. Resour. **12**: 247-258.

Chernomor, O., Von Haeseler, A., Minh, B.Q. (2016): Terrace aware data structure for phylogenomic inference from supermatrices. Syst. Biol. **65**: 997-1008.

Deng, K., He, Y.-X., Wang, X.-P., Wang, T.-L., Wang, J.-C., Chen, Y.-H., Cui, J.-G. (2024): Hainan frilled treefrogs' calls partially conform to Menzerath–Altmann's law, but oppose Zipf's law of abbreviation. Anim. Behav. **213**: 51-59.

Dufresnes, C., Litvinchuk, S.N. (2022): Diversity, distribution and molecular species delimitation in frogs and toads from the Eastern Palaearctic. Zool. J. Linn. Soc. **195**: 695-760.

Edgar, R.C. (2004): MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. **32**: 1792-1797.

Elith, J., Phillips, S.J., Hastie, T., Dudík, M., Chee, Y.E., Yates, C.J. (2011): A statistical explanation of MaxEnt for ecologists: Statistical explanation of MaxEnt. Divers. Distrib. **17**: 43-57.

Entiauspe-Neto, O.M., Dervanoski, D., Abegg, A.D. (2024): Can fieldwork driven by predictive species distribution models yield new rare or relevant geographic records? A case study with Neotropical snakes. Austral Ecol. **49**: e70013.

Farr, T.G., Rosen, P.A., Caro, E., Crippen, R., Duren, R., Hensley, S., Kobrick, M., Paller, M., Rodriguez, E., Roth, L., Seal, D., Shaffer, S., Shimada, J., Umland, J., Werner, M., Oskin, M., Burbank, D., Alsdorf, D. (2007): The shuttle radar topography mission. Rev. Geophys. **45**: 2005RG000183.

Feng, X., Park, D.S., Liang, Y., Pandey, R., Papeş, M. (2019): Collinearity in ecological niche modeling: Confusions and challenges. Ecol. Evol. **9**: 10365-10376.

Fick, S.E., Hijmans, R.J. (2017): WorldClim 2: new 1-km spatial resolution climate surfaces for global land areas. Int. J. Climatol. **37**: 4302-4315.

Frost, D.R. (2024a): Amphibian species of the world 6.2, an online reference. American Museum of Natural History, New York. Available online at: https://amphibiansoftheworld.amnh.org/. [*Accessed 13 September 2024*].

Frost, D.R. (2024b): *Kurixalus odontotarsus*. American Museum of Natural History, New York. Available online at: https://amphibiansoftheworld.amnh.org/Amphibia/Anura/Rhacophoridae/Rhacophorinae/Kurixalus/Kurixalus-odontotarsus. [*Accessed 19 November 2024*].

Frost, D.R., Grant, T., Faivovich, J., Bain, R.H., Haas, A., Haddad, C.F.B., De Sá, R.O., Channing, A., Wilkinson, M., Donnellan, S.C., Raxworthy, C.J., Campbell, J.A., Blotto, B.L., Moler, P., Drewes, R.C., Nussbaum, R.A., Lynch, J.D., Green, D.M., Wheeler, W.C. (2006): The amphibian tree of life. Bull. Am. Mus. Nat. Hist. **297**: 1-291.

GBIF.org (2024a): Occurrence Download: Anura. The Global Biodiversity Information Facility. https://doi.org/10.15468/DL.CXHN8Z.

GBIF.org (2024b): Occurrence Download: *Kurixalus idiootocus*. The Global Biodiversity Information Facility. https://doi.org/10.15468/DL.XPSDR7.

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O. (2010): New algorithms and methods to estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. Syst. Biol. **59**: 307-321.

Guo, C.-P., Zhong, M.-J., Wah Leung, K., Wang, X.-Y., Hu, J.-H. (2022): A new species of the genus *Kurixalus* (Anura, Rhacophoridae) from Sichuan Province, southwestern China. Zool. Res. **43**: 90-94.

Hijmans, R.J. (2024): raster: Geographic data analysis and modeling.

Hijmans, R.J., Barbosa, M., Ghosh, A., Mandel, A. (2024): 'geodata': Download geographic data.

Hoang, D.T., Chernomor, O., Von Haeseler, A., Minh, B.Q., Vinh, L.S. (2018): UFBoot2: Improving the ultrafast bootstrap approximation. Mol. Biol. Evol. **35**: 518-522.

Hofmann, E.P., Nicholson, K.E., Luque-Montes, I.R., Köhler, G., Cerrato-Mendoza, C.A., Medina-Flores, M., Wilson, L.D., Townsend, J.H. (2019): Cryptic diversity, but to what extent? Discordance between single-locus species delimitation methods within mainland anoles (Squamata: Dactyloidae) of Northern Central America. Front. Genet. **10**: 11.

Hou, M., Peng, X., Miao, J., Liu, S., Li, P., Orlov, N.L. (2021): Description a new species of genus *Kurixalus* (Amphibia: Anura: Rhacophoridae) from Chengdu Prefecture, Sichuan Province, China. Anim. Mol. Breed. **11**: 1-16.

Hou, J., Xiang, J., Li, D., Liu, X. (2023): Prediction of potential suitable distribution areas of *Quasipaa spinosa* in China based on MaxEnt optimization model. Biology **12**: 366.

IUCN Standards and Petitions Committee (2024): Guidelines for using the IUCN Red List categories and criteria. Gland, Switzerland.

Kass, J.M., Muscarella, R., Galante, P.J., Bohl, C.L., Pinilla-Buitrago, G.E., Boria, R.A., Soley-Guardia, M., Anderson, R.P. (2021): ENMeval 2.0: Redesigned for customizable and reproducible modeling of species' niches and distributions. Methods Ecol. Evol. **12**: 1602-1608.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A. (2012): Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics **28**: 1647-1649.

Kidov, A.A., Litvinchuk, S.N. (2021): Distribution and conservation status of the Hyrcanian Wood Frog (*Rana pseudodalmatina*) in Azerbaijan. Russ. J. Herpetol. **28**: 97-107.

Kohler, D.B., Zhang, X., Messenger, K.R., Chin Yu An, K., Ghosh, D., Othman, S.N., Wang, Z., Amin, H., Prasad, V.K., Wu, Z., Borzée, A. (2024): At home in Jiangsu: Environmental niche modeling and new records for five species of amphibian and reptile in Jiangsu, China. Herpetozoa **37**: 85-93.

Kramer-Schadt, S., Niedballa, J., Pilgrim, J.D., Schröder, B., Lindenborn, J., Reinfelder, V., Stillfried, M., Heckmann, I., Scharf, A.K., Augeri, D.M., Cheyne, S.M., Hearn, A.J., Ross, J., Macdonald, D.W., Mathai, J., Eaton, J., Marshall, A.J., Semiadi, G., Rustam, R., Bernard, H., Alfred, R., Samejima, H., Duckworth, J.W., Breitenmoser-Wuersten, C., Belant, J.L., Hofer, H., Wilting, A. (2013): The importance of correcting for sampling bias in MaxEnt species distribution models. Divers. Distrib. **19**: 1366-1379.

Lanfear, R., Calcott, B., Ho, S.Y.W., Guindon, S. (2012): PartitionFinder: Combined selection of partitioning schemes and substitution models for phylogenetic analyses. Mol. Biol. Evol. **29**: 1695-1701.

Lanfear, R., Frandsen, P.B., Wright, A.M., Senfeld, T., Calcott, B. (2017): PartitionFinder 2: New methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. Mol. Biol. Evol. **34**: 772-773.

Li, J. (2023): iNaturalist observation 186300362. iNaturalist.org. Available online at: https://www.inaturalist.org/observations/186300362.

Li, J., Che, J., Bain, R.H., Zhao, E., Zhang, Y. (2008): Molecular phylogeny of Rhacophoridae (Anura): A framework of taxonomic reassignment of species within the genera *Aquixalus*, *Chiromantis*, *Rhacophorus*, and *Philautus*. Mol. Phylogenet. Evol. **48**: 302-312.

Li, J.-T., Li, Y., Klaus, S., Rao, D.-Q., Hillis, D.M., Zhang, Y.-P. (2013): Diversification of rhacophorid frogs provides evidence for accelerated faunal exchange between India and Eurasia during the Oligocene. Proc. Natl. Acad. Sci. **110**: 3441-3446.

Li, S., Dai, W., Wang, Z., Wu, Z., Wang, J. (2024): Detecting range shrinking from historical amphibian species occurrences under influence of human impacts: A case study using the Chinese giant salamander, *Andrias davidianus*. Ecol. Evol. **14**: e70595.

Lin, Y.-S., Kam, Y.-C. (2008): Nest choice and breeding phenology of an arboreal-breeding frog, *Kurixalus eifingeri* (Rhacophoridae), in a bamboo forest. Zool. Stud. **47**: 129-137.

Liu, C., Newell, G., White, M., Machunter, J. (2025): Improving the estimation of the Boyce index using statistical smoothing methods for evaluating species distribution models with presence-only data. Ecography **2025**: e07218.

Luedtke, J.A., Chanson, J., Neam, K., Hobin, L., Maciel, A.O., Catenazzi, A., Borzée, A., Hamidy, A., Aowphol, A., Jean, A., Sosa-Bartuano, Á., Fong G., A., De Silva, A., Fouquet, A., Angulo, A., Kidov, A.A., Muñoz Saravia, A., Diesmos, A.C., Tominaga, A., Shrestha, B., Gratwicke, B., Tjaturadi, B., Martínez Rivera, C.C., Vásquez Almazán, C.R., Señaris, C., Chandramouli, S.R., Strüssmann, C., Cortez Fernández, C.F., Azat, C., Hoskin, C.J., Hilton-Taylor, C., Whyte, D.L., Gower, D.J., Olson, D.H., Cisneros-Heredia, D.F., Santana, D.J., Nagombi, E., Najafi-Majd, E., Quah, E.S.H., Bolaños, F., Xie, F., Brusquetti, F., Álvarez, F.S., Andreone, F., Glaw, F., Castañeda, F.E., Kraus, F., Parra-Olea, G., Chaves, G., Medina-Rangel, G.F., González-Durán, G., Ortega-Andrade, H.M., Machado, I.F., Das, I., Dias, I.R., Urbina-Cardona, J.N., Crnobrnja-Isailović, J., Yang, J.-H., Jianping, J., Wangyal, J.T., Rowley, J.J.L., Measey, J., Vasudevan, K., Chan, K.O., Gururaja, K.V., Ovaska, K., Warr, L.C., Canseco-Márquez, L., Toledo, L.F., Díaz, L.M., Khan, M.M.H., Meegaskumbura, M., Acevedo, M.E., Napoli, M.F., Ponce, M.A., Vaira, M., Lampo, M., Yánez-Muñoz, M.H., Scherz, M.D., Rödel, M.-O., Matsui, M., Fildor, M., Kusrini, M.D., Ahmed, M.F., Rais, M., Kouamé, N.G., García, N., Gonwouo, N.L., Burrowes, P.A., Imbun, P.Y., Wagner, P., Kok, P.J.R., Joglar, R.L., Auguste, R.J., Brandão, R.A., Ibáñez, R., Von May, R., Hedges, S.B., Biju, S.D., Ganesh, S.R., Wren, S., Das, S., Flechas, S.V., Ashpole, S.L., Robleto-Hernández, S.J., Loader, S.P., Incháustegui, S.J., Garg, S., Phimmachak, S., Richards, S.J., Slimani, T., Osborne-Naikatini, T., Abreu-Jardim, T.P.F., Condez, T.H., De Carvalho, T.R., Cutajar, T.P., Pierson, T.W., Nguyen, T.Q., Kaya, U., Yuan, Z., Long, B., Langhammer, P., Stuart, S.N. (2023): Ongoing declines for the world's amphibians in the face of emerging threats. Nature **622**: 308-314.

Luo, T., Zhao, X., Lan, C., Li, W., Deng, H., Xiao, N., Zhou, J. (2023): Integrated phylogenetic analyses reveal the evolutionary, biogeographic, and diversification history of Asian warty treefrog genus *Theloderma* (Anura, Rhacophoridae). Ecol. Evol. **13**: e10829.

Lv, Y.-Y., He, K., Klaus, S., Brown, R.M., Li, J.-T. (2018): A comprehensive phylogeny of the genus *Kurixalus* (Rhacophoridae, Anura) sheds light on the geographical range evolution of frilled swamp treefrogs. Mol. Phylogenet. Evol. **121**: 224-232.

Lyu, Z., Li, M., Wang, G., Liu, G., Liu, M., Jiang, K., Jiang, D., Li, J. (2024): Taxonomic status of three recently proposed species of the genus *Kurixalus* (Anura, Rhacophoridae), with discussion on the ZooBank registrations for electronical publication. Asian Herpetol. Res. **15**: 31-40.

Madhushanka, S., Manamendra-Arachchi, K. (2021): Morphometry and habitat selection of the mountain hourglass treefrog [*Taruga eques* (Günther, 1858)] (Amphibia, Rhacophoridae) in the Horton Plains National Park, Sri Lanka. Univ. Colombo Rev. **3**: 64.

Messenger, K.R., Othman, S.N., Chuang, M.-F., Yang, Y., Borzée, A. (2022): Description of a new *Kurixalus* species (Rhacophoridae, Anura) and a northwards range extension of the genus. ZooKeys **1108**: 15-49.

Mo, Q., Sun, T., Chen, H., Yu, G., Du, L. (2023): Biogeographic origin of *Kurixalus* (Anura, Rhacophoridae) on the east Asian islands and tempo of diversification within *Kurixalus*. Animals **13**: 2754.

Nguyen, T.T., Matsui, M., Duc, H.M. (2014): A new tree frog of the genus *Kurixalus* (Anura: Rhacophoridae) from Vietnam. Curr. Herpetol. **33**: 101-111.

Nguyen, T.V., Duong, T.V., Luu, K.T., Poyarkov, N.A. (2020): A new species of *Kurixalus* (Anura: Rhacophoridae) from northern Vietnam with comments on the biogeography of the genus. J. Nat. Hist. **54**: 195-223.

Pan, T., Wang, H., Duan, S., Ali, I., Yan, P., Cai, R., Wang, M., Zhang, J., Zhang, H., Zhang, B., Wu, X. (2019): Historical population decline and habitat loss in a critically endangered species, the Chinese alligator (*Alligator sinensis*). Glob. Ecol. Conserv. **20**: e00692.

Pang, C., Tang, S., Yu, G., Zhou, J.-J. (2024): First description of the female and morphological variations with range extension of *Kurixalus lenquanensis* (Anura, Rhacophoridae). Biodivers. Data J. **12**: e130411.

Phillips, S.J., Anderson, R.P., Dudík, M., Schapire, R.E., Blair, M.E. (2017): Opening the black box: an open-source release of Maxent. Ecography **40**: 887-893.

Proosdij, A.S.J. van, Sosef, M.S.M., Wieringa, J.J., Raes, N. (2016): Minimum required number of specimen records to develop accurate species distribution models. Ecography **39**: 542-552.

QGIS.org (2023): QGIS. QGIS Association.

R Core Team (2022): R: A Language and environment for statistical computing. Vienna, Austria, Foundation for Statistical Computing.

Rhoden, C.M., Peterman, W.E., Taylor, C.A. (2017): Maxent-directed field surveys identify new populations of narrowly endemic habitat specialists. PeerJ **5**: e3632.

Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P. (2012): MrBayes 3.2: Efficient bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. **61**: 539-542.

Sarker, G.C., Wostl, E., Thammachoti, P., Sidik, I., Hamidy, A., Kurniawan, N., Smith, E.N. (2019): New species, diversity, systematics, and conservation assessment of the puppet toads of Sumatra (Anura: Bufonidae: *Sigalegalephrynus*). Zootaxa **4679**: 365-391.

Shin, Y., Min, M., Borzée, A. (2021): Driven to the edge: Species distribution modeling of a clawed salamander (Hynobiidae: *Onychodactylus koreanus*) predicts range shifts and drastic decrease of suitable habitats in response to climate change. Ecol. Evol. **11**: 14669-14688.

Trifinopoulos, J., Nguyen, L.-T., von Haeseler, A., Minh, B.Q. (2016): W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. Nucleic Acids Res. **44**: W232–W235.

Vignali, S., Barras, A.G., Arlettaz, R., Braunisch, V. (2020): *SDMtune*: An R package to tune and evaluate species distribution models. Ecol. Evol. **10**: 11488-11506.

Warren, D.L., Seifert, S.N. (2011): Ecological niche modeling in Maxent: the importance of model complexity and the performance of model selection criteria. Ecol. Appl. **21**: 335-342.

Wilkinson, J.A., Drewes, R.C., Tatum, O.L. (2002): A molecular phylogenetic analysis of the family Rhacophoridae with an emphasis on the Asian and African genera. Mol. Phylogenet. Evol. **24**: 265-273.

Wu, S.-P., Huang, C.-C., Tsai, C.-L., Lin, T.-E., Jhang, J.-J., Wu, S.-H. (2016): Systematic revision of the Taiwanese genus *Kurixalus* members with a description of two new endemic species (Anura, Rhacophoridae). ZooKeys **557**: 121-153.

Xu, W., Wu, Y.-H., Zhou, W.-W., Chen, H.-M., Zhang, B.-L., Chen, J.-M., Xu, W., Rao, D.-Q., Zhao, H., Yan, F., Yuan, Z., Jiang, K., Jin, J.-Q., Hou, M., Zou, D., Wang, L.-J., Zheng, Y., Li, J.-T., Jiang, J., Zeng, X.-M., Chen, Y., Liao, Z.-Y., Li, C., Li, X.-Y., Gao, W., Wang, K., Zhang, D.-R., Lu, C., Yin, T., Ding, Z., Zhao, G.-G., Chai, J., Zhao, W.-G., Zhang, Y.-P., Wiens, J.J., Che, J. (2024): Hidden hotspots of amphibian biodiversity in China. Proc. Natl. Acad. Sci. **121**: e2320674121.

Yu, G., Hui, H., Rao, D., Yang, J. (2018): A new species of *Kurixalus* from western Yunnan, China (Anura, Rhacophoridae). ZooKeys **770**: 211-226.

Yu, G., Rao, D., Matsui, M., Yang, J. (2017a): Coalescent-based delimitation outperforms distance-based methods for delineating less divergent species: the case of *Kurixalus odontotarsus* species group. Sci. Rep. **7**: 16124.

Yu, G., Wang, J., Hou, M., Rao, D., Yang, J. (2017b): A new species of the genus *Kurixalus* from Yunnan, China (Anura, Rhacophoridae). ZooKeys **694**: 71-93.

Yu, G.-H., Du, L.-N., Wang, J.-S., Rao, D.-Q., Wu, Z.-J., Yang, J.-X. (2020): From mainland to islands: colonization history in the tree frog *Kurixalus* (Anura: Rhacophoridae). Curr. Zool. **66**: 667-675.

Zanaga, D., Van De Kerchove, R., De Keersmaecker, W., Souverijns, N., Brockmann, C., Quast, R., Wevers, J., Grosu, A., Paccini, A., Vergnaud, S., Cartus, O., Santoro, M., Fritz, S., Georgieva, I., Lesiv, M., Carter, S., Herold, M., Li, L., Tsendbazar, N.-E., Ramoino, F., Arino, O. (2021): ESA WorldCover 10 m 2020 v100. Zenodo. https://doi.org/10.5281/ZENODO.5571936.

Zeng, J., Wang, J.-S., Yu, G.-H., Du, L.-N. (2021): A new species of *Kurixalus* (Anura, Rhacophoridae) from Guizhou, China. Zool. Res. **42**: 227-233.

Zhao, E., Wang, L., Shi, H., Wu, G., Zhao, H. (2005): Chinese rhacophorid frogs and description of a new species of *Rhacophorus*. Sichuan J. Zool. **24**: 297-300.

Zhu, B., Wang, J., Sun, Z., Yang, Y., Wang, T., Brauth, S.E., Tang, Y., Cui, J. (2017): Competitive pressures affect sexual signal complexity in *Kurixalus odontotarsus*: insights into the evolution of compound calls. Biol. Open **6**: 1913-1918.

Zhu, G., Qiao, H. (2016): Effect of the Maxent model's complexity on the prediction of species potential distributions. Biodivers. Sci. **24**: 1189-1196.

Acta Herpetologica 20(1): 17-28, 2025
DOI: 10.36253/a_h-16266

Can artificial rearing benefit the natural breeding of European pond turtle (*Emys orbicularis*) in its northern distribution range?

Vytautas Rakauskas[1,*], Kristė Stravinskaitė[2], Rasa Jautakienė[2], Jonas Šimkus[2], Inga Čitavičienė[3], Alma Pikūnienė[2], Gintarė Stankevičė[2], Eglė Šidagytė-Copilas[4]

[1] *Laboratory of Fish Ecology, State Scientific Research Institute Nature Research Centre, Verkiu str. 98, LT-12201 Vilnius, Lithuania*
[2] *Lithuanian Zoological Gardens, Radvilėnu rd. 21, LT-50299 Kaunas, Lithuania*
[3] *Meteliai Regional Park Directorate, Kampeliu str. 10, Aleknoniu v., LT-64351, Alytus r., Lithuania*
[4] *Laboratory of Evolutionary Ecology of Hydrobionts, Nature Research Centre, Akademijos str. 2, LT-12201 Vilnius, Lithuania*
**Corresponding author.* Email: vytautas.rakauskas@gamtc.lt

Submitted on: 2024, 14th June; revised on: 2025, 27th February; accepted on: 2025, 17th March
Editor: Ilaria Bernabò

Abstract. The European pond turtle (*Emys orbicularis*) is a protected and endangered species in Europe experiencing a drastic demographic decrease over most of its range. In Lithuania, at its northern distribution range, *E. orbicularis* populations are small and fragmented, making them highly vulnerable to decline and deserving of active protection measures. The main reasons for the decrease in *E. orbicularis* populations are habitat loss due to changes in land use and the destruction of nesting sites. The survival of healthy *E. orbicularis* populations depends on the survival of eggs and the first overwintering of hatchlings. The aim of this study was to compare the hatching success of naturally vs artificially incubated *E. orbicularis* eggs and further artificially reared hatchling survival during their first year. We also provided an overview of the main nesting measurements, including clutch size and depth, from the small *E. orbicularis* populations in Lithuania. Finally, we furnished detailed descriptions of the artificial egg incubation and subsequent hatchling-rearing methodologies that can be used to preserve and increase the size of local small *E. orbicularis* populations in their northern distribution range.

Keywords. Turtle conservation, nesting ecology, Natura 2000.

INTRODUCTION

The European pond turtle, *Emys orbicularis* (Linnaeus, 1758), is listed as Near Threatened in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species and is protected in Europe by the Bern Convention and Habitats Directive 92/43/EEC (Luiselli and Vamberger, 2024). Being native to most European countries, *E. orbicularis* is considered extinct in the Netherlands and Belgium. In some European countries, such as Czech, Denmark and the United Kingdom, the species has been reintroduced (Luiselli and Vamberger, 2024). Lithuania and Latvia mark the northern edge of its distribution in Europe (Meeske et al., 2006). While it was widespread in Lithuania at the beginning of the 20th century, it is now found in only a few populations in the south and in a few single individuals in the north (Bastytė-Cseh, 2021). The current population of *E. orbicularis* in Latvian waters is even sparser and located mainly in the southern part of the country (Pupins and Pupina, 2008). Nowadays, *E. orbicularis* is classified as an Endangered species and included in the Red Data Book of Lithuania (Bastytė-Cseh, 2021).

The decline of the *E. orbicularis* population is predominantly of anthropogenic origin, due to direct destruction of their nesting sites, habitat loss from chang-

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

www.fupress.com/ah

es in land use (Schneeweis and Breu, 2013), environmental pollution (Savic, 2010), collecting for trade (Meeske and Pupins, 2009; Mollov et al., 2013), accidental killing by traffic (Isailovic and Mesaroš, 2013), and introduction of alien species and predators (Fritz and Chiari, 2013; Liuzzo et al., 2023; Purger et al., 2023). Finally, climate change is also a factor that has a clear impact on the survival and distribution of *E. orbicularis* (Joos et al., 2017; Cerasoli et al., 2019; Nekrasova et al., 2021) followed by genetic fragmentation (Vecchioni et al., 2020).

Previous studies have shown that predators attack *E. orbicularis* at all stages of life: eggs, hatchlings, juvenile turtles and adults (Fritz and Chiari, 2013; Ayaz et al., 2017; Nekrasova et al., 2021; Purger et al., 2023). However, the species is most vulnerable in the early stages of life, such as incubation of eggs and overwintering of hatchlings. Reducing mortality rates in these early stages is crucial to increase the population size of *E. orbicularis* (Mitrus and Zemanek, 1998). There are several strategies to achieve this. One effective method is to cover turtle nests with wire mesh, which protects eggs and hatchlings from predators in their natural environment (Schindler et al., 2017; Kiss et al., 2021). Additionally, newly hatched juveniles can be collected and raised artificially during their first year. Alternatively, freshly laid eggs can be collected and artificially incubated, followed by rearing the hatchlings for their first year. After a wintering period, carefully screened juvenile turtles, evaluated by veterinarians, can be released back into their natural habitats. This process can contribute to increasing the size of vulnerable *E. orbicularis* populations.

This study aimed to compare the hatching success of artificially vs naturally incubated *E. orbicularis* eggs and hatchling survival during their first year. We expected that hatching success would be higher under artificial incubation compared to natural conditions. The study also provides valuable insights into the characteristics of *E. orbicularis* clutches from Lithuanian populations, including nest depth, herbaceous coverage, mean clutch size, and average egg weight. Furthermore, it presents a comprehensive methodology for the artificial incubation and subsequent rearing of hatchlings.

MATERIAL AND METHODS

Sampling sites

E. orbicularis eggs and hatchlings were collected from the wild in Southern Lithuania, mostly in Natura 2000 sites: Juodabalė Zoological Reserve (LTLAZ0010), Kučiuliskė village surroundings (LTLAZ0001), Drapalai village surroundings (LTDRU0004), Margiai village surroundings (LTLAZ0035), Petroškiai forest (LTLAZ0020), Paveisėjai village, and Stankūnai village. The study was performed in the 2015–2022 year's period.

Collection and transportation of eggs

Eggs for the artificial incubation experiment were excavated in early summer (in June) within the first 24 hours after they were laid to minimise embryo lethality at this stage. *E. orbicularis* clutch measurements, such as the mean clutch size and average egg weight, were recorded for each excavated clutch. After excavation, the eggs were placed in plastic boxes filled with a 100 mm thick layer of moist sand. Two thirds of each egg were immersed in the sand to prevent moisture loss, and the layer of sand protected the eggs from possible smashes during transportation. The eggs were not repositioned in the chamber because the embryos begin to develop at the top, very close to the shell, and repositioning them would cause the embryo to die.

Collection and transportation of hatchlings

In the spring, we selected nests for the experiments and covered them with wire netting (10 × 25 mm mesh size) as soon as the female laid eggs and walked away from their nests. We attached the wire nets tightly to the ground with 5 mm diameter metal hooks (at least 25 cm long) so that predators could not tear or dig them out from the sides. Hatchlings for the experiments were dug out at the beginning of autumn, when the air temperatures start to decrease but still are > 10 °C. Small metal spades were used for digging, and as the clutches were reached, digging was finished by hand to avoid injuring the hatchlings. All hatchlings were active when taken from the nests and were placed in boxes with moss (*Sphagnum* sp.) and taken to the Lithuanian Zoological Gardens. During the excavation, the following measurements of *E. orbicularis* nests were taken: nest depth, herbaceous coverage, mean clutch size, and average egg weight for non-hatched eggs. A wooden frame (50 × 50 cm) was used to assess the herbaceous coverage. It was positioned above the selected nest, and the percentage of herbaceous coverage was visually evaluated.

Morphometric measurements and sex determination

We measured *E. orbicularis* juveniles' plastron length (PL) and weight five times for each individual: one day after hatching, at 30, 60 and 90 days of age, and ten months of age, before releasing them back into the wild.

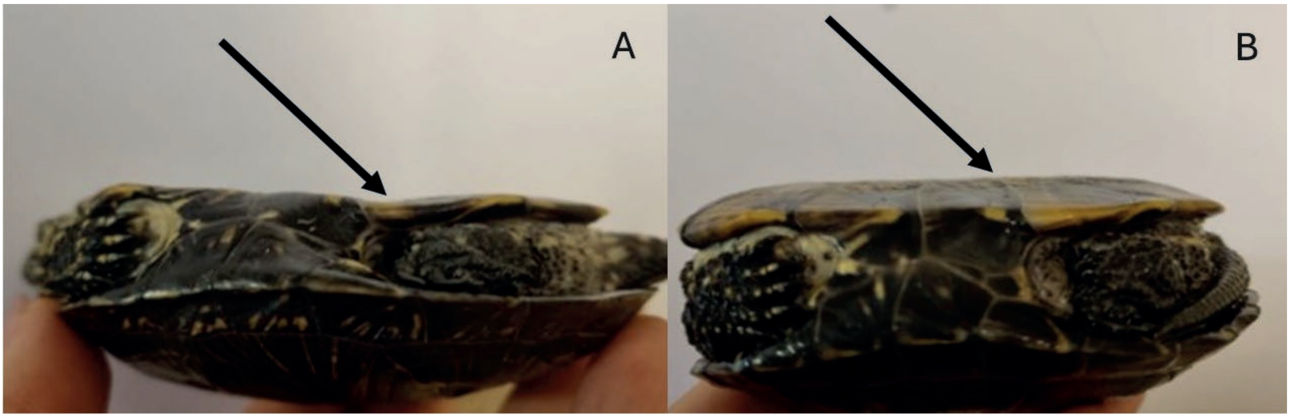

Fig. 1. Plastron shape difference used to determine sex. (A) Male plastron with a slightly concave posterior part and (B) female plastron with a flat (smooth) profile.

We used scales (Romansas, model KB, Lithuania) to measure the weight, with an accuracy of 0.01 g and an error of ± 0.1 g. We used a digital caliper (Carbon Fiber Composites, model CTCF1506, China) with a resolution of 0.1 mm and an accuracy of ± 0.1 mm to measure length. Each juvenile turtle has unique plastron patterns (Salom-Oliver et al., 2022), so photos of each individual pattern were taken to help identify individuals before taking repeated measurements.

The gender of the reared turtle was determined by the concavity of the lower part of the plastron: females have a flat (smooth) section of the lower plastron, whereas males have a slightly concave posterior part of the plastron (Fig. 1). While other methods exist for determining the sex of turtles, such as the colour of the iris or the length of the nails (Avanzi and Millfanti, 2003; Berthomieu and Vermeer, 2021), these methods are unreliable due to the young age of the turtles.

Release into the wild

We successfully reared juvenile turtles and released them in the same Natura 2000 sites where we collected them. At the start of the summer, when the sun was shining, the air temperature was >20 °C and the water temperature was >15 °C, we carefully screened *E. orbicularis* juveniles by veterinarians and handed them over to the responsible specialists from the protected area for release into the wild. Two to three days before release, the turtles were kept a few degrees cooler and not fed. It was difficult to predict the outside air temperature, and excess food in the gastrointestinal tract of the turtles could have spoiled and killed them when the air cooled down suddenly. The turtles from each egg clutch were released into a natural water course that met all the requirements of a suitable *E. orbicularis* habitat. These water courses are under reserve protection in the same territories of Meteliai and Veisiejai Regional Parks where the turtles were collected.

The entire protocol including all the details about the eggs incubation, rearing conditions of hatchlings, feeding and overwintering procedures, are presented in Supplementary Materials.

Statistical analyses

All contingency tables (annual variation in nesting herbaceous cover level; incubation treatment-wise hatching success and survival, as well as overall and treatment-wise sex ratio) were tested using Chi-squared tests. The annual variation in nesting depth and clutch size were analysed using one-way ANOVAs.

Generalized linear models (GLMs) with binomial (or beta binomial in case of significant overdispersion) error distribution were fitted to test the annual variation in hatching success and sex ratio (as female percentage) using clutch-wise data. Mixed general linear models (GLMMs) with a binomial (or beta binomial under overdispersion) error distribution and a random effect of the year were fitted to explore the hatching success (per fertilised clutch) and sex ratio (as female percentage per viable clutch) as functions of the additive effects of nesting depth and herbaceous cover level (the interaction term was dropped according to an insignificant partial test). Similar GLMMs were also fitted to test the differences in the same two endpoints among incubation treatments.

The weight of eggs was compared between females and males (as identified later) by fitting a linear mixed model (LMM) with a random effect of clutch (initially nested in year factor, which was later removed, see Results). The growth of naturally and artificially incubated turtles was analysed by log-transformed body length and weight. The two growth LMMs were built in a forward-stepwise extension procedure. The null model only included a random effect of an individual turtle nested within the random effect of the clutch. The pool of potential fixed effects included the measurement date, sex, and incubation treatment factors with all possible interactions. Within each step, the most informative fixed term, associated with the largest decrease in model Akaike Information Criterion (AIC) value, would be added, respecting the hierarchy of interactions. The minimum threshold of AIC decrease to significantly improve the model was considered to be 2.

The analyses were performed using R v. 4.3.1 software. Mixed models were fitted using the package *glmmTMB* v.1.1.10. Appropriate *post hoc* analyses were aided by the packages *rcompanion* v. 2.4.36, *emmeans* v. 1.8.8, and *multcomp* v. 1.4-25. Compliance with the assumptions of all linear models was inspected using the functions from package *performance* v. 0.12.4. The significance level of $P < 0.05$ was specified for all statistical analyses *a priori*.

RESULTS

Nesting sites and clutch size

We collected 126 clutches of *Emys orbicularis* between 2015 and 2021 (Table 1). In the autumn, 96 clutches were excavated and naturally incubated, while 30 clutches were excavated at the beginning of summer for artificial egg incubation. The herbaceous cover of the studied nests was generally low, with 47.9% shaded by 21–50%, 42.7% by less than 20%, and 9.4% by 51–80% (none under more than 80% cover). However, the shading of studied nests varied significantly among years (Chi-squared test: $\chi^2_{12} = 35.7$, $P = 0.0004$; Fig. 2A). The mean nest depth was 9.7 ± 2.3 (range 4–15) cm and varied annually (one-way ANOVA: $F_{6,89} = 5.7$, $P < 0.0001$; Fig. 2B). On average, there were 11.4 ± 3.0 (range 2–23) eggs per clutch, but this number also varied significantly among years (one-way ANOVA: $F_{6,119} = 2.8$, $P = 0.015$; Fig. 2C). According to the available data, excavated eggs weighed 9.2 ± 0.5 (range 7.1–10.9).

Hatching success and sex ratio

In the autumn, a total of 1092 eggs/hatchlings from 96 clutches were excavated. Of these, 712 (65.2%) were viable juveniles, 239 (21.9%) were non-viable hatchings, 133 (12.2%) were non-fertilised, and 8 (0.7%) eggs were physically damaged (most likely by the activity of predators). When considering only the 845 fertilised cases, 74.9% were viable. The hatching success (per fertilised clutch) varied among years (beta binomial GLM: $P < 0.0001$; Fig. 3A). After considering the random year effect, it was negatively related to both nest depth and herbaceous cover level (beta binomial GLMM: $P < 0.046$).

Viable juveniles were brought to the laboratory for artificial rearing. Of these hatchlings, 394 (55.3%) were identified as females, while 318 (44.7%) were recognised as males, giving an overall sex ratio of 1.2:1, which was

Table 1. Number of *Emys orbicularis* eggs/hatchlings collected, and overall reared individuals released in LT Natura 2000 territories during the study period (2015–2022).

Collection year	Egg incubation type (natural, artificial)	N° excavated clutches	N° collected eggs/ hatchlings	Alive hatchlings	Released number
2015	natural	11	133	103	101
	artificial	2	23	18	18
2016	natural	10	109	57	58
	artificial	4	43	28	28
2017	natural	16	170	20	17
	artificial	5	50	34	36
2018	natural	18	191	124	152
	artificial	15	174	145	145
2019	natural	10	142	92	90
	artificial	2	23	18	18
2020	natural	13	162	128	114
	artificial	2	25	10	10
2021	natural	18	185	159	157
Total		126	1430	970	943

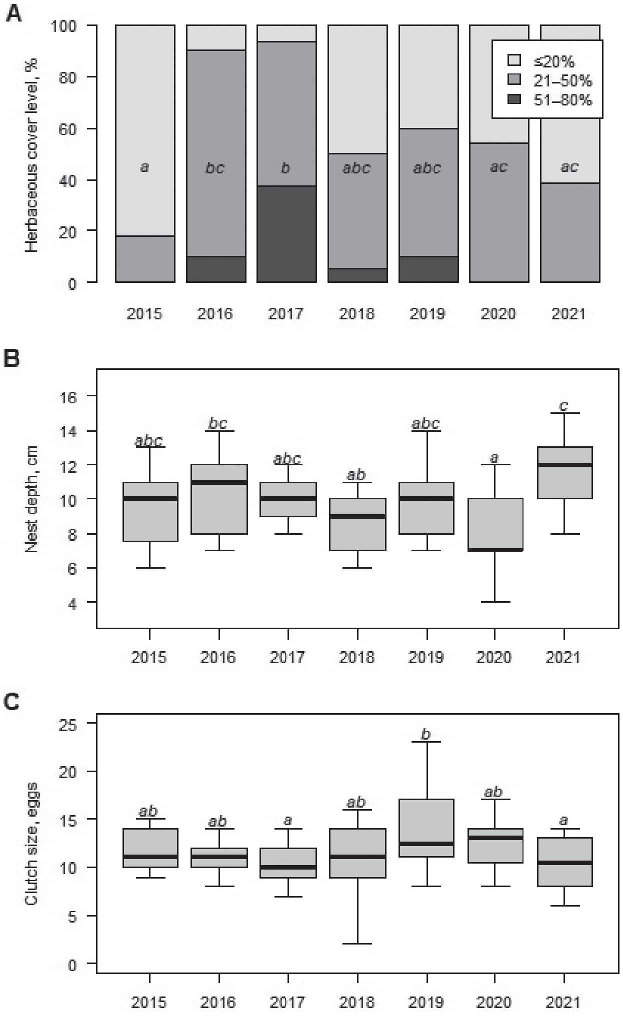

Fig. 2. Herbaceous cover level frequency (A), depth (B) and number of eggs (C) of studied *Emys orbicularis* nests by year. Letters denote homogenous groups according to *post hoc* analysis with Bonferroni-adjusted *G* tests (A) or Tukey method for P-value adjustment (B, C).

significantly female-skewed (Chi-squared test: $\chi^2_1 = 8.1$, $p = 0.004$). There was no significant variation in female percentage (per viable clutch) among years (binomial GLM: $P = 0.30$; Fig. 3B). Nest depth and herbaceous cover had no effect on female percentage (binomial GLMM: $P \geq 0.37$).

In summer, 338 eggs from 30 clutches were excavated for artificial incubation. Of these, 253 (74.8%) successfully hatched into viable juveniles, with only 8 (2.4%) being non-viable, 72 (21.3%) being non-fertilised, and 5 (1.5%) being physically damaged. This means that out of 261 fertilised eggs, 96.9% successfully hatched. The hatching success (per fertilised clutch) under artificial incubation was consistent across years (binomial GLM: $P = 0.38$; Fig. 3C). Of those successfully artificially hatched, 177 (70.0%) were females, while 76 (30.0%) were identified as males, showing a 2.3/1 sex ratio, which was even more female-skewed (Chi-squared test: $\chi^2_1 = 40.3$, $P < 0.0001$). There was no significant annual variation in female percentage per viable clutch (binomial GLM: $P = 0.32$; Fig. 3D).

Artificially incubated eggs generally hatch more successfully (Chi-squared test: $\chi^2_1 = 60.1$, $p < 0.001$) and exhibit a significant female-skewed sex ratio (Chi-squared test: $\chi^2_1 = 15.9$, $p < 0.001$). After accounting for the random year effect, both differences remained significant (beta binomial GLMM for hatching success per fertilised clutch and binomial GLMM for female percentage per viable clutch: $P \leq 0.0018$).

Growth

A total of 957 hatchlings were taken for further artificial rearing: 704 were of natural origin, while 253 were of artificial incubation. Twelve deaths occurred: 10 of natural origin (one upon hatching and nine before the 300-day measurement) and 2 of artificial incubation (both before the 300-day measurement). The mortality during rearing was low (1.2%) and did not differ between the two treatments (Chi-squared test: $\chi^2_1 = 0.54$, $P = 0.46$). Further growth analyses were conducted using only data from surviving turtles. It is noteworthy that even at the egg stage, “female” eggs were significantly heavier than “male” eggs (LMM: $F_{1,\,224} = 5.6$, $P = 0.019$), after accounting for the significant random effect of clutch ($P < 0.001$). Both random effects of turtle and clutch were significant in terms of turtle length and weight ($p < 0.001$) (the nesting random year factor was insignificant ($p = 0.8$) and thus removed).

In the null LMMs of turtle growth, both random effects of turtle and clutch were significant in terms of turtle length and weight ($P < 0.00001$). The forward stepwise extension of models indicated that changes in length and weight significantly varied only between sexes (LMM, Data: Sex effect: $F_{4,3772} \geq 33{,}6$, $P \leq 0.00001$) but not by incubation. Females were generally larger, and these increasing differences were consistent across all measurement dates (Fig. 4 and Table 2).

DISCUSSION

Nesting habitats

Many scientific studies are dedicated to *Emys orbicularis* nesting ecology (Drobenkov, 2000; Ayaz et al., 2017; Mitrus et al., 2018), however, there is a lack of

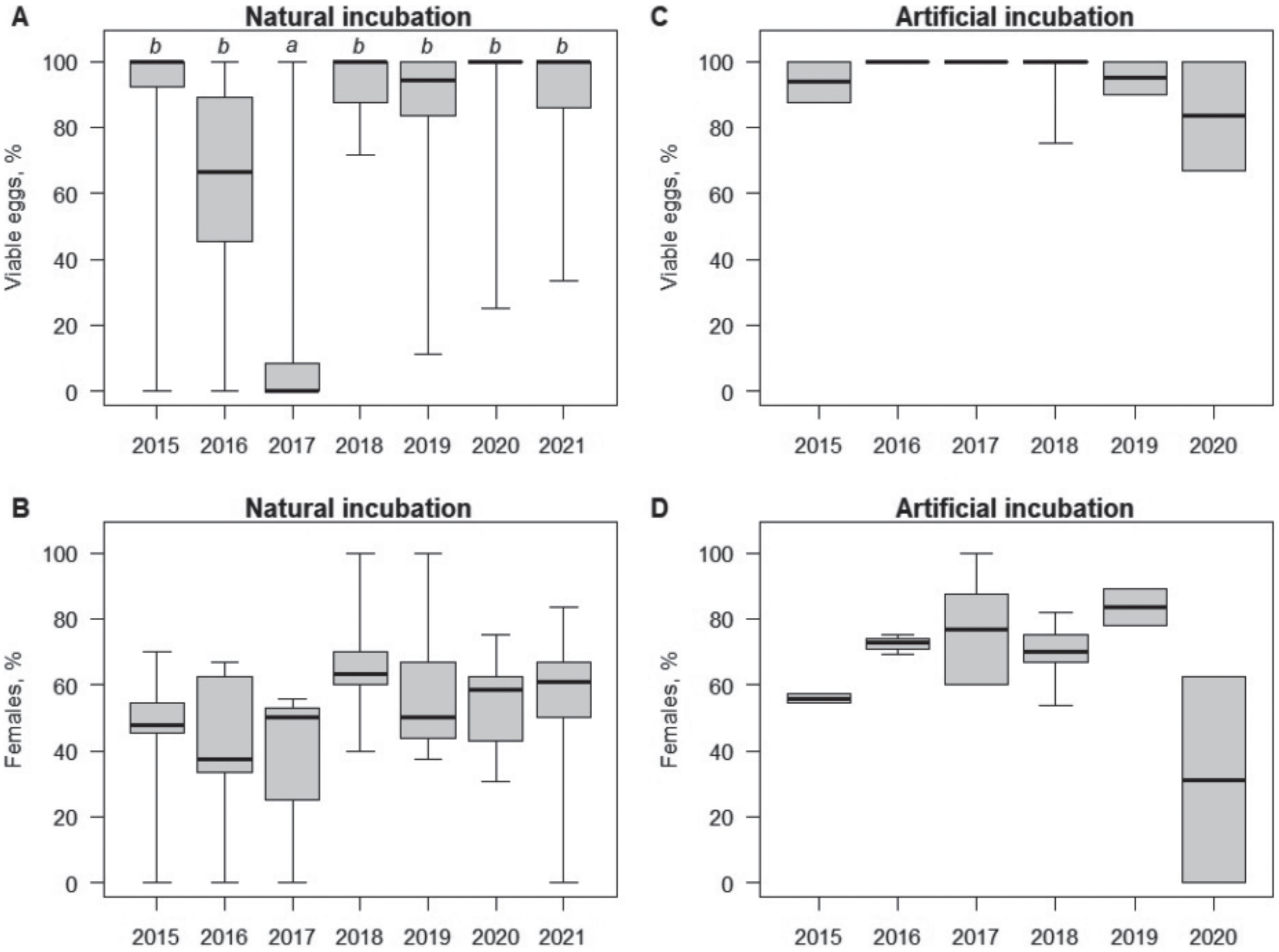

Fig. 3. Percentage of viable eggs among fertilised clutch (A, C) and of females per viable clutch (B, D) in naturally (A, B) and artificially (C, D) incubated clutches of *Emys orbicularis* by year. Letters denote homogenous groups according to *post hoc* analysis with Tukey method for P-value adjustment.

such information from known Lithuanian populations (Meeske, 1997). This study revealed that most (91%) of *E. orbicularis* females lay eggs in rather open areas with little (up to 50%) herbaceous vegetation in Lithuania. Similar results were obtained in other countries, including Poland (Meeske, 1997), Slovenia (Novotny et al., 2004), Italy (Zuffi and Rovina, 2006) and Spain (Diaz-Paniagua et al., 2014). The shores of small water bodies are usually covered by dense vegetation, so *E. orbicularis* females sometimes have to migrate hundreds of metres or even kilometres to find such an area. As suitable nesting places are rare, females may lay eggs in unsuitable areas more distant from water bodies, such as forest and gravel roads, cultivated fields or any other open soil (Mitrus and Zemanek, 2004). Eggs left in such nests do not survive until spring, especially if there is a lack of snow in winter and thus low nest temperatures (Najbar and Szuszkiewicz, 2005). Furthermore, females from northern populations usually lay a single clutch of eggs only once a year (Mitrus and Zemanek, 2004), so if these clutches are placed in areas where individuals have little chance of survival, small *E. orbicularis* populations can decrease significantly in a relatively short period. Furthermore, juveniles are exposed to higher risks of predation due to the lack of a suitable nesting habitat near water bodies (Tetzlaff et al., 2020). Turtles use human-altered environments for egg-laying (Joyal et al., 2001; Purger et al., 2023), making them vulnerable to generalist predators. The presence of potential predators, such as mammals or bird species, is generally prevalent within natural and semi-natural environments (Chelazzi et al., 2000; Rössler, 2000; Zuffi, 2000). It was hypothesised that these predators may impact approximately 75-95% of undisturbed nesting sites (Rovero and Chelazzi, 1996; Zuffi and

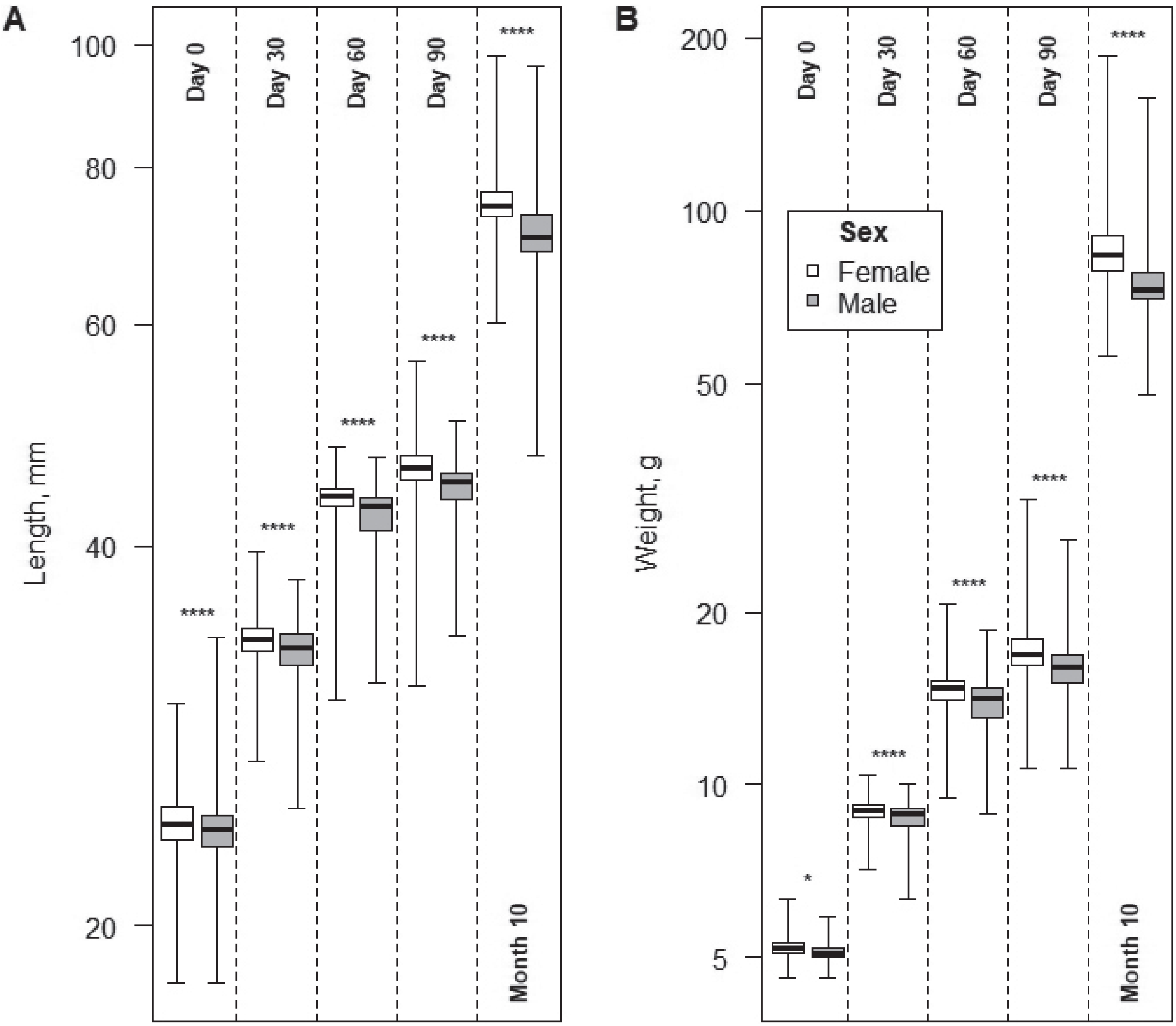

Fig. 4. Variation (median, quartiles, range) of *Emys orbicularis* length (A) and weight (B) during rearing by measurement date (day since hatching), and sex. Stars denote significant differences within each measurement date: * $P \leq 0.05$, and ****$P \leq 0.00001$. Note the *log*-scale of the *y* axes, and see Table 3 for descriptive statistics of raw group data by incubation.

Odetti, 1998; Rössler, 2000). Therefore, it was emphasised (Mitrus and Zemanek, 1998), that to increase small *E. orbicularis* population size, the mortality of the individuals must be reduced in the first year of their life.

In this study, we took two active measures to reduce mortality of the most vulnerable *E. orbicularis* life stages. Firstly, *E. orbicularis* nests were covered with wire mesh to protect eggs and hatchlings from predators in the natural environment. Secondly, eggs were artificial incubated, and hatchlings reared for their first year before being released back into the wild.

Hatching success

Covering nests with wire mesh is one of the simplest tools for protecting eggs and hatchlings from predators and it has been effectively used in many European countries (Schindler et al., 2017; Kiss et al., 2021). In this study, nest protection resulted in only two clutches affected by predators, and eight eggs physically damaged from the overall 1092 eggs laid within protected clutches. In a similar study in Hungary, Kiss et al. (2021) observed that egg-hatching success in wire mesh-protected *E. orbicularis* nests varied from 67.7 to 84.3% under natural condi-

Table 2. Summary of *Emys orbicularis* length and weight during rearing by measurement date (day since hatching), sex, and natural or artificial incubation. Values represent mean ± SD (IQR - interquartile range).

Date	Length (mm)		Weight (g)	
	Females	Males	Females	Males
Natural incubation				
Day 0	24.1 ± 0.9 (23.5–24.6)	23.8 ± 1.1 (23.2–24.4)	5.2 ± 0.2 (5.1–5.3)	5.1 ± 0.2 (5.0–5.2)
Day 30	33.7 ± 1.1 (33.1–34.2)	33.0 ± 1.5 (32.4–34.0)	8.9 ± 0.5 (8.6–9.2)	8.7 ± 0.7 (8.2–9.1)
Day 60	43.3 ± 2.0 (43.0–44.2)	42.1 ± 2.7 (41.1–43.8)	14.9 ± 1.2 (14.2–15.5)	14.0 ± 1.5 (13.2–14.8)
Day 90	46.0 ± 2.1 (45.1–47.0)	44.5 ± 2.1 (43.9–45.8)	17.9 ± 2.7 (16.4–18.1)	16.4 ± 2.3 (15.2–16.8)
Month 10	75.8 ± 4.9 (73.2–76.8)	71.0 ± 5.0 (68.5–73.3)	88.2 ± 17.5 (77.9–91.5)	75.0 ± 11.1 (70.2–76.5)
Artificial incubation				
Day 0	24.3 ± 1.9 (23.0–25.3)	23.8 ± 1.8 (22.8–25.0)	5.2 ± 0.3 (5.1–5.4)	5.1 ± 0.2 (5.0–5.3)
Day 30	33.5 ± 2.1 (32.5–35.0)	33.0 ± 2.1 (31.6–34.3)	9.1 ± 0.5 (9.0–9.3)	9.0 ± 0.5 (8.9–9.3)
Day 60	43.8 ± 1.5 (43.0–44.8)	42.7 ± 2.1 (42.2–44.2)	14.2 ± 1.1 (13.6–14.8)	13.5 ± 1.1 (12.8–14.2)
Day 90	46.9 ± 1.7 (45.7–47.9)	44.8 ± 2.3 (43.5–46.0)	16.8 ± 1.2 (16.0–17.6)	15.4 ± 1.3 (14.5–16.4)
Month 10	74.2 ± 2.9 (72.5–75.8)	70.6 ± 4.0 (68.5–72.8)	85.1 ± 7.7 (80.2–88.7)	75.7 ± 6.0 (70.3–80.1)

tions. Previous studies have shown that the abundance of *E. orbicularis* juveniles in areas with wire mesh protection increased by 50% (Schmidt, 2017).

We excavated 30 clutches of *E. orbicularis* immediately after the eggs were laid for artificial incubation in the laboratory. Previous attempts to apply the clutch relocation method to protect eggs have not been successful (Marchand and Litvaitis, 2004; Mitrus, 2008; Bona et al., 2012). It was concluded that this method is too risky because the initial stages of egg development are sensitive to environmental changes and relocation can have a negative impact. However, this study proved that this protective measure is highly effective, as only 2.4% of the collected and artificially incubated eggs failed to hatch.

Clutch parameters

It is vital to understand the natural clutch parameters of small *E. orbicularis* populations at the northern distribution edge when applying active protection measures. We observed 126 natural clutches of *E. orbicularis* with a mean of 11 eggs per clutch and some significant difference among years. The number of eggs in a single clutch is similar to the average number of eggs per clutch reported from neighbouring countries: 11-15 eggs in Poland (Jablonski and Jablonska, 1998; Najbar and Szuszkiewicz, 2005), 9–13 eggs in Latvia (Pupins et al., 2019). Our observations confirmed, the average egg number of *E. orbicularis* clutch tends to differ across different latitudes (Zuffi et al., 2017). In southern regions, clutches of *E. orbicularis* are generally smaller than in northern populations. On average, the clutch size in Hungary is 9 eggs (Kiss et al., 2021), 7 eggs in Turkey (Ayaz et al., 2017), and 6 eggs in Italy (Liuzzo et al., 2024). This is explained by the fact that females in the Southern European populations lay two clutches per year, while those from Central and East European populations usually lay only one clutch per year (Fritz, 2003). Also, our study found that the average weight of *E. orbicularis* eggs is 9.2 g. Similarly, 8.1 g was reported as the average egg weight from the neighbouring country of Belarus (Drobenkov, 2000), whereas, in Ukraine, the average weight of deposited *E. orbicularis* eggs was lower at 7.4 g (Zinenko, 2004).

The mean depth of our studied nests was 9.7 cm (ranging from 4 to 15 cm), which is consistent with the nest depths reported by other authors: 10.1 cm in Italy (Liuzzo et al., 2024) and 8 cm in Turkey (Ayaz et al., 2017), whereas in Spain Diaz-Paniagua and colleagues (2014) found nest depth ranging from 4 to 6.8 cm. There was a significant year effect on the studied nest depth in this study, probably related to the different temperature profiles each year. The depth of the nests is crucial for the development of embryos and the survival of hatchlings in the northern *E. orbicularis* populations. Our research clearly shows that the nesting depth and herbaceous cover negatively impact hatching success. If eggs are placed in a nest that is too deep or overgrown by plants, there is a lower chance of them hatching. Schneeweiß (2004) explains that survival of northern populations depends on suitable summer temperatures for incubation and the duration of solar radiation at the nest site. Higher or lower nest temperatures can cause higher mortality of embryos. *E. orbicularis* embryos develop most successfully at temperatures ranging from 18 to 33 °C (Pieau and Dorizzi, 1981). However, if the clutch is placed in a nest that is too shallow, there is a higher risk of the hatchlings freezing to death during their first winter in the northern *E. orbicularis* populations, where the newly born hatchlings overwinter in their nests.

Hatchlings size and sex ratio

Our study revealed that the mean body mass of newly hatched turtles was 5.2 g, similar to hatchling weights reported from neighbouring countries: 5.2 g found in the Latvian population (Pupins et al., 2019), 6.1 g in the Belarusian population (Drobenkov, 2000), and 6.9 g in the Ukrainian populations (Pupins et al., 2019). However, lower hatchling weights were reported from the southern *E. orbicularis* populations: 4.9 g in Hungary (Kiss et al., 2021), 4.8 g in Spain (Diaz-Paniagua, 2014), and 3.6 g in Turkey (Ayaz et al., 2017). Our studied hatchlings had a bigger mean plastron length (24.0 mm) compared to hatchlings of Turkish (19.6 mm; Ayaz et al., 2017) or Spanish populations (22.3 mm; Diaz-Paniagua et al., 2014). Our results clearly confirm the tendency described by Pupins et al. (2019), who compared *E. orbicularis* hatchlings from different geographic regions and found that hatchlings in the northern parts of the range are larger than in the southern parts. Joss et al. (2017) also found a correlation between latitude and *E. orbicularis* body size. This and the aforementioned studies support Bergmann's rule (Bergmann, 1848), which states that animals in colder climates have larger body sizes than those in warmer climates.

The size and weight of *E. orbicularis* hatchlings vary due to many factors, including the age of the nesting female, the size of the clutch, and the incubation temperature of eggs (year factor) (Pupins et al., 2019). It is vital to understand the size and sex ratio of hatchlings, especially when comparing artificial vs natural incubation and further hatchlings rearing. This study found that hatchlings attain larger weight if incubated naturally vs. artificially. This is because the incubation time of artificial eggs is much shorter (57-80 days) than natural eggs (Pupins et al., 2019) with 85-113 days reported in Poland (Mitrus and Zemanek, 2000), 90-117 days in Austria (Rössler, 2000), and 83 days in Spain (Diaz-Paniagua et al., 2014). However, the initially observed hatchling weight differences disappeared by the end of the 10-month rearing period.

Moreover, newly hatched females were bigger in both weight and length compared to juvenile males. It is important to note that even at egg stage, “female” eggs were significantly larger than “male” eggs. The observed initial gender differences remained unchanged until the end of rearing. Other studies have also reported larger *E. orbicularis* females (Zuffi et al., 1999; Fediras et al., 2017; Liuzzo et al., 2021).

The study established a clear female-dominated bias in hatchlings from both artificial and natural incubation methods, with a pronounced female-skewed ratio observed in the artificially incubated eggs. *E. orbicularis*, like other thermophilous species, exhibits a discernible sensitivity to temperature fluctuations (Sommer et al., 2007; 2009; Joos et al., 2017; Cerasoli et al., 2019; Nekrasova et al., 2021). The incubation temperature has a huge impact on hatchlings, determining their sex and influencing survival. At higher temperatures (more than 29.5 °C), hatches occur exclusively of female turtles, while at lower temperatures (below 27.5 °C), hatches occur exclusively of male turtles. At an intermediate temperature (28.5 °C), the hatchling ratio of females and males is equal (Zaborski et al., 1988). In our research, *E. orbicularis* eggs were incubated at 25-27 °C during the night and at 28-29.5 °C during the day. We observed that on these temperature conditions, the sex ratio of hatchlings was 2.3/1 (female/male). Individuals excavated from nests that experienced lower temperatures during the incubation period under natural conditions had an almost equal female/male ratio (1.2/1).

Success of artificial rearing

We experimented with raising *E. orbicularis* hatchlings in an artificial environment for their first year to reduce the high winter mortality rate in the wild. The hatchlings were raised in either an artificial or natural incubation process, and their survival rates were the same. The survival rate of the reared hatchlings in this experiment was an impressive 98.8%, which is significantly higher than the 77.8% survival rate of artificially reared hatchlings in Slovakia (Bona et al., 2012) and much higher than the 7.1% survival rate of *E. orbicularis* overwintering hatchlings in nests under natural conditions (Bona et al., 2012).

Concluding remarks

The decrease in *E. orbicularis* populations in Europe, especially in its northern distribution edge, requires special protection measures, primarily for the critical early life stages. The methodologies for artificial *E. orbicularis* egg incubation and further hatchling rearing we fine-tuned resulted highly effective and significantly increased *E. orbicularis* hatching success and survival. Artificially reared *E. orbicularis* individuals could be released to the wild in equal numbers of males and females, which would help balance the skewed sex ratio in their small, threatened populations as well as help to protect the overall genetic diversity of *E. orbicularis* in its northern distribution range.

ACKNOWLEDGEMENTS

The research was funded by the project “Implementation of Nature Conservation and Management Measures by Preserving and Increasing the Populations of European Pond Turtles and European Fire-bellied Toad” (Nr. 05.4.1-APVA-V-018-01-0004).

State laws on handling wild animals were followed, with six permits (Nos. 50, 14, 15, 22, 15, 100) from the state Environmental Protection Agency being issued for collecting, handling, rearing and releasing reared individuals of pond turtles back into the wild during the whole study period.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <http://www.unipv.it/webshi/appendix> Manuscript number 16266.

REFERENCES

Avanzi, M., Millfanti, M. (2003): Le grand livre des tortues terrestres et aquatiques. De Vecchi Edition.

Ayaz, D., Çiçek, K., Bayrakci, Y., Tok, C.V. (2017): Reproductive ecology of the European pond turtle, *Emys orbicularis* (Linnaeus, 1758), from Mediterranean Turkey. Acta Zool Bulg. **10**: 23-29.

Bastytė-Cseh, D. (2021): Balinis vėžlys *Emys orbicularis* (Linnaeus, 1758). In: Red Data Book of Lithuania. Animals, plants, fungi, p. 212. Rašomavičius, V. Ed., Vilnius, Lututė.

Bergmann, C. (1848): Über die Verhältnisse der Wärmeökonomie der Thiere zu ihrer Grösse. Vandenhoeck und Ruprecht.

Bona, M., Novotny, M., Danko, S., Buresova, A. (2012): Headstarting in a small population of European pond turtles (*Emys orbicularis*) in Central European conditions: first results. Herpetol. Notes **5**: 547-550.

Berthomieu, L., Vermeer, J. (2021): EAZA Best Practice Guidelines for the European pond turtle *(Emys orbicularis),* European Association of Zoos and Aquariums, Amsterdam, The Netherlands.

Cerasoli, F., Iannella, M., Biondi, M. (2019): Between the hammer and the anvil: how the combined effect of global warming and the non-native common slider could threaten the European pond turtle. Manag. Biol. Invasions **10**: 428-448.

Chelazzi, G., Lebboroni, M., Tripepi, S., Utzeri, C., Zuffi, M.A.L. (2000): A primer on the conservation biology of the European pond turtle, *Emys orbicularis*, of Italy. In: Proc. II Int. Symp. on *Emys orbicularis*, Blanc, Brenne. Chelonii **2**: 101-104

Diaz-Paniagua, C., Andreu, A.C., Marco, A., Nuez, M., Hidalgo-Vila, J., Perez-Santigosa, N. (2014): Data on nesting, incubation, and hatchling emergence in the two native aquatic turtle species (*Emys orbicularis* and *Mauremys leprosa*) from Doñana National Park. Basic Appl. Herpetol. **28**: 147-151.

Drobenkov, S.M. (2000): Reproductive ecology of the pond turtle (*Emys orbicularis* L.) in the northeastern part of the species range. Russ. J. Ecol. **31**: 49-54.

Fediras, S., Rouag, R., Ziane, N., Olivier, A., Béchet, A., Benyacoub, S. (2017): Population structure and morphometrics of the European pond turtle (*Emys orbicularis* (Linnaeus, 1758)) at Lake Tonga, Algeria. Russ. J. Herpetol. **25**: 88-96

Frıtz, U. (2003): Die Europäische Sumpfschildkröte. Supplement der Zeitschrift für Feldherptologie No. 1. Bielefeld: Laurenti-Verlag. 224 p.

Fritz, U., Chiari, Y. (2013): Conservation actions for European pond turtles–a summary of current efforts in distinct European countries. Herpetol. Notes **6**: 105.

Isailovic, J.C., Mesaroš G. (2013): Conservation activities for the European pond turtle (*Emys orbicularis*) in Serbia. Herpetol. Notes **6**: 119-121.

Jablonski, A., Jablonska, S. (1998): Egg-laying in the European pond turtle, *Emys orbicularis* (L.). Leczynsko–Wlodawskie Lake District (east Poland). Mertensiella **10**: 141-146.

Joyal, L.A., McCollough, M., Hunter Jr., M.L. (2001): Landscape ecology approaches to wetland species conservation: a case study of two turtle species in southern Maine. Con. Biol. **15**: 1755-1762.

Joos, J., Kirchner, M., Vamberger, M., Kaviani, M., Rahimibashar, M. R., Fritz, U., Müller, J. (2017): Climate and patterns of body size variation in the European pond turtle, *Emys orbicularis*. Biol. J. Linn. Soc. **122**: 351-365.

Kiss, I., Erdelyi, G., Szabo, B. (2021): Nesting activity and reproductive success of *Emys orbicularis* in Babat Valley (Gödöllő, Hungary). Herpetol. Conserv. Biol. **16**: 624-638.

Liuzzo, M., Borella, S., Ottonello, D., Arizza, V., Malavasi, S. (2021): Population abundance, structure and movements of the European pond turtle, *Emys orbicularis*

(Linnaeus 1758) based on capture-recapture data in a Venice Lagoon wetland area, Italy. Ethol. Ecol. Evol. **33**: 561–575.

Liuzzo, M., Fantinato E., Malavasi S. (2023): Successful reproduction of feral *Trachemys scripta* (Schoepff, 1792) in an inland wetland of the Veneto region, Le Basse del Brenta, Italy. Herpotol. Notes **16**: 465-469.

Liuzzo, M., Spada, A., Facca, C., Borella, S., Malavasi, S. (2024): Nesting habitat characteristics and predation patterns in the European pond turtle *Emys orbicularis* (L., 1758): implications for management and conservation measures. Glob. Ecol. and Conserv. **52**: e02975

Luiselli, L., Vamberger, M. (2024): *Emys orbicularis* (Europe assessment). The IUCN Red List of Threatened Species 2024: e.T7717A207667247.

Marchand, M.N., Litvaitis, J.A. (2004): Effects of landscape composition, habitat features, and nest distribution on predation rates of simulated turtle nests. Biol. Conserv. **117**: 243-251.

Meeske, A.C.M., Pupins, M. (2009): The European pond turtle in Latvia. Pp. 214-216. In: European Pond Turtles. The genus *Emys*. Rogner, M., Ed, Frankfurt, Chimaira.

Meeske, M. (1997): Nesting ecology of European pond turtle (*Emys orbicularis*) in South Lithuania. Acta Zool. Lit. **7**: 138-142.

Meeske, M., Pupins, M., Rybczynski, K. (2006): First results on the distribution and condition of the European pond turtle (*Emys orbicularis*) at the northern edge of its distribution in Lithuania and Latvia (in German). Z. Feldherpetol. **13**: 71-99.

Mitrus, S., Zemanek, M. (2004): Body size and survivorship of the European pond turtle *Emys orbicularis* in Central Poland. Biol. Brat. **59**: 103-107.

Mitrus, S. (2008): Relocation of the European pond turtle *Emys orbicularis* clutches did not influence the hatching rate. Acta Biol. Univ. Daugavp. **8**: 63-66.

Mitrus, S., Najbar, B., Kotowicz, A., Najbar, A. (2018): The body size of headstarted and wild juvenile European pond turtles (*Emys orbicularis*). Russ. J. Herpetol. **25**: 161-164.

Mitrus, S., Zemanek, M. (1998): Reproduction of *Emys orbicularis* (L.) in central Poland. Mertensiella **10**: 187-191.

Mitrus, S., Zemanek, M. (2000): Distribution and biology of *Emys orbicularis* (L.) in Poland. Stapfia **69**: 107-118.

Mollov, I., Naumov, B., Dobrev, D., Popgeorgiev, G. (2013): Conservation activities for European pond turtles (*Emys orbicularis*) in Bulgaria. Herpetol. Notes **6**: 135-138.

Najbar, B., Szuszkiewicz, E. (2005): Reproductive ecology of the European pond turtle *Emys orbicularis* (LINNAEUS, 1758) (Testudines: Emydidae) in Western Poland. Acta Zool. Cracov. **48**: 11-19.

Nekrasova, O., Marushchak, O., Pupins, M., Skute, A., Tytar, V., Čeirāns, A. (2021): Distribution and potential limiting factors of the European pond turtle (*Emys orbicularis*) in Eastern Europe. Diversity **13**: 280.

Novotny, M., Danko, S., Havaš, P. (2004): Activity cycle and reproductive characteristics of the European pond turtle (*Emys orbicularis*) in the Tajba National Nature Reserve, Slovakia. Biol. Brat. **59**: 113-121.

Pieau, C., Dorizzi, M. (1981): Determination of temperature sensitive stages for sexual differentiation of the gonads in embryos of the turtle, *Emys orbicularis*. J. Morphol. **170**: 373-382.

Pupins, M., Nekrasova, O., Marushchak, O., Dubyna, A., Neizhko, I. (2019): Morphological features of European pond turtles' *Emys orbicularis* (Linnaeus, 1758) hatchlings at the northern edge (Latvia) and central part (Ukraine) of its distribution range. Pp. 11-13. In: Proceedings of the II Congresso Nazionale Testuggini e Tartarughe, Italy, Albenga.

Pupins, M., Pupina, A. (2008): Distribution of European pond turtle *Emys orbicularis* (Linnaeus, 1758) on the northern edge of its area in Latvia. Rev. Esp. Herp. **22**: 149-157.

Purger, J.J., Molnár, T.G., Lanszki, Z., Lanszki, J. (2023): European pond turtle (*Emys orbicularis*) nest predation: A study with artificial nests. Biology **12**: 342.

Rovero, F., Chelazzi, G. (1996): Nesting migrations in a population of the European pond turtle *Emys orbicularis* (L.) (Chelonia Emydidae) from central Italy. Ethol. Ecol. Evol. **8**: 297-304.

Rössler, M. (2000): Der Lebensraum der Europaischen Sumpfschildkröte *Emys orbicularis* (L.) in den niederösterreichischen Donau-Auen. Stapfia **69**: 145-156.

Salom-Oliver, M., Amengual, A.R.H., Aguiló-Zuzama, A., Ribas-Serra, A., Vallespir, J., Tejada-Gavela, S., Pinya, S. (2022): Plastron color patterns allow for individual photo-identification in two different chelonian species. Can. J. Zool. **100**: 789-798.

Savic, A. (2010): Distribution of and potential factors of threat for *Emys orbicularis* (Linne 1758) within city of Niš and surrounding area. B.Sc. thesis, Faculty of Sciences and Mathematics, University of Niš (In Serbian, English summary).

Schindler, M., Frötscher, H., Hille, A., Bruck, M.R., Schmidt, M., Kornilev, Y.V. (2017): Nest protection during a long-term conservation project as a tool to increase the autochthonous population of *Emys orbicularis* (L., 1758) in Austria. Acta Zool. Bulg. **10**: 147-154.

Schmidt S. D. (2017): Evaluation of habitat features influencing turtle abundance in the Donau-Auen. Wissenschaftliche Reihe Nationalpark Donau-Auen **70**: 1–211. Orth an der Donau: Nationalpark Donau-Auen.

Schneeweiß, N. (2004): Climatic impact on reproductive success of *Emys orbicularis* at the northwestern border of the species range. Biologia **59**: 131-137.

Schneeweiß, N., Breu, H. (2013): Conservation activities for the European pond turtle (*Emys orbicularis*) in Germany. Herpetol. Notes **6**: 113-115.

Sommer, R.S., Lindqvist, C., Persson, A., Bringsøe, H., Rhodin, A.G., Schneeweiss, N., Široky, P., Bachmann, L., Fritz, U. (2009): Unexpected early extinction of the European pond turtle (*Emys orbicularis*) in Sweden and climatic impact on its Holocene range. Mol. Ecol. **18**: 1252-1262.

Sommer, R.S., Persson, A., Wieseke, N., Fritz, U. (2007): Holocene recolonisation and extinction of the pond turtle, *Emys orbicularis* (L., 1758), in Europe. Quat. Sci. Rev. **26**: 3099-3107.

Tetzlaff, S.J., Estrada, A., DeGregorio, B.A., Sperry, J.H. (2020): Identification of factors affecting predation risk for juvenile turtles using 3D printed models. Animals **10**: 275.

Vecchioni, L., Marrone, F., Arculeo, M., Fritz, U., Vamberger, M., 2020. Stand out from the crowd: small-scale genetic structuring in the endemic sicilian pond turtle. Diversity **12**: 343.

Zaborski, P., Dorizzi, M., Pieau, C. (1988): Temperature-dependent gonadal differentiation in the turtle *Emys orbicularis*: concordance between sexual phenotype and serological HY antigen expression at threshold temperature. Differentiation **38**: 17-20.

Zinenko, O. (2004): Notes on egg-laying, clutch size and hatchling feeding of *Emys orbicularis* in the Kharkiv region, Ukraine. Biol. Brat. **59**: 149-151.

Zuffi, M.A.L., Odetti, F. (1998): Double egg deposition in the European pond turtle, *Emys orbicularis*, from central Italy. Ital. J. Zool. **65**: 187-189.

Zuffi, M. A. L., Odetti, F., Meozzi, P. (1999): Body size and clutch size in the European pond turtle (*Emys orbicularis*) from central Italy. J. Zool., **247**: 139-143.

Zuffi, M.A.L. (2000): Biology of the conservation of the European pond turtle, *Emys orbicularis*, of Italy. Stapfia **69**: 219-228.

Zuffi, M.A.L., Mangiacotti, M., Masucci, G. D., Sacchi, R., Scali, S., Sannolo, M. (2017): Stable or plastic body shape? *Emys orbicularis* hatchlings-juveniles growth patterns under different ecological conditions. North-West. J. Zool. **13**: 262-270.

Zuffi, M.A.L., Rovina, L. (2006): Habitat characteristics of nesting areas and of predated nests in a Mediterranean population of the European pond turtle, *Emys orbicularis*. Acta Herpetol. **1**: 37-50.

Acta Herpetologica 20(1): 29-39, 2025
DOI: 10.36253/a_h-16881

A comparative analysis of female genitalia of seven Old World snake species using a silicone modeling technique

Kostadin Andonov[1,*], Angel Dyugmedzhiev[1], Borislav Naumov[1], Nikolay Todorov[2], Vladislav Vergilov[1]

[1] *Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 2 Gagarin Street, 1113 Sofia, Bulgaria*
[2] *Independent researcher, Krakra 30 street, Dragichevo, Bulgaria*
**Corresponding author.* Email: kandonov91@gmail.com

Submitted on: 2024, 26th November; revised on: 2024, 6th December; accepted on: 2025, 12th May
Editor: Francesco Paolo Faraone

Abstract. Genitalia diversity has long been an object of evolutionary and functional morphology studies, with a primary focus on male copulatory organs. Despite extensive studies on snake genitalia, particularly hemipenes, female copulatory organs remain understudied. This research aims to fill this gap by modifying a recently introduced silicone modeling methodology for preparation and by describing female snake genitalia for seven previously undescribed species. The methodology is based on employing a two-component condensation silicone into snakes' genitalia to create internal models for intersexual comparative morphology. We conducted a comprehensive examination of seven Old World snake species – Boidae: *Eryx jaculus*, Colubridae: *Dolichophis caspius*, *Zamenis longissimus*, *Elaphe quatuorlineata*, *Coronella austriaca*, and Viperidae: *Vipera ammodytes*, and *V. berus*. Our results reveal significant variations in female genitalia morphology and we speculate that this is also reflected in copulation adjustment between the different types of species' genitalia.

Keywords. Copulation, morphology, cloaca, urodaeum, pouches.

1. INTRODUCTION

Closely related taxa often exhibit morphologically distinctive genitalia (Arnqvist, 1998; Gredler et al., 2014). Understanding genital diversity has long been an object of functional morphology and evolutionary research, with most studies focused mainly on male copulatory organs (i.e., Dufour, 1844; Darwin, 1871; Mayr, 1963; Thornhill, 1983; Eberhard, 1985; Shapiro and Porter, 1989; East et al., 1993; Hosken and Stockley, 2004; Cohn, 2011; Gredler et al., 2014; Brennan and Prum, 2015; Langerhans et al., 2016; Brennan, 2022). Since the pioneering work of Dufour (1844), who proposed the lock-and-key mechanism, researchers have concentrated not only on the morphology of male copulatory organs but their evolution as well. However, in the past several decades, the lock-and-key mechanism has been confronted (Eberhard, 1985, 2010; Shapiro and Porter, 1989). A strong argument against the lock-and-key hypothesis is the lack of the supposed female "locks" in many groups (Eberhard 1985; Shapiro and Porter, 1989), and the expected pattern of character displacement in males within zones of sympatry among closely related species is often absent (i.e., Ware and Opell, 1989; Eberhard, 2010). Nevertheless, most of the studies have focused mainly on male copulatory organs, while female genitalia are still heavily understudied (Ah-King et al., 2014), and thus, more studies on female genitalia with various techniques are required.

Snake genitalia, in particular, have been studied for many years. After the fundamental study of Cope (1895) who describes the hemipenial morphology of more than 200 species, the general morphology of hemipenes and female genitalia are considered species-specific (Dowling and Savage, 1960; Keogh, 1999; Zaher, 1999, Siegel

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

www.fupress.com/ah

et al., 2012). Male snakes possess two copulatory organs known as hemipenis, which are often ornamented with various structures (Cope, 1895; Dowling and Savage, 1960; Keogh, 1999; Andonov et al., 2017). The hemipenes are inverted inside a hemipenial sheath in the proximal part of the tail, close to the cloacal opening and are everted during copulation or sometimes as a defense behavior (i.e., Cope, 1895; Dowling and Savage, 1960). The female reproductive tract consists of two oviducts (pouch, non-glandular uterus, glandular uterus, posterior infundibulum, anterior infundibulum), and two ovaries, with sperm storage primarily occurring in the non-glandular uterus and posterior infundibulum (Siegel et al., 2012; Jurkfitz et al., 2023). However, there is an ongoing debate whether the pouch is derivative of the urodeaeum (Blackburn 1988) or the oviducts (Siegel et al., 2011). The two paired oviducts are enclosed in a thin visceral pleuroperitoneum and are suspended in the coelomic cavity by a dorsal mesentery. The non-glandular uterus features a thin lamina propria and is lined with an epithelium mainly made up of ciliated cells (Siegel et al., 2011). From a macroscopic perspective, the cloaca can be segmented into three to four distinct regions, varying by species: the proctodaeum, urodaeum, an anterior extension of the urodaeum (which may not exist in all species), and a coprodaeal complex consisting of two regions (Siegel et al., 2011, 2012). Despite the prevalence of studies on male genitalia, over the last century that have been a number of studies on female genitalia as well (i.e., Pope, 1941; Edgren, 1953; Inger and Marx, 1962; Gabe and Saint-Girons, 1965; Pisani, 1976; Siegel et al., 2011, 2012; Showalter et al., 2014, Granados et al., 2022). Still, there are only a few studies analyzing the potential alignment between hemipenes and pouch (Pope, 1941; Inger and Marx, 1962, Granados et al., 2022).

Female genitalia have been described mainly *in situ* after dissection of certain individuals with very few exceptions (Granados et al., 2022), while male hemipenes are usually described in their everted and inflated state. Such two-dimensional observation of female genitalia restricts the potential for further investigation of copulatory adjustment of male and female genitalia. We believe that using a better methodology for female snake genitalia preparation and their respective description, with a three-dimensional (3D) view of the organs following Granados et al. (2022), is necessary for a proper comparison to the common hemipenial descriptions, so further analyses for potential copulatory adjustments could be performed. Similar methodology for three-dimensional genitalia models has been used not only for snakes, but also for caimans (Moore et al., 2022), and marine mammals (Orbach et al., 2018; 2021).

In the present study, we use a recently proposed methodology that allows a description of female snake genitalia in a shape that presumably has the closest resemblance to its form during copulation (Granados et al., 2022) with a few minor additions. Such descriptions can give useful information for comparative morphology between male and female genitalia and provide valuable knowledge for testing one of the main hypotheses for genital evolution, i.e., lock-and-key mechanism. Moreover, we provide the first descriptions of female genitalia for seven Old World snake species, based on this methodology.

2. MATERIAL AND METHODS

We investigated the female genitalia morphology of seven Old World snake species from three families. For the purposes of the study and intersexual comparative morphology, we used 16 adult female individuals belonging to the following species: Boidae: *Eryx jaculus* (Linnaeus, 1758) (n = 1); Colubridae: *Dolichophis caspius* (Gmelin, 1789) (n = 2), *Zamenis longissimus* (Laurenti, 1768) (n = 2), *Elaphe quatuorlineata* (Lacepède, 1789) (n = 2), *Coronella austriaca* Laurenti, 1768 (n = 1); Viperidae: *Vipera ammodytes* (Linnaeus, 1758) (n = 5), *V. berus* (Linnaeus, 1758) (n = 1). Since there are records for ontogenetic differences in the female genitalia shape (Showalter et al., 2014), only adult individuals were used. Individuals were considered adults based on species-specific total length (Ltot) threshold, which is the sum of the snout-vent length (SVL) and the tail-length: *E. jaculus*: Ltot >40 cm, *D. caspius* and *E. quatuorlineata*: Ltot >100 cm; *Z. longissimus*: Ltot >90 cm; *C. austriaca* and *V. ammodytes*: Ltot >46 cm; *V. berus*: Ltot >35 cm (Biserkov et al., 2007; Stojanov et al., 2011; the authors' unpublished data). The individuals that we used were either from the collection of the National Museum of Natural History, Bulgarian Academy of Sciences in Sofia, or freshly found dead specimens (i.e., dead on the road individuals) (see Supplementary file 1).

The posterior end (tail with part of the abdomen) of each tested snake was immersed in 2% KOH solution for 3-6 hours, depending on the level of fixation and the size of the individual (Pesantes, 1994). After the tissue softened and became rubber-like, a two-component condensation dental silicone was injected into the individuals' cloaca.

For the preparation of the genitalia models, we followed a methodology proposed by Granados et al. (2022) with a few adjustments and changes, such as not tying the oviducts or the intestine immediately after the cloaca, but further, in order to receive additional information about key structures, and not removing the cloaca from the individual. Each specimen was securely tied approximately 5-6 cm anterior to the cloacal opening to ensure the silicone enters the vaginal pouch first and does not spread to the abdomen and intestines. We used Stomaflex™ Light as a solid silicone component and Stomaflex™ Gel Catalyst to harden the solid component. Each filling hardened for 3-5 minutes, after which a small incision was made at the base of the cloacal opening, followed by removal of the silicone model (Fig. 1). The models were washed with water and kept in plastic tubes. The snake specimens were also washed with water and re-preserved in 90% ethanol.

For the description of the female genitalia, we follow (with some additions) the terminology adopted by Siegel et al. (2011, 2012) where the bifurcated cloacal area, that receives the distal part of the hemipenis, is named "vaginal pouch" (or only "pouch"). Considering that Seigel et al. (2011, 2012) describe the states of bifurcation of the pouch histologically, while we observe them only morphologically, we do not follow the four-state categorization of the pouches (bifurcated pouch, separated pouch arms with no urodaeal divide, separated pouch arms with urodaeal divide, and simple pouch), but classify them as follows: simple – no bifurcation, slightly separated – when the silicone model arms are less than 25% of the total model length, separated – when the model arms are between 25%-50% of the total model length, and bifurcated – when the model arms are more than 50% of the total model length. This classification has a close resemblance to the respective term for the general shape of hemipenes, allowing the comparisons to be as accurate as possible. For the description of hemipenes, we followed Andonov et al. (2017), where the terminology is mainly based on Dowling and Savage (1960) and Zaher (1999) with a few additions and clarifications. Following the characteristic for male genitalia introduced in Andonov et al. (2017) – hemipenial proportion index (HPI), we suggest a similar index for the female genitalia description – female genital proportion index (FGPI) where the maximal width of the silicone model is divided by its total length. Female genitalia models with FGPI>0.5 were considered "stubby"; those with FGPI between 0.5 and 0.25— "medium formed"; those with FGPI< 0.25— "elongated".


Fig. 1. Removal of the silicone model from female genitalia of *Coronella austriaca.*

Considering that we have tested five female *V. ammodytes* individuals, we conducted descriptive statistics for the species.

All photos of the models were made with Sony DSC-HX400V Ver. 2.10 on a black background, and the hemipenes described by Andonov et al. (2017) have been photographed using a high-resolution digital camera (Nikon COOLPIX P510) by placing the object on a glass slide positioned about 20 cm above a black background to reduce unwanted shadows.

The research was carried out in accordance with permit № 861/13.01.2021 provided by the Ministry of Environment and Waters.

3. RESULTS

The models showed that female cloaca and vaginal pouches were prominent and some silicon has entered the intestines as well (Fig. 2).

In this section, we provide a detailed morphological description of the female cloaca. All figures present the silicone models provided during the current research, as well as pictures of the species' hemipenes as described in Andonov et al. (2017).

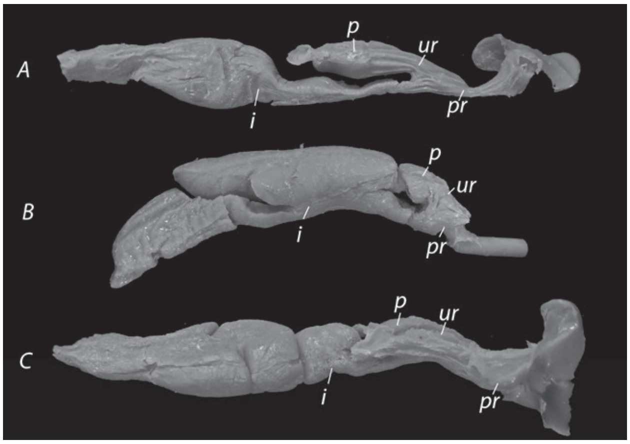

Fig. 2. Lateral view of silicone models of female genitalia and their positioning relative to the intestine for A) *Coronella austriaca*, B) *Eryx jaculus*, and C) *Vipera berus* (pr – proctodaeum; ur – urodaeum; p – pouch; i – intestine).

Family Boidae

Eryx jaculus. The silicone model (n = 1) is short and the pouch arms are almost absent. However, there is a very slight bifurcation present; the genitalia model is not bifurcated to slightly bifurcated and stubby – FGPI > 0.5 (Fig. 3). It differs from all other models significantly, being the only stubby model. On the contrary, the hemipenis is medium formed according to Andonov et al., 2017, with an undivided *sulcus spermaticus* and with not much ornamentation, lacking any calcified structures or calyces, with only a few flounces with scalloped edges present.

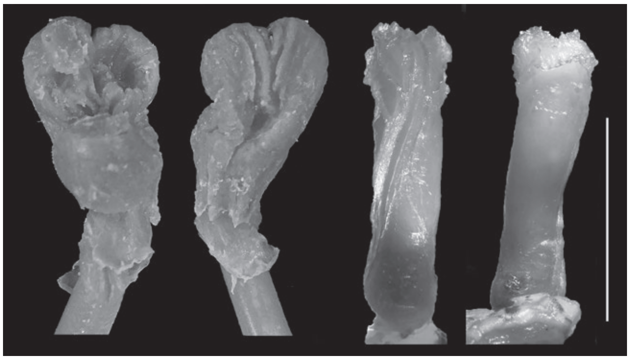

Fig. 3. Ventral and dorsal view of female genitalia model of *Eryx jaculus* (NMNHS III-17-35) – left, and sulcate and asulcate side of the species hemipenis (NMNHS III-17–38 in Andonov et al., 2017). Scale = 1 cm.

Family Colubridae

Coronella austriaca. The model (n = 1) is relatively long with prominent bifurcation and long pouch arms, being elongated (FGPI = 0.22) and slightly separated (Fig. 4). It has a similarity with the hemipenis general shape of the species where one of the hemipenial lobes is slightly shorter than the other, although this similarity might be due to a preparation imperfection or an artifact in the intestine (see *Discussion*). The hemipenis has an undivided *s. spermaticus.*

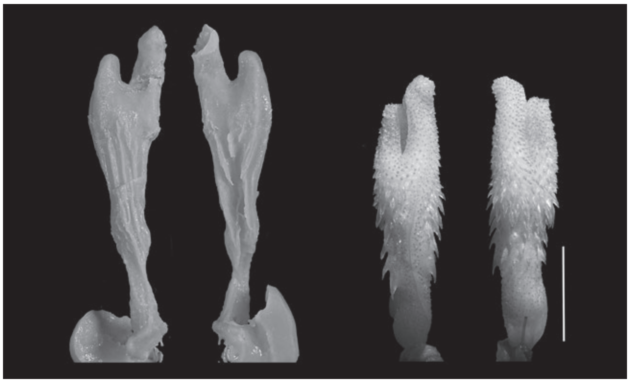

Fig. 4. Ventral and dorsal view of female genitalia model of *Coronella austriaca* (NMNHS III-13-80) – left, and sulcate and asulcate side of the species hemipenis (NMNHS III-13–48 in Andonov et al., 2017). Scale = 1 cm.

Dolichophis caspius. The models (n = 2) are elongated (FGPI = 0.33-0.36) with prominent bifurcation and long, separated pouch arms (Fig. 5). There is a distinctive crease in the base of the vaginal pouch arms, which shows a conspicuous similarity with the species general hemipenial shape. The hemipenis, as described in Andonov et al. (2017) is simple and bulbous and descriptively fits to one of the vaginal pouch arms.

Elaphe quatuorlineata. The models (n = 2) are elongated with prominent bifurcation and very long pouch arms, being elongated (FGPI = 0.16-0.28) and bifurcated (Fig. 6). This model, along with the one of *Z. longissimus* has the longest pouches in relation to the total length. The hemipenis is slightly bilobed, bulbous, medium-formed, and does not possess many calcified or non-calcified structures.

Zamenis longissimus. The models (n = 2) are elongated to medium (FGPI = 0.25-0.35), separated, with prominent bifurcation and very long pouch arms (Fig. 7). This is the model with the longest pouches in relation to the total length. The hemipenis is slightly bilobed, bulbous, medium-formed, and does not possess many calcified or non-calcified structures.

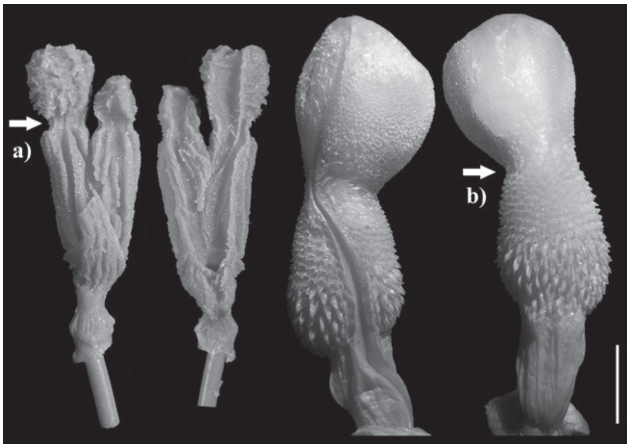

Fig. 5. Ventral and dorsal view of female genitalia model of *Dolichophis caspius* (NMNHS III-12-17) – left, and sulcate and asulcate side of the species hemipenis (NMNHS III-12–36 in Andonov et al., 2017); a) crease of the vaginal pouch arms; b) crease of the hemipenial body. Scale = 1 cm.

Fig. 6. Ventral and dorsal view of female genitalia model of *Elaphe quatuorlineata* (NMNHS III-4-9) – left, and sulcate and asulcate side of the species hemipenis (NMNHS III-4–4 in Andonov et al., 2017). Scale = 1 cm.

Fig. 7. Ventral and dorsal view of female genitalia model of *Zamenis longissimus* (no museum number was available, the specimen was found dead on the road on 10.05.2018) – left, and sulcate and asulcate side of the species hemipenis (NMNHS III-9–14 in Andonov et al., 2017). Scale = 1 cm.

Family Viperidae

Vipera ammodytes. The models (n = 5) are medium formed (FGPI = 0.36-0.62, M = 0.459, SD = 0.104) and slightly separated, with very short vaginal pouch arms (Fig. 8). The species hemipenis on the other side is divided and subcylindrical in shape. The ornamentation of the male copulatory organ is pronounced with multiple calcified structures present (i.e., spines, calyces).

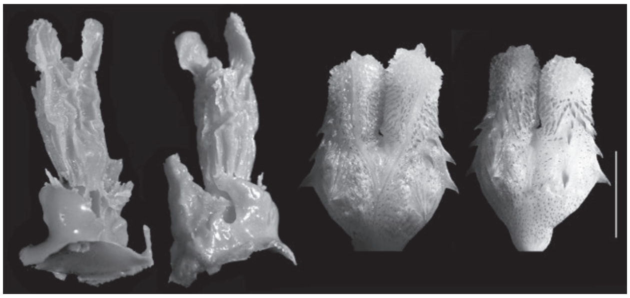

Fig. 8. Ventral and dorsal view of female genitalia model of *Vipera ammodytes* (NMNHS III-1-116) – left, and sulcate and asulcate side of the species hemipenis (NMNHS III-1–52 in Andonov et al. 2017). Scale = 1 cm.

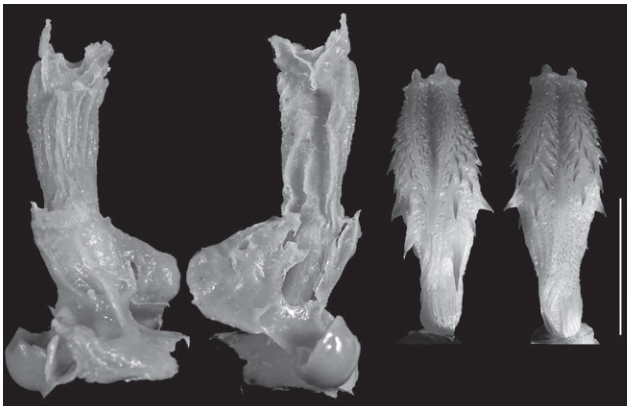

Fig. 9. Ventral and dorsal view of female genitalia model of *Vipera berus* (NMNHS III-2-34) – left, and sulcate and asulcate side of the species hemipenis (NMNHS III-2–60 in Andonov et al. 2017). Scale = 1 cm.

Vipera berus. The model (n = 1) is medium formed (FGPI = 0.32) and slightly separated, with very short pouch arms (Fig. 9). The characteristics are similar to those of *V. ammodytes*, and the species hemipenis is also similarly divided, with subcylindrical shape resembling the male organ of *V. ammodytes*.

In general, most of the models of the representatives of the family Colubridae show a much higher level of bifurcation and the pouches are longer, in comparison to the base of the cloaca (Fig. 4-7). In the representatives of the family Viperidae (Fig. 8-9), the pouches are significantly shorter, and the female cloaca shape in *Eryx jaculus* (Fig. 3) differs from the species of both other families. The female genitalia models of species where hemipenes are divided are much less bifurcated, while the genitalia models of species for which the hemipenes are typically simple (no bilobation observed) or slightly bilobed (the bilobation is very low with the body of the hemipenis being more than 75% of the total hemipenial length) have a significant bifurcation and very prominent pouch.

4. DISCUSSION

In this study, we describe for the first time the female genital morphology of seven snake species (*E. jaculus*, *D. caspius*, *Z. longissimus*, *E. quatuorlineata*, *C. austriaca*, *V. ammodytes*, and *V. berus*), using a recently proposed methodology (Granados et al., 2022) with a few modifications. The results show significant variation between some species and suggest different types of adjustment of male and female genitalia during copulation (see *Genitalia morphology*). The methodology used in the present study also has the potential to reveal valuable information about the process of copulation and might shed light on some of the hypotheses for genital evolution in snakes. Female genital morphology in snakes is considered species-specific (Siegel et al., 2011, 2012), although Showalter et al. (2014) also suggest some intraspecific variation. We did not find intraspecific variation in species where multiple models were made. In particular, analyses of the bigger sample of *V. ammodytes* models reveal that their general shape does not vary and the proportions between them are similar (see *Results*). However, a larger sample is required to draw a definitive conclusion. For the current discussion, we assume that the general morphology of female genitalia is species-specific, following Siegel et al. (2012), which is corroborated by the lack of variation in *V. ammodytes*.

There is a different level of asymmetry in some of the models of the bifurcated pouches. This could be due to uneven inflation by the silicone, although some minor differences between the pouch arms could be expected, considering the similar asymmetry observed in the simple pouch of *Afrotyphlops punctatus* (Leach, 1819), where only the right pouch arm is present (Gabe and Saint-Girons, 1965). Further studies on more and fresher specimens could reveal additional variation.

Methodology discussion

With a few exceptions in the early years of research of hemipenial morphology, where male snake copulatory organs are described *in situ* (i.e., Cope, 1895), hemipenes are usually described in an everted and fully expanded state (i.e., Dowling and Savage, 1960; Pesantes, 1994; Keogh, 1999; Zaher, 1999; Zaher and Prudente, 2003). On the contrary, most descriptions of the female snake cloaca are made by dissecting individuals and examining the morphology *in situ* (e.g., Pisani, 1976; Siegel et al., 2011, 2012; Showalter et al., 2014), which does not provide a complete 3-D perspective of the examined objects. However, Granados et al. (2022) recently proposed a new method using silicone modeling, which we believe could be modified for the purposes of comparative research on snakes' male and female genitalia. The silicone models can show the cloacal morphology in detail and reveal information about some of the main genital characteristics, such as the size and topology of the pouch, that might go unnoticed when the genitalia are described *in situ*. The models also present a clear 3-D perspective on the morphology of the main genital regions. When the genitals are filled with two-component condensation silicone, the filling first enters the cloaca and the pouch. Thus, by suppressing the posterior intestine and coprodaeum, the main part of the filling enters the pouch, and once it hardens, the rest of the silicone fills in the intestine. Since the uterus is narrower and presumably tight, we believe that silicone enters only the pouch region. Unlike Granados et al. (2022), we chose not to tie the oviducts or the intestine. While tying the intestine could seem advantageous – potentially preventing silicone from entering and hardening in a way that might alter the genitalia model – we opted against it to preserve the natural structure and dynamics of the system. In addition, the posterior parts of the oviducts are part of the pouch (Showalter, 2014), and it is speculated that they could have a role in the copulatory adjustment as well (Giacomini, 1893; Ludwig and Rahn, 1943). Therefore, tying the oviducts might lead to losing significant information. Thus, we believe that it is important to model the entire female genitalia, potentially revealing important insights for the male-female genitalia copulatory adjustment. By not tying the intestine and the oviducts, immediately after the cloaca, but further (see *Methodology*), we are also able to see the full three-dimensional morphology and topology of the organs in this part of the body. Silicone is a semi-liquid paste, and if it is under optimal levels of thickness, a portion of it could enter the intestine. Although this could be considered a disadvantage of the methodology, we believe that it can also reveal important information on the general morphology of the whole internal body region. Without such immediate isolation of the intestine, the general position of all of the organs can be examined (Fig. 2) similar to the lateral descriptions of internal organs made by Gabe and Saint-Girons (1965). We emphasize that the models are extracted from both old fixed museum specimens and recently found dead individuals, and soaking them in KOH cannot completely reverse the tissues to their original state. Still, we think that preserved individuals are suitable enough to be used for the purpose of the study. The lack of visible differences in the general female genital morphology between preserved and fresh specimens in this study corresponds to the results of Andonov et al. (2017), who report no major differences in the hemipenial morphology between old fixed individuals and freshly dead animals.

It should be noted also that the proposed approach reveals the general morphology of female genitalia, allowing comparative studies between sexes, as well as studies on the level of genital adjustment during copulation. However, for a precise investigation of the functionality of genital morphology, combined methodologies should be used (e.g., a combination of histological approach and macroscopic observation of silicone models).

Pouch morphology

Our study reveals that most of the models of Colubridae species have a deeper level of bifurcation and the pouch arms are longer compared to the base of the cloaca, whereas the pouches of Viperidae species are significantly shorter. The *Eryx jaculus* female genitalia has a different shape from the representatives of the other families. The female genitalia models in species with bilobed hemipenes are substantially less bifurcated, whereas genitalia models in species with simple or slightly bilobed hemipenes show a significant bifurcation and a highly conspicuous pouch (Fig. 3-9).

Although we only observed gross morphology and macroscopy morphology of female genitalia, our results partially confirm the conclusion made by Siegel et al. (2012), who state that snake female genitalia have pouches that bifurcate , and the level of separation and pouch length might differ. One small difference is that we observe a slight bifurcation in the *Eryx jaculus* vaginal pouch (Fig. 3), unlike Siegel et al. (2012), who did not report such bifurcation. We attribute it to the methodological differences, and this is an additional reason to further explore the maximum potential of the herein-proposed methodology.

An interesting trend we observed is that species with bilobed and divided male genitalia (*V. ammodytes* and *V. berus*) have a very slightly bifurcated vaginal pouch while the opposite is valid for species with slightly bilobed hemipenes (*D. caspius*, *Z. longissimus*, and *E. quatuorlineata*). We consider that this might reveal an important insight into the anatomy of the snake copulation and could provide information about genital adjustment during the act. In general, a close alignment of the male and female cloaca during copulation, allows the hemipenis to penetrate the cloaca (i.e., Pope, 1941; Pisani, 1976). In some species, this could also be supported by basal hemipenial spines, where they not only serve as an anchor but also help the male to open the female cloaca (Pisani 1976; Friesen et al. 2013). An additional erection of the hemipenis inside the female cloaca follows this opening. Ludwig and Rahn (1943) suggest that after successful penetration, the hemipenis distends and fully closes the vaginal pouches and cloaca, allowing sperm to be deposited by the most apical part of the *sulcus spermaticus*, based on a study on *Crotalus viridis* Rafinesque, 1818.

Only a few studies are commenting on the exact adjustment between the different types of species' genitalia (i.e., Cope, 1898; Pope, 1941; Ludwig and Rahn, 1943; Inger and Marx, 1962). In Pope (1941), the author shows a perfect fit between hemipenis and female genitalia of *Erythrolamprus poecilogyrus* (Wied-Neuwied, 1824) during copulation. The hemipenis of this species is bilobed and each end of the bifurcated *sulcus spermaticus* points into each bifurcation of the vaginal pouch and touches the non-glandular uterus. Although not described in detail, from the picture provided, appears that the bifurcation of the pouch is not that prominent. A similar correlation is found in *Calamaria lumbricoidea* Boie, 1827 (Inger and Marx, 1962), although the conclusion is made after *in situ* description of female genitalia, therefore it is unclear what the actual genitalia alignment would look like during copulation. In addition, Inger and Marx (1962) find some interspecific variation as well. However, it should be mentioned that the authors study both adults and subadults, so the already described ontogenetic variation in hemipenes by Jadin and King (2012), might have affected the results. Edgren (1953) described a close alignment between the male hemipenis and female genitalia of *Heterodon platirhinos* Latreille, 1801, but not as aligned as Pope's (1941) description. Edgren (1953) suggested that the unoccupied part of the area of the simple tall columnar epithelium of the cloaca is later filled with sperm and secretions that likely form a copulatory plug.

Our findings, based on silicone genitalia models, suggest that a similar mechanism might be relevant not only for species described by Pope (1941) and Inger and Marx (1962), but also for species with divided hemipenes, that we studied (*V. ammodytes*, and *V. berus*). We might assume, that the hemipenis of these species enters the cloaca during copulation, distends, and fully closes the vaginal pouches and cloaca and the tips of the two lobes touch the non-glandular uterus. Thus, having the divided *sulcus spermaticus* tightly surrounded by the female genitalia, the semen can be delivered to each oviduct easily with the lobes pointing towards them, as suggested by Ludwig and Rahn (1943).

We suggest that a similar adjustment can be observed in *C. austriaca* (Fig. 4), a species with bilobed hemipenes, and undivided *sulcus spermaticus*. Considering the elongation of the vaginal pouches, the lobes of the hemipenis might enter deeper into the pouches during copulation, and the semen is directed into one of the oviducts only, unlike the described adjustment in *V. ammodytes* and *V. berus*.

Having a simple or slightly bilobed hemipenis coupled with an undivided *sulcus spermaticus* is also observed (Andonov et al., 2017). Some of the species that we studied also have such hemipenial morphology (*E. jaculus*, *D. caspius*, *Z. longissimus, E. quatuorlineata*). If the copulation of these species follows the same mechanics and adjustment as for the species with bilobed hemipenis and divided *sulcus spermaticus*, a perfect closure of the vaginal pouch and cloaca will most likely not be possible and the semen might not be delivered to the pouch directly . This could lead to a reduction in the effectiveness of copulation. Therefore, another mechanism for effective semen delivery might be present. It is possible that during copulation those hemipenes do not constrain penetration to the proctodaeum and urodaeum but enter one of the vaginal pouch branches deeply, reaching the non-glandular uterus very closely. This could ensure successful copulation and semen delivery. An argument for that can be found in the morphology of the apical part of those simple and slightly bilobed hemipenes. All herein studied hemipenes lack any big and visible structures as part of the ornamentation of the apical part or bear just a few structures there. A similar correlation is discussed by Cope (1898), who observed that when the hemipenis is spiny, the cloacal walls are thick, and in cases when the male organ lacks ornamentation, the cloaca walls are thin. The latter is further confirmed by Pope (1941) with his observation on the genitalia of *Trimeresurus albolabris* Gray, 1842, and *T. stejnegeri* Schmidt, 1925. An additional argument for our hypothesis, regarding the non-bifurcated hemipenes, can be found by closely observing the genitalia of *D. caspius.* There is a very close resemblance between the hemipenial shape and one of the vaginal pouch branches, although more individuals should be examined, for making a solid conclusion. It should be added also that the asymmetry of the pouch might be a result of a non-well-inflated pouch (Fig. 4). Actually, the hypothesis that hemipenial lobes can enter the enlarged pouches has been already suggested (Giacomini, 1893; Ludwig and Rahn, 1943). Considering the species studied, we agree with it, but we believe that such penetration is much more likely to happen for species with simple or slightly bilobed hemipenes, rather than for those with divided copulatory organs.

Most of the studies investigate the hemipenial morphology, after removal of the organ. This causes some morphological changes and hinders complete morphological examination (i.e., a lot of the muscle tissue is removed). Thus, it is still uncertain to what extent the sulcus is closed when semen is transferred, and therefore additional methods for hemipenial observation should also be developed and applied, for example everting and inflating the hemipenis while still attached to the body, revealing its original topology. It should be also researched whether there is an additional function of the variable hemipenial morphology, such as stimulative. De-Lima et al. (2019) have explained the function of the deeply bilobed (divided) hemipenes (with structural folds on the apical tips) of the lizard species *Tropidurus torquatus* (Wied-Neuwied, 1820). They suggest stimulatory function, as the males stimulate the secretion of the female genitalia with the apical parts of the hemipenial lobes. The types of adjustment between snake genitalia could have similar functions as well, and not simply physical fit for semen transportation, but this is a matter that requires deeper investigation.

Our findings suggest that snake species may exhibit distinct patterns of genital alignment during copulation, with possible functional implications. We tentatively present different types of copulatory adjustment in species studied here as follows: adjustment in species with divided hemipenes and slightly bifurcated vaginal pouches, adjustment in species with bilobed hemipenes and highly bifurcated vaginal pouches, and species with simple or slightly bilobed hemipenes and slightly or highly bifurcated vaginal pouches. However, the limitations of the current study do not allow a definitive conclusion or categorization of the types of copulatory adjustment. Thus, more congeneric species should be studied for further confirmation of the herein-described adjustment types. We also assume that the copulation adjustment types are not limited to those observed in this study. It should be noted that there are also various species posing a simple hemipenis with divided *sulcus. spermaticu*s such as *Adelphostigma occipitalis* (Jan, 1863), *Xenopholis undulatus* (Jensen, 1900), *Dipsas jamespetersi* (Orcés and Almendáriz, 1989), etc. (Zaher, 1999; Cadle, 2007). We hypothesize that this could be another group of species with potentially different adjustments, but further research is needed.

However, there are still only a few species examined with the used methodology, so deeper research is needed to either confirm or reject this hypothesis, which could either group genital fit types as a general rule or on the contrary, reveal a higher variation. Additionally, a higher representative sample is needed for statistical tests, because even though the individuals were very well preserved, internal changes due to decaying processes should not be excluded entirely and should be further investigated. The function and exact adjustment during copulation should also be further studied with more complementary methods (e.g., under high-resolution X-rays), preferably using a single-species approach (Arnqvist, 1997).

CONCLUSIONS

In conclusion, our study confirms the importance of the currently proposed silicone model technique for comprehensive analysis of snake genitalia, particularly focusing on the underexplored female cloaca. In addition, the current research proposes important additions to this methodology that might reveal more information about female genitalia morphology. The approach gives extensive insights into female genital morphology, making it a useful tool for comparative studies on male and female reproductive organs. Our findings, which address the scant knowledge of female snake genitalia, provide a groundwork for future research into copulatory adaptations and the co-evolution of male and female genitalia. Furthermore, our suggestion of an adjustment between male hemipenis shape and vaginal pouch bifurcation implies a possible link to snake copulation dynamics. We propose that the significant genital variations observed among the snake species studied here contribute to diverse copulation adjustments, with implications for successful semen delivery and the reproductive strategies of these species.

ACKNOWLEDGMENTS

We thank Dr. Boyan Zlatkov (IBER-BAS, Sofia) for his guidance and helpful comments on the preprint. We thank Dr. Simeon Lukanov and Dr. Simeon Borissov for the English review and improvement of the text. We thank the National Museum of Natural History at the Bulgarian Academy of Sciences for providing the museum specimens. We thank two anonymous reviewers for their detailed and constructive comments and suggestions, which significantly improved the quality of the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <http://www-9.unipv.it/webshi/appendix/index.html> manuscript number 16881.

REFERENCES

Ah-King, M., Barron, A.B., Herberstein, M.E. (2014): Genital evolution: why are females still understudied? PLOS Biol. **12**: e1001851.

Andonov, K., Natchev, N., Kornilev, Y.V., Tzankov, N. (2017): Does sexual selection influence ornamentation of hemipenes in Old World snakes? Anat. Rec. **300**: 1680-1694.

Arnqvist, G. (1997): The evolution of animal genitalia: distinguishing between hypothesis by single species studies. Biol. J. Linn. Soc. **60**: 365-379.

Arnqvist, G. (1998): Comparative evidence for the evolution of genitalia by sexual selection. Nature **393**: 784-786.

Biserkov, V., Naumov, B., Tsankov, N., Stoyanov, A., Petrov, B., Dobrev, D., Stoev, P. (2007): Guide to the amphibians and reptiles in Bulgaria. Sofia, Green Balkans.

Blackburn, D.G. (1998): Structure, function, and evolution of the oviducts of squamate reptiles, with special reference to viviparity and placentation. Jour. of Exp. Zool. **282**: 560-617.

Brennan, P.L., Prum, R.O. (2015): Mechanisms and evidence of genital coevolution: The roles of natural selection, mate choice, and sexual conflict. Cold Spring Harb. Perspect. Biol. **7**: a017749.

Brennan, P.L. (2022): Evolution and morphology of genitalia in female amniotes. Integr. Comp. Biol. **62**: 521-532.

Cadle, J. (2007): The snake genus *Sibynomorphus* (Colubridae: Dipsadinae: Dipsadini) in Peru and Ecuador, with comments on the systematics of Dipsadini. Bull. Mus. Comp. Zool. **158**: 183-283.

Cohn, M.J. (2011): Development of the external genitalia: conserved and divergent mechanisms of appendage patterning. Dev. Dyn. **240**: 1108-1115.

Cope, E.D. (1895): The classification of the Ophidia. Trans. Amer. Philos. Soc. **18**: 186-219.

Cope, E.D. (1898): The Crocodilians, Lizards, and Snakes of North America. Kept. US Nat. Mus. **1898**: 153-1270.

Darwin, C. (1871): The descent of man and selection in relation to sex. Modern Library, New York.

De-Lima, A.K.S., Paschoaletto, I.P., Pinho, L.dO., Benmamman, P., Klaczko, J. (2019): Are hemipenial traits under sexual selection in *Tropidurus* lizards? Hemipenial development, male and female genital morphology, allometry and coevolution in *Tropidurus torquatus* (Squamata: Tropiduridae). PLOS ONE **14**: e0219053.

Dowling, H.G., Savage, J.M. (1960): A guide to the snake hemipenis: a survey of basic structure and systematic characters. Zool. **45**: 17-28.

Dufour, L. (1844): Anatomie générale des diptères. Ann. Sci. Nat. **1**: 244-264.

East, M.L., Hofer, H., Wickler, W. (1993): The erect 'penis' is a flag of submission in a female-dominated society: greetings in Serengeti spotted hyenas. Behav. Ecol. Sociobiol. **33**: 355-370.

Eberhard, W.G. (1985): Sexual selection and the evolution of animal genitalia. Harvard University Press, Cambridge, MA.

Eberhard, W.G. (2010): Evolution of genitalia: theories, evidence, and new directions. Genetica **138**: 5-18.

Edgren, R.A. (1953): Copulatory adjustment in snakes and its evolutionary implications. Copeia **1953**: 162-164.

Friesen, C.R., Uhrig, E.J., Squire, M.K., Mason, R.T., Brennan, P.L.R. (2013): Sexual conflict over mating in red-sided garter snakes (*Thamnophis sirtalis*) as indicated by experimental manipulation of genitalia. P. Roy. Soc. B. Biol. Sci. **281**: 20132694.

Gabe, M., Saint-Girons, H. (1965): Contribution à la morphologie comparée du cloaque et des glandes épidermoïdes de la région cloacale chez les lépidosauriens. Mém. Mus. Hist. Nat. **XXXIII**: 149-332

Giacomini, E. (1893): Sull'ovidutto del Sauropsidi. Monit. Zool. Ital. **4**: 202-265.

Granados, G.L., Greenwood, L., Secor, S., Shan, S., Hedrick, B.P., Brennan, P.L. (2022): Examining the shape and size of female and male genitalia in snakes using three-dimensional geometric morphometrics, Biol. J. Linn. Soc. **136**: 466-476.

Gredler, M.L., Larkins, C.E., Leal, F., Lewis, A.K., Herrera, A.M., Perriton, C.L., Sanger, T.J., Cohn, M.J. (2014): Evolution of external genitalia: insights from reptilian development. Sex. Dev. **8**: 311-326.

Hosken, D.J., Stockley, P. (2004): Sexual selection and genital evolution. Trends. Ecol. Evol. **19**: 87-93.

Inger, R.F., Marx, H. (1962): Variation of hemipenis and cloaca in the colubrid snake *Calamaria lumbricoidea*. Syst. Zool. **11**: 32-39.

Jadin, R.C., King, R.B. (2012): Ontogenetic effects on snake hemipenial morphology. J. Herpetol. **46**: 393-395.

Jurkfitz, R.C., Silva, K.M.P., Almeida-Santos, S.M. (2023): Sperm storage in *Crotalus durissus* (Serpentes: Crotalinae): histological insights about the female reproductive tract of pit vipers. Zoomorphology, **142**: 487-496.

Keogh, J.S. (1999): Evolutionary implications of hemipenial morphology in the terrestrial Australian elapid snakes. Zool. J. Linn. Soc. Lond. **125**: 239-278.

King, R.B., Jadin, R.C., Grue, M., Walley, H.D. (2009): Behavioural correlates with hemipenis morphology in New World natricine snakes. Biol. J. Linn. Soc. **98**: 110-120.

Langerhans, R.B., Anderson, C.M., Heinen-Kay, J.L. (2016): Causes and consequences of genital evolution. Integr. Comp. Biol. **56**: 741-751

Ludwig, M., Rahn, H. (1943): Sperm storage and copulatory adjustment in the prairie rattlesnake. Copeia **1943**: 15-18.

Mayr, E. (1963): Animal species and evolution. Cambridge, Mass: Harvard University Press.

Moore, B.C., Kelly, D.A., Piva, M., Does, M., Kim, D.K., Simoncini, M., Leiva, P.M.L., Pina, C.I. (2022): Genital anatomy and copulatory interactions in the broad snouted Caiman (*Caiman latirostris*). The. Anat. Rec. **305**: 3075-3087.

Myers, C.W., McDowell, S.B. (2014): New taxa and cryptic species of neotropical snakes (Xenodontinae), with commentary on hemipenes as generic and specific characters. Bull. A. Mus. Nat. His. **385**: 1-112.

Orbach, D.N., Hedrick, B.P., Würsig, B., Mesnick, S.L., Brennan, P.L.R. (2018): The evolution of genital shape variation in female cetaceans. Evolution **72**: 261-273

Orbach, D.N., Brassey, C.A., Gardiner, J.D., Brennan, P.L.R. (2021): 3D genital shape complexity in female marine mammals. Ecol. Evol. **11**: 3210-3218.

Pesantes, O.S. (1994): A method for preparing the hemipenis of preserved snakes. J. Herpetol. **28**: 93-95.

Pisani, G.R. (1976): Comments on the courtship and mating mechanics of *Thamnophis* (Reptilia, Serpentes, Colubridae). J. Herpetol. **10**: 139-142.

Pope, C.H. (1941): Copulatory adjustment in snakes. Fieldiana Zool. **24**: 149-152.

Shapiro, A.M., Porter, A.H. (1989): The lock-and-key hypothesis: evolutionary and biosystematic interpretation of insect genitalia. Annu. Rev. Entomol. **34**: 231-245.

Showalter, I., Todd, B.D., Brennan, P.L.R. (2014): Intraspecific and interspecific variation of female genitalia in two species of watersnake. Biol. J. Linn. Soc. **111**: 183-191.

Siegel, D.S., Miralles, A., Chabarria, R.E., Aldridge, R.D. (2011): Female reproductive anatomy: cloaca, oviduct, and sperm storage. Chapter 9. In: Reproductive biology and phylogeny of snakes, pp 347-409. Aldridge R., Sever D.M. Eds, Boca Raton, Florida, CRC Press.

Siegel, D.S., Miralles, A., Trauth, S.E., Aldridge, R.D. (2012) The phylogenetic distribution and morphological variation of the 'pouch' in female snakes. Acta Zool. **93**: 400-408.

Stojanov, A., Tzankov, N., Naumov, B. (2011): Die Amphibien und Reptilien Bulgariens. Chimaira, Frankfurt am Main.

Thornhill, R. (1983): Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. Am. Nat. **122**: 765-788.

Ware, A., Opell, B.D. (1989): A test of the mechanical isolation hypothesis in two similar spider species. J. Arachnol. **17**: 149-162.

Zaher, H. (1999): Hemipenial morphology of the South American xenodontine snakes: with a proposal for a monophyletic Xenodontinae and a reappraisal of colubroid hemipenes. Bull. Am. Mus. Nat. **240**: 1-168.

Zaher, H., Prudente, A.L.C. (2003): Hemipenes of *Siphlophis* (Serpentes, Xenodontinae) and techniques of hemipenial preparation in snakes: a response to Dowling. Herpetol. Rev. **34**: 302-307.

Acta Herpetologica 20(1): 41-47, 2025
DOI: 10.36253/a_h-15323

Growth and metamorphosis of *Bufo gargarizans gargarizans* larvae: effects of water volume and food diversity

Tong Lei Yu*, Ying Xie, Xin Mei Sun, Cheng Le Feng, Jiang Xue Fan, Yu Qi Liu, Tong Xian Chen, Xue Ke Ma, Bing Yao Wang, Meng Zhang

College of Life Science, Xinyang Normal University, SD 464000, China
**Corresponding author.* Email: yutonglei_00000@163.com

Submitted on: 2023, 6th November; revised on: 2023, 13th November; accepted on: 2025, 21th January
Editor: Tian Zhao

Abstract. Organisms can evolve and produce different phenotypes in respond to local environmental conditions. Abiotic factors, such as water volume and food diversity in breeding ponds, also play significant roles in the survival and age at metamorphosis of amphibians. Here, we experimentally examined the plasticity of growth rate, survival rate, larval mass, age, and body size at metamorphosis in *Bufo gargarizans gargarizans* in response to varying combinations of water volume and food diversity. The interaction between water volume and food diversity had no effect on larval growth and development. However, food diversity had a significant impact on the age at metamorphosis, with two food resources resulting in a shorter larval period and earlier metamorphosis, particularly in larger water volumes. Our findings demonstrated that tadpoles raised in larger water volumes had larger body sizes at metamorphosis compared to those in smaller water volumes. Additionally, tadpoles in larger water volumes exhibited higher growth rates and shorter larval periods than those in smaller water volumes. We suggest that the increased frequency of physical encounters between tadpoles and the vessel walls in smaller volumes, akin to high-density conditions, causes psychological stress due to crowding, which hinders larval growth and development.

Keywords. *Bufo gargarizans gargarizans*, water volume, food diversity, phenotypic plasticity, metamorphic size.

INTRODUCTION

Organisms have the ability to adapt to specific environmental conditions by changing their phenotype (Pigliucci, 2001; DeWitt and Scheiner, 2004). Biotic factors such as population density (Kehr et al., 2014), competition, and predation can impact the composition of tadpole populations (e.g., tadpole size, developmental stage, distribution, richness, and diversity). Additionally, abiotic factors like hydroperiod, habitat size, or water volume can affect the survival and timing of metamorphosis in amphibians (Semlitsch et al., 1996; Wilbut, 1997).

For organisms with complex life cycles, such as amphibians, the growth and developmental rate of the larval stage can change according to different environmental conditions (Richter-Boix et al., 2011). Although excessive water volume can reduce the growth of certain species (Pearman, 1993; Kehr et al., 2014), growth rates increase with larger absolute water volume in most cases (Pearman, 1993; Montealegre-Delgado et al., 2013). Several studies have examined the potential impact of abiotic factors, such as habitat space or water volume, on the density effects (biotic factor) in organisms. As a result, the crowding effect caused by high population density often reduces the growth and development of larvae (Smith-Gill and Berven, 1979; Semlitsch and Caldwell, 1982; Relyea and Hoverman, 2003). To date, four primary factors have been suggested to explain the decrease in individual growth and the lengthening of the larval period. These factors include a decrease in food ration

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

www.fupress.com/ah

(Wilbur, 1977; Hota and Dash, 1981), competition for limited food resources (Brockelman, 1969; Wilbur and Collins, 1973; DeBenedictis, 1974), social interactions (Gromko et al., 1973), and stress (Lynn and Edelman, 1936; John and Fenster, 1975).

Most anuran tadpoles are herbivorous or omnivorous, but there are a few that are carnivorous and cannibalistic (Kamat, 1962; Costa and Balasubramaniam, 1965; Sabnis and Kolhatkar, 1977; Sabnis and Kuthe, 1978; Sekar, 1992; Altig et al., 2007). For instance, *Bufo stomaticus* larvae showed optimal growth when provided with a diet containing a combination of animal and plant proteins. However, when these larvae were only provided with goat meat, their growth rate declined, and the timing of metamorphosis was delayed (Saidapur, 2001). Similarly, *Bufo melanostictus* larvae showed comparable growth rates when fed a diet of spinach and *Chironomous* larvae. However, their growth was stunted and metamorphosis was delayed when provided with only *Chironomous* larvae (Sabnis and Kuthe, 1978). This suggests that competition among larvae may be reduced when multiple food sources are available in breeding sites, allowing individuals to utilize alternative resources (Martin and Garnett, 2013).

Extensive studies have been conducted on the relationships between crowding and growth rates (Goetsch, 1924; Gromko et al., 1973; Steinwascher, 1978; Fixari III et al., 2017), but little attention has been paid to the impact of confining solitary tadpoles and the potential effects of water volume and food diversity on tadpole growth and development. In this study, we investigated the potential interactive effects of food diversity and water volume on the plasticity of metamorphic traits in *Bufo gargarizans gargarizans*, including the length of larval period, survival, the size at metamorphosis, and growth rate.

MATERIALS AND METHODS

Field procedures

Bufo gargarizans gargarizans exhibits sexual dimorphism and has a wide distribution in East Asia. They are explosive breeders (Wells, 2007), typically breeding between 6 to 14 days (Yu and Sharma, 2012) and tadpoles hatch after two weeks. Ponds used as breeding sites are often rich in *Spirogyra* and pondweed (e.g., *Potamogeton crispus*), which serve as a food source for tadpoles (Wei et al., 2011).

During the peak breeding period in mid-February 2018, we captured 5 mating pairs of *B. g. gargarizans* in Shihe County (32°08’39”N, 114°02’37”E, altitude 84 m), central plains of China. Each pair was then placed individually in a plastic container (20 L) filled with approximately 12-15 cm of pond water until females laid their eggs. Once the oviposition process was completed, we collected 50 eggs from each of the 5 egg masses. All eggs were placed separately into five plastic containers (5 L) filled with approximately 10 cm of tap water. Tap water has been previously stored in two large buckets (100 L) for several days before use. After the breeding was finished, we transported all toads and the remaining eggs back to their original spawning site.

Laboratory procedures

We employed a randomized block design to test the effect of food diversity (two food resources: *Potamogeton crispus* and fish food with high protein content (>45%), lipids (>12%), algae (>12%), fiber (>4%), and ash (<10%); single food resource: *P. crispus*), and water volume (250 mL, 500 mL, 2000 mL, 8000 mL) on the growth and development of *B. g. gargarizans* tadpoles. Each tadpole (Gosner stage 25, absorption of external gills and formed spiracle, Gosner, 1960) was placed individually in a series of numbered opaque tanks. A total of 120 tanks were used, with the tank diameters ranging from approximately 9.49 cm to 26.99 cm, representing the four different water volumes. Water temperature was recorded using a mercury thermometer.

The experiment was conducted using 120 tanks, with each of the eight treatment combinations replicated 15 times. In each treatment combination, three tadpoles from the same families were used to diminish intra-specific competition in accordance with the principles of kin selection theory. Then, olfactory cues were provided during the larval period (before Gosner stage 43) by adding 25-800 mL of water from other families when changing water, minimizing maternal influence. Throughout the study period, tadpoles were subjected to a 13L:11D photoperiod. The room temperature was maintained at 27.72 ± 1.12 °C, and any temperature variations were consistent across all parts of the experiment. To ensure cleanliness, we used a plastic straw to remove faeces and excess food from the tanks once a day prior to feeding. In smaller containers (250 mL and 500 mL), half of the water was replaced twice a week. For the 2000 mL containers, water replacement occurred once a week, while for the 8000 mL containers, it was done biweekly.

When the first metamorph (Gosner stage 42) was observed, tanks were checked daily. All metamorphs were then carefully collected and placed individually in plastic vials with sand and 1 mm of water until the end of metamorphosis (Gosner stage 46). We measured five variables. The length of the larval period was determined by counting the number of days from the beginning of the experiment until Gosner stage 42. Mass at metamorphosis was measured using a balance (to the nearest 0.001 g), and snout-vent length (SVL) was measured using a digital caliper (to the nearest 0.01 mm). Growth rate was calculated by dividing the mass at metamorphosis by the age at metamorphosis (Gosner stage 42), and the survival rate was determined by calculating the percentage of tadpoles that successfully underwent metamorphosis.

Data analysis

We analyzed the length of the larval period, SVL, mass at metamorphosis, and growth rate using univariate two-way ANOVAs with type III sum of mean squares, treating water volume, food diversity, and their interaction as fixed factors. We also used a log-linear model to examine the survival rate. In cases where the univariate two-way ANOVAs yielded significant results, we performed ANOVAs or one-way ANOVAs with post-hoc comparisons (Fisher's LSD) to assess differences between water volume or food diversity. However, given the non-significant findings from the log-linear model, no further analyses were performed to investigate differences in survival rates across water volume or food diversity. Statistical analyses were conducted using IBM SPSS Statistics 20.0 (IBM Corp, Armonk, NY, USA).

RESULTS

The effects of water volume and food diversity on the length of the larval period were significant (Table 1). However, their interaction was not significant (Table 1). Tadpoles raised at 8000 mL took less time to metamorphose than those raised at other volumes (Fisher's LSD's post hoc tests, all $P < 0.001$, Fig 1), while no difference was found across other water volume treatments (all $P > 0.060$). Tadpoles feeding on two resources reached metamorphosis earlier than those raised at single-resource treatments ($P = 0.028$ for all 1 of 4 LSD's post hoc tests, Fig 1).

The mass at metamorphosis was significantly influenced by water volume (Table 1, Fig. 1). However, there was no significant impact of food diversity or the interaction between water volume and food diversity (Table 1). Tadpoles reared in 8000 ml and 2000 ml had significantly larger mass at metamorphosis compared to those raised in 250 mL and 500 mL (all $P < 0.001$), but not between 8000 mL and 2000 mL ($P = 0.807$) or 250 mL and 500 mL ($P = 0.992$).

Water volume significantly affected SVL at metamorphosis (Table 1, Fig. 1), but food diversity, as well as water volume × food diversity interaction, did not (Table 1). The SVL of tadpoles reared at 8000 ml and 2000 ml was significantly larger at metamorphosis compared to those reared at 250 mL and 500 mL (all $P < 0.003$). However, there was no significant difference in SVL between tadpoles reared at 8000 mL and 2000 mL ($P = 0.904$), or between those reared at 250 mL and 500 mL ($P = 0.777$).

In addition to water volume (Table 1, Fig.1), food diversity, as well as the interaction between water volume and food diversity did not significantly affect growth rate (Table 1). Tadpoles raised at 8000 mL had a greater growth rate than those raised at other treatments (all $P < 0.038$, Fig. 1), while tadpoles raised at 250 mL and 500

Table 1. Two-way ANOVA tables for the effects of water volume and food diversity on metamorphic traits in a *Bufo gargarizans gargarizans* population.

Response variable	Source of variation	df	MS	F-value	P-value
Length of larval period	Water volume	3	100.014	16.594	<0.001
	Food diversity	1	30.539	5.067	0.027
	Water volume × Food diversity	3	12.225	2.028	0.115
	Error	94	6.027		
SVL	Water volume	3	4.92	7.306	<0.001
	Food diversity	1	0.5	0.742	0.391
	Water volume × Food diversity	3	0.532	0.79	0.502
	Error	94	0.673		
Body mass	Water volume	3	0.015	19.447	<0.001
	Food diversity	1	<0.001	0.044	0.835
	Water volume × Food diversity	3	0.0004	0.561	0.642
	Error	94	0.001		
Growth rate	Water volume	3	20.244	32.709	<0.001
	Food diversity	1	0.391	0.631	0.429
	Water volume × Food diversity	3	0.46	0.743	0.529
	Error	94	0.619		

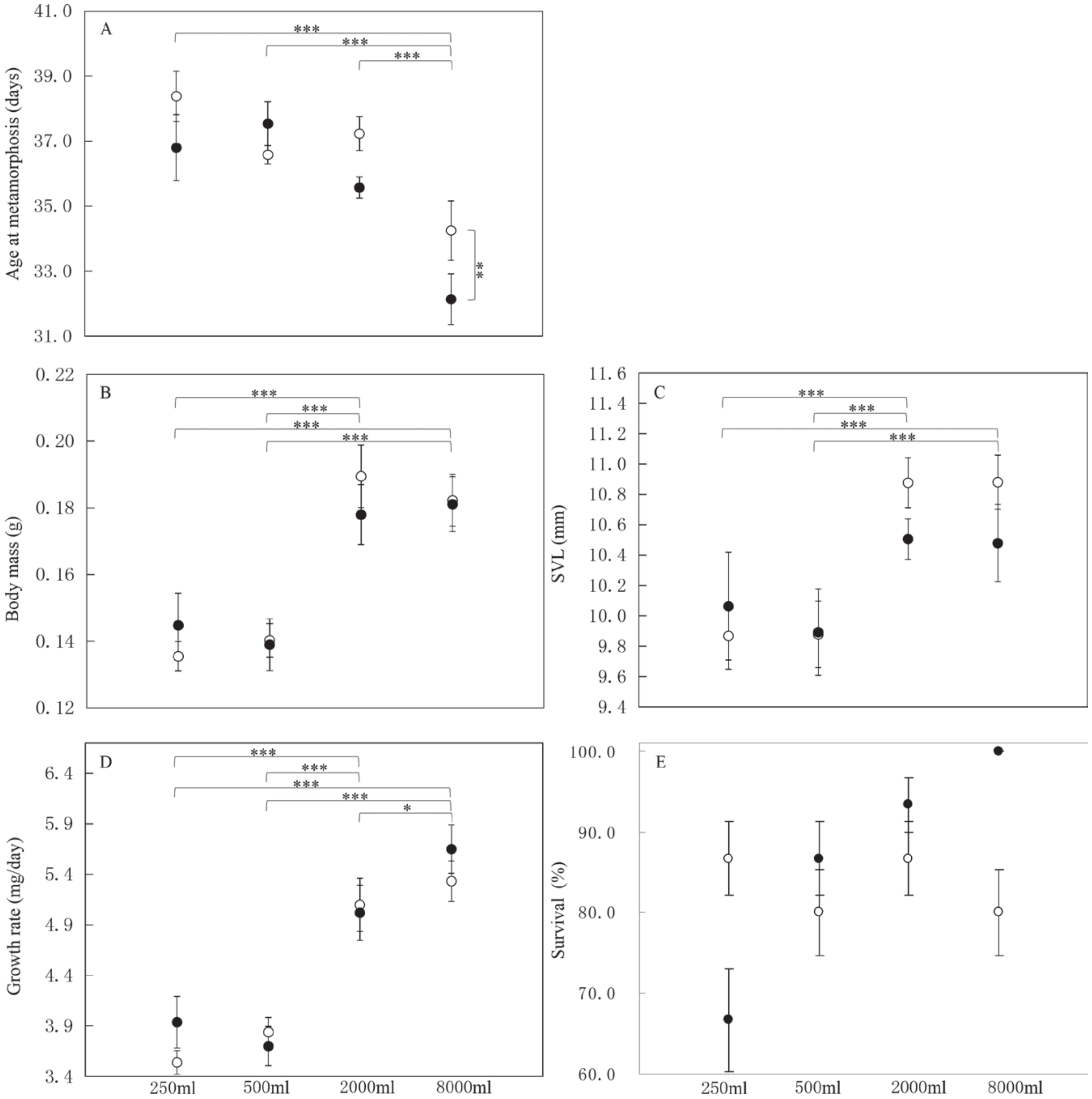

Fig. 1. Influences of water volume and food diversity on (A) age at metamorphosis, (B) SVL, (C) body mass, (D) growth rate and (E) survival of *Bufo gargarizans gargarizans* (black circles, two resources; open circles, single resource; Error bars indicate standard errors).

mL had a smaller growth rate than those raised at 2000 mL (both $P < 0.001$), but not between 250 mL and 500 mL ($P = 0.809$).

Water volume and food diversity did not affect survival at metamorphosis (water volume, $Z = -0.97$, $P = 0.330$; food diversity, $Z = -0.57$, $P = 0.571$), neither the interaction between water volume and food diversity ($Z = 0.83$, $P = 0.405$, Fig. 1).

DISCUSSION

Increasing the absolute volume of water increases the growth and developmental rate of tadpoles (Gromko et al., 1973; Golay and Durrer, 1994; Smith, 1998; Durnin and Smith, 2001; Kehr et al., 2014). Our study found that single tadpoles reared in larger water volume had a larger body size at metamorphosis compared to those raised in smaller water volume. Additionally, our results demonstrated that single *B. g. gargarizans* tadpoles raised in larger water volume grew at a higher rate and had a shorter larval period than those raised in smaller water volume. Previous studies have also shown that smaller water volume decreased tadpole growth (Smith, 1998; Durnin and Smith, 2001), which is consistent with our results.

In this study, all tadpoles were individually placed in opaque glass beakers and received the same amount of food, suggesting that the effects of water volume could occur independently of food limitation, chemical accumulation, and social interactions. These findings support the idea that mechanisms operating in smaller water volumes can lead to crowding effects. One of the mechanisms responsible for the effects of water volume is an increase in the number of collisions among individuals confined to limited volumes, which in turn leads to a decrease in growth or development rates (John and Fenster, 1975; John and Fusaro, 1981; Rot-Nikcevic et al., 2005; Fixari III et al., 2017). Nevertheless, in our experiment there was an increased possibility of physical contact between tadpoles and vessel walls due to the smaller water volume. Additionally, we observed higher activity levels in tadpoles in smaller water volumes. This increased activity can be attributed to the increased collisions and agitation, resulting in an expenditure of energy (Rugh, 1934). Crowding diminishes the appetite of tadpoles and leads to slower growth (Adolph, 1931). Therefore, we suggest that the frequency of physical encounters between tadpoles and the vessel walls may cause psychological stress due to crowding (John and Fenster, 1975).

The surface area played a crucial role in restricting growth by regulating the availability of oxygen. Likewise, Yung (1885) discovered that tadpoles attained a larger size in tanks with a greater surface area. In this study, the surface and bottom areas of the container increase proportionally with the increase in container volume, but we speculated that the oxygen levels in smaller containers may not have been sufficient to restrict growth or had minimal inhibitory effects due to the increased frequency of water changes as the tank volume decreased. Additionally, in tanks with smaller water volumes, but with the same number of tadpoles, there was a higher accumulation of fecal material on the tank bottom, which could potentially impact growth (Podhradsky, 1932; Adolph, 1931). In this case, we employed plastic straws to clean up feces once a day throughout the experiments, thus minimizing the potential impact of feces on growth.

While the interaction between water volume and food diversity did not impact larval growth and development, we found that food diversity plays a significant role in determining the length of the larval period. Specifically, larger bodies of water with two food resources resulted in a shorter larval period and earlier metamorphosis. Within natural populations, tadpoles that undergo early metamorphosis can escape pond drying and predators (Loman, 2002). Additionally, they acquire a larger body size before hibernation, increasing their chances of survival during the longer winter period (Valenzuelasanchez et al., 2015).

In conclusion, both water volume and food diversity had an impact on larval growth and development, but these factors acted independently. The small volume of water had a more pronounced effect on limiting larval growth and development compared to food diversity. This could be attributed to the increased physical encounters between tadpoles and vessel walls, resulting in stress from crowding or psychological factors. Additionally, the crowded conditions in a small volume of water could lead to limited oxygen supply, which may be a secondary factor in reducing larval growth. Further studies are needed to explore the amount of available oxygen and gain a better understanding of its role in this process.

ACKNOWLEDGEMENTS

The field research on the plateau brown frog was funded by National Natural Science Foundation of China (Grant no. 31741019) and Natural Science Foundation of Henan Province of China (Grant no. 252300421429). This study was approved by the Animal Ethics Committee at Xinyang Normal University, Xinyang, Hunan, China, approval code: XYEC-2018-008, approval date: 1 January 2018.

REFERENCES

Adolph, E.F. (1931): The size of the body and the size of the environment in the growth of tadpoles. Biol Bull. **61:** 350-375.

Altig, R., Whiles, M.R., Taylor, C.L. (2007): What do tadpoles really eat? Assessing the trophic status of an understudied and imperiled group of consumers in freshwater habitats. Freshw. Biol. **52:** 386-395.

Brockelman, W.Y. (1969): An analysis of density effects and predation in *Bufo americanus* tadpoles. Ecology **50:** 632-644.

Costa, H.H., Balasubramaniam, S. (1965): The food of the tadpoles of *Rhacophorus cruciger* (Blyth). Ceylon J. Sci. **5:** 105-109

Debenedictis, P.A. (1974): Interspecific competition between tadpoles of *Rana pipiens* and *Rana sylvaticu*: an experimental field study. Ecol. Monogr. **44:** 129-151.

DeWitt, T.J., Scheiner, S.M. (2004): Phenotypic Plasticity: functional and conceptual, approaches. New York: Oxford University Press.

Durnin, K.B., Smith, G.R. (2001): Effects of changing water volume on the tadpoles of two anuran species (*Pseudacris triseriata* and *Rana blairi*). J. Freshw. Ecol. **16:** 411-414.

Fixari, J.E. III., Mysiw, A.W., Sorenson, D.R., Smith, G.R., Rettig, J.E. (2017): Crowding effects on growth rate of *Lithobates sphenocephalus* tadpoles (Amphibia: Anura: Ranidae). Salamandra **53:** 466-468.

Gillespie, G.R. (2002): Impacts of sediment loads, tadpole density, and food type on the growth and development of tadpoles of the spotted tree frog *Litoria spenceri*: an in-stream experiment. Biol. Conserv. **106:** 141-150.

Goetsch, W. (1924): Lebensraum und Korpergrosse. Biol. Zentralbl. **44:** 529-560.

Golay, N., Durrer, H. (1994): Volume d'eau effectif, volume d'eau accessible et effet de masse chez les têtards de *Bufo calamita*. Alytes **12:** 64-74.

Gosner, K.L. (1960): A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica **16**: 183-190.

Gromko, M.H., Mason, F. S., Smith-Gill, S. J. (1973): Analysis of the crowding effect in *Runa pipiens* tadpoles. J. Exp. Zool. **186:** 63-72.

Hota, A.K., Dash, M.C. (1981): Growth and metamorphosis of *Rana tigrina* larvae: effects of food level and larval density. Oikos **37:** 349-352.

John, K.R., Fusaro, J.M. (1981): Growth and metamorphosis of solitary *Rana pipiens* tadpoles in confined space. Copeia **1981:** 737-741.

John, K.R., Fenster, D. (1975): The effects of partitions on the growth rate of crowded *Rana pipiens* tadpoles. Am. Midl. Nat. **93:** 123-130.

Kamat, N.D. (1962): On the intestinal contents of tadpoles and algae of small ponds. Curr. Sci. **31:** 300-301.

Kehr, A.I., Schaefer, E.F., Duré, M.I., Gómez, V.I. (2014): Influence of light intensity, water volume and density in tadpoles raised in mesocosm experiments. J. Zool. **293:** 33-39.

Licht, L.E. (1967): Growth inhibition in crowded tadpoles: Intraspecific and interspecific effects. Ecology **48:** 736-745.

Loman, J. (2002): *Rana temporaria* metamorph production and population dynamics in the field–effects of tadpole density, predation and pond drying. J. Nat. Conserv. **10:** 95-107.

Lynn, W.G., Edelman, A. (1936): Crowding and metamorphosis in the tadpole. Ecology **17:** 104-109.

Martin, R.A., Garnett, S.C. (2013): Relatedness and resource diversity interact to influence the intensity of competition. Biol. J. Linn. Soc. **110:** 689-695.

Montealegre-Delgado, X.K., Avendaño-Casadiego, K., Bernal, M.H. (2013): Efecto del tamaño del habitat en la supervivencia, desarrollo y crecimiento en renacuajos de *Engystomops pustulosus* (Anura: Leiuperidae) y *Rhinella humboldti* (Anura: Bufonidae). Pap. Avulsos Zool. **53:** 309-314.

Pearman, P.B. (1993): Effects of habitat size on tadpole populations. Ecology **74:** 1982-1991.

Pigliucci, M. (2001): Phenotypic plasticity: beyond nature and nurture. Maryland: Johns Hopkins University Press.

Podhiradsky, J. (1932): tjber den Einfluss des Lebensriumes auf das Wachstum der Tiere. Arch. F. Entwickl. **127:** 251-82.

Relyea, R.A., Hoverman, J.T. (2003): The impact of larval predators and competitors on the morphology and fitness of juvenile tree frogs. Oecologia **134:** 596-604.

Richter-Boix, A., Tejedo, M., Rezende, E.L. (2011): Evolution and plasticity of anuran larval development in response to desiccation. A comparative analysis. Ecol. Evol. **1:** 15-25.

Rot-Nikcevic, I., Denver, R.J., Wassersug, R.J. (2005): The influence of visual and tactile stimulation on growth and metamorphosis in anuran larvae. Funct. Ecol. **19:** 1008-1016.

Rugh, R. (1934): The space factor in the growth rate of tadpoles. Ecology **15:** 407-411.

Sabnis, J.H., Kuthe, S.N. (1978): Observation on food and growth of *Bufo melanostictus* tadpole. J. Bombay Nat. Hist. Soc. **77:** 21-25

Sabnis, J.H., Kolhatkar, B.L. (1977): Observations on the food preference of *Rana cyanophlyctis* tadpole. Comp. Physiol. Ecol. **2:** 232-233.

Saidapur, SK. (2001): Behavioral ecology of anuran tadpoles: the Indian scenario. Proc. Indian Natn. Sci. Acad. (PINSA) **67**: 311-322.

Sekar, A.G. (1992): A study of the food habits of six anuran tadpoles. J. Bombay Nat. Hist. Soc. **89:** 9-16.

Semlitsch, R.D., Caldwell, J.P. (1982): Effects of density on growth, metamorphosis, and survivorship in tadpoles of *Scuphiopus holbrooki*. Ecology **63:** 905-911.

Semlitsch, R.D., Scott, D.E., Pechmann, J.H.K., Gibbons, J.W. (1996): Structure and dynamics of an amphibian community: Evidence from a 16-year study of a natural pond. In: Long-term studies of vertebrate communities, pp. 217-247. Cody, M.L., Small-Wood, J.A., Eds, San Diego: Academic Press Inc.

Smith, G.R. (1998): Abundance and water volume effects in amphibian larvae. Bull. Maryland Herpetol. Soc. **34:** 113-117.

Smith-Gill, S.J., Berven, K.A. (1979): Predicting amphibian metamorphosis. Am. Nat. **113:** 563-585.

Steinwascher, K. (1978): Interference and exploitation competition among tadpoles of *Rana utricularia*. Ecology **59:** 1039-1046.

Székely, P., Cogălniceanu, D., Tudor, M. (2010): Effect of habitat drying on the development of the Eastern spadefoot toad (*Pelobates syriacus*) tadpoles. Amphib.-Reptil. **31:** 425-434.

Valenzuelasanchez, A., Cunningham, A.A., Sotoazat, C. (2015): Geographic body size variation in ectotherms: effects of seasonality on an anuran from the southern temperate forest. Front. Zool. **12:** 37-37.

Wei, G., Xia P.H., Cheng, G., Wang, S., Zhang, J.Z., Lu, J.C., Xu, N. (2011): Predatory effect of tadpoles of *Bufo gargarizans gargarizans* and *Microhyla pulchra* on Algae. Guizhou Agr. Sci. **39:** 150-151.

Wells, K.D. (2007): The ecology and behavior of amphibians. University of Chicago Press, Chicago.

Wilbur, H.M. (1977): Interactions of food level and population density in *Rana sylvatica*. Ecology **58:** 206-209.

Wilbur, H.M. (1997): Experimental ecology of food webs: complex systems in temporary ponds. Ecology **78**: 2279-2303.

Wilbur, H. M., Collins, J. P. (1973): Ecological aspects of amphibian metamorphosis. Science **58:** 1305-1314.

Yu, T.L., Sharma, M.D. (2012): Sex recognition and mate choice by male *Bufo gargarizans* in Central China. Zool. Sci. **29:** 347-350.

Yung, E. (1885): Influence du nombre des individus contenus dans un meme vase, et de la forme de ce vase, sur le dleveloppment des larues de Grenouille. Comp. Rend. A cad. Sci. **101:** 1018-1020.

Acta Herpetologica 20(1): 49-58, 2025
DOI: 10.36253/a_h-17047

Anuran life histories remain consistent across contrasting precipitation regimes in Northeastern Brazilian forests

Francis Luiz Santos Caldas[1,2,3*], Adrian Antonio Garda[4], Cássio Rachid Meireles de Almeida Simões[5], Edinaldo Leite-Filho[6], Renato Gomes Faria[2,3], Daniel Oliveira Mesquita[7]

[1] *Instituto Federal de Sergipe, Campus Propriá, CEP 49900-000, Propriá, Brasil*
[2] *Departamento de Biologia, Laboratório de Répteis e Anfíbios, Universidade Federal de Sergipe, CEP 49100-000, São Cristovão, Brasil*
[3] *Programa de Pós Graduação em Ecologia e Conservação, Universidade Federal de Sergipe, CEP 49100-000, São Cristovão, Brasil*
[4] *Departamento de Botânica, Ecologia e Zoologia, Universidade Federal do Rio Grande do Norte, CEP 59078-900, Natal, Brasil*
[5] *Programa de Pós-Graduação em Biodiversidade e Evolução, Instituto de Biologia, Universidade Federal da Bahia, CEP 40170-115, Salvador, Brasil*
[6] *Centro de Ciências Agrárias, Laboratório de Microbiologia, Universidade Federal da Paraíba – Campus II, CEP 58397-000, Areia, Brasil*
[7] *Departamento de Sistemática e Ecologia, Universidade Federal da Paraíba, CEP 58059-900 João Pessoa, Paraíba, Brasil*
**Corresponding author.* Email: francisluiz_bio@hotmail.com

Submitted on: 2024 16th December; revised on: 2025 20th March; accepted on:2025, 27th March
Editor: Andrea Costa

Abstract. Amphibians are inherently dependent on water, and many species characteristics are influenced by this resource, particularly in ecoregions with contrasting precipitation regimes, such as the dry Caatinga and the moist Atlantic Forests of Northeastern Brazil. We compared these environments and hypothesized that anurans, regardless of the species, would be smaller in the Caatinga due to unpredictable duration of the rainy season, which accelerates metamorphosis, and the resulting fluctuations of resources, which limit energy reserves for growth. Furthermore, we anticipated that female reproductive strategies in the Caatinga would be more focused on quality rather than quantity, with small clutches and large oocytes. To test these hypotheses, we sampled areas of Caatinga and Atlantic Forest (two of each) in northeastern Brazil through 15-day consecutive campaigns conducted during the dry and rainy seasons. We combined active searches and pitfall traps to collect the anurans. We performed intraspecific comparisons using morphometric measurements and number and volume of oocytes. Contrary to our expectations, body size did not differ between forests for either sex. In moist environments, the extended reproductive period and the associated energetic demands may constrain growth, imposing costs similar to those in the semiarid environment, resulting in comparable body sizes. Similarly, few differences were observed in oocyte numbers and volumes. *Leptodactylus macrosternum* and *Rhinella diptycha* showed fewer and smaller oocytes, respectively, in the Caatinga. Biological factors such as lifespan and body size, and environmental factors such as the duration of the hydroperiod, predation, and insolation can influence oocyte size and numbers in different conditions. However, they likely interact in minimizing energy expenditure, resulting in similar reproductive strategies in contrasting environments.

Keywords. Frogs, local adaptation, abiotic conditions, selective pressures, Ecophysiology, life history, precipitation seasonality.

INTRODUCTION

Understanding how abiotic factors shape species' life histories is a central question in ecology (Blaustein et al., 1999; Saenz et al., 2006; Pavón-Vázquez et al., 2022). This issue is particularly relevant for amphibians, given their critical dependence on water for essential biological functions (Pough et al., 1983; Dayton and Fitzgerald,

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

www.fupress.com/ah

2006; Brandão et al., 2020). Consequently, life history of these organisms can be directly affected by hydrological dynamics and precipitation regimes (Sullivan and Fernandez, 1999; Navas et al., 2004; Li et al., 2023).

External morphology evolves under selective pressures tied to specific ecological demands, such as increased efficiency in competitive interactions or survival in environments with fluctuating resource availability (Demetrius, 2000; Schäuble, 2004). Consequently, marked morphological variation may arise across populations subject to the selective pressures of different environments. These modifications in life history traits (e.g. body size, clutch size) can result from phenotypic plasticity or local evolutionary adaptation (Pfennig and Murphy, 2002; García et al., 2021; Tiar-Saadi et al., 2022). Howard (1981) suggested that adult body size in amphibians is directly related to reproductive effort, responding to ecological constraints that influence development trajectories of each sex.

In stable environments, with limited resource fluctuations, body size of both sexes increases with the rise in resource acquisition, and sexual selection favors larger individuals, even though dimorphism is preserved (Howard, 1981). Additionally, physiological performance during the larval phase directly influences adult body size (Werner, 1986). Conversely, in unstable environments with high resource fluctuations, anurans encounter sharp increases in prey availability, mainly invertebrates, during the brief and irregular rainy season (Bento et al., 2016; Silva et al., 2021). As a result, post-metamorphic individuals may endure extended periods of reduced resource availability, limiting energy intake for growth and ultimately leading to smaller adults.

Harsh environments can also compromise reproductive output, affecting reproductive investment by imposing significant losses on clutches due to high temperature, solar radiation rates or irregular water availability (Blaustein et al., 1995; Neckel-Oliveira, 2004; Kissel et al., 2019). Life history theory posits that in unstable environments investing in larger oocytes reduces clutch sizes but increases chances of survival of both reproductive females and juveniles (Roff, 1992; Roff, 2002). For example, clutch size in *Bufo andrewsi* increases with female body size in seasons that favor growth, making them more enduring (Liao et al., 2016). Meanwhile, oocyte size increases during shorter, less favorable periods for resource acquisition regardless of body size (Liao et al., 2016).

In the Neotropical realm, the dry Caatinga and the moist Atlantic Forest of Brazil are ecoregions with contrasting abiotic conditions. While the former experiences an extended dry season, semiarid climate, reduced and irregular precipitation regimes, and predominance of temporary aquatic environments (Nimer, 1989; Ab'Saber, 1999; Silva et al., 2017), the latter has a humid tropical climate with high and evenly distributed precipitation throughout the year and greater predominance of permanent aquatic environments, due to its proximity to the coast (Ab'Saber, 1977; Tonhasca-Júnior, 2005; Joly et al., 2014). Anurans occurring in these contrasting environments exhibit shorter reproductive periods in the Caatinga, as a result of the reduced spatial and temporal availability of water (Caldas et al., 2016; Caldas et al., 2019). Given the contrasting conditions occupied by frogs in these quite different environments, we hypothesized that: 1) Anuran males and females are smaller in the Caatinga compared to the Atlantic Forest, as water irregularity and the predominance of temporary aquatic environments would accelerate metamorphosis, and marked seasonal fluctuations in resources availability in the post-metamorphic period would reduce the energy stock necessary for growth; 2) Females in the Caatinga are expected to prioritize reproductive quality over quantity, producing fewer but larger oocytes compared to their counterparts in the Atlantic Forest. These strategies could ensure higher chances of survival for larvae in harsh and unstable environments.

To test the hypotheses, we measured morphometric traits of reproductive males and females of species occurring both in Caatinga and Atlantic Forest and counted the mature oocytes of females from both ecoregions. Four species were selected for the morphometric analysis: *Boana raniceps* (Chaco tree frog), *Leptodactylus vastus* (Northeastern Pepper Frog), *Scinax x-signatus* (Venezuela snouted treefrog), and *Leptodactylus troglodytes* (Pernambuco white-lipped frog). Three species were selected for the reproductive analysis [*B. raniceps, Leptodactylus macrosternum* (Miranda's White-lipped Frog) and *Rhinella diptycha* (cururu toad)]. All species have aquatic reproductive modes and the entire larval period occurs in the water (Nunes-de-Almeida et al., 2021) making them interesting models to evaluate both questions tested in this paper.

MATERIAL AND METHODS

Study areas

The study was conducted with anuran specimens from four protected areas in the Northeast region of Brazil: the Benjamin Maranhão Botanical Garden Permanent Preservation Area, the Seridó and Aiuaba Ecological Stations and the Guaribas Biological Reserve (Fig. 1). The Seridó and Aiuaba Ecological Stations are situated in the Northern Sertaneja Depression, located in the Caatinga ecoregion, part of the biome "Tropical & Subtropical Dry

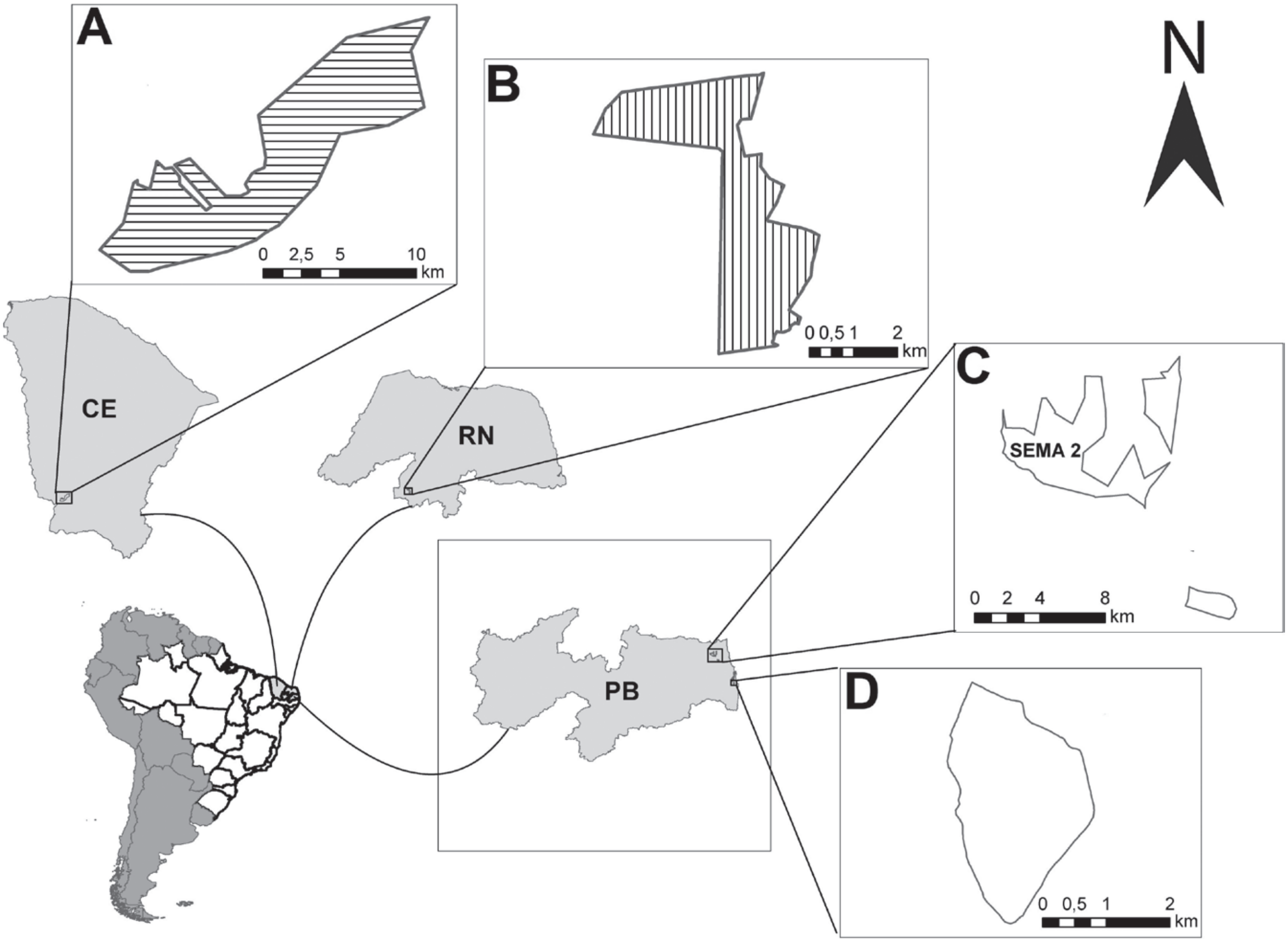

Fig. 1. Study areas: Aiuaba Ecological Station – CE (A), Seridó Ecological Station – RN (B), Guaribas Biological Reserve – PB (C), and Benjamin Maranhão Botanical Garden– PB (D). Caatinga are filled with lines, and Atlantic Forest areas are without lines.

Broadleaf Forests" (Dinerstein et al., 2017). The climate is hot and semi-arid, and the average precipitation is below 800 mm (Velloso et al., 2002). The Guaribas Biological Reserve and the Benjamin Maranhão Botanical Garden are located in the Atlantic Forest ecoregion, part of the biome "Tropical & Subtropical Moist Broadleaf Forests" (Dinerstein et al., 2017) with a tropical rainy climate and a short dry season (Köppen type Am) and have average precipitation above 1500 mm (Nimer, 1979).

The Seridó Ecological Station (Fig. 1) is located in the state of Rio Grande do Norte (06°35' to 06°40'S, 37°20' to 37°39'W), in the municipality of Serra Negra do Norte, totaling an area of approximately 1,166 ha, mainly composed of herbs and shrubs, predominating genus such as *Amburana* (Fabaceae), *Mimosa* (Fabaceae), among others, and an endemic species, *Gossypium mustelinum* (Malvaceae) (Velloso et al., 2002). The Aiuaba Ecological Station (Fig. 1) located in the state of Ceará (06°36'01" to 06°44'35" S and 40°07'15" to 40°19'19" W), in the municipality of Aiuaba, totaling an area of 11,525 ha (Velloso et al., 2002). It includes an arboreal Caatinga remnant with predominantly open vegetation formations with *Cnidoscolus–Bursera–Caesalpinia* (Andrade-Lima, 1981).

The Guaribas Biological Reserve and Benjamin Maranhão Botanical Garden (Fig. 1) are located in Paraíba state. The former is located in the municipalities of Mamanguape (91.59%) and Rio Tinto (8.41%), encompassing areas termed SEMA 01, 02, and 03. We sampled SEMA 2 (06°40'40" a 06°44'59" S e 41°12'47" a 41°07'11" O) which has an area of approximately 3,378 ha (Barbosa et al., 2011). This protected area is highly heterogeneous, comprising tree formations, typical coastal restingas savannas, and an arboreal formation of semideciduous seasonal forest (Oliveira-Filho and Carvalho, 1993). In turn, the Botanic Garden is a remnant of forest located in the urban area of the municipality of João Pessoa (07°06'S and 34°52'W). The area is divided by the BR-230 highway, resulting in a larger continuous portion of 471 ha (sampling site) and 11 small fragments located within Campus I of the Federal University of Paraíba. This environment is typical of the Northeast coastal moist forest, featuring secondary growth and a flora of trees, shrubs, and herbaceous plants, with a low sub-canopy layer and few lianas (Dias et al., 2006).

Data collection

Sampling for this study was conducted in 2013 across the four protected areas. We conducted surveys during the dry season in January (Guaribas), September (Aiuaba), and December (Seridó), while rainy season surveys were made in May (Seridó), April (Aiuaba), and June (Guaribas). For the Benjamin Maranhão Botanical Garden, sampling took place between 2011 and 2012. These data were obtained using the same collection methods as the other sites over a period of 12 months. Since it was possible to filter data covering a similar range of days as the other areas in a seasonal approach, no inconsistencies were caused in the sampling. We selected May, June and July (three of the rainiest months) and October, November, and December (three of the driest months). These intervals showed rainfall averages similar to those of the Guaribas REBIO in 2013 (see National Institute of Meteorology, INMET, 2012). These months were chosen based on seasonal definitions established by Lima and Heckendorff (1985), Aguiar and Martins (2002) and Velloso et al. (2002): in the Benjamim Maranhão Botanic Garden, the period of highest rainfall concentration typically occurs between March and August, in the Guaribas Biological Reserve between April and October, and in the Northern Sertaneja Depression, rainfall occurs from October to April. These patterns have been corroborated by 2013 monthly precipitation averages for each site (data from the Brazilian National Institute of Meteorology, INMET, 2012; INMET, 2013).

Each survey site was sampled for 15 consecutive days per season, resulting in 30 sampling days per site and a total of 90 days across sites. Two methodologies were used for field sampling. The main survey approach involved systematic visual and auditory inspection of the environments for anurans following standard methods (Crump and Scott, 1994), conducted between 18:00 and 22:00 hours. The active search has always been performed by two scientists, totaling 360 hours per person, and after being located, the specimens were manually collected. We tried to survey as many aquatic environments of aquatic environments based on the availability of each site: Guaribas Biological Reserve – five temporary ponds, one perennial stream and one perennial lake; Benjamim Maranhão Botanic Garden – a dammed section of the Jaguaribe River (7°08’42’ ’S and 34°51’54” W); Seridó Ecological Station – three temporary ponds, one intermittent stream, and one perennial lake; Aiuaba Ecological Station – four temporary ponds and one perennial lake. We believe the surveyed environments were adequate to record the species used in the comparisons. Their structural variation among sites was not considered problematic, as the species do not have riparian habits, and all but one (*Corythomantis greeningi*) reproduce in lentic habitats (Arzabe, 1999; Vieira et al., 2007; Santana et al., 2008).

The complementary survey approach involved the use of pitfall traps. We selected two aquatic environments with distinct conditions (temporary and perennial) in each survey site and install three trapping systems at the margins of each (except at the Botanical Garden, where two areas on the banks of the Jaguaribe River were used). The system consisted of four 30-L buckets buried in the ground and connected by 5-m plastic drift fences arranged in a Y pattern (Cechin and Martins, 2000; Enge, 2001). The traps were checked twice daily for 15 days to prevent animal mortality.

Morphometry

We selected four anuran species, *B. raniceps, L. vastus*, *S. x-signatus*, and *L. troglodytes*. The collected specimens were transported to the laboratory, euthanized with 2% lidocaine, fixed in 10% formalin, and then preserved in 70% ethanol (Caldas et al., 2016; Caldas et al., 2019), with each specimen assigned an identification number. After fixation, we measured 11 morphometric traits for each individual using a digital caliper (precision of 0.01 mm): snout-vent length (SVL), head length, head width, inter-eye distance, eye-nostril distance, inter-nostril distance, thigh length, tibia length, foot length, eye diameter, and inter-orbital distance (Napoli and Pimenta, 2009).

Next, we dissected the sampled individuals to determine their sex through direct observation of the gonads. The reproductive condition was assessed by examining the reproductive organs in females and the vocal sacs in males. Females were considered reproductive and suitable for comparison if they had highly convoluted oviducts and developed ovaries, while males were considered reproductive if they displayed fully evident vocal sacs (Mesquita et al., 2004). Individuals lacking these characteristics were classified as non-reproductive and excluded from the analyses.

Reproduction

We selected three species for these analyses: *B. raniceps*, *L. macrosternum*, and *R. diptycha*. They were the only ones that had females with oocytes and occurred in both of the compared ecoregions. We obtained large samples for each species (*B. raniceps*, n = 73; *L. macrosternum*, n = 210; *R. diptycha*, n = 86). However, the proportions of females were low [*B. raniceps* = 25 (34%); *L. macrosternum* = 81 (39%); *R. diptycha* = 24 (28%)], a situation that commonly occur in some anurans (Braun and Braun, 1977; Wiens et al., 2011). Few of these cataloged females contained oocytes (*B. raniceps*, n = 8 (32%); *L. macrosternum*, n = 31 (38%); *R. diptycha*, n = 9 (37%). Due to the smaller number of females and the occasional presence of oocytes in them, it was not possible to obtain information for the last two species at one of the Atlantic Forest sites (APP JBBM). In the laboratory, we dissected the specimens and removed the mature ovarian oocytes (post-vitellogenic) from females. These oocytes were identified by their well-differentiated hemispheres, with the animal pole displaying a dark brown coloration and the vegetative pole appearing milky and ivory in color (Melchiors et al., 2004). The material was then preserved in 70% ethanol.

We counted oocytes using a graduated cylinder filled with water up to a fixed measurement (properly recorded). We submerged the entire mass of oocytes into the container and recorded the displacement of the water column. Subsequently, we removed the mass and reintroduced only enough to displace 1.5 mm^3. Next, the oocytes present in this small mass were dissociated by immersing them in a 10% sodium hypochlorite solution for 20 minutes (Melchiors et al., 2004). The dissociation allowed for the individual counting of the oocytes using a stereoscopic magnifying glass. With the total volume of the oocyte mass and the number of oocytes present in 1.5 mm^3, it was possible to estimate each oocyte's total number and average volume through a simple proportion. After completing the procedures, we layed all specimens and oocytes in the Coleção Herpetológica da Universidade Federal da Paraíba.

Data analysis

We initially assessed the normality of the data using the Shapiro-Wilk test to determine whether parametric or nonparametric analyses would be appropriate. Body size was treated as an isometric variable (Rohlf and Bookstein, 1987), following the protocol described by Somers (1986). An isometric eigenvector was calculated with predefined values of p-0.5, where "p" represents the number of variables (Jolicoeur, 1963). The scores for this eigenvector, referred to as "composite body size," were obtained by multiplying the n x p matrix of log10-transformed data, where "n" is the number of observations. To evaluate differences in composite body size between individuals of the same species across ecoregions, we conducted separate analyses of variance (ANOVA) for males and females.

For the oocyte masses, we calculated the average number of oocytes for species from both the Caatinga and Atlantic Forests. To test whether the total number and average individual volume of oocytes differed significantly between specimens from the two ecoregions, we performed an analysis of variance (ANOVA) (Shapiro-Wilk, $p > 0,05$) or Mann-Whitney test (Shapiro-Wilk, $p < 0,05$), depending on the normality of the data. All analyses were conducted using the R program for Windows (R Development Core Team, 2018): package Rcmdr version 2.9-5 for Shapiro-Wilk, ANOVA, and Mann-Whitney (Fox et al., 2020).

RESULTS

We did not find significant differences in body size in the four species between the two ecoregions, regardless of sex. Males and females exhibit similar body sizes in both forests: males of *B. raniceps* (ANOVA, $F_{1,46}$: 0.60; P = 0.44), females of *B. raniceps* (ANOVA, $F_{1,23}$: 0.01; P = 0.91), and other males: *S. x-signatus* (ANOVA, $F_{1,47}$: 0.41; P = 0.52), *L. vastus* (ANOVA, $F_{1,33}$: 2.70; P = 0.11), and *L. troglodytes* (ANOVA, $F_{1,18}$: 2.10; P = 0.16) (Table 1).

Table 1. Descriptive statistics and body size comparisons for amphibians from the Caatinga (within parentheses) and the Atlantic Forest. NS = not significant.

Variables	*B. raniceps* n = 24 (49)		*S. x-signatus* n = 22 (27)	*L. vastus* n = 15 (20)	*L. troglodytes* n = 6 (15)
	males n = 15 (33)	females n = 9 (16)	males n = 22 (27)	males n = 15 (20)	males n = 6 (15)
SVL	68.66 ± 3.42	72.57 ± 11.15	34.89 ± 2.78	135.25 ± 31.87	48.33 ± 1.59
	(63.00 ± 10.35)	(68.91 ± 7.82)	(33.19 ± 4.12)	(153.30 ± 20.37)	(43.42 ± 6.74)
Composite Body Size	4.15 ± 0.08	4.23 ± 0.30	3.07 ± 0.17	5.11 ± 0.61	3.65 ± 0.09
	(4.08 ± 0.30)	(4.24 ± 0.14)	(3.10 ± 0.16)	(5.34 ± 0.14)	(3.55 ± 0.15)
	$F_{1,46} = 0.60^{NS}$	$F_{1,23} = 0.01^{NS}$	$F_{1,47} = 0.41^{NS}$	$F_{1,33} = 2.70^{NS}$	$F_{1,18} = 2.10^{NS}$

Table 2. Descriptive statistics and comparisons of reproductive traits for amphibians from the Caatinga (within parentheses) and the Atlantic Forest. *P < 0.05; NS = not significant.

Variables	*B. raniceps* n = 4 (4)	*L. macrosternum* n = 10 (21)	*R. diptycha* n = 4 (5)
Average number of oocytes	4,765 ± 4,634 (3,728 ± 2,487) $F_{1,6} = 0.15^{NS}$	5,255 ± 2,051 (2,877 ± 1,723) $F_{1,29} = 11.41^{*}$	24,818 ± 10,013 (18,024 ± 17,440) $U_{1,7} = 0.97^{NS}$
Average volume of oocytes	0.0029 ± 0.0017 mm^3 (0.0013 ± 0.0002 mm^3) $F_{1,6} = 3.55^{NS}$	0.0018 ± 0.0005 mm^3 (0.0019 ± 0.0010 mm^3) $F_{1,29: =} 0.20^{NS}$	0.0036 ± 0.0013 mm^3 (0.0015 ± 0.0008 mm^3) $F_{1,7} = 7.77^{*}$

Similarly, the number of oocytes did not differ between ecoregions for *B. raniceps* and *R. diptycha* (Table 2). However, we observed differences in the number of oocytes for *L. macrosternum*, which was lower in the Caatinga (mean = 2,877 ± 1,723) and higher in the Atlantic Forest (mean = 5,255 ± 2,051); (ANOVA, $F_{1,29}$: 11.41; p = 0.02) (Table 2). The other species did not show variations: *B. raniceps* (ANOVA, $F_{1,6}$: 0.15; p = 0.70) and *R. diptycha* (Mann-Whitney, $U_{1,7}$: 0.97; p = 0.32) (Table 2).

The average volume of each oocyte did not differ for *B. raniceps* (Table 2). However, we observed differences for *R. diptycha*, which had significantly smaller oocytes in the Caatinga (mean = 0.0015 ± 0.0008 mm^3) compared to the Atlantic Forest (mean = 0.0036 ± 0.0013 mm^3); (ANOVA, $F_{1,7}$: 7.76; p = 0.03) (Table 2). The other species did not show variations: *B. raniceps* (ANOVA, $F_{1,6}$: 3.55; p = 0.11) and *L. macrosternum* (ANOVA, $F_{1,29}$: 0.20; p = 0.66) (Table 2).

DISCUSSION

Contrary to our expectations, neither males nor females differed in size between Caatinga and Atlantic Forest. Similarly, reproductive efforts in the Caatinga did not differ significantly from the Atlantic Forest, except for *L. macrosternum* and *R. diptycha*, which showed a lower number and a smaller size of oocytes, respectively. Adult frogs were not smaller in environments with irregular precipitation regimes and reduced resources availability during the extended dry season (Caatinga), suggesting that hydroperiod and prey availability may not be limiting factors for growth. Possibly to avoid compromising growth due to resource limitations, other strategies to minimize energy loss in the dry season seem to have been selected in amphibians from Caatinga, such as aestivation and use of subterranean shelters in periods of reduced activity (Varjão and Ribeiro, 2018; Jared et al., 2019; Moreira et al., 2021).

In the Caatinga, lower precipitation and shorter hydroperiods can hypothetically accelerate metamorphosis and restrict resource availability for adults. Conversely, in the Atlantic Forest the hydrological stability can allow extended reproductive activity, but at the cost of expending more energy in vocalization, territory defense, partner encounter, courtship, and investment in oocytes (Arzabe et al., 1998; Afonso and Eterovick, 2007; Caldas et al., 2016). In both cases, energy deprivation would impact body size, as predicted by life history theory. Given limitations and finite resources, reproduction can incur costs that would ultimately reduce growth rate (Bradshaw and McMahon, 2008). In other words, body size patterns in the two ecoregions would be similar because growth would not be the main focus of energy investment, and thus, selective pressures would not promote an increase in size. In the Caatinga, the limited availability of trophic resources during the short rainy season would provide just enough energy for a rapid reproductive event. In the Atlantic Forest, the greater availability of trophic resources, ensured by annual water stability, would support a prolonged reproductive event. In both cases, the primary objective would be reproduction, and energy investment would be prioritized for this activity.

The expected patterns for hostile environments (Caatinga) and stable environments (Atlantic Forest) were not corroborated for most of the species' reproductive investment. The number of oocytes did not change in *B. raniceps*. In contrast, the volume of oocytes in *R. diptycha* was smaller in the Caatinga, contrary to some expectations for their sizes under harsh conditions (Morrison and Hero, 2003; Liao et al., 2016). Alternatively, harsh environments with short seasons for resource acquisition can result in low annual growth rates. Thus, smaller oocytes or clutches would be compensated by more reproductive events and greater longevity (Sinsch et al., 2015), a pattern common for large-size species (Stark and Meiri, 2018), like *Rana temporaria* (Sinsch et al., 2015). Indeed, *R. diptycha* is a large species (Sousa et al., 2022), and longevity has already been indicated as a key factor for the persistence of some bufonids in arid environments (Tevis Jr, 1966). Therefore, slow growth rates and higher longevity resulting in more reproductive events in the Caatinga in *R. diptycha* may account for its smaller oocytes.

Leptodactylus macrosternum presented a lower number of oocytes in the Caatinga. However, contrary to what would be expected if the species in both regions allocated the same amount of energy for reproduction, these oocytes were not larger than those in the Atlantic Forest. Although it is not clear what drives this difference among areas, other factors can be driving egg number and size in this species (see Horato et al., 2024). The eggs of *L. macrosternum* are layed in the water (Heyer, 1969), and harsh insolation in arid environments can more severely affect eggs layed outside of water (Caldas et al., 2016). Similarly, there is less predation pressure in temporary aquatic environments subject to prolonged droughts (Woodward, 1983; Dayton and Fitzgerald, 2001; Schiwitz et al., 2020). The combination of such factors could prevent natural selection from favoring an increase in oocyte volume, resistance, and larval size. Thus, the qualitative nature of *L. macrosternum* reproduction would prevail. This hypothesis needs to be tested for the cause-and-effect relationships we discussed to be confirmed.

Studies focusing on the possible effects of environmental variables on life history traits in amphibian species from the Brazilian Forests remain scarce. Despite our moderate sample sizes, our data suggest that the differences between precipitation regimes are probably insufficient to promote significant reproductive and morphological adjustments in species that can thrive in both Caatinga and Atlantic Forest. However, the observed patterns require further investigation to be completely understood. In particular, future research should focus on other environmental factors and taxa, as well as increasing the number of samples analyzed.

ACKNOWLEDGMENTS

We thank the Biodiversity Information and Authorization System (Sistema de Autorização e Informação em Biodiversidade – SISBIO) for granting the collection license (number 36095-8). This study received support from the managers and staff of all conservation units, especially J. L. Nascimento, G. Freitas, M. Kluppel (all from Guaribas Biological Reserve), G. Stephenson (Seridó Ecological Station), and M. Souza (Aiuaba Ecological Station). The Federal University of Paraíba (Universidade Federal da Paraíba–UFPB) provided logistical support during the study, and the Federal University of Rio Grande do Norte (Universidade Federal do Rio Grande do Norte–UFRN) and the Federal University of Sergipe (Universidade Federal de Sergipe–UFS) provided significant assistance during the project. Finally, we thank the Coordination for the Improvement of Higher Education Personnel – CAPES for the research scholarship and CNPq/ICMBIo for the financial support to AAG, which partially funded this study (# 552031/2011-9). AAG and DOM thank CNPq for financial support (research productivity grants numbers 309531/2015-2 and 303610/2014-0/306541/2017-3, 304715/2021-2, respectively). DOM acknowledges FAPESQ-PB for financial support (PRONEX and Universal). FLSC also thanks CNPQ for financial support in the postdoctoral period (numbers 150827/2018-0 and 150063/2022-9).

REFERENCES

Ab'Saber, A.N. (1977): Os domínios morfoclimáticos na América do Sul: primeira aproximação. Geomorfologia **52**: 1-121.

Ab'Saber, A.N. (1999): Sertões e sertanejos: uma geografia humana sofrida. Estud. Av. **13**: 7-59.

Afonso, L.G., Eterovick, P.C. (2007): Spatial and temporal distribution of breeding anurans in streams in southeastern Brazil. J. Nat. Hist. **41**: 949-963.

Aguiar, A.J.C., Martins, C.F. (2002): Abelhas e vespas solitárias em ninhos-armadilha na Reserva Biológica Guaribas (Mamanguape, Paraíba, Brasil). Rev. Bras. Zool. **19**: 101-116.

Andrade-Lima, D. (1981): The caatingas dominium. Rev. Bras. Bot. **4**: 149-163.

Arzabe, C., Carvalho, C.X., Costa, M.A.G. (1998): Anuran assemblages in Crasto Forest ponds (Sergipe State, Brazil): comparative structure and calling activity patterns. Herpetol. J. **8**: 111-113.

Arzabe, C. (1999): Reproductive activity patterns of anurans in two different altitudinal sites within the Brazilian Caatinga. Rev. Bras. Zool. **16**: 851-864.

Barbosa, M.R.V., Thomas, W.W., Zárate, E.L.P., Lima, R.B., Agra, M.F., Lima, I.B., Pessoa, M.C.R., Lourenço, A.R.L., Delgado Júnior, G.C., Pontes, R.A., Chagas, E.C.O., Viana, J.L., Neto, P.C.G., Araújo, C.M.L.R., Araújo, A.A.M., Freita, G.B., Lima, J.R., Silva, F.O., Vieira, L.A.F., Pereira, L.A., Costa, R.M.T., Duré, R.C., Sá, M.G.V. (2011): Checklist of the vascular plants of the Guaribas Biological Reserve, Paraíba, Brazil. Rev. Nord. Biol. **20**: 79-106.

Bento, D.M., Ferreira, R.L., Prous, X., Souza-Silva, M., Bellini, B.C., Vasconcellos, A. (2016): Seasonal variations in cave invertebrate communities in the semiarid Caatinga, Brazil. J. Cave Karst Stud. **78**: 61-71.

Blaustein, A.R., Edmond, B., Kiesecker, J.M., Beatty, J.J., Hokit, D.G. (1995): Ambient ultraviolet radiation causes mortality in salamander eggs. Ecol. Appl. **5**: 740-743.

Blaustein, L., Garb, J.E., Shebitz, D., Nevo, E. (1999): Microclimate, developmental plasticity and community structure in artificial temporary pools. Hydrobiologia **392**: 187-196.

Bradshaw, C.J.A., McMahon, C.R. (2008): Fecundity. In: Encyclopedia of Ecology, pp. 1535-1543. Amsterdam, Elsevier Inc.

Brandão, R.A., Fenker, J., Lopes, B.E.P.C., Sena, V.M.A., Vasconcelos, B.D. (2020): Diet of terrestrial anurans in an ephemeral and simplified habitat during the dry season in the Brazilian Cerrado. Ethol. Ecol. Evol. **1**: 1-24.

Braun, P.C., Braun, C.A.S. (1977): Nova espécie de *Hyla* do Estado do Rio Grande do Sul, Brasil (Anura, Hylidae). Rev. Bras. Biol. **37**: 853-857.

Caldas, F.L.S., Silva, B.D., Santos, R.A., De-Carvalho, C.B., Santana, D.O., Gomes, F.F.A., Faria, R.G. (2016): Autoecology of *Phyllomedusa nordestina* (Anura: Hylidae) in areas of the Caatinga and Atlantic Forest in the State of Sergipe, Brazil. North-West. J. Zool. **12**: 271-285.

Caldas, F.L.S., Garda, A.A., Cavalcanti, L.B.Q., Leite-Filho, E., Faria, R.G., Mesquita, D.O. (2019): Spatial and trophic structure of anuran assemblages in environments with different seasonal regimes in the Brazilian Northeast Region. Copeia **107**: 567-584.

Cechin, S.Z., Martins, M. (2000): Eficiência de armadilhas de queda (pitfall traps) em amostragens de anfíbios e répteis no Brasil. Rev. Bras. Zool. **17**: 729-740.

Crump, M.L., Scott, N.J.J. (1994): Measuring and monitoring biological diversity: standard methods for amphibians. In: Standard Techniques for Inventory and Monitoring, pp. 76-141. Heyer, W.R., Donnelly, M.A., Mcdiarmid, R.W., Hayek, L.C., Foster, M.S., Eds., Washington, D.C., Eds, Smithsonian Institution Press.

Dayton, G.H., Fitzgerald, L.A. (2001): Competition, predation, and the distributions of four desert anurans. Oecologia **129**: 430-435.

Dayton, G.H., Fitzgerald, L.A. (2006): Habitat suitability models for desert amphibians. Biol. Cons. **132**: 40-49.

Demetrius, L. (2000): Directionality theory and the evolution of body size. Proc. R. Soc. B. **267**: 2385-2391.

Dias, S.C., Candido, D.M., Brescovit, A.D. (2006): Scorpions from Mata do Buraquinho, João Pessoa, Paraíba, Brazil, with ecological notes on a population of *Ananteris mauryi* Lourenço (Scorpiones, Buthidae). Rev. Bras. Zool. **23**: 707-710.

Dinerstein, E., Olson, D., Joshi, A., Vynne, C., Burgess, N., Wikramanayake, E., Saleem, M. (2017): Ecoregions 2017. Resolve. https://ecoregions.appspot.com/.

Enge, K.M. (2001): The pitfalls of pitfall traps. J. Herpetol. **35**: 467-478.

Fox, J., Marquez, M.M., Bouchet-Valat, M. (2024). Rcmdr: R Commander. R package version 2.9-5, https://github.com/RCmdr-Project/rcmdr.

García, J., Arizaga, J., Rodríguez, J.I., Alonso, D., Suárez-Seoane, S. (2021): Morphological differentiation in a migratory bird across geographic gradients in mountains of southern Europe. J. Biogeogr. **48**: 2828-2838.

Heyer, W.R. (1969): The adaptive ecology of the species groups of the genus *Leptodactylus* (Amphibia, Leptodactylidae). Evolution **23**: 421-428.

Horato, M.N., Almeida-Santos, M., Rocha, C.F.D., Sabagh, L.T. (2024): Reproductive ecology of treefrogs: egg size promotes reproductive effort differences between females. Evol. Ecol. **38**: 481-493.

Howard, R.D. (1981): Sexual dimorphism in bullfrogs. Ecology **62**: 303-310.

INMET (2012): Instituto Nacional de Meteorologia. https://portal.inmet.gov.br.

INMET (2013): Instituto Nacional de Meteorologia. https://portal.inmet.gov.br.

Jared, C., Mailho-Fontana, P.L., Mendelson, J., Antoniazzi, M.M. (2019): Life history of frogs of the Brazilian semi-arid (Caatinga), with emphasis in aestivation. Acta Zool. **101**: 302-310.

Jolicoeur, P. (1963): The multivariate generalization of the allometry equation. Biometrics **19**: 497-499.

Joly, C.A., Metzger, J.P., Tabarelli, M. (2014): Experiences from the Brazilian Atlantic Forest: ecological findings and conservation initiatives. New Phytol. **204**: 459-473.

Kissel, A.M., Palen, W.J., Ryan, M.E., Adams, M.J. (2019): Compounding effects of climate change reduce population viability of a montane amphibian. Ecol. Appl. **29**: 1-12.

Li, H., Cui, C., Shen, H., Zhu, Y., Chen, Z., Chen, X. (2023): Morphological variation and its correlation with bioclimatic factors in *Odorrana graminea* sensu stricto. Front. Ecol. Evol. **11**: 1-11.

Liao, W.B., Luo, Y., Lou, S.L., Lu, D., Jehle, R. (2016): Geographic variation in life-history traits: growth season affects age structure, egg size and clutch size in Andrew's toad (*Bufo andrewsi*). Front. Zool. **13**: 1-9.

Lima, P.J., Heckendorff, W.D. (1985): Climatologia. In: Atlas Geográfico do Estado da Paraíba, pp. 34-43. UFPB, Ed., João Pessoa, Governo do Estado da Paraíba.

Melchiors, J., Di-Bernardo, M., Pontes, G.M.F., de Oliveira, R.B., Solé, M., Kwet, A. (2004): Reproduction of *Pseudis minuta* (Anura, Hylidae) in southern Brazil. Phyllomedusa **3**: 61-68.

Mesquita, D.O., Costa, G.C., Zatz, M.G. (2004): Ecological aspects of the casque-headed frog *Aparasphenodon brunoi* (Anura, Hylidae) in a Restinga habitat in southeastern Brazil. Phyllomedusa **3**: 51-59.

Moreira, D.C., Carvajalino-Fernández, J.M., Navas, C.A., Carvalho, J.E., Hermes-Lima, M. (2021): Metabolic and redox biomarkers in skeletal muscle underlie physiological adaptations of two estivating anuran species in a South American semi-arid environment. Front. Physiol. **12**: 1-13.

Morrison, C., Hero, J.M. (2003): Geographic variation in life-history characteristics of amphibians: a review. J. Anim. Ecol. **72**: 270-279.

Napoli, M.F., Pimenta, B.V.S. (2009): A new species of the *Bokermannohyla circumdata* group (Anura: Hylidae) from the coastal forests of Bahia, Northeastern Brazil. Copeia **2009**: 674-683.

Navas, C.A., Antoniazzi, M.M., Jared, C. (2004): A preliminary assessment of anuran physiological and morphological adaptation to the Caatinga, a Brazilian semiarid environment. Int. Congr. Ser. **1275**: 298-305.

Neckel-Oliveira, S. (2004): Effects of landscape change on clutches of *Phyllomedusa tarsius*, a neotropical treefrog. Biol. Cons. **118**: 109-116.

Nimer, E. (1979): Climatologia do Brasil. Rio de Janeiro, IBGE.

Nimer, E. (1989): Climatologia da região Nordeste. In: Climatologia do Brasil, p. 315-361. IBGE, DRNEA, Eds., Rio de Janeiro, Fundação Instituto Brasileiro de Geografia e Estatística.

Nunes-de-Almeida, C.H.L., Haddad, C.F.B., Toledo, L.F. (2021): A revised classification of the amphibian reproductive modes. Salamandra, **57**: 413-427.

Oliveira-Filho, A., Carvalho, D. (1993): Floristica e fisionomia da vegetação no extremo norte do litoral da Paraíba. Rev. Bras. Bot. **16**: 115-130.

Pavón-Vázquez, C.J., Brennan, I.G., Skeels, A., Keogh, J.S. (2022): Competition and geography underlie speciation and morphological evolution in Indo-Australasian monitor lizards. Evolution **76**: 476-495.

Pfennig, D.W., Murphy, P.J. (2002): How fluctuating competition and phenotypic plasticity mediate species divergence. Evolution **56**: 1217-1228.

Pough, F.H., Taigen, T.L., Stewart, M.M., Brussard, P.F. (1983): Behavioral modification of evaporative water loss by a Puerto Rican frog. Ecology **64**: 244-252.

R Development Core Team (2018): R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Roff, D.A. (1992): Evolution of Life Histories: theory and Analysis. New York, Chapman e Hall.

Roff, D.A. (2002): Life-history Evolution. Sunderland, Sinauer Associates.

Rohlf, F.J., Bookstein, F.L. (1987): A comment on shearing as a method for "size correction". Syst. Biol. **36**: 356-367.

Saenz, D., Fitzgerald, L.A., Baum, K.A., Conner, R.N., Adams, D. (2006): Abiotic correlates of anuran calling phenology: the importance of rain, temperature, and season. Herpetol. Monogr. **20**: 64-82.

Santana, G.G., Vieira, W.L., Pereira-Filho, G.A., Delfim, F.R., Lima, Y.C., Vieira, K.S. (2008): Herpetofauna em um fragmento de Floresta Atlântica no estado da Paraíba, Região Nordeste do Brasil. Biotemas **21**: 75-84.

Schäuble, C.S. (2004): Variation in body size and sexual dimorphism across geographical and environmental space in the frogs *Limnodynastes tasmaniensis* and *L. peronii*. Biol. J. Linn. Soc. **82**: 39-56.

Schiwitz, N.C., Schalk, C.M., Saenz, D. (2020): Activity level-predation risk tradeoff in a tadpole guild: implications for community organization along the hydroperiod gradient. Am. Midl. Nat. **183**: 223-232.

Silva, I.S., Lucena, E.F., Moura, F.M.S., Vasconcellos, A. (2021): Termite flights seasonally promote nutrient pulses in the Caatinga dry forest in northeastern Brazil. Appl. Soil Ecol. **166**: 1-6.

Silva, J.M.C., Barbosa, L.C.F., Leal, I.R., Tabarelli, M. (2017): The Caatinga: understanding the challenges. In: Caatinga: the Largest Tropical Dry Forest Region in South America, pp. 3-19. Silva, J.M.C., Leal, I.R., Tabarelli, M., Eds, Gewerbestrasse, Springer International Publishing.

Sinsch, U., Pelster, B., Ludwig, G. (2015): Large-scale variation of size- and age-related life-history traits in the common frog: a sensitive test case for macroecological rules. J. Zool. **297**: 32-43.

Somers, K.M. (1986): Multivariate allometry and removal of size with principal components analysis. Syst. Biol. **35**: 359-368.

Sousa, T.R., Benício, R.A., Fonseca, M.G. (2022): Polimorfismo em *Rhinella diptycha* (Anura: Bufonidae) em uma área de Caatinga, estado do Piauí, Nordeste do Brasil. Biosphere **1**: 16-22.

Stark, G., Meiri, S. (2018): Cold and dark captivity: Drivers of amphibian longevity. Glob. Ecol. Biogeogr. **27**: 1384-1397.

Sullivan, B.K., Fernandez, P.J. (1999): Breeding activity, estimated age-structure, and growth in sonoran desert anurans. Herpetologica **55**: 334-343.

Tevis Jr, L. (1966): Unsuccessful breeding by desert toads (*Bufo punctatus*) at the limit of their ecological tolerance. Ecology **47**: 766-775.

Tiar-Saadi, M., Tiar, G., Bouslama, Z., Široký, P. (2022): Mechanisms determining body size and shape difference in Algerian spur-thighed tortoises (*Testudo graeca*). Animals **12**: 1-21.

Tonhasca-Júnior, A. (2005): Ecologia e História Natural da Mata Atlântica. Rio de Janeiro, Interciência.

Varjão, I.C.G., Ribeiro, L.B. (2018): Field record of aestivation with formation of cocoon in the frog *Leptodactylus fuscus* (Anura: Leptodactylidae) in a semiarid region of northeastern Brazil. Phyllomedusa **17**: 135-138.

Velloso, A.L., Sampaio, E.V., Giulietti, A.M., Barbosa, M.R.V., Castro, A.A.J.F., Queiroz, L.P., Fernandes, A., Oren, D.C., Cestaro, L.A., Carvalho, A.J.E. (2002): Ecorregiões Propostas para o Bioma Caatinga. Recife, Flamar Gráfica e Editora.

Vieira, W.L.S., Arzabe, C., Santana, G.G. (2007): Composição e distribuição espaço-temporal de anuros no Cariri paraibano, Nordeste do Brasil. Oecol. Bras. **11**: 383-396.

Werner, E.E. (1986): Amphibian metamorphosis: growth rate, predation risk, and the optimal size at transformation. Amer. Nat. **128**: 319-341.

Wiens, J.J., Pyron, R.A., Moen, D.S. (2011): Phylogenetic origins of local-scale diversity patterns and the causes of Amazonian megadiversity. Ecol. Lett. **14**: 643-652.

Woodward, B.D. (1983): Predator-prey interactions and breeding-pond use of temporary-pond species in a desert anuran community. Ecology **64**: 1549-1555.

Acta Herpetologica 20(1): 59-67, 2025
DOI: 10.36253/a_h-16116

New record and dietary ecology of a poorly known amphibian species, *Micryletta menglienica* (Yang and Su, 1980), from Vietnam

Anh Van Pham[1,*], Truong Quang Nguyen[2,3], Nenh Ba Sung[4], Thuy Van Tran[1], Minh Duc Le[1,5,6], Anh Ngoc Thi Ho[1], Hai Ngoc Ngo[2,*]

[1] *Faculty of Environmental Sciences, University of Science, Vietnam National University, Hanoi, 334 Nguyen Trai Road, Hanoi, Vietnam*
[2] *Institute of Biology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Hanoi 10072, Vietnam*
[3] *Graduate University of Science and Technology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay, Hanoi 10072, Vietnam*
[4] *Tay Bac University, Son La City, Son La Province, Vietnam*
[5] *Central Institute for Natural Resources and Environmental Studies, Vietnam National University, 19 Le Thanh Tong, Hanoi, Vietnam*
[6] *Department of Herpetology, American Museum of Natural History, Central Park West at 79th Street, New York, New York*
**Corresponding authors.* Email: ngohai2709@gmail.com; phamanh@hus.edu.vn

Submitted on: 2024, 4th May; revised on: 2024, 4th June; accepted on: 2025, 6th May
Editor: Wei Chen

Abstract. Recent field surveys revealed a new population of the Menglian Paddy Frog (*Micryletta menglienica*) from Son La Province, northwestern Vietnam based on molecular and morphological analyses. The species has been listed as "Data Deficient" in the IUCN Red List. To date, knowledge about the natural history of this species is scarce, including data on its dietary ecology. Using the stomach-flushing method, we analyzed stomach contents of 45 individuals (21 males and 24 females) from a total of 52 captured frogs because stomachs of six males and one female (13.5%) were empty. The food spectrum of *M. menglienica* comprises 12 types of 691 prey items (687 invertebrate items and four unidentified items), belonging to six insect orders, larvae and Araneae. Similar to other species of the family Microhylidae, *M. menglienica* is also identified as an ant (Formicidae) – termite (Rhinotermitidae) eating specialist with distinctly higher percentages of occurrence frequency (F = 65 %), number of prey items (N = 80 %), and importance (Ix = 75 %) than those of remaining types. The Simpson diversity index of 0.939 shows a high abundance of food prey in the wild, and the Evenness index of 0.536 suggests biased consumption of feeding selection for *M. menglienica*.

Keywords. Formicidae, prey items, Rhinotermitidae, Son La Province, stomach contents.

INTRODUCTION

Inhabiting both freshwater and terrestrial ecosystems, amphibians play a paramount role in maintaining the stability of food webs and energy flow between their habitats (Duellman and Trueb, 1994; Wells, 2007). In particular, amphibians transfer the energy intake by consuming many invertebrates, even small vertebrate species, to higher trophic levels by serving as an essential food source for several predatory animal groups (such as reptiles and mammals), accounting for a major part of animal biomass in ecosystems (Burton and Likens, 1975; Toledo et al., 2007; Oliveira et al., 2013). Thus, understanding the trophic niche may provide additional insights into species' natural history and biotic interactions (Schoener, 1974; Toft, 1980; Prado et al., 2005;

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

www.fupress.com/ah

Wells, 2007; Duré et al., 2009). To investigate this matter in amphibians, the stomach content is often obtained to identify which prey was eaten and their relative importance in the species' diet (Maneyro et al., 2004; Caldart et al., 2012; Le et al., 2018). In terms of conservation assessments, these analyses provide crucial data on conditions and resources required for species survival under human impacts (e.g., habitat loss, degradation, and overexploitation for trade and food consumption) that can imperil the stability in animal communities with intermittent trophic chains (Clavel et al., 2011). However, detailed information on the diet niche of many amphibian species is still lacking.

The paddy frogs of the genus *Micryletta* Dubois 1987 currently consist of 13 species, distributed from northeastern India and China through the Indochina region and expanding southward to west Malaysia and Sumatra (Frost, 2024). Remarkably, as many as eight species of *Micryletta* have been discovered in the last five years (Frost, 2024). The Menglian Paddy Frog, *Micryletta menglienica* (Yang and Su, 1980) was originally described based on type series collected in Menglien, Yunnan Province, southern China (Yang and Su, 1980; Liu et al., 2021) and subsequently recorded from Lang Son and Cao Bang provinces, northern Vietnam (Ohler and Grosjean, 2005). It has recently been reassigned to the genus *Micryletta* after having been placed in the genus *Kalophrynus* (Fei, 2020; Liu et al., 2021). The species is currently classified as Data Deficient in the IUCN Red List because little data on its population status and natural history are available (IUCN, 2023).

As a result of our field surveys in Son La Province, northwestern Vietnam, individuals of *Micryletta* were collected and their morphological characteristics closely resemble those of *Micryletta menglienica* as reported by Liu et al. (2021). We herein confirm the record of the species for the first time from Son La Province based on analyses of molecular and morphological data. In addition, to better understand the natural history of dietary ecology of the poorly known species, we analyzed the stomach content of 52 captured individuals to identify prey items and determined their relative importance in the species' diet.

MATERIALS AND METHODS

Field surveys and sampling

Field surveys were conducted in June, July, and September 2017 in two localities of Son La Province, northwestern Vietnam: the first site in Son La City (21°18.659'N, 103°46.956'E, at an elevation of 550 m) and the second one in Phong Lai Commune, Thuan Chau District (21°36.458'N, 103°33.980'E, at an elevation of 680 m) (Table 1, Fig. 1). The survey transects were set up with lengths ranging from 2.0 to 3.0 km, in limestone karst forests and open areas of grass and shrubs. We captured frogs by hand between 20:00 and 24:00h and used a stomach-flushing technique to obtain stomach contents without sacrificing them (Griffiths, 1986; Leclerc and Courtois, 1993; Solé et al. 2005; Norval et al., 2014). Prey items were preserved in 70% ethanol and deposited at the Faculty Environmental of Science, University of Science, Vietnam National University, Hanoi, Vietnam. Frogs were subsequently released at the collecting site after measurements of snout-vent length (SVL) and mouth width (MW) with a digital caliper to the nearest 0.01 mm taken. In total, 52 frogs, including 27 males and 25 females, were caught at two sites (Table 2).

Table 1. The number of paddy frogs were captured from Son La Province, northwestern Vietnam.

Locality	Time	Number of individuals		
		Male	Female	Total
Son La City	10-13 June 2017	5	3	8
	6-9 July 2017	3	4	7
	5-7 September 2017	2	0	2
Phong Lai Commune	16-19 June 2017	6	9	15
	15-18 July 2017	8	6	14
	11-13 September 2017	3	3	6
Total		27	25	52

Species identification

For taxonomic identification, four individuals were collected for voucher specimens. After having been photographed in life, animals were anesthetized and euthanized in a closed vessel with a piece of cotton wool containing ethyl acetate (Simmons, 2002), fixed in 85% ethanol and subsequently stored in 70% ethanol. Determination of species was based on morphology following Liu et al. (2021). We also sequenced two new samples of *Micryletta menglienica* collected from Son La Province. We used the protocols of Le et al. (2006) for DNA extraction, amplification, and sequencing. A fragment of 16S rRNA gene, approximately 480bp was amplified and sequenced using a primer pair 16Sar and 16Sbr (Palumbi et al. 1991). Sequences were compared with those available from GenBank using Basic Local Alignment Search Tool (BLAST) searches.

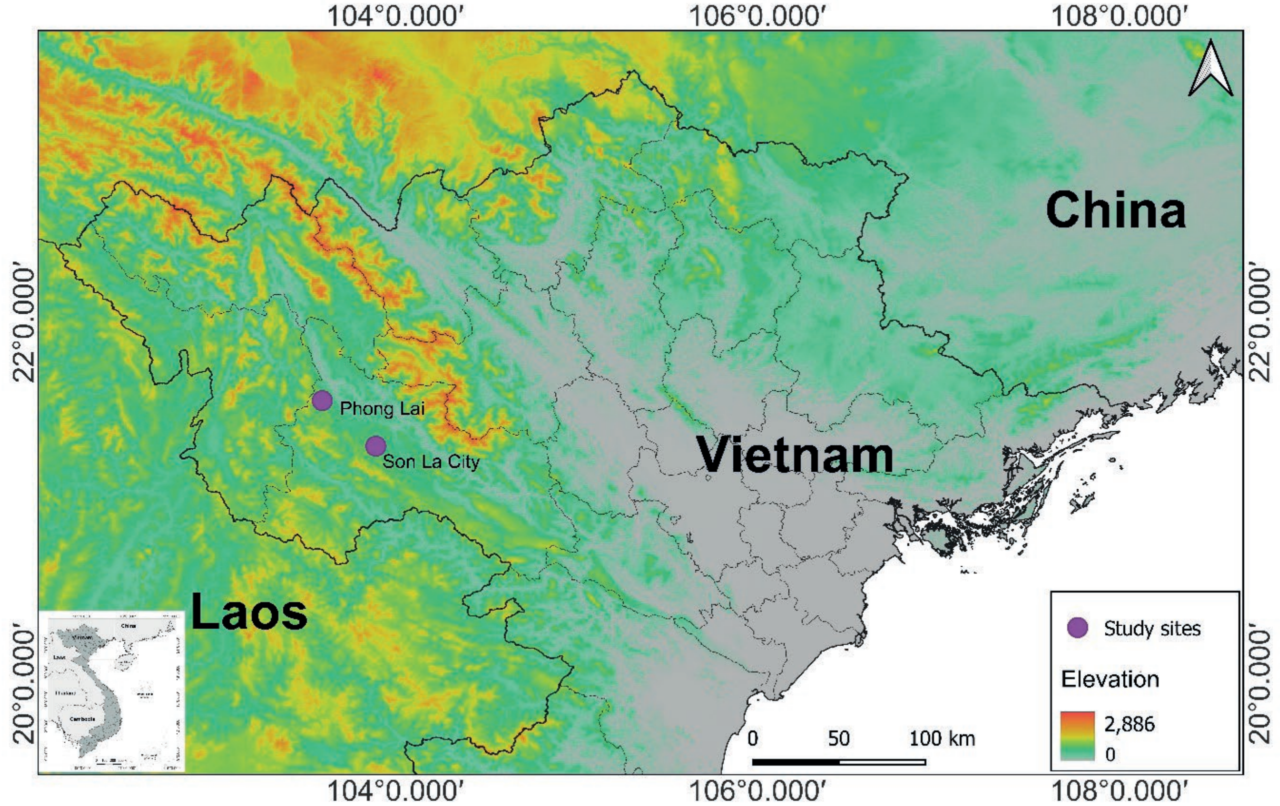

Fig. 1. Map of surveyed sites in Son La Province, northwestern Vietnam: (1) Son La City and (2) Phong Lai Commune.

Table 2. Sizes and volume of prey consumed by *Micryletta menglienica* in relation to morphological characteristics (SVL: snout-vent length, MW: mouth width, Wm: item-mean width, Wt: item-total width, Lm: item-mean length, and Lt: item-total length (Lt) of prey items; and asterisks (*) indicating the significant difference with the P-value < 0.05).

	Total	Female	Male	Test (Sex)	Test (Locality)	Test (Sex + Locality)	SVL	MW
Wm (mm)	1.33 ± 0.12 (0.2-5.0)	1.11 ± 0.09 (0.5-2.67)	1.58 ± 0.23 (0.2-5.0)	F = 4.4; P = 0.04 < 0.05*	F = 3.05; P = 0.09 > 0.05	F = 4.3; P = 0.04 < 0.05*	r = -0.18; P = 0.25 > 0.05	r = 0.02; P = 0.92 > 0.05
Wt (mm)	15.7 ± 2.56 (0.5-66.0)	18.5 ± 4.28 (0.5-66.0)	12.5 ± 2.4 (1.0-50.7)	F = 1.4; P = 0.24 > 0.05	F = 1.8; P = 0.19 > 0.05	F = 0.9; P = 0.3 > 0.05	r = 0.14; P = 0.34 > 0.05	r = 0.05; P = 0.72 > 0.05
Lm (mm)	2.8 ± 0.19 (0.64-7.23)	2..81 ± 0.25 (1.07-7.23)	2.78 ± 0.31 (0.64-5.8)	F = 0.008; P = 0.9 > 0.05	F = 0.01; P = 0.91 > 0.05	F = 0.4; P = 0.55 > 0.05	r = 0.06; P = 0.69 > 0.05	r = 0.24; P = 0.11 > 0.05
Lt (mm)	35.24 ± 6.6 (2.0-184.8)	46.32 ± 11.52 (2.0-184.8)	22.6 ± 4.01 (2.0-72.0)	F = 3.4; P = 0.07 > 0.05	F = 1.6; P = 0.2 > 0.05	F = 0.4; P = 0.54 > 0.05	r = 0.23; P = 0.14 > 0.05	r = 0.14; P = 0.35 > 0.05
Volume (mm^3)	44.02 ± 8.41 (0.26-224.6)	40.8 ± 10.9 (0.26-188.4)	47.7 ± 13.3 (0.3-224.8)	F = 0.16; P = 0.69 > 0.05	F = 0.27; P = 0.61 > 0.05	F = 1.9; P = 0.18 > 0.05	r = -0.06; P = 0.7 > 0.05	r = -0.02; P = 0.91 > 0.05

Stomach content analysis

In the laboratory, prey items were identified under microscopes (Olympus SZ 700) following taxonomic literature of invertebrates (i.e., Naumann et al., 1991; Johnson and Triplehorn, 2005). The maximum length (L) and width (W) of each prey item were measured to the nearest 0.01 mm using either a digital caliper or a calibrated ocular micrometer fitted to a microscope (Hirai and Matsui, 2001). The volume (V, mm^3) of prey items was calculated using the formula for a prolate spheroid ($\pi = 3.14$; Magnusson et al., 2003): $V = 4\pi/3 \times (L/2) \times (W/2)^2$. The index of relative importance (Ix), was used to determine the importance of each food type, which was calculated following the formula: Ix = (%F + %N + %V)/3 (Caldart et al., 2012), where %F (F – Frequency of occurrence) is a percentage of stomach containing each prey type, %N (N – Number) is a percentage of number of each prey item in all.

We used the reciprocal Simpson's heterogeneity index, 1-D, to calculate dietary heterogeneity: $D = [n_i(n_i - 1)]/[N(N - 1)]$; where n_i is the number of food items in the i^{th} taxon category and *N* is the total number of prey items (Krebs, 1999). We used another index to estimate prey evenness. The evenness index is calculated from the equation: $J' = H'/H_{max} = H'/\ln S$; where *S* is the total number of prey taxa and *H'* is the index of taxon diversity. The value of *H'* is calculated from the equation: $H' = -(p_i \times \ln p_i)$; where p_i is the proportion of food items belonging to the i^{th} taxon for the total food items of the sample (Magurran, 2004; Muñoz-Pedreros and Merino, 2014).

Two-way ANOVA tests were performed to determine differences or similarities in morphological characteristics of the species, sizes, and volume of ingested food between males and females, between populations, and the combined effects of "Sex + Location". Post hoc Tukey tests (TukeyHSD) were afterwards applied to check the difference between all pairs of sex groups in each population, and location groups in each sex after the ANOVA tests. Females were identified by having longer snout-vent length (SVL ≥ 21.0 mm), whereas males were characterized by a single internal vocal sac and shorter SVL (< 21.0 mm). All statistical analyses were performed by using the software environment R.3.1.2 (RStudio Team, 2018).

RESULTS

A new record of Micryletta menglienica *in Son La Province*

Pham et al. (2016) reported *Micryletta inornata* (Boulenger, 1890) from Co Ma Commune, Thuan Chau District, Son La Province, Vietnam. However, the specimens were re-identified herein as *M. menglienica* because two sequences (GenBank accession numbers PQ634813 and PQ634814) obtained from the collected samples in Son La Province were identical to each other and 99.35% similar to those with accession numbers OK335186 and OK335187 of *M. menglienica* available on GenBank. This is also the first record of *M. menglienica* from Son La Province, northwestern Vietnam.

Morphological characteristics of paddy frogs collected in Son La Province match well with the diagnosis of *Micryletta menglienica* (Liu et al., 2021): size small (SVL: 14.8-27.7 mm, n = 52); head wider than long; snout obtusely round in profile; the interorbital distance wider than upper eyelid; tympanum indistinct; supratympanic fold distinct; vomerine teeth absent; tibiotarsal articulation of adpressed limb reaching the level of eye; dorsal surface granular with small round tubercles; subtle longitudinal median ridge present on dorsum; supratympanic fold present; dorsolateral fold absent; outer metatarsal tubercle absent; dorsum coloration purple brown, blueish gray or dark brown; body flanks brown with dark-brown to black patches or spots edged with white, a large black streak extending on each side from tip of the snout to crotch; ventral surface greyish brow, whitish with light-gray marbling patterns on chest and belly (Fig. 2).

Regarding sexual dimorphism, males have a shorter snout-vent length (SVL: 19.03 ± 1.95 mm, ranging 14.8-20.8 mm, n = 27; ANOVA-test, F = 124.2, P-value < 0.001) and a narrower mouth width (MW: 5.6 ± 0.73 mm, ranging 4.4-6.7 mm, n = 27; F = 52.1, P-value < 0.001) than those of females (SVL: 24.73 ± 1.4 mm, ranging 21.5-27.7 mm and MW: 6.77 ± 0.49 mm, ranging 6.0-7.8 mm, n = 25, respectively). Together with the effect of locations, the tests did not approve the general difference in both snout-vent length and head width (P-values > 0.05). In particular, the intra-sexual variations in both morphological traits of *M. menglienica* were not presented between two populations of Son La City and Phong Lai Commune (Tukey's HSD tests, P-values < 0.001), whereas males' traits were significantly different to females', noted in each population (Tukey's HSD tests, P-values < 0.001).

In terms of natural history, paddy frogs were found between 19:00 and 24:00 at elevations between 550 and 680 m a.s.l. The surrounding habitat was the limestone karst forest of small hardwoods, shrubs and grasses (Fig. 2). The relative humidity was approximately 70-85% and the air temperature ranged from 25 to 30°C. Other sympatric amphibian species were also observed, including *Leptobrachella* sp., *Microhyla butleri* Boulenger 1900, *M. heymonsi* Vogt 1911, and *Polypedates megacephalus* Hallowell 1861.

Dietary ecology

A total of 52 individuals (27 males and 25 females) of *M. menglienica* were captured in Son La City (17 individuals) and Thuan Chau District (35 individuals) of Son La Province. All caught individuals were checked for stomach contents, except for seven frogs (13.5%), whose stomachs were empty. Based on the obtained contents, we identified a total of 691 ingested food items (687 invertebrates and four unidentified items) belonging to 12 different prey types of six insect orders: Blattodea, Coleoptera (Coccinel-

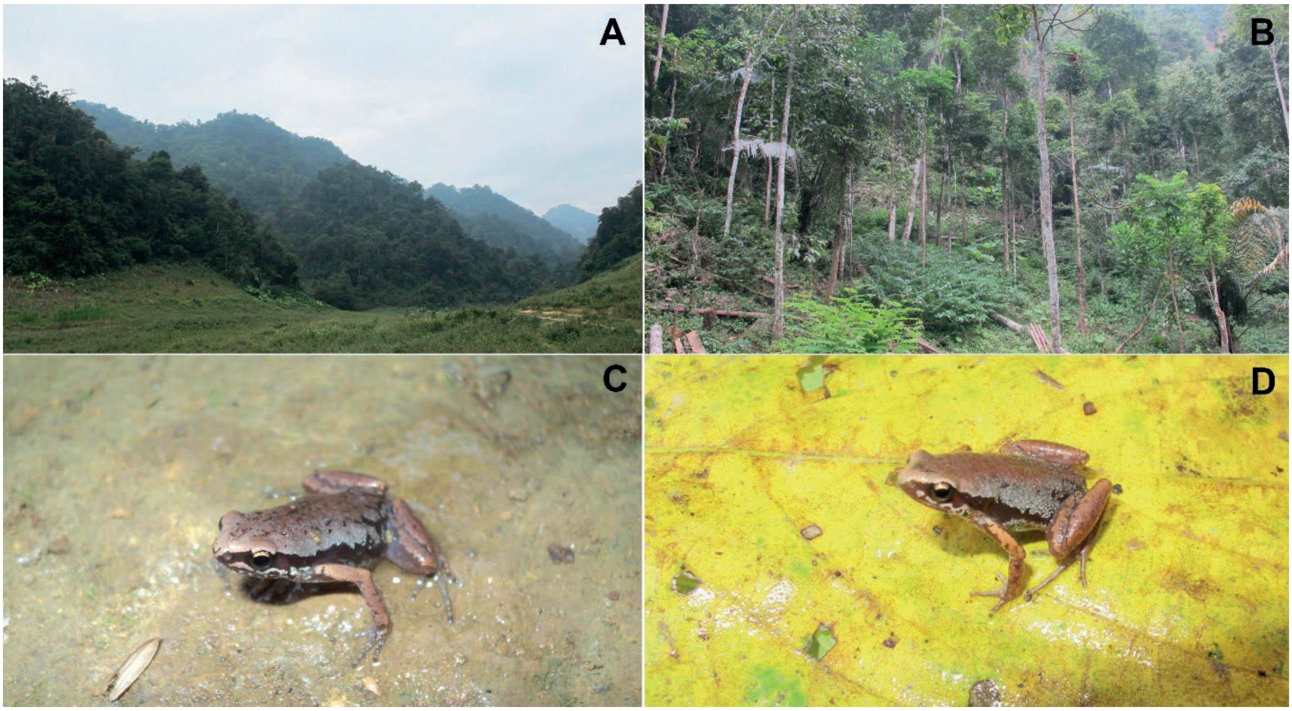

Fig. 2. Natural habitat and an individual of *Micryletta menglienica* A, C. in Phong Lai Commune, B, D. in Son La City.

lidae, Staphylinidae, Tenebrionidae, and Other Coleoptera), Diptera (Syrphidae, Other Diptera), Hymenoptera (Formicidae), Isoptera (Rhinotermitidae), and Orthoptera (Gryllidae), Insect larvae, and Spiders (Araneae).

In terms of size measurements, the item-mean width (Wm) of ingested prey was 1.33 ± 0.12 mm (0.2-5.0 mm), the item-total width (Wt) was 15.7 ± 2.56 (0.5-66.0 mm), the item-mean length (Lm) was 2.8 ± 0.19 mm (0.64-7.23 mm), and the item-total length (Lt) was 35.24 ± 6.6 (2.0-184.8 mm) (Fig. 3, Table 2). We calculated the mean volume of 44.02 ± 8.41 mm^3 (0.26-224.6 mm^3). There was only a significant difference in the item-mean width (Wm) of consumed prey between males and females, and the interaction of "Sex + Locality" (Two-way ANOVA tests, P-values = 0.04 < 0.05), whereas remaining prey characteristics (i.e., Wt, Lm, Lt and volume) were relatively similar between intraspecific sexes, localities and combined effect of "sex + locality" (P-values > 0.05; Fig. 3, Table 2). Our multiple linear regression analyses showed that the sizes and volume of ingested prey are not significantly correlated with morphological traits of SVL and MW of the species (Table 2).

The prey of *M. menglienica* mainly comprised two invertebrate families of Formicidae and Rhinotermitidae, which accounted for more than 65% of the frequency of occurrence (F) and 80% of the number of prey items (N) (Table 3). The two invertebrate families were also the most important prey of the species, accounting for more than 75% of Ix (Table 3).

The dietary breadth of *M. menglienica* from Son La Province, expressed by the Simpson's index of diversity was 0.939, and evenness was 0.536 (Table 4). Males of *M. menglienica* had higher values of both Simpson index (0.926) and evenness (0.783) than those of females (Table 4). The population of *M. menglienica* in Son La City consumed less diverse prey than the population in Thuan Chau District (Table 4).

DISCUSSION

In this study, *M. menglienica* was recorded for the first time in Son La Province, which is approximately 350 km from the type locality of the species in Puer City, Yunnan Province, China. With the new record of *M. menglienica* in Son La Province, we recommend additional field surveys be conducted in other karst ecosystems in northern Vietnam to look for new populations of the species and better understand the species distribution range.

A total of 12 different prey types belonging to six invertebrate orders, larvae and spiders (Araneae) were identified as the food spectrum of *M. menglienica*, in which ants (Formicidae) and termites (Rhinotermiti-

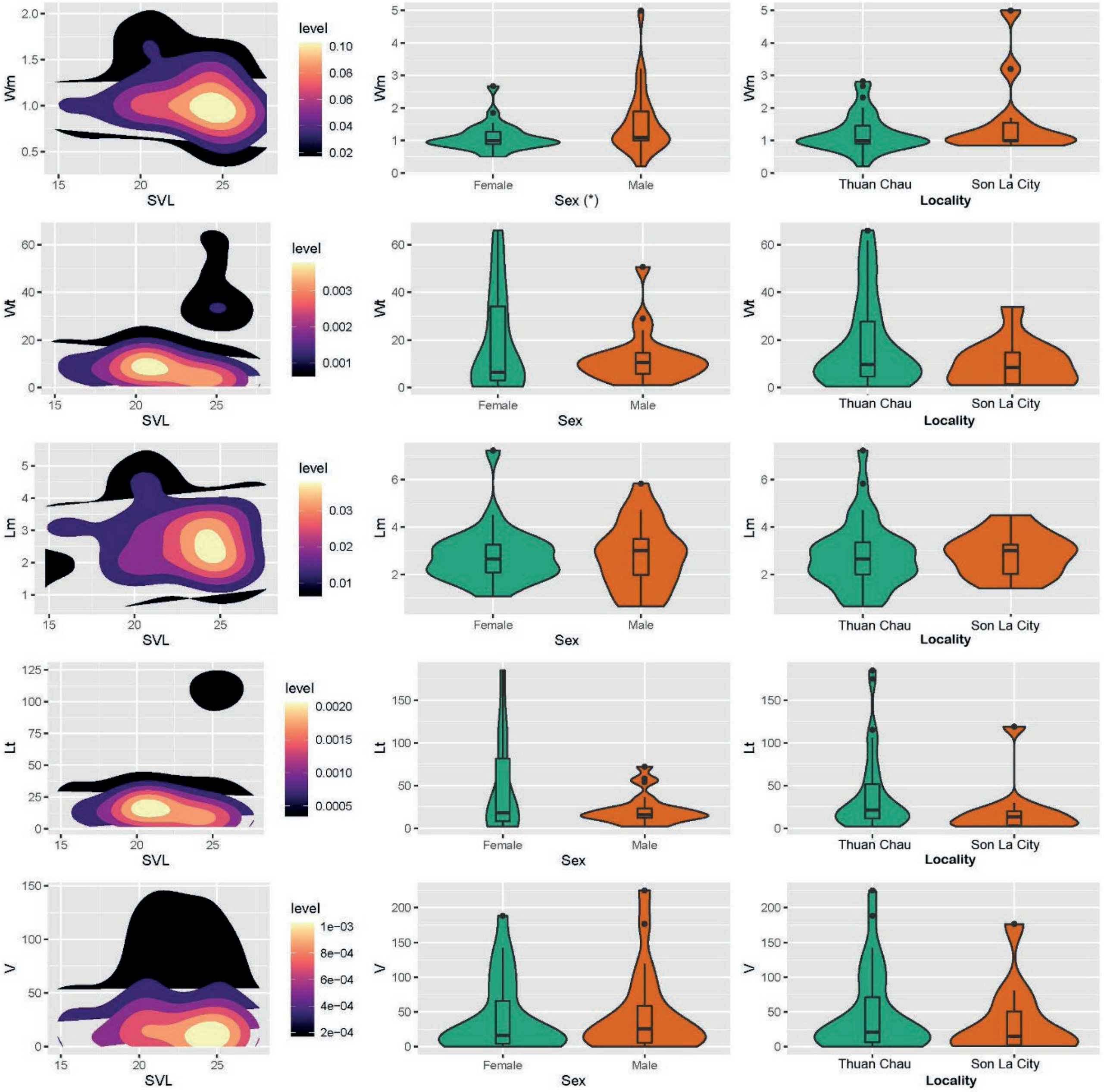

Fig. 3. Size and volume of prey consumed by *Micryletta menglienica* in relation to snout-vent length (left column), to sex (middle column), and locality (right column) (Wm: item-mean width, Wt: item-total width, Lm: item-mean length, Lt: item-total length, and V: volume of prey items; and asterisks (*) indicating the significant difference with the P-value < 0.05).

dae) were determined as the most dominant and important prey of the species. Anurans, especially in the family Microhylidae, have been widely recognized as ant or sometimes termite specialists (Toft, 1980; Isacch and Barg, 2002; Sole et al., 2002; Berazategui et al., 2007; Atencia et al., 2017; Lopes et al., 2017).

In this study, we did not find any intersexual difference in the sizes and volume of prey eaten by *M. menglienica*. Competitive interactions among conspecifics for sharing resources could take place between males and females (Schoener, 1974; Irschick et al., 2005; van Schingen et al., 2015). Our estimation based on the number of food items for indices of Simpson diversity (more than 0.88) showed a high abundance of prey at the studied sites, but not evenly among food types (0.536 – Evenness index). Furthermore, females' prey (Evenness index

Table 3. Dietary composition of *Micryletta menglienica* in Son La Province: F = frequency of occurrence, N = number of items, V = volume (mm3), Ix = index of relative importance of each prey type (n = 45 stomach contents)

Prey type	F	%F	N	%N	V	%V	Ix
Araneae	1	1.41	1	0.14	1.57	0.08	0.54
Blattodea	1	1.41	1	0.14	20.12	1.02	0.86
Coleoptera							
Coccinellidae	2	2.82	2	0.29	9.42	0.48	1.19
Staphylinidae	3	4.23	3	0.43	4.31	0.22	1.63
Tenebrionidae	1	1.41	1	0.14	2.74	0.14	0.56
Other Coleoptera	6	8.45	9	1.30	219.35	11.07	6.94
Diptera							
Syrphidae	1	1.41	1	0.14	5.65	0.29	0.61
Other Diptera	1	1.41	3	0.43	36.24	1.83	1.22
Hymenoptera							
Formicidae	36	50.70	476	68.89	632.57	31.94	50.51
Isoptera							
Rhinotermitidae	11	15.49	91	13.17	901.84	45.53	24.73
Orthoptera							
Gryllidae	2	2.82	2	0.29	12.04	0.61	1.24
Insect larvae	2	2.82	97	14.04	14.26	0.72	5.86
Unidentified	4	5.63	4	0.58	120.67	6.09	4.10
Total	71	100	691	100	1980.77	100	100

Table 4. Prey diversity and evenness indices of *Micryletta menglienica,* expressed with value (95 % confidence limits).

	Simpson 1-D	Evenness
Species (*Micryletta menglienica*)	0.939 (0.930-0.9438)	0.536 (0.495-0.573)
Male	0.926 (0.909-0.933)	0.783 (0.684-0.825)
Female	0.8896 (0.877-0.899)	0.50 (0.471-0.562)
Son La City	0.761 (0.670-0.818)	0.566 (0.466-0.697)
Phong Lai Commune	0.9255 (0.9158-0.932)	0.5647 (0.516-0.599)

= 0.5) was more biased than those in males (0.783). All of these can reduce intersexual competition. Pham et al. (2022) studied the diet niche of *Microhyla butleri* and *M. heymonsi* in Son La Province and showed that both sympatric species primarily consumed ants and termites as well. However, there were significant differences in the trophic niche between them to limit interspecific competition. In particular, 11 prey types of *M. butleri* were not found in the food spectrum of *M. heymonsi* and nine prey types were not presented in vice versa. Furthermore, they preferred to consume significantly different prey in the sizes and volume (Pham et al. 2022).

Regarding human impacts, karstic forest degradation caused by infrastructure development (e.g., road expansion, development of residential areas, and limestone quarrying) has been documented in Son La City (Pham pers. obs), which could explain the low number of *M. menglienica*. Only 17 individuals of *M. menglienica* were captured in Son La City during three surveys, compared to half of captured animals (n = 35) in less disturbed habitats in Phong Lai Commune. Such human impacts particularly destabilize its wild populations and food availability. However, similar to what is happening in Son La Province, unsustainable human activities (particularly quarrying and tourist activities) over karst ecosystems in northern Vietnam, can extensively impact undiscovered populations of the species in unique karstic habitats (Clemens et al. 2006). Therefore, conservation measures are needed to protect the species, although *M. menglienica* has not been targeted for the pet trade and food consumption, and its geographical distribution is potentially extended. However, to date, no conservation plan has been developed to safeguard the poorly known species as well as its natural habitats. Besides the investigation of dietary ecology, we highly recommend further studies to identify the entire geographical extent, assess population status, and determine other ecological traits of *M. menglienica*. Only when the baseline data becomes available, appropriate conservation measures can be designed to better safeguard the species from current extinction risks.

ACKNOWLEDGMENTS

We thank T.V. Dau and Q.T. Bui (Son La) for their assistance in the field. Fieldwork and collection of specimens were approved by the Forest Protection Department of Son La Province (permit No. 22/GT-SL).

REFERENCES

Atencia, P., Conde, Ó., Solano, L. (2017): Use of biological resources by *Elachistocleis pearsei* (Ruthven, 1914) (Anura: Microhylidae) in a lentic ecosystem from the department of Sucre, Colombia. Acta Zool. Mex. **33**: 130-132.

Berazategui, M., Camargo, A., Maneyro, R. (2007): Environmental and seasonal variation in the diet of *Elachistocleis bicolor* (Guérin-Méneville 1838) (Anura: Microhylidae) from northern Uruguay. Zool. Sci. **24**: 225-231.

Burton, T.M., Likens, G.E. (1975): Energy flow and nutrient cycling in salamander populations in the Hubbard Brook Experimental Forest, New Hampshire. Ecology. **56**: 1068-1080.

Caldart, V.M., Lop, S., Bertaso, T.R.N., Zanini, C. (2012): Feeding ecology of *Crossodactylus schmidti* (Anura: Hylodidae) in southern Brazil. Zool. Stud. **51**: 484-493.

Clavel, J., Julliard, R., Devictor, V. (2011): Worldwide decline of specialist species: toward a global functional homogenization? Front. Ecol. Environ. **9**: 222-228.

Clements, R., Sodhi, N. S., Schilthuizen, M., Ng, P. K. (2006): Limestone karsts of Southeast Asia: imperiled arks of biodiversity. Bioscience **56**: 733-742

Duellman, W.E., Trueb, L. (1994): Biology of Amphibians. The John Hopkins University Press, Baltimore, USA.

Duré, M.I., Kehr, A.I., Schaefer, E.F. (2009): Niche overlap and resource partitioning among five sympatric bufonids (Anura, Bufonidae) from northeastern Argentina. J. Herpetol. **8**: 27-39.

Fei, L. (2020): Atlas of Amphibians in China (Field Edition). Zhengzhou, Henan science and Technology Press.

Frost, Darrel. R. (2024): Amphibian Species of the World: an online reference. American Museum of Natural History, New York, USA. Available: https://amphibiansoftheworld.amnh.org/index.php. [Accessed: 02 February 2024]

Griffiths, R.A. (1986): Feeding niche overlap and food selection in Smooth and Palmate newts, *Triturus vulgaris* and *T. helveticus*, at a pond in mid-Wales. J. Anim. Ecol. **55**: 201-214.

Hirai, T., Matsui, M. (2001): Diet composition of the Indian rice frog, *Rana limnocharis*, in rice fields of central Japan. Curr. Herpetol. **20**: 97-103.

Irschick, D.J., Vanhooydonck, B., Herrel, A., Meyers, J.J. (2005): Intraspecific correlations among morphology, performance, and habitat use within a green anole lizard (*Anolis carolinensis*) population. Biol. J. Linn. Soc. **85**: 211-221.

Isacch, J.P., Barg, M. (2002): Are bufonid toads specialized ant feeders? A case test from the Argentinian flooding pampa. J. Nat. Hist. **36**: 2005-2012.

IUCN. (2023): The IUCN Red List of Threatened Species. Available: https://www.iucnredlist.org [Accessed: 10 January 2023]

Johnson, N.F., Triplehorn, C.A. (2005): Borror and Delong's Introduction to the Study of Insects. Belmont, California, USA, Thomson Learning Inc.

Krebs, C.J. (1999): Ecological Methodology. Menlo Park, California, Addison Wesley Longman.

Le, D.M., Raxworthy, C.J., McCord, W.P., Mertz, L. (2006): A molecular phylogeny of tortoises (Testudines: Testudinidae) based on mitochondrial and nuclear genes. Mol. Phylogenet. Evol. **40:** 517-531.

Le, T.T.D., Rowley, J.L.J., Tran, T.A.D., Vo, N.T., Hoang, D.H. (2018): Diet composition and overlap in a montane frog community in Vietnam. Herpetol. Conserv. Biol. **13**: 205-215.

Leclerc, J., Courtois, D. (1993): A simple stomach flushing method for ranid frogs. Herpetol. Rev. **24**: 142-143.

Liu, S., Yang, B., Wang, Q., Hou, M. (2021): Taxonomic reassessment of the poorly known microhylid, *Kalophrynus menglienicus* Yang & Su, 1980. Herpetozoa **34**: 223-232.

Lopes, M.S., Bovendorp, R.S., Moraes, G.J.D., Percequillo, A.R., Bertoluci, J. (2017): Diversity of ants and mites in the diet of the Brazilian frog *Chiasmocleis leucosticta* (Anura: Microhylidae). Biota Neotrop. **17**: 1-5.

Magnusson, W.E., Lima, A.P., Silva, W.A., Araújo, M.C. (2003): Use of geometric forms to estimate volume of invertebrates in ecological studies of dietary overlap. Copeia **1**: 13-19.

Magurran, A.E. (2004): Measuring Biological Diversity. Malden, Massachusetts, USA, Blackwell Science.

Maneyro, R.D.E., Naya, I.R., Canavero, A., Camargo, A. (2004): Diet of the South American frog *Leptodactylus ocellatus* (Anura, Leptodactylidae) in Uruguay. Iheringia Sér. Zool. **94**: 57-61.

Muñoz-Pedreros, A., Merino, C. (2014): Diversity of aquatic bird species in a wetland complex in southern Chile. J. Nat. Hist. **48**: 1453-1465.

Naumann, I.D., Came, P.B., Lawrence, J.F., Nielsen, E.S., Spradbery, J.P., Taylor, R.W., Whitten, M.J., Littlejohn, M.J., Eds. (1991): The Insects of Australia. 2 Vols, Division of Entomology, CSIRO, Australia. Melbourne University Press, Melbourne, Australia, and Cornell University Press, Ithaca, New York.

Norval, G., Huang, S.C., Mao, J.J., Goldberg, S.R., Yang, Y.J. (2014): Notes on the diets of five amphibian species from southwestern Taiwan. Alytes. **30**: 69-77.

Ohler, A., Grosjean, S. (2005): Color pattern and call variation in *Kalophrynus* from south-east Asia. Herpetozoa **18**: 99-106.

Oliveira, M.C.L.M., Santos, M.B., Loebmann, D., Hartman, A., Tozetti, A.M. (2013): Diversity and associations between coastal habitats and anurans in southernmost Brazil. An. Acad. Bras. Cienc. **85**: 575-584.

Palumbi, S.R., Martin, A., Romano, S., McMillan, W.O., Stice, L., Grabowski, G. (1991): The simple fool's guide to PCR. Department of Zoology and Kewalo Marine Laboratory, Hawaii, USA.

Pham, V.A., Ngo, D.C., Nguyen, Q.T., Ngo, V.B. (2022): Diet of *Microhyla butleri* and *M. heymonsi* from Son La Province, Northwest, Vietnam. Herpetol. Conserv. Biol. **17**: 217-224.

Pham, V.A., Nguyen, Q.T., Nguyen, L.H.S., Tu, V.H., Song, B.N., Giang, A. H. (2016): Diversity of the Narrow-mouth frogs (Amphibia: Anura: Microhylidae) from Son La Province. Proceedings of the 2st National scientific conference on Biological research and teaching in Vietnam. **2**: 37-42.

Prado, C.P.A., Uetanabaro, M., Haddad, C.F.B. (2005): Breeding activity patterns, reproductive modes, and habitat use by anurans (Amphibia) in a seasonal environment in the Pantanal, Brazil. Amphib.-Reptil. **26**: 211-221.

RStudio Team. (2018): RStudio: Integrated Development for R. RStudio, Inc., Boston, MA, USA. Available at: https://www.rstudio.com/.

Schoener, T.W. (1974): Resource partitioning in ecological communities. Science **185**: 27-39.

Simmons, J.E. (2002): Herpetological Collecting and Collections Management, 2nd ed. Lawrence, Kansas: Society for the Study of Amphibians and Reptile.

Solé, M., Beckmann, O., Pelz, B., Kwet, A., Egels, W. (2005): Stomach-flushing for diet analysis in anuran: an improved protocol evaluated in a case study in Araucaria forests, southern Brazil. Stud. Neotrop. Fauna Environ. **40**: 23-28.

Solé, M., Ketterl, J., Di-Bernardo, M., Kwet, A. (2002): Ants and termites are the diet of the microhylid frog *Elachistocleis ovalis* (Schneider, 1799) at an Araucaria forest in Rio Grande do Sul, Brazil. Herpetol. Bull. **79**: 14-17.

Toft, C.A. (1980): Feeding ecology of thirteen syntopic species of anuran in a seasonal tropical environment. Oecologia **45**: 131-141.

Toledo, L.F., Silva, R.R., Haddad, C.F.B. (2007): Anurans as prey: an exploratory analysis and size relationships between predators and their prey. J. Zool. **271**: 170-177.

van Schingen, M., Pham, C.T., An, H.T., Nguyen, T.Q., Bernardes, M., Bonkowski, M., Ziegler, T. (2015): First ecological assessment of the endangered crocodile lizard *Shinisaurus crocodilurus* Ahl, 1930 in Vietnam: Microhabitat characterization and habitat selection. Herpetol. Conserv. Biol. **10**: 948-958.

Wells, K.D. (2007): The Ecology and Behavior of Amphibians. Chicago, Illinois, USA, University of Chicago Press.

Yang, D.T., Su, C.Y. (1980): A new species of the family Microhylidae frog from Yunnan. Zool. Res. **1**: 257-260.

Acta Herpetologica 20(1): 69-74, 2025
DOI: 10.36253/a_h-16853

Estimation of the body condition of European cave salamanders (genus *Speleomantes*) from digital images

Eleonora Cialente[1,*], Ben Oetken[2], Luca Coppari[1,3], Enrico Lunghi[1,3]

[1] *Dipartimento di Medicina clinica, Sanità pubblica, Scienze della Vita e dell'Ambiente (MeSVA), Università degli Studi dell'Aquila, via Vetoio, Coppito (AQ), 67100 - L'Aquila, Italy*
[2] *Zoologisches Institut – Technische Universität Braunschweig, Mendelssohnstraße 4, 38106 Braunschweig - Braunschweig, Germany*
[3] *Aquila, via dell'Aringo, Preturo (AQ), 67100 - L'Aquila, Italy*
**Corresponding author.* Email: eleonora.cialente@student.univaq.it

Submitted on: 2024, 11th November; revised on: 2024, 3rd December; accepted on: 2025, 3rd March
Editor: Giacomo Rosa

Abstract. Species monitoring is a key activity for conservation studies. Some of the monitoring methods require individual handling, which may provoke negative effects on animal fitness. We here present a new non-invasive method that allows to estimate the body condition of the European cave salamanders (genus *Speleomantes*) using the tail area as a proxy, since these species usually accumulate fat tissue in their tail. We selected 915 high-quality images of individuals belonging to the eight *Speleomantes* species. Using the ImageJ program we calculated the tail measurements (length and area) from which we obtained the Fat Tail Index (FTI). The FTI was then correlated with the Scaled Mass Index (SMI) of individuals. We used GLMM to assess whether SMI is correlated to FTI, individual sex, species identity and the type of inhabited environment. We observed a significant correlation between SMI and FTI ($R^2 = 0.62$). The GLMM analysis showed a significant effect on SMI due to sex, species and the type of environment. Females and individuals from surface environments showed the highest SMI. Among species, we observed a significant variability in their body condition and in the correlation between SMI and FTI. This study provided a reliable and non-invasive method that allows to estimate the body condition for terrestrial salamanders of the genus *Speleomantes*.

Keyword. Scaled mass index, conservation, *Hydromantes*, monitoring, morphometry, photography.

INTRODUCTION

Monitoring is a key activity for species conservation (Beebee and Griffiths, 2005). It consists of repeated sampling activities that allow us to collect important information on the status of the species and on the ecological features that characterise their environment (McCravy, 2018; Lunghi et al., 2020a). Species monitoring provides direct information on the population consistency and the individuals' fitness (Ficetola et al., 2018a; Lunghi et al., 2022). It can be performed by adopting non-invasive methods such as counting the observed individuals (Ficetola et al., 2018a), or methods that require animals' handling (Dunn and Ralph, 2004; Lunghi and Bruni, 2018). Capture-mark-recapture and the record of biometrics parameters represent powerful tools for collecting important data on species; however, they may provide negative side effects on individuals. For example, invasive marking methods (e.g., toe-clipping) can affect individuals' behaviour and negatively impact their fitness (Golay and Durrer, 1994; Davis and Ovaska, 2001). Negative effects could also be provoked by low-impact techniques such as handling. Individual handling can be a direct source of pathogen transmission, and it can also represent a source of stress that weakens the individual immune system and increases its exposure to potential pathogens (Bliley and Woodley, 2012; Lunghi et al., 2016).

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

www.fupress.com/ah

During the last decades, there has been a tendency to adopt less invasive methods aiming to reduce the negative effects on individuals without compromising the quality of data (Soto-Azat et al., 2009; Perry et al., 2011; Gabor et al., 2013). For example, biocompatible compounds (e.g., Visible Implant Elastomers) or distinctive individual patterns are preferred over invasive toe-clipping to individually recognize wild animals (Speybroeck and Steenhoudt, 2017; Lunghi and Bruni, 2018). The use of digital photography in conservation studies further contributes to reducing the negative effects on individuals. Indeed, digital datasets composed of high-quality images have been demonstrated to be a valuable source of information for species monitoring and conservation (Husain et al., 2017).

In this study, we aimed to test a new method that allows to predict the body condition (defined as the amount of fat stored; Wilder et al., 2016) in European cave salamanders (genus *Speleomantes*) from digital images. The genus *Speleomantes* includes eight strictly protected amphibian species that are endemic (or subendemic) to Italy (Lanza et al., 2006; Rondinini et al., 2022). *Speleomantes* are fully terrestrial troglophile species (see Howarth and Moldovan, 2018) inhabiting both surface and subterranean environments (Costa et al., 2016; Ficetola et al., 2018b). They are generalist predators that prey on a wide range of invertebrate species (Cianferoni and Lunghi, 2023) and use their tails as organs to accumulate fat tissue (Wake and Dresner, 1967; Fitzpatrick, 1973). Accordingly, the area of the tail should provide reliable information on the amount of fat stored by individuals, which can be considered a fitness-related trait (Wilder et al., 2016). Previous studies have shown a positive correlation between tail width and body condition in urodeles (Bendik and Gluesenkamp, 2013); however, this seemed to not apply to *Speleomantes* (Rosa et al., 2021). In both studies only the width at the tail base was considered, overlooking the potential that the rest of the tail may have in energy storage. We here specifically tested the use of the overall tail area as a reliable method for predicting the body condition of individuals.

MATERIALS AND METHODS

We analysed high-quality images of *Speleomantes* from different datasets (Lunghi et al., 2020c; Lunghi et al., 2021b; Coppari et al., 2024). Pictures were taken directly in the field and showed a dorsal view of individuals in their natural position (Fig. 1) (Lunghi et al., 2021a). We randomly selected photos of 915 individuals (Table 1), for which sex, weight, and total length were known. To avoid introducing bias, we did not include pictures of individuals with tail issues and gravid females with visible eggs in our dataset. Due to the possibility of recognizing individuals of *Speleomantes* from the dorsal pattern (Lunghi et al., 2019), pictures taken on the same population but during different periods were checked to avoid pseudoreplication. We used the program ImageJ to obtain measurements of the tail, which extends from the end of the cloaca (tail base) to the tip of the tail (Fig. 1) (Lunghi et al., 2020b). We measured the tail length and area. We divided the tail area for its length to produce a standardized Fat Tail Index (FTI), which should be linked to the amount of adipose tissue the individual stored in its tail (Wake and Dresner, 1967; Fitzpatrick, 1973). We used the Scaled Mass Index (SMI) as a reference to evaluate the reliability of the FTI in predicting *Speleomantes*' body condition. The SMI is a reliable index used to estimate fat stored in amphibians (MacCracken and Stebbings, 2012; Rosa et al., 2021), and it is based on the relationship between the body mass of individuals and a linear predictor of body size that accounts for allometric growth (Peig and Green, 2009). Considering the size difference between juveniles and adults and

Fig. 1. An example of the image used in this study; in red the section of the tail area used to estimate individuals' body condition (scale bar = 10 mm).

Table 1. Summary of the images used in this study.

Species	Number of juveniles	Number of males	Number of females	Total
S. italicus	30	56	55	141
S. supramontis	46	31	44	121
S. sarrabusensis	36	21	17	74
S. ambrosii	62	29	49	140
S. strinatii	47	41	35	123
S. imperialis	41	30	15	86
S. genei	41	41	40	122
S. flavus	29	34	45	108
Total	332	283	300	915

Table 2. Parameters related to model selection for testing the significant correlation between SMI and FTI in *Speleomantes*. We here compare the full model (SMI ~ FTI *Domain + FTI *Species + FTI *Sex) with a series of reduced models to evaluate the usefulness of each interaction. The best model (lowest AIC) failed the collinearity test (see text), and therefore, we chose the second-best model (bold), which does not have the same issue. Df = degree of freedom; AIC = Akaike information criterion; BIC = Bayesian information criterion; ΔDf = difference of degrees of freedom.

Model	Df	AIC	BIC	Log-Likelihood	Deviance	Chi-Square	ΔDf	*P*-value
FTI*Domain + Species + FTI*Sex	15	566.89	639.17	-268.445	536.89			
Domain + Species + Sex	15	566.89	639.17	-268.445	536.89	0	0	
FTI*Domain + Species + Sex	16	539.94	617.41	-253.971	507.94	28.949	1	< 0.001
Domain + Species + FTI*Sex	17	567.86	649.78	-266.929	533.86	0	1	1
Domain + FTI*Species + Sex	**22**	**237.08**	**343.09**	**-96.538**	**193.08**	**340.781**	**5**	**< 0.001**
FTI*Domain + FTI*Species + Sex	23	239.07	349.91	-96.537	193.07	0.001	1	0.970
Domain + FTI*Species + FTI*Sex	24	225.96	341.62	-88.981	177.96	15.113	1	< 0.001
FTI*Domain + FTI*Species + FTI*Sex	25	227.43	347.90	-88.714	177.43	0.534	1	0.465

between the different *Speleomantes* species (Lanza et al., 2006; Lunghi, 2022), we estimated the SMI for each age class (adult vs juveniles) and species separately. We used Generalized Linear Mixed Models (GLMM) implemented in R Studio to evaluate how well FTI correlates with SMI. The SMI was the dependent variable, while FTI, individuals' sex, species, and habitus (surface vs. subterranean) were independent factors. The use of sex and species allowed us to evaluate the potential divergences occurring between sexes or between species (Lanza et al., 2006; Rosa et al., 2021). Including the habitus among independent variables allowed us to assess whether subterranean populations might show higher efficiency in fat storage, a feature observed in other cave vertebrates (Lunghi and Zhao, 2020). Population identity and the sampling period were included as random factors to account for geographical and seasonal variance in individuals' body condition (Lunghi et al., 2022). We added the interactions between FTI and the other three variables (sex, species, habitus) as additional predictors. The FTI variable was log-transformed and then centred on its mean to mitigate collinearity. Model selection was performed based on the AIC criterion and through the Likelihood Ratio Test. The model with the lowest AIC showed severe collinearity issues (Variance Inflation Factor, VIF > 10), making parameter estimates unreliable. Therefore, we selected a slightly higher AIC model with acceptable collinearity levels (VIF < 7) to ensure stability and interpretability (Table 2).

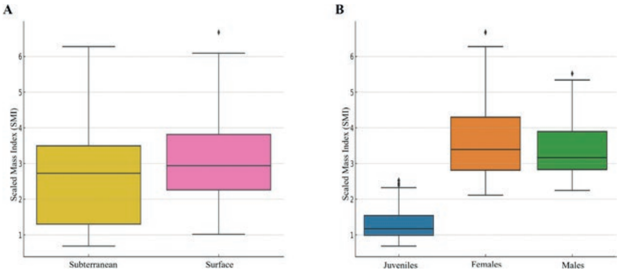

Fig. 2. Results of the GLMM showing the effects of the environment (A) and of the individual sex (juveniles, adult females, adult males) (B) on the individual SMI. Boxes delimit the 2nd and 3rd percentile; bars inside boxes represent the median; the whiskers represent the standard deviation; the asterisks indicate outliers.

RESULTS

We identified a significant correlation of SMI with FTI (χ^2 (1) = 204.07, $P < 0.001$), habitus (χ^2 (1) = 5.73, $P = 0.017$), species (χ^2 (7) = 110.64, $P < 0.001$), sex (χ^2 (2) = 5400.25, $P < 0.001$) and with the interaction between FTI and species (χ^2 (7) = 424.90, $P < 0.001$). The SMI was higher in surface populations (β =0.159, SE = 0.07, $P = 0.017$) (Fig. 2A) and in females (males, β = -0.05, SE = 0.01, $P < 0.001$; juveniles, β = -0.93, SE = 0.01, $P < 0.001$) (Fig. 2B). The correlation between SMI and FTI significantly diverged between *Speleomantes* species (Fig. 3, Supplementary Materials Fig. S1). This model showed a very high explanatory power (marginal R^2 = 0.95, conditional R^2 = 0.96).

DISCUSSION

Our study identified the Fat Tail Index (FTI) as a reliable method to estimate individual body condition regarding the amount of energy reserves. The correlation between FTI and SMI was strong and relatively high (R^2 = 0.62), indicating that this index can be reliably

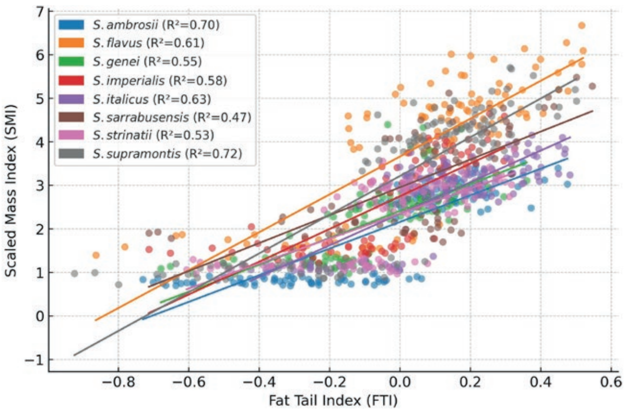

Fig. 3. Results of the GLMM showing the divergent correlation between SMI and FTI for each *Speleomantes* species. On the plot, FTI is log-transformed and centred on its mean as used in the analysis.

employed to estimate the body condition of *Speleomantes* from digital images, even in the absence of individual weight. This outcome further highlights the usefulness of digital photos in conservation studies and the potential contribution that citizen science could provide for the monitoring of animal population.

Our findings opposed the results of previous studies where the use of the tail width was not recommended for the estimation of the body condition for this genus. In their study, Rosa et al. (2021) considered the width at the tail base as a proxy of individual fitness in *S. imperialis* (using the Scale Mass Index, SMI). Instead, we propose using the overall tail area (FTI) because adipose tissue is likely stored throughout the tail length and not just at its base (Fitzpatrick, 1973). Our method was highly supported by the analysis, confirming the hypothesis that fat tissue is accumulated not only in a specific section but throughout the tail organ. In some circumstances, the enlargement at the tail base might be considered a proxy for the overall tail enlargement due to fat storage (Bendik and Gluesenkamp, 2013). Still, it does not always work (Rosa et al., 2021). Indeed, in their study, Rosa et al. (2021) assessed the correlation between SMI and the tail base only in *S. imperialis*. Considering the variability of the correlation between SMI and FTI observed here (Fig. 3, but see also Fig. S1), it may be possible that the tail base may be more diagnostic for some other *Speleomantes* species. On the other hand, the assessment of the overall increase in the tail volume seems more reliable and informative than the use of specific tail parts (Rosa et al., 2021). Although the proposed method already represents a reliable alternative for estimating the body condition in *Speleomantes*, considering the overall tail volume might further increase the accuracy of the estimation.

Rosa et al. (2021) found a significant correlation between tail width and SMI in females but not males. In our analysis, we observed a strong effect of sex on SMI, where females showed the highest correlation and juveniles the lowest (Fig. 2B). Sexually mature females need to store large amounts of energy to carry out highly demanding reproduction and consequently usually have a higher body condition than males of similar size (Lunghi et al., 2018b). On the other hand, the low correlation with SMI observed for juveniles can also be easily explained. Juvenile *Speleomantes* likely invest more in growth than in fat storage to quickly reach a size that makes them unsuitable for some predators (Lunghi and Corti, 2021). This was also hypothesized in other studies where juveniles often occurred in sub-optimal environmental conditions to find the highest prey availability (Ficetola et al., 2013; Lunghi et al., 2015).

We observed a divergence in SMI among the eight *Speleomantes* species (Fig. 3). The group of species that showed the highest correlation with SMI was composed of four Sardinian species (*S. flavus*, *S. imperialis*, *S. supramontis*, *S. sarrabusensis*) and *S. italicus*. The four Sardinian species showed the highest divergence (β = 0.30-0.51), while that for *S. italicus* was moderate (β = 0.15). This group of Sardinian *Speleomantes* is defined as "giant" due to their larger size compared to the other congeneric species (Lanza et al., 2006). The larger size of these species may allow them to increase the proportion of fat stored in their body (Fitzpatrick, 1973), or they can have access to bigger prey that provide a more significant amount of nutrients (Lunghi et al., 2018a). On the other hand, hypothesizing the reasons for the higher SMI observed in *S. italicus* compared to the other similar-sized species is more challenging, and, considering the lack of supporting information, we save this for future assessments to avoid falling into mere speculations.

The correlation between FTI and SMI showed significant variability between species, ranging from 0.47 for *S. sarrabusensis* to 0.72 for *S. supramontis* (Fig. 2). This means that, although FTI is a good proxy to estimate *Speleomantes* body condition, for some species this prediction seems to be stronger. Unfortunately, we do not have supporting information to explain this result. Further analyses aiming to evaluate potential physiological and morphological interspecific divergences are needed to shed light on this interesting case.

We observed a higher SMI in surface populations of *Speleomantes*. This result does not support the hypothesis that subterranean populations of *Speleomantes* show improved abilities in fat storage (Lunghi and Zhao, 2020) but rather raises alternative hypotheses. *Speleomantes* from surface environments are mostly active during wet seasons, meaning that their main foraging activity likely occurs only during limited periods (Costa et al., 2016; Salvidio et al., 2017). Contrarily, subterranean populations can buffer hostile seasons (too hot and/or dry) thanks to the underground microclimate (Culver and Pipan, 2019), which allows them, and their prey as well, to be more or less active all year round (Lunghi et al., 2022). Therefore, in this circumstance, the opposite could occur, and the surface populations may show enhanced abilities for fat storage. On the other hand, although being active for less time, surface populations have at disposal higher prey diversity and availability compared to those living in subterranean environments (Culver and Pipan, 2019), a key factor that can compensate for the reduced foraging activity and provide more nutrients that can be translated into a higher body condition of individuals. These hypotheses need to be explored to shed light on the dynamics behind the observed divergence in SMI between surface and subterranean populations.

In conclusion, our study demonstrated the validity of the correlation between the tail area and the individuals' body condition in *Speleomantes*, making this a valuable proxy for implementing conservation and ecological studies.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <http://www-9.unipv.it/webshi/appendix/index.html> manuscript number 16853

REFERENCES

Beebee, T.J.C., Griffiths, R.A. (2005): The amphibian decline crisis: a watershed for conservation biology? Biol. Conserv. **125**: 271-285.

Bendik, N.F., Gluesenkamp, A.G. (2013): Body length shrinkage in an endangered amphibian is associated with drought. J. Zool. **290**: 35-41.

Bliley, J.M., Woodley, S.K. (2012): The effects of repeated handling and corticosterone treatment on behavior in an amphibian (Ocoee salamander: *Desmognathus ocoee*). Physiol. Behav. **105**: 1132-1139.

Cianferoni, F., Lunghi, E. (2023): Inferring on *Speleomantes* foraging behavior from gut contents examination. Animals **13**: 2782.

Coppari, L., Di Gregorio, M., Corti, C., Merilli, S., Mulargia, M., Cogoni, R., Manenti, R., Ficetola, G.F., Lunghi, E. (2024): Four years monitoring of the endangered European plethodontid salamanders. Sci. Data **11**: 706.

Costa, A., Crovetto, F., Salvidio, S. (2016): European plethodontid salamanders on the forest floor: local abundance is related to fine-scale environmental factors. Herpetol. Conserv. Biol. **11**: 344-349.

Culver, D.C., Pipan, T. (2019). The biology of caves and other subterranean habitats. New York, Oxford University Press.

Davis, T.M., Ovaska, K. (2001): Individual recognition of amphibians: effects of toe clipping and fluorescent tagging on the salamander *Plethodon vehiculum*. J. Herpetol. **35**: 217-225.

Dunn, E.H., Ralph, C.J. (2004): Use of mist nets as a tool for bird population monitoring. Stud. Avian Biol. **29**: 1-6.

Ficetola, G.F., Barzaghi, B., Melotto, A., Muraro, M., Lunghi, E., Canedoli, C., Lo Parrino, E., Nanni, V., Silva-Rocha, I., Urso, A., Carretero, M.A., Salvi, D., Scali, S., Scarì, G., Pennati, R., Andreone, F., Manenti, R. (2018a): *N*-mixture models reliably estimate the abundance of small vertebrates. Sci. Rep. **8**: 10357.

Ficetola, G.F., Lunghi, E., Canedoli, C., Padoa-Schioppa, E., Pennati, R., Manenti, R. (2018b): Differences between microhabitat and broad-scale patterns of niche evolution in terrestrial salamanders. Sci. Rep. **8**: 10575.

Ficetola, G.F., Pennati, R., Manenti, R. (2013): Spatial segregation among age classes in cave salamanders: habitat selection or social interactions? Popul. Ecol. **55**: 217-226.

Fitzpatrick, L.C. (1973): Energy allocation in the Allegheny mountain salamander, *Desmognathus ochrophaeu*. Ecol. Monogr. **43**: 43-58.

Gabor, C.R., Bosch, J., Fries, J.N., Davis, D.R. (2013): A non-invasive water-borne hormone assay for amphibians. Amphib.-Reptil. **34**: 151-162.

Golay, N., Durrer, H. (1994): Inflammation due to toe-clipping in natterjack toads (*Bufo calamita*). Amphib.-Reptil. **15**: 81-96.

Howarth, F.G., Moldovan, O.T. (2018). The ecological classification of cave animals and their adaptations. In: Cave Ecology, pp. 41-67. Moldovan, O.T., Kováč, L., Halse, S., Eds., Berlin, Springer.

Husain, N., Roy, P., Hussain Trak, T. (2017): Photography as a conservation tool in science. Trends Biosci. **10**: 9317-9321.

Lanza, B., Pastorelli, C., Laghi, P., Cimmaruta, R. (2006): A review of systematics, taxonomy, genetics, biogeography and natural history of the genus *Speleomantes* Dubois, 1984 (Amphibia Caudata Plethodontidae). Atti Mus. civ. stor. nat. Trieste **52**: 5-135.

Lunghi, E. (2022): Doubling the lifespan of European plethodontid salamanders. Ecology **103**: 1-4.

Lunghi, E., Bacci, F., Zhao, Y. (2021a): How can we record reliable information on animal colouration in the wild? Diversity **13**: 356.

Lunghi, E., Bruni, G. (2018): Long-term reliability of Visual Implant Elastomers in the Italian cave salamander (*Hydromantes italicus*). Salamandra **54**: 283-286.

Lunghi, E., Cianferoni, F., Ceccolini, F., Veith, M., Manenti, R., Mancinelli, G., Corti, C., Ficetola, G.F. (2018a): What shapes the trophic niche of European plethodontid salamanders? PLOS ONE **13**: e0205672.

Lunghi, E., Cianferoni, F., Corti, C., Zhao, Y., Manenti, R., Ficetola, G.F., Mancinelli, G. (2022): The trophic niche of subterranean populations of *Speleomantes italicus*: a multi-temporal analysis. Sci. Rep. **12**: 18257.

Lunghi, E., Cianferoni, F., Giachello, S., Zhao, Y., Manenti, R., Corti, C., Ficetola, G.F. (2021b): Updating salamander datasets with phenotypic and stomach content information for two mainland *Speleomantes*. Sci. Data **8**: 150.

Lunghi, E., Corti, C. (2021): Predation of European cave salamanders (*Speleomantes*) by the spider *Meta bourneti*. Spixiana **44**: 54.

Lunghi, E., Corti, C., Manenti, R., Barzaghi, B., Buschettu, S., Canedoli, C., Cogoni, R., De Falco, G., Fais, F., Manca, A., Mirimin, V., Mulargia, M., Mulas, C., Muraro, M., Murgia, R., Veith, M., Ficetola, G.F. (2018b): Comparative reproductive biology of European cave salamanders (genus *Hydromantes*): nesting selection and multiple annual breeding. Salamandra **54**: 101-108.

Lunghi, E., Corti, C., Mulargia, M., Zhao, Y., Manenti, R., Ficetola, G.F., Veith, M. (2020a): Cave morphology, microclimate and abundance of five cave predators from the Monte Albo (Sardinia, Italy). Biodivers. Data J. **8**: 1-16.

Lunghi, E., Giachello, S., Manenti, R., Zhao, Y., Corti, C., Ficetola, G.F., Bradley, J.G. (2020b): The post hoc measurement as a safe and reliable method to age and size plethodontid salamanders. Ecol. Evol. **10**: 11111-11116.

Lunghi, E., Giachello, S., Zhao, Y., Corti, C., Ficetola, G.F., Manenti, R. (2020c): Photographic database of the European cave salamanders, genus *Hydromantes*. Sci. Data **7**: 171.

Lunghi, E., Manenti, R., Canciani, G., Scarì, G., Pennati, R., Ficetola, G.F. (2016): Thermal equilibrium and temperature differences among body regions in European plethodontid salamanders. J. Therm. Biol. **60**: 79-85.

Lunghi, E., Manenti, R., Ficetola, G.F. (2015): Seasonal variation in microhabitat of salamanders: environmental variation or shift of habitat selection? PeerJ **3**: e1122.

Lunghi, E., Romeo, D., Mulargia, M., Cogoni, R., Manenti, R., Corti, C., Ficetola, G.F., Veith, M. (2019): On the stability of the dorsal pattern of European cave salamanders (genus *Hydromantes*). Herpetozoa **32**: 249-253.

Lunghi, E., Zhao, Y. (2020): Do Chinese cavefish show intraspecific variability in morphological traits? Ecol. Evol. **10**: 7723-7730.

MacCracken, J.G., Stebbings, J.L. (2012): Test of a body condition index with amphibians. J. Herpetol. **46**: 346-350.

McCravy, K.W. (2018): A review of sampling and monitoring methods for beneficial arthropods in agroecosystems. Insects **9**: 170.

Peig, J., Green, A.J. (2009): New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. Oikos **118**: 1883-1891.

Perry, G., Wallace, M.C., Perry, D., Curzer, H., Muhlberger, P. (2011): Toe clipping of amphibians and reptiles: science, ethics, and the law. J. Herpetol. **45**: 547-555.

Rondinini, C., Battistoni, A., Teofili, C. (2022). Lista Rossa IUCN dei vertebrati italiani 2022. Roma, Comitato Italiano IUCN e Ministero dell'Ambiente e della Sicurezza Energetica.

Rosa, G., Costa, A., Renet, J., Romano, A., Roner, L., Salvidio, S. (2021): Energy storage in salamanders' tails: the role of sex and ecology. Sci. Nat. **108**: 27.

Salvidio, S., Pasmans, F., Bogaerts, S., Martel, A., van de Loo, M., Romano, A. (2017): Consistency in trophic strategies between populations of the Sardinian endemic salamander *Speleomantes imperialis*. Anim. Biol. **67**: 1-16.

Soto-Azat, C., Clarke, B.T., Fisher, M.C., Walker, S.F., Cunningham, A.A. (2009): Non-invasive sampling methods for the detection of Batrachochytrium dendrobatidis in archived amphibians. Dis. Aquat. Org. **84**: 163-166.

Speybroeck, J., Steenhoudt, K. (2017): A pattern-based tool for long-term, large-sample capture-mark-recapture studies of fire salamanders *Salamandra* species (Amphibia: Urodela: Salamandridae). Acta Herpetol. **12**: 55-63.

Wake, D.B., Dresner, I.G. (1967): Functional morphology and evolution of tail autotomy in salamanders. J. Morphol. **122**: 265-306.

Wilder, S.M., Raubenheimer, D., Simpson, S.J. (2016): Moving beyond body condition indices as an estimate of fitness in ecological and evolutionary studies. Funct. Ecol. **30**: 108-115.

Acta Herpetologica 20(1): 75-81, 2025
DOI: 10.36253/a_h-16673

On the maxillary dentition of the Western whip snake, *Hierophis viridiflavus* (Lacépède, 1789): heterodonty is not opisthoglyphy

Matteo R. Di Nicola[1,2,*], Massimo Delfino[3,4]

[1] *Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Via Bologna 148, 10154 Turin, Italy*
[2] *Faculty of Veterinary Medicine, Department of Pathobiology, Pharmacology and Zoological Medicine, Wildlife Health Ghent, Ghent University, 9820 Merelbeke, Belgium*
[3] *Dipartimento di Scienze della Terra, Università degli Studi di Torino, via Valperga Caluso 35, 10125 Turin, Italy*
[4] *Institut Català de Paleontologia Miquel Crusafont (ICP-CERCA), Edifici ICTA-ICP, c/ Columnes s/n, Campus de la UAB, 08193 Cerdanyola del Vallès, Barcelona, Spain*
**Corresponding author.* Email: matteoriccardo.dinicola@izsplv.it

Submitted on: 2024, 30th September; Revised on: 2024, 28th November; Accepted on: 2024, 3rd December
Editor: Andrea Villa

Abstract. The Western whip snake (*Hierophis viridiflavus*) is a colubroid snake typically considered aglyphous and non-venomous, although this is currently a subject of debate. Indeed, the maxillary dentition of this species has recently been described as opisthoglyphous due to the presence of enlarged rear maxillary teeth, reportedly characterised by prominent grooves and ridges. Aiming to test the actual presence of these structures and provide a clearer characterisation of the maxillary dentition of the species, we analysed 17 dry-prepared and disarticulated *H. viridiflavus* specimens using both scanning electron and optical microscopy. The imaging confirmed the presence of posterior, enlarged maxillary teeth displaying a distal carina, highlighted by shallow longitudinal inflections on the disto-labial and disto-lingual tooth surfaces. Nonetheless, the analysis revealed the consistent absence of the meso-labially placed groove typical of opisthoglyphous taxa. Based on the results of our study, the maxillary dentition of the Western whip snake is heterodont, specifically opisthomegadont, and is confirmed to be aglyphous.

Keywords. Aglyphous, Colubrid, non-front-fanged snakes, opisthoglyphous, proteroglyphous, rear-fanged, solenoglyphous, tooth morphology.

INTRODUCTION

Snakes exhibit a wide variety of dental morphologies and adaptations that are crucial for their survival and feeding strategies. Broadly speaking, snakes are carnivorous reptiles, and their teeth are variably long, slender, and slightly curved, making them ideal for piercing and, in most cases, holding onto prey. Snake teeth are attached to the jaws in a modified pleurodont manner, where each tooth is set within a shallow depression (Lillywhite, 2014; Pough et al., 2016).

In members of the clade Alethinophidia Nopcsa, 1923, encompassing all snakes other than blind snakes (Scolecophidia Cope, 1864), teeth are present on the maxillae, palatines, and pterygoids in the upper jaw, and on the dentary in the lower jaw. The premaxilla bears teeth in some basal species (Lee and Scanlon, 2002; Mahler and Kearney, 2006; Pough et al., 2016; Berkovitz and Shellis, 2023).

Teeth replacement in snakes is a continuous process throughout their lives, characterised by a unique mechanism where functional teeth are replaced by new ones developing in successive waves along the jaw. Unlike most other amniotes, snakes lack external resorption pits during this process; instead, tooth replacement involves internal resorption within the pulp cavity. This adapta-

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

www.fupress.com/ah

tion allows snakes to maintain a constant supply of functional teeth, essential for their feeding efficiency and survival, as damaged or lost teeth are replaced rapidly. This mechanism supports their ability to ingest large or resistant prey and minimises the risk of collateral damage to neighbouring teeth during replacement (Lillywhite, 2014; LeBlanc et al., 2023).

Heterodonty, the presence of differently shaped teeth, is evident in many species. For example, venomous snakes (all belonging to the clade Caenophidia Hoffstetter, 1939) tend to have heterodont dentitions, characterised by the presence of differently shaped teeth. Specifically, these snakes typically exhibit enlarged, specialised teeth (i.e., fangs) with remarkable morphological features (e.g., grooves, hollow centres, ridges along the fang channels) that aid in venom delivery (Triep et al., 2013; Broeckhoven and du Plessis, 2017; Avella et al., 2021). In contrast, nonvenomous snakes tend to homodonty (i.e., all teeth present the same shape), although variations in tooth size and morphology are common (Young and Kardong, 1996; Pough et al., 2016; Berkovitz and Shellis, 2023). In any case, the differences in the shape and function of the teeth within each snake species, even when minimal, make it overly simplistic to distinguish solely between heterodonty and homodonty (see Ryerson and Valkenburg, 2021; Segall et al., 2023). Indeed, Westeen et al. (2020) recently highlighted the presence of a wide range of dentition phenotypes in snakes, particularly in rear-fanged ones, consistent with adaptation to different, specialised diets and prey capture modes. Therefore, for many snake species indicated as homodonts, it would be more accurate to describe them as having low degrees of heterodonty rather than true homodonty.

The diversity of maxillary dentition phenotypes in colubroid snakes is traditionally categorised into four main types: i) aglyphous, ii) opisthoglyphous, iii) proteroglyphous, and iv) solenoglyphous (e.g., Boulenger, 1893, 1894, 1896; Pough et al., 2016; Gower and Zaher, 2022; Delfino and Villa, 2024).

These terms are commonly used to reflect the following characteristics on an etymological basis (e.g., Merriam-Webster, 2024; Treccani, 2024): aglyphous (Greek: '*a-*' for 'without' + '*gluphḗ*' for 'carving' or 'engraving') snakes lack any grooved fangs; opisthoglyphous (Greek: '*ópisthen-*' for 'behind' + '*gluphḗ*') refers to snakes with grooved fangs positioned at the rear of the maxilla; proteroglyphous (Greek: '*próteros-*' for 'first' or 'earliest' + '*gluphḗ*') indicates snakes with grooved hollow, generally non-movable fangs at the front of the maxilla; solenoglyphous (Greek: '*solén-*' for 'pipe' or 'tube' + '*gluphḗ*') denotes snakes with non-grooved hollow, retractable, independently movable fangs at the front of the maxilla (for differences in fang canaliculation between solenoglyphous and proteroglyphous snakes, refer to Broeckhoven and du Plessis, 2017). A further term, endoglyphous (Greek: '*éndon-*' for 'within' or 'inside' + '*gluphḗ*'), is rarely used for lumping together the proteroglyphous and solenoglyphous conditions in order to underline the presence of fangs with an inner cavity (see Golay et al., 1993; Pin, 2009).

This classification clearly has limitations, as not all snake species possess dentition that perfectly and exclusively matches one of these categories. For instance, non-front-fanged snakes (NFFS), exhibit different dental types, also in terms of tooth surface (see Young and Kardong, 1996). According to Weinstein et al. (2022), the term 'non-front-fanged' is preferred over 'opisthoglyphous' because the broad range of modifications in the maxillary teeth of these snakes resists simple classification under traditional terms.

A number of snake species possess enlarged, but not grooved rear maxillary teeth (e.g., Natricidae Bonaparte, 1838 and some Colubridae Oppel, 1811 snakes; see Edmund, 1969; Berkovitz and Shellis, 2017, 2023; Weesten et al., 2020). For some of them, the term 'opisthoglyphous' has sometimes been imprecisely used solely with reference to the larger size of the posterior teeth (as for Natricidae in Westeen et al., 2020: fig. 1).

The dentition of species with enlarged rear maxillary teeth, regardless of whether the tooth surface is grooved or not, should be referred to as opisthomegadont (sensu Edmund, 1969). The distinction between grooved and ungrooved teeth may not always be unambiguous (see Young and Kardong, 1996), and thus it is challenging to have categorising terms for each intermediate condition. In agreement with Berkovitz and Shellis (2023), who equate opisthoglyphy with the 'Type 3' categorisation by Young and Kardong (1996), it is thus reasonable to use the term 'opisthoglyphous' to identify taxa that possess enlarged rear maxillary teeth with a well-defined groove on the meso-labial side, such as *Boiga* Fitzinger, 1826, *Dispholidus* Duvernoy, 1832, *Erythrolamprus* Boie, 1826, *Galvarinus* Trevine et al., 2022, *Malpolon* Fitzinger, 1826, and *Telescopus* Wagler, 1830 (for the morphology of these teeth, see: Fig. 1; Young and Kardong, 1996; Broeckhoven and du Plessis, 2017; Modahl and Mackessy, 2019; Sánchez et al., 2019; Herrera et al., 2022; Weinstein et al., 2022).

Regardless of instances where 'opisthoglyphous' is used broadly as a synonym for 'non-front fanged' or 'rear fanged' (e.g., Westeen et al., 2020; Gower and Zaher, 2022), a recent manuscript has defined the Western whip snake as opisthoglyphous in the strict sense, indicating that its enlarged posterior maxillary teeth are characterised by the presence of 'prominent grooves and ridges' (Paterna, 2023: p. 126).

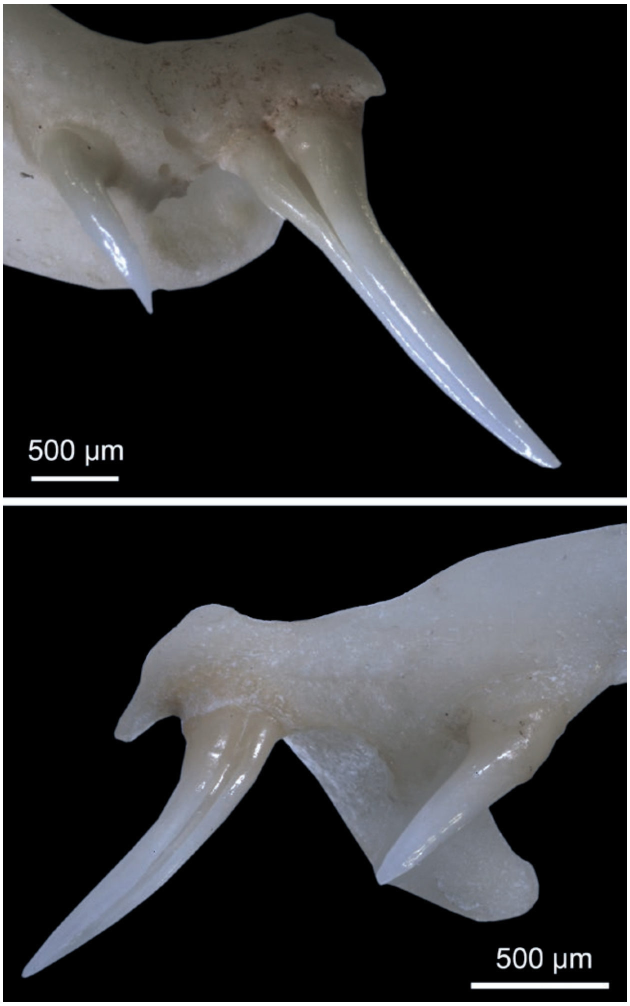

Fig. 1. Stereomicroscope images of a rear enlarged maxillary tooth of *Malpolon insignitus* (Geoffroy Saint-Hilaire, 1827) MDHC 400 (above) and *Telescopus fallax* (Fleischmann, 1831) MDHC 303 (below) in labial view. In both instances, an evident, deep groove meso-labially oriented is visible. The morphological and size differences compared to the teeth that precede them anteriorly are significant. Photos taken with a Leica M205C stereomicroscope.

Using both optical microscopy and Scanning Electron Microscopy (SEM), we investigated whether the enlarged rear maxillary teeth of the Western whip snake are indeed characterised by pronounced grooves and ridges, and aimed to clarify the classification of the maxillary dentition of the species.

MATERIALS AND METHODS

The focus species

The Western whip snake, *Hierophis viridiflavus* (Lacépède, 1789), is a colubroid snake with an average total length between 110 and 150 cm (Vanni and Zuffi, 2011; Di Nicola et al., 2021a). Its range extends across south-central Europe, from northeastern Spain to coastal Croatia, and includes the entire Italian Peninsula, as well as Sardinia, Sicily, and numerous smaller islands and islets (Avella et al., 2017; Di Nicola et al., 2021b). Currently, two subspecies are recognised: *Hierophis viridiflavus viridiflavus* (Lacépède, 1789), in the western part of the species range, typically characterised by a pattern of black and yellow dots and stripes; and *Hierophis viridiflavus carbonarius* (Bonaparte, 1833), in the eastern part, often predominantly or completely melanistic (Di Nicola et al., 2021a, b; Senczuk et al., 2021; Sindaco and Razzetti, 2021; Storniolo et al., 2023).

The species is highly defensive and prone to biting when cornered/handled (Bea, 1998; Di Nicola et al., 2021a; Avella et al., 2024). Early research identified the presence of Duvernoy's glands in *H. viridiflavus* (Phisalix, 1922; Taub, 1967). Additionally, some bite reports have described the onset of symptoms in humans possibly indicating envenomation (e.g., Bédry et al., 1998; Dutto et al., 2015; Avella et al., 2024), although the clinical interpretations of some of them is questionable (see Weinstein et al., 2022; Avella et al., 2024; Paolino et al., 2024). There is a lack of recent research on the oral glands of the Western whip snake, as well as proteomic studies aimed at identifying the components of its saliva and oral secretions, which could provide valuable insights into the biochemical potential of *H. viridiflavus* and its classification as 'non-venomous' (Avella et al., 2024).

The dentition of the Western whip snake, though sometimes described as homodont (e.g., Dutto et al., 2015), features two enlarged teeth at the rear of each maxilla (Fig. 2), which are less curved compared to other maxillary teeth (Racca et al., 2020; Avella et al., 2024). In light of this, the species dentition is clearly heterodont.

Morphological observations

Focusing on the morphology of the enlarged rear teeth, we observed the maxillary dentition of 17 disarticulated, dry prepared skeletons of morphologically mature and immature Western whip snakes from both the subspecies *H. v. carbonarius* and *H. v. viridiflavus* (MDHC 9, 34, 69, 74, 80, 118, 198, 199, 219, 265, 298, 306, 328, 442, 458, 460, 502; Collection acronym: MGPUT MDHC - Massimo Delfino Herpetological Collection, Museo di Geologia e Paleontologia, Dipartimento di Scienze della Terra, Università degli Studi di Torino). The sample included both females and males.

For optical microscopy, a Leica M205C stereomicroscope equipped with a Leica DMC 2900 camera was

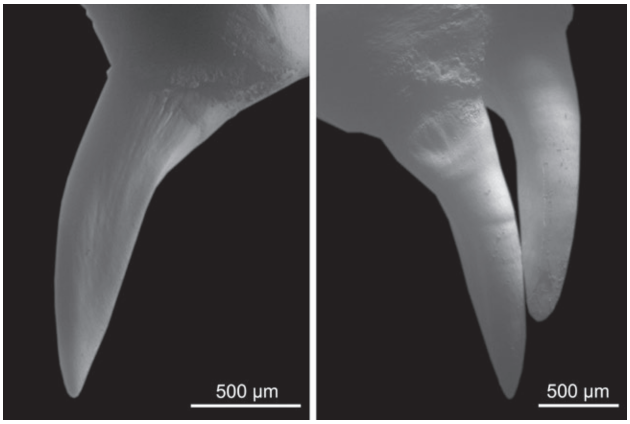

Fig. 2. SEM images of the enlarged posterior maxillary teeth of two adult *H. viridiflavus* specimens. Left tooth in labial view (left; code MDHC 219): the right, straight edge corresponds to the distal carina, which is associated to a weak depression barely visible in this SEM image. Right teeth in labial-mesial view (right; code MDHC 118): not showing the distal carina and associated depression.

used. SEM observations were performed with a Tescan Essence microscope. The samples were not coated with a conductive material to preserve their original surface characteristics; for this reason, we operated in Single Vac conditions using a Back Scattered Electron Detector. The system was configured with an accelerating voltage of 15 keV and a beam current of 30 nA.

RESULTS

In all analysed specimens, the last two maxillary teeth are different in size and morphology from those preceding the diastema. These rear teeth are characterised by a distal carina, which is bordered on both sides by a shallow depression extending from the distal region to the disto-labial and disto-lingual surfaces. These are inflections of the surface and do not constitute an actual groove (see Figs 2-3**)**. It can be excluded the presence of any meso-labial (antero-lateral) grooves comparable to those of snake traditionally considered opisthoglyphous (e.g., genera *Malpolon* and *Telescopus*; compare Figs 2-3 with Fig. 1).

DISCUSSION

Given the considerable diversity in snake dentition, the term ‘non-front-fanged snakes’ is currently to be preferred over more common yet overgeneralising terms like ‘rear-fanged’ and ‘aglyphous’ when referring to snakes lacking anterior fangs. For instance, NFFS commonly exhibit more or less pronounced heterodonty. In this case, if the posterior maxillary teeth are larger, it is appropriate to describe the heterodont dentition as opisthomegadont. This condition may involve enlarged maxillary teeth that are grooved to varying extents, or not at all, potentially in combination with other superficial morphological features.

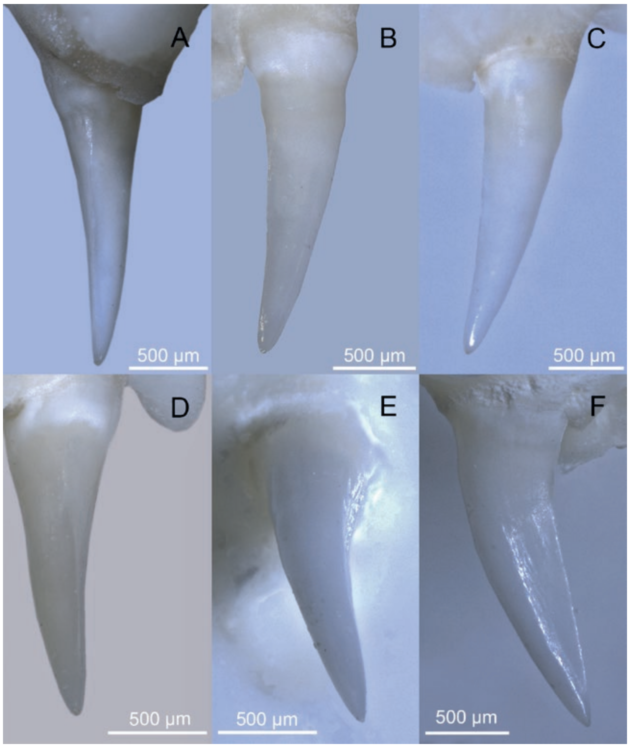

Fig. 3. Stereomicroscopic images of the enlarged posterior maxillary teeth of two adult *H. viridiflavus* sspp. specimens. *Hierophis v. carbonarius* (MDHC 198): right tooth in distal view (A); left tooth in distal view (B); right tooth in labial view (C); left tooth in labial-distal view (D). *Hierophis v. viridiflavus* (MDHC 219): right tooth in mesial view (E); right tooth in lingual view (F). The distal carina is clearly visible in A, D, and F; the slight depression associated to the carina is better visible in D and especially in F.

Based on our results, the dentition of the Western whip snake is heterodont, opisthomegadont. While the rear maxillary teeth display a complex morphology including sulci and carinae, these features are weakly expressed and can be variably perceived, for instance depending on the light orientation under microscopy. From our perspective, these characteristics do not correspond to the deep and very-well-marked anterolateral groove characteristic of teeth from snakes traditionally classified as opisthoglyphous in the strict etymological sense. Nevertheless, we agree with Weinstein et al. (2022) that the dichotomy between aglyphous and opisthoglyphous dentition fails to adequately capture the extensive morphological diversity observed in NFFS, and that each case should be analysed individually.

It is worth noting that a recent paper, published after the submission of our manuscript, suggests that even *Dolichophis caspius* (Gmelin, 1789), along with several other taxa, is opisthoglyphous (Paterna and Grano, 2024). However, our direct inspection of *D. caspius* specimens MDHC 518 and 519 confirmed that the dentition of this species is clearly aglyphous, with the morphology of the posterior maxillary teeth closely resembling that described here for *H. viridiflavus*. Through the application of optical and scanning electron microscopy we were able to determine the absence of prominent grooves in the posterior, enlarged maxillary teeth of *H. viridiflavus*. In light of this, we do not support classifying this species as opisthoglyphous in the morphological sense. Further investigations, employing tools such as micro-CT scanners, will enable a more detailed analysis of the tooth morphology of this and other NFFS species.

ACKNOWLEDGEMENTS

The authors wish to thank Simona Cavagna and Marco Pavia for their assistance with the scanning electron microscopy and optical imaging at the Department of Earth Sciences, University of Turin, and Ignazio Avella and Marco Sassoè for their comments, which have improved our manuscript.

Massimo Delfino is supported by Fondi di Ricerca Locale UNITO (2021-2023). This is publication number 383 of the Museo di Geologia e Paleontologia collections at the Università degli Studi di Torino.

REFERENCES

Avella, I., Barajas-Ledesma, E., Casewell, N.R., Harrison, R.A., Rowley, P.D., Crittenden, E., Wüster, W., Castiglia, R., Holland, C., Van Der Meijden, A. (2021): Unexpected lack of specialisation in the flow properties of spitting cobra venom. J. Exp. Biol. **224**: jeb229229.

Avella, I., Castiglia, R., Senczuk, G. (2017): Who are you? The genetic identity of some insular populations of *Hierophis viridiflavus* s.l. from the Tyrrhenian Sea. Acta Herpetol. **12**: 209-214.

Avella, I., Savini, F., Di Nicola, M.R. (2024): A prolonged bite by a Western Whip Snake, *Hierophis viridiflavus carbonarius* (Bonaparte 1833) (Serpentes, Colubridae), resulting in pronounced local oedema. RandA **31**: e21448.

Bea, A. (1998): *Coluber viridiflavus* Lacépède, 1789. In: Reptiles, Fauna Ibérica, Vol. 10, pp. 358-363. Salvador, A., Ramos, M.A., Eds, Madrid, Museo Nacional de Ciencias Naturales.

Bédry, R., Hilbert, G., Goyffon, M., Laffort, P., Parrens, E. (1998): Is the saliva of the European whip snake (*Coluber viridiflavus*) neurotoxic? Toxicon **12**: 1729-1730.

Berkovitz, B.K.B., Shellis, R.P. (2017): The teeth of non-mammalian vertebrates - 1st ed. London, Academic press.

Berkovitz, B.K.B., Shellis, R.P. (2023): The teeth of non-mammalian vertebrates: form, function, development and growth. Amsterdam, Academic Press.

Boie, H. (1826): Merkmale einiger japanischer Lurche. Isis von Oken **2**: 203-216.

Bonaparte, C.L.J.L. (1833): Iconographia della fauna Italica per le quattro classi degli animali vertebrati. Roma, Salviucci.

Bonaparte, C.L.J.L. (1838): Amphibiorum tabula analytica. Nuovi Ann. Sci. Nat. **1**: 391-393.

Boulenger, G.A. (1893): Catalogue of the snakes in the British Museum (Natural History). Vol. I. London, British Museum (Natural History).

Boulenger, G.A. (1894): Catalogue of the snakes in the British Museum (Natural History). Vol. II. London, British Museum (Natural History).

Boulenger, G.A. (1896): Catalogue of the snakes in the British Museum (Natural History). Vol. III. London, British Museum (Natural History).

Broeckhoven, C., Du Plessis, A. (2017): Has snake fang evolution lost its bite? New insights from a structural mechanics viewpoint. Biol. Lett. **13**: 20170293.

Cope, E.D. (1864): On the characters of the higher groups of Reptilia Squamata: And especially of the Diploglossa. Proc. Acad. Nat. Sci. Philadelphia **16**: 224-231.

Delfino M., Villa A. (2024). An overview of the skeleton of reptiles. In: Handbook of Zoology, Reptilia, Volume 1: General Biology, Archosauria, Chelonia, pp. 39-56. Joger, U., Ed., Berlin/Boston, Walter de Gruyter GmbH.

Di Nicola, M.R., Cavigioli, L., Luiselli, L., Andreone, F. (2021a): Anfibi & Rettili d'Italia. Edizione aggiornata. Latina, Edizioni Belvedere.

Di Nicola, M.R., Di Rosa, D., Mariacher, A., Zabbia, T. (2021b): *Hierophis viridiflavus carbonarius* (Western whip snake). Coloration. Herpetol. Rev. **52**: 873-874.

Dutto, M., Ineich, I., Serre-Collet, F., Goyffon, M., Bédry, R. (2015): Trois cas de morsures du Colubridé *Hierophis viridiflavus* (Lacépède, 1789). Bull. Soc. Herp. Fr. **156**: 55-62.

Duvernoy, D.M. (1832): Memoire sur le caracteres tires de l'anatomie pour distinquer les serpens venimeux des serpens non venimeux. Ann. Sci. Nat., **26**: 113-160.

Edmund, A.G. (1969): Dentition. In: Biology of the Reptilia, pp. 117-200. Vol. 1. Gans, C., Parsons, T.S., Bellairs, A.d'A., Eds, London, Academic Press.

Fitzinger, L.J.F.J. (1826): Neue Classification der Reptilien nach ihren natürlichen Verwandtschaften: nebst einer Verwandtschafts-Tafel und einem Verzeichnisse der Reptilien-Sammlung des K. K. Zoologischen Museum zu Wien. Wien, J.G. Heubner.

Fleischmann, F.L. (1831): Dalmatiae nova serpentum genera. Erlangae, C. Heyderi.

Geoffroy Saint-Hilaire, I. (1827): Description des reptiles qui se trouvent en Égypte. In: Description de l'Egypte ou recueil des observations et des recherches qui ont été faites en Égypte pendant l'expedition de l'Armée française, publié par les ordres de sa Majesté-L'Empereur Napoléon le Grand. Histoire Naturelle. Tome premier. Première partie, pp. 121-160. Savigny, M.J.C.L., Ed., Paris, Imprimerie Impériale.

Gmelin, J.F. (1789): Caroli a Linné, Systema Naturae per Regna Tria Naturae, Secundum Classes, Ordines, Genera, Species; cum Characteribus, Differentiis, Synonymis, Locis. Editio Decima Tertia, Aucta, Reformata. Vol. I, part 3. Leipzig, G.E. Beer.

Golay, P., Smith, H. M., Broadley, D. G., Dixon, J. R., McCarthy, C., Rage, J. C., Schätti B., Toriba, M. (1993). Endoglyphs and other major venomous snakes of the world. A checklist. Geneva, Azemiops herpetological data center.

Gower, D.J., Zaher, H. (2022): The origin and early evolutionary history of snakes. Cambridge, Cambridge University Press.

Herrera, Y., Fuentes-Retamal, S., Kemmerling, U., Peichoto, M.E., Ortiz, J.C., Urra, F.A. (2022): Shedding light on the dentition and venom delivery system of the rear-fanged snake, *Galvarinus chilensis chilensis* (Serpentes: Dipsadidae: Tachymenini) from Chile. Biology **11**: 1788.

Hoffstetter, R. (1939): Contribution a l'étude des Elapidae actuels et fossiles et de l'ostéologie des ophidians. Arch. Mus. Hist. Nat. Lyon **15**: 1-78.

Lacepède, B.G.É.d.L. (1789): Histoire naturelle des quadrupèdes ovipares et des serpens, vol. 2. Paris, Hôtel de Thou.

LeBlanc, A.R.H., Palci, A., Anthwal, N., Tucker A.S., Araujo R., Pereira M.F.C., Caldwell M.W. (2023): A conserved tooth resorption mechanism in modern and fossil snakes. Nat. Commun. **14**: 742

Lee, M.S., Scanlon, J.D. (2002): Snake phylogeny based on osteology, soft anatomy and ecology. Biol. Rev. **77**: 333-401.

Lillywhite, H.B. (2014): How snakes work: structure, function and behavior of the world's snakes. Oxford, Oxford University Press.

Mahler, D.L., Kearney, M.R. (2006): The palatal dentition in squamate reptiles: Morphology, development, attachment, and replacement. Fieldiana Zool. **2006**: 1-61.

Merriam-Webster (2024): Merriam-Webster.com dictionary. Retrieved September 1, 2024, from https://www.merriam-webster.com/.

Modahl, C.M., Mackessy, S.P. (2019): Venoms of rear-fanged snakes: New proteins and novel activities. Front. Ecol. Evol. **7**: 279.

Nopcsa, F. (1923): Die Familien der Reptilien. Fortschritte der Geologie und Paläontologie, vol. 2. Berlin, Verlag Gebrüder Borntraeger.

Oppel, M. (1811): Die Ordnungen, Familien und Gattungen der Reptilien als Prodrom einer Naturgeschichte derselben. Munich, J. Lindauer.

Paolino, G., Di Nicola, M. R., Ballouard, J. M., Bonnet, X., Damm, M., Le Roux, G., Lüddecke, T., Marini, D., Weinstein, S. A., Avella, I. (2024). A review of bites by non-front-fanged snakes (NFFS) of Europe. Toxicon **250**: 108116.

Paterna, A. (2023): The role of modified teeth in the function of prolonged bites in *Hierophis viridiflavus* (Serpentes: Colubridae). Phyllomedusa **22**: 121-130.

Paterna, A., Grano, M. (2024). Morphology of the maxillary bones in the Caspian whip-snake *Dolichophis caspius* (Serpentes Colubridae) supports the opisthoglyphous model within western palearctic whip-snakes. Biodivers. J. **15**: 693-700.

Phisalix, M. (1922): Animaux venimeux et venins: la fonction venimeuse chez tous les animaux; les appareils venimeux; les venins et leurs propriétés; les fonctions et usages des venins; l'envenimation et son traitement. Paris, Masson & cie.

Pin, T.C. (2009): The study of the mediation of Ohanin, a king cobra (*Ophiophagus hannah*) toxin through the central nervous system. Unpublished MSc thesis. National University of Singapore.

Pough, F.H., Andrews, R.M., Crump, L.C., Savitzky, A.H., Wells, K.D., Brandley, M.C. (2016): Herpetology. Sunderland, Sinauer associates Inc.

Racca, L., Villa, A., Wencker, L.C.M., Camaiti, M., Blain, H., Delfino, M. (2020): Skull osteology and osteological phylogeny of the Western whip snake *Hierophis viridiflavus* (Squamata, Colubridae). J. Morphol. **281**: 808-833.

Ryerson, W.G., Van Valkenburg, T. (2021): Linking tooth shape to strike mechanics in the *Boa constrictor*. Integr. Comp. Biol. **61**: 759-771.

Sánchez, M.N., Teibler, G.P., Sciani, J.M., Casafús, M.G., Maruñak, S.L., Mackessy, S.P., Peichoto, M.E. (2019): Unveiling toxicological aspects of venom from the Aesculapian false coral snake *Erythrolamprus aesculapii*. Toxicon **164**: 71-81.

Segall, M., Houssin, C., Delapré, A., Cornette, R., Herrel, A., Milgram, J., Shahar, R., Dumont, M. (2023): Armed to the teeth: The underestimated diversity in tooth shape in snakes and its relation to feeding behavior and diet. Ecol. Evol. **13**: e10011.

Senczuk, G., Gramolini, L., Avella, I., Mori, E., Menchetti, M., Aloise, G., Castiglia, R. (2021): No association between candidate genes for color determination and color phenotype in *Hierophis viridiflavus,* and characterization of a contact zone. J. Zool. Syst. Evol. Res. **59**: 748-759.

Sindaco, R., Razzetti, E. (2021): An updated check-list of Italian amphibians and reptiles. Nat. Hist. Sci. **8**: 35-46.

Storniolo, F., Mangiacotti, M., Zuffi, M.A.L., Scali, S., Sacchi, R. (2023): Large scale phenotypic characterisation of *Hierophis viridiflavus* (Squamata: Serpentes): climatic and environmental drivers suggest the role of evolutionary processes in a polymorphic species. Evol. Ecol. **37**: 419-434.

Taub, A.M. (1967): Comparative histological studies on Duvernoy's gland of colubrid snakes. Bull. Am. Mus. Nat. Hist. **138**: 1-50.

Treccani (2024): Enciclopedia Italiana. Accessed August 28, 2024. https://www.treccani.it/.

Trevine, V.C., Grazziotin, F.G., Giraudo, A., Sallaberry-Pincheira, N., Vianna, J.A., Zaher, H. (2022): The systematics of Tachymenini (Serpentes, Dipsadidae): An updated classification based on molecular and morphological evidence. Zool. Scr. **51**: 643-663.

Triep, M., Hess, D., Chaves, H., Brücker, C., Balmert, A., Westhoff, G., Bleckmann, H. (2013): 3D flow in the venom channel of a spitting cobra: Do the ridges in the fangs act as fluid guide vanes? PLOS One **8**: e61548.

Vanni, S., Zuffi, M.A.L. (2011): *Hierophis viridiflavus* (Lacépède, 1789). In: Fauna d'Italia, vol. 45, Reptilia, pp. 509-516. Corti, C., Capula, M., Luiselli, L., Razzetti, E., Sindaco, R., Eds, Bologna, Calderini.

Wagler, J.G. (1830): Natürliches System der Amphibien: mit vorangehender Classification der Säugethiere und Vögel: ein Beitrag zur vergleichenden Zoologie. Munich, J.G. Cotta.

Weinstein, S.A., Warrell, D.A., Keyler, D. (2022): 'Venomous' bites from non-venomous snakes. Second edition. Amsterdam, Elsevier.

Westeen, E.P., Durso, A.M., Grundler, M.C., Rabosky, D.L., Davis Rabosky, A.R. (2020): What makes a fang? Phylogenetic and ecological controls on tooth evolution in rear-fanged snakes. BMC Evol. Biol. **20**: 80.

Young, B.A., Kardong, K.V. (1996): Dentitional surface features in snakes (Reptilia: Serpentes). Amphib.-Reptil. **17**: 261-276.

Acta Herpetologica 20(1): 83-91, 2025
DOI: 10.36253/a_h-16715

Home range analysis of *Teratoscincus roborowskii* in the Turpan Basin, Northwestern China: insights from VHF tagging technology

Wenjuan Jing[1,2,4], Han Yan[2,4], Xuejun Ma[2,3,4], Feng Xu[2,*]

[1] *Key Laboratory of Special Environment Biodiversity Application and Regulation in Xinjiang, Xinjiang Key Laboratory of Special Species Conservation and Regulatory Biology, International Center for the Collaborative Management of Cross-border Pest in Central Asia, School of Life Sciences, Xinjiang Normal University, Urumqi 830054, China*
[2] *State Key Laboratory of Ecological Safety and Sustainable Development in Arid Lands, China-Tajikistan Belt and Road Joint Laboratory on Biodiversity Conservation and Sustainable Use, Xinjiang Key Laboratory of Biodiversity Conservation and Application in Arid Lands, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, Urumqi 830011, China*
[3] *Xinjiang Key Laboratory for Ecological Adaptation and Evolution of Extreme Environment Biology, College of Life Sciences, Xinjiang Agricultural University, Urumqi, Xinjiang 830052, China*
[4] *These authors contributed equally and should be treated as the co-first authors*
* *Corresponding author.* Email: xufeng@ms.xjb.ac.cn

Submitted on: 2024, 17th October; revised on: 2024, 21th October; accepted on: 2025, 3rd May
Editor: Wei Chen

Abstract. The home range (HR) of an animal encompasses the area utilized during activities such as foraging, mating, and other routine behaviors, with its size reflecting behavioral patterns and ecological niche. Factors influencing HR size include sex, body size, and diet, with sex and body size being the most significant determinants. The *Teratoscincus roborowskii*, or Turpan Wonder Gecko, is endemic to the Turpan Basin of Xinjiang, northwestern China, yet its HR during the breeding period remains understudied. This research employed radio-tracking methods to evaluate the HR of *T. roborowskii* during the breeding season, focusing on influences from sex and body size. Our study involved radio telemetry of 11 individuals from June to July in 2020 and 2021. We quantified total and core HR sizes using the Minimum Convex Polygon (MCP) and Kernel Density Estimation (KDE) methods. Results via MCP revealed total and core HR sizes of 7894.06 ± 2672.87 m^2 and 4852.41 ± 2045.55 m^2, respectively. Males exhibited larger HRs than females; however, the difference was not statistically significant (df = 3, p = 0.196, n = 11). A significant correlation was found between Snout-vent Length (SVL) and HR size (r = 55, p = 0.002, n = 11), indicating that lizards with larger SVLs occupied larger HRs, regardless of sex. Comparison with mark-recapture methods showed that HR sizes were 4-5 times larger when measured via radio-tracking. This study provides critical insights into the activity range and influencing factors of *T. roborowskii* during the breeding period, contributing essential data for its conservation efforts.

Keywords. Kernel density estimation method, Minimum Convex Polygon, radio-tracking, movement ecology, reproduction period.

INTRODUCTION

Home range refers to the area traversed by an animal during natural activities such as foraging, mating, nurturing young, and other routine behaviors (Burt, 1943; Powell and Mitchell, 2012). It constitutes a critical habitat that offers essential shelter and food resources, with environmental conditions and potential mates being the primary resources of interest in most ecological studies (Kearney et al., 2018; Ryberg et al., 2019; Ariano-

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

www.fupress.com/ah

Sánchez et al., 2020; Balouch et al., 2022; Clement et al., 2022; Renet et al., 2022; Ventura et al., 2022). The size of the HR is often viewed as an indicator of the energetic and physiological needs or ecological niche of a species (Huey et al., 1989; Warner and Shine, 2008; Verwaijen and Van Darnme, 2008; Kusaka and Valdivia, 2021; Sillero et al., 2021; Zhong et al., 2021). Consequently, researchers have extensively investigated the relationships between various ecological factors and HR area across different lizard species (Huey et al., 1989; Perry and Garland, 2002; Salido and Vicente, 2019).

Several factors may influence the size of a lizard's HR. Larger-bodied lizards typically require greater distances to satisfy their energetic needs, thereby necessitating larger HRs for effective foraging (Armstrong, 1965; Schoener, 1968; Turner et al., 1969; Perry and Garland, 2002; Garcia-Rosales et al., 2021; Zhao et al., 2022). Additionally, HR size can be influenced by foraging strategies and the specific types of prey available within the ecological hierarchy (Nunn and Barton, 2000; Mysterud et al., 2001). Furthermore, studies indicate that male lizards often exhibit larger HRs than females during the breeding season, a phenomenon attributed to differences in mating behaviors (Aragon et al., 2001; Mysterud et al., 2001; Li et al., 2013; Ventura et al., 2022).

Two primary methods are employed to calculate HR: the Minimum Convex Polygon (MCP) method and Kernel Density Estimation (KDE). The MCP method is widely recognized for its ease of use and comparability across studies (Anderson, 1982; Laver and Kelly, 2008). While it does not appropriately account for the unique distribution patterns of observations, it provides a straightforward description of the HR (Seaman and Powell, 1996). Conversely, the KDE method has gained favor for its ability to generate utilization distributions (UD) and assess the degree of HR overlap among individuals (Worton, 1995; Mitchell and Powell, 2004; Gitzen et al., 2006). This technique requires the careful selection of an appropriate bandwidth for calculating UD, with least squares cross-validation (hLSCV) and reference bandwidth (href) methods being common choices. The hLSCV method is often recommended due to its capacity for smoothness and fit, rendering it more suitable than the href approach (Powell, 2000; Gitzen et al., 2006).

Over the years, methodologies for recording animal occurrence locations have transitioned from labor-intensive techniques to automated systems (Harris et al., 1990; Cagnacci et al., 2010; Kie et al., 2010). Very High-Frequency (VHF) radio telemetry devices enable the real-time monitoring and recording of an animal's sequential locations (Harris et al., 1990; Marzluff et al., 2004; Mitchell and Powell, 2004; Moorcroft and Barnett, 2008; Williams et al., 2020). These devices incorporate transmitters that emit signals at specific radio frequencies, allowing tracking by nearby radio receivers. However, the use of VHF technology necessitates close proximity to the studied animals, which can interfere with their natural behavior and habitat use.

2. MATERIALS AND METHODS

2.1 Study site

The Turpan Eremophyte Botanical Garden (TEBG) is located in the southeastern part of the Turpan Basin in Xinjiang, China (89°11'E, 42°51'N). This garden is unique as the only botanical garden globally dedicated to the Asian desert plant subregion, situated at altitudes ranging from -105 to -76 meters. The TEBG experiences an arid continental climate, characterized by an annual minimum temperature of -9.5 °C and a maximum temperature of 49.6 °C. The annual effective accumulated temperature is 5454.5 °C, with approximately 3000 hours of sunshine per year. Annually, the area receives an average precipitation of 16.4 mm and maintains an average humidity of 41.0% (Yin, 2004). The garden is home to a diverse array of flora, encompassing over 200 plant genera and 60 families, including species such as *Tamarix* spp., *Calligonum* spp., *Capparis spinosa* L., *Ammopiptanthus* spp., and various insect families, including Formicidae, Carabidae, and Tenebrionidae. Additionally, notable populations of reptiles and birds inhabit the garden, such as *Vulpes corsac*, *Lepus* spp., *Eremias velox*, *Cyrtopodion* spp., *Phrynocephalus grumgrzimailoi*, and *Eryx tataricus*.

2.2 Data collection using radio telemetry

During the breeding periods of June to July in 2020 and 2021, a radio-telemetry survey was conducted to track a total of 13 individuals of the target lizard species, comprising 5 females, 7 males, and 1 juvenile. In 2020, 7 lizards (3 females and 4 males) were monitored, while 6 lizards (2 females, 3 males, and 1 juvenile) were observed in 2021. The lizards were manually captured and equipped with VHF transmitters (model: Lotek's CTx Connectivity VHF tags) weighing 0.9 g, representing less than 1% of the minimum body mass of the lizards. The transmitters were affixed to the lizards' dorsum using a back-loading method. The snout-to-vent length (SVL) of all captured individuals was measured using a vernier caliper, and sex was recorded.

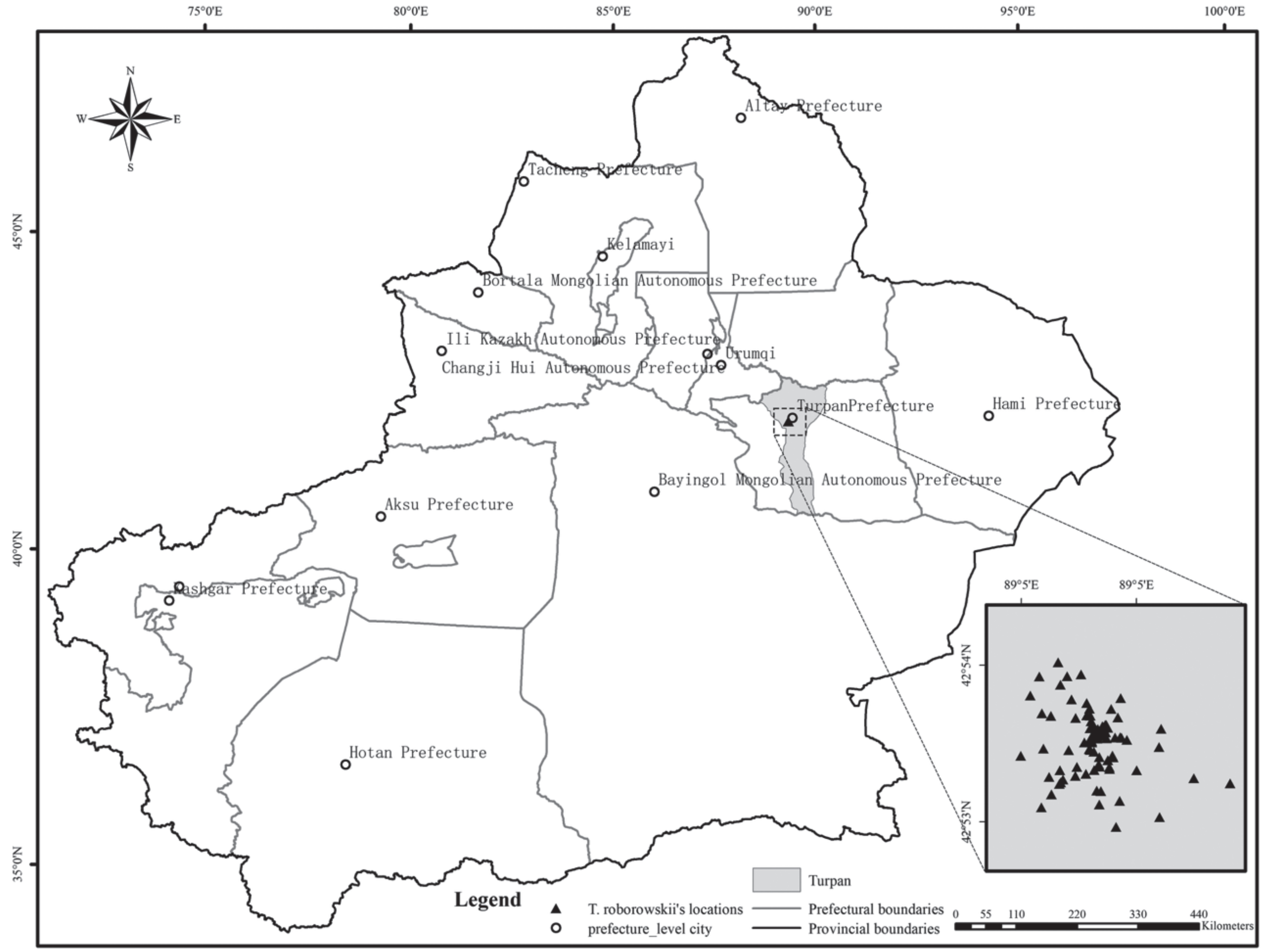

Fig. 1. Map showing the locations of *T. roborowskii*.

Following their release, the lizards' positions (longitude and latitude) were tracked using a Lotek VHF biotracker equipped with a three-element BNC antenna. To mitigate the effects of temporal autocorrelation on home range estimations, a tracking schedule was established to record one GPS location within a 60-minute interval each day from 00:00 to 05:00 h, aligning with the active foraging period of the species during the night. The lizards were recaptured to retrieve the transmitters, and positional data were subsequently downloaded for analysis. Telemetry individuals were designated as F1-F6 for females, M1-M6 for males, and J1 for the juvenile. During the study, 230 effective location points for the lizards were recorded; however, less than five location points for individuals M5 and M6 could not be included in the home range model analysis. Consequently, only 11 individuals were analyzed for home range results.

2.3 Data analyses

To determine the home ranges of the 11 individuals, we employed two methods: the Minimum Convex Polygon (MCP) and Kernel Density Estimation (KDE), utilizing the adehabitatHR package in R version 4.1.2 (R Core Team, 2019). The KDE method was selected due to its widespread application among researchers for home range estimation (Silverman,1986; Worton, 1995). The 95% home ranges of the lizards were calculated using both the MCP and KDE methods, while the 50% home ranges were specifically derived from the KDE method. The 95% MCP and KDE probability contours represent the overall home range of the lizards, whereas the 50% MCP and KDE probability contours delineate the core area within their home range (Powell, 2000).

$$h_{(\mathrm{epa})} = 1.77\sigma n^{-\frac{1}{6}}$$

Table 2. Results of individual home range analysis using MCP and KDE methods.

ID	95%MCP/m^2	95%KDE/m^2	50%MCP/m^2	50%KDE/m^2
M1	4175.48	10700.52	198.07	2393.54
M2	7915.51	22711.50	781.09	5402.25
M3	20717.65	40166.70	532.41	1673.61
M4	1747.42	4120.18	458.73	682.32
M6	25669.02	85977.76	3374.15	19050.47
M1-M6 Mean±SE	12045.02±4721.50	32735.33±14654.04	786.7±504.30	5840.44±3395.19
F1	4778.08	11958.08	597.44	1839.71
F2	1182.72	30242.78	45.08	3636.23
F4	1194.22	4479.96	277.13	762.55
F5	1879.77	11207.34	241.35	2819.91
F6	16418.05	58503.87	2772.50	11446.41
F1-F6 Mean±SE	5090.57±2908.36	23278.41±9789.22	1068.89±583.76	4100.96±1898.30
Mean±SE	8567.79±2859.55	28006.87±8455.754	927.80±366.68	4970.7±1856.47
J1	1156.75	4449.44	29.84	458.03

$$\hat{f}(x) = \frac{1}{nh}\sum_{i=1}^{n} K\left(\frac{x - X_i}{h}\right)$$

The KDE method for calculating the home ranges of the lizards relies on the bandwidth (h), the number of locations (n), and a unimodal bivariate probability density function (K) as described by Silverman (1986).

Seaman et al. (1999) recommended the least-squares cross-validation (LSCV) method for bandwidth selection in KDE. However, recent studies have indicated that the LSCV bandwidth may not be suitable for many lizard species. Consequently, we opted for the reference bandwidth (href), which offers improved fitting performance for small sample sizes. For the kernel function, we selected the Epanechnikov (epa) method, known for its ability to accurately fit multiple central areas and produce reliable results (Silverman, 1986). To facilitate comparisons with other studies, we also employed the MCP model. Additionally, we identified the activity centers for each individual using the KDE method (Bertrand et al., 1996).

Statistical analysis involved assessing the normality of the home range size and snout-vent length (SVL) variables using the Shapiro-Wilk normality test. The results indicated that home range size was not normally distributed (W = 0.808, p = 0.018). Consequently, we log-transformed the home range size and employed F test to evaluate variance homogeneity. After confirming the normality and homogeneity of variance of the data, we utilized the Student's t-test to compare home range sizes and SVL between sexes. In cases where no significant difference in SVL was observed between the sexes, SVL data were combined to analyze the effect of gender on home range size. To assess the correlation between home range size and SVL, we calculated Pearson's correlation coefficient. It is important to note that for juvenile individual J1, we performed home range calculations without conducting further statistical analyses. All statistical analyses were conducted using R (R Core Team, 2019), and data are presented as Mean ± standard error (Mean ± SE). The significance level was set at $p \leq 0.05$.

3. RESULTS

3.1 Home range estimations

During the survey period, we collected a total of 230 location points for the 11 lizards, yielding an average of 20.91 ± 2.36 points per individual. The 95% home range sizes for each lizard were calculated using both the Minimum Convex Polygon (MCP) and Kernel Density Estimation (KDE) methods. The areas ranged from a minimum of 1182.72 m^2 to a maximum of 25669.02 m^2 for the MCP method, and from 4120.18 m^2 to 85977.76 m^2 for the KDE method. The mean total home range size for the lizards was 8567.79 ± 2859.55 m^2 as determined by the 95% MCP method and 28006.87 ± 8455.75 m^2 as calculated using the 95% KDE method. Furthermore, we identified the core area within the home range for each individual, finding mean core sizes of 927.80 ± 366.68 m^2 (50% MCP) and 4970.70 ± 1856.47 m^2 (50% KDE).

The results of the Student's t-tests indicated that male lizards exhibited larger home range sizes compared to female lizards. However, there were no significant differences in snout-vent length (SVL) between

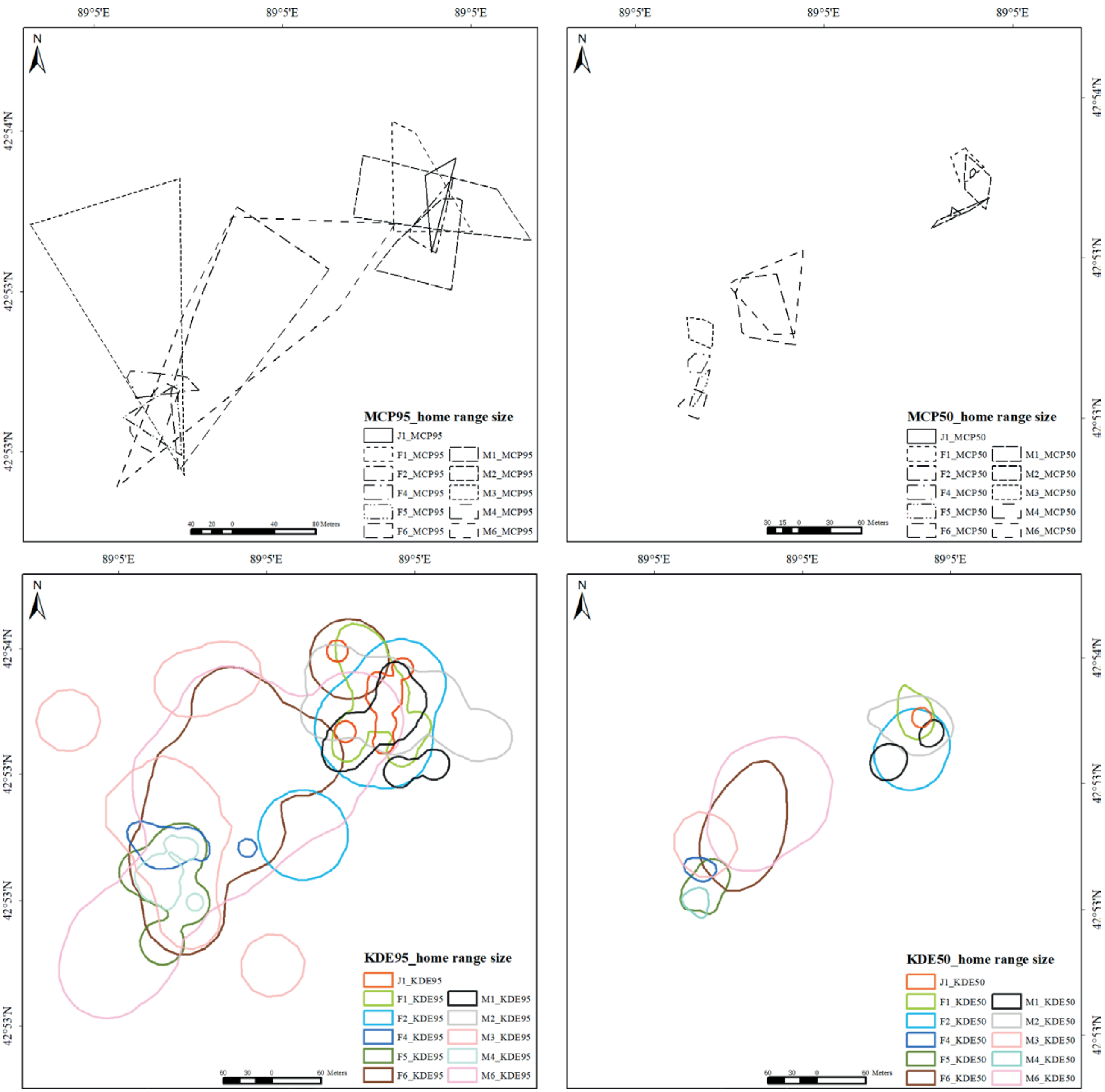

Fig. 2. Home range plot.

adult male and female lizards (t = -0.858, df = 7.519, p = 0.418). When different SVL categories were combined and sex was the sole consideration, no significant differences were observed between male and female lizards regarding total home range size as assessed by both the 95% Minimum Convex Polygon (MCP) method (t = 1.410, df = 7.999, p = 0.196) and the 95% Kernel Density Estimation (KDE) method (t = 0.344, df = 7.778, p = 0.740). Similarly, for core home range size, no significant differences were found using the 50% MCP method (t = 0.795, df = 7.128, p = 0.452) or the 50% KDE method (t = 0.1495, df = 7.577, p = 0.885). Furthermore, all variables met the assumptions of normality and homogeneity of variance.

A correlation analysis was performed to investigate the relationship between snout-vent length (SVL) and home range size among individuals. The analysis revealed a significant positive correlation between the 50% Minimum Convex Polygon (MCP) core areas and SVL, suggesting that individuals with greater SVL

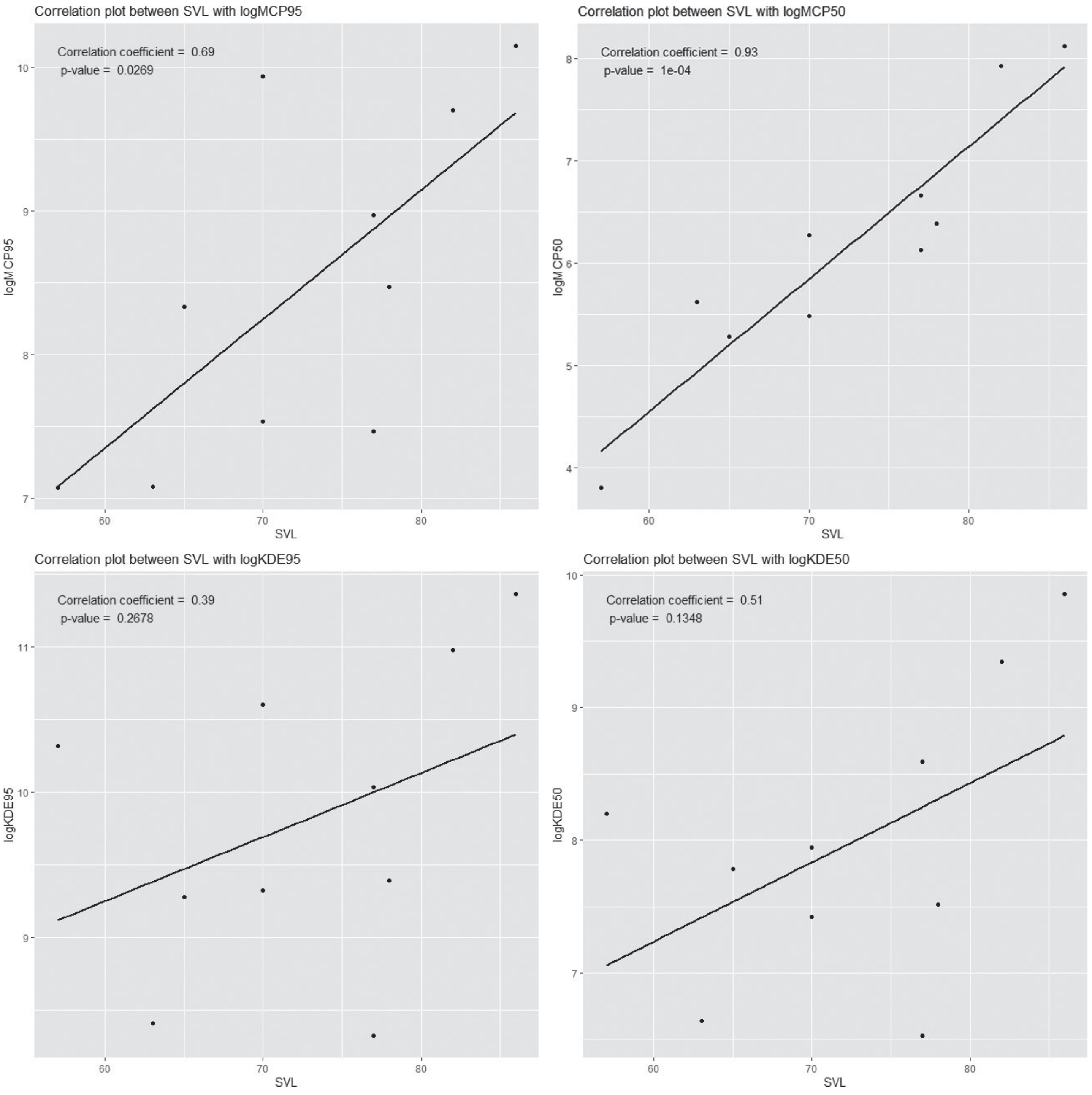

Fig. 3. Correlation plot between SVL and home range size.

tend to occupy larger home ranges. In contrast, the results obtained from the Kernel Density Estimation (KDE) method did not demonstrate a strong correlation trend. This discrepancy may be attributed to the inherent variability associated with the KDE method, which can lead to fluctuations in the estimated home range sizes.

4. DISCUSSION

4.1 Comparison between mark-recapture and radio telemetry methods

The mark-recapture method can be significantly influenced by the configuration of the capture grid and the distance from the capture point. Continuous tagging may also interfere with the behavior of the animals involved (Lira and dos Santos Fernandez, 2009). Consequently, this method has faced scrutiny owing to its propensity to underestimate home range sizes (Gurnell et al., 1989). Previous research consistently indicates that radio tracking methods yield larger home range estimates compared to the mark-recapture technique. For instance, both Sunquist (1987) and Bradshaw (2002) reported that the radio telemetry method produced significantly larger home ranges for *Didelphis marsupialis* and *Tarsipes rostratus*, respectively. Bergstrom et al. (1988) documented that radio telemetry estimates for chipmunks' home ranges were six times greater than those derived from the mark-recapture method. Similar findings have been observed in studies concerning lizards, where the radio tracking method generated home range sizes between four and five times larger than those obtained through mark-recapture (Tisell et al., 2019). Specifically, Li et al. (2013) reported home range sizes for *T. roborowskii* calculated via the mark-recapture method as 337.37 ± 185.95 m^2 for males, 187.80 ± 90.09 m^2 for females, and 191.57 ± 52.4 m^2 for juveniles, confirming that males possessed larger home ranges than females and juveniles during the breeding season.

4.2 Effects of sex on home range

Adult sexual dimorphism in lizards is categorized into three types: (1) males larger than females, (2) females larger than males, and (3) no significant size difference between sexes (Powell and Russell, 1985). Generally, larger lizards require more extensive home ranges to meet their energy demands, thereby securing food resources and obtaining a competitive edge in mating scenarios. *T. roborowskii* falls into the third category, exhibiting no substantial size disparity between sexes, aside from males having a significantly broader head width than females (Harestad and Bunnell, 1979; Liu et al., 2010). The absence of a significant difference in home range size between sexes may stem from the negligible differences in body size and reproductive investment. Male lizards often overlap their home ranges with multiple females as a strategy to maximize reproductive success. During the breeding season, increased territoriality in males, coupled with the reproductive behaviors that elevate movement and survival costs for females, further complicates home range dynamics (Payne et al., 2022; Utsumi et al., 2022; Zhao et al., 2022). This study found that larger individuals of *T. roborowskii* tended to occupy more expansive home ranges, with males exhibiting larger HRs than females, likely attributable to the inconsequential differences in body size and reproductive effort between the sexes (Liu, 2010). Previous research corroborates these findings; for instance, male *Leiolepis reevesii* displayed significantly larger home ranges than females, and a positive correlation was noted between home range size and snout-vent length (SVL) (n=11, r=0.815, P=0.004). Although both males and females displayed intrasexual territoriality, females exhibited significantly higher territoriality than males (Yang et al., 2019). In another study, the home range of male *Phrynocephalus vlangalii* was reported to be 7.6 times larger than that of females, independent of SVL (Wang et al., 2004). Similarly, male *S. crocodilurus* demonstrated a significantly larger linear home range compared to females, with no apparent influence from body weight. Gender and age emerge as significant factors influencing home range dynamics, as both sexes display territorial behaviors (Qing, 2019). Furthermore, artificially elevated testosterone levels in *Uta stansburiana* have been shown to significantly increase home range size and territoriality (DeNardo et al., 1994).

Silverman (1986) assert that bandwidth selection considerably impacts the KDE method's outcomes. Seaman and Powell (1996) recommend utilizing least squares cross-validation (hLSCV) as the bandwidth selection technique for KDE fitting. However, our findings indicated that employing hLSCV with small sample sizes resulted in excessive smoothness and overestimation of home range sizes, leading to fragmented home range representations, particularly in individuals with multiple activity centers and clustered distributions. For example, the smoothing value for individuals M1 (Loci=26) and A1 (Loci=27) was inadequate, making the hLSCV bandwidth non-nested and introducing considerable bias (Seaman et al., 1998). In contrast, the href method is generally viewed as appropriate for Gaussian-distributed sites and may offer advantages for estimating home range sizes (Bowman et al., 1999).

CONCLUSION

Our study demonstrates that male *T. roborowskii* have larger home range sizes than females during the breeding period, with larger individuals occupying more extensive home ranges. Additionally, we found that the radio tracking method produced larger home range estimates compared to the mark-recapture method, thereby providing a valuable reference for method selection in future research.

ACKNOWLEDGEMENT

The authors are grateful to Yunyun Zhang, Liying Yin and Yixin Yu for participation in field research and two anonymous reviewers for their helpful comments on an earlier draft of this paper. This study is supported by the Third Xinjiang Scientific Expedition Program (grant number 2021xjkk0600) and the China Biodiversity Observation Network (Sino-BON).

REFERENCE

Anderson, D.J. (1982): The Home Range - a new nonparametric-estimation technique. Ecology **63**: 103-112.

Aragon, P., Lopez, P., Martin, J. (2001): Seasonal changes in activity and spatial and social relationships of the Iberian rock lizard, *Lacerta Monticola*. Can. J. Zool.-Rev. Can. Zool. **79**: 1965-1971.

Arano-Sanchez, D., Mortensen, R.M., Reinhardt, S., Rosell, F. (2020): Escaping drought: Seasonality effects on home range, movement patterns and habitat selection of the Guatemalan beaded lizard. Glob. Ecol. Conserv. **23**: E01178.

Armstrong, J.T. (1965): Breeding home range in the nighthawk and other birds - Its evolutionary and ecological significance. Ecology **46**: 619-629.

Baird, T.A., Timanus, D.K., Sloan, C.L. (2003): Intra- and intersexual variation in social behavior: Effects of ontogeny, phenotype, resources, and season. In: Lizard Social Behavior, pp. 7-46. Fox, S.F, McCoy, J.J, Baird, T.A., Eds, Baltimore, John Hopkins Univerisity Press.

Balouch, D., Driscoll, D.A., Naseer, A. (2022): Impacts of land cover on reptile movement and habitat use in farming landscapes. Anim. Conserv. **25**: 837-848.

Bergstrom, B.J. (1988): Home ranges of 3 species of chipmunks (*Tamias*) as assessed by radiotelemetry and grid trapping. J. Mammal. **69**: 190-193.

Blundell, G.M., Maier, J.A.K., Debevec, E.M. (2001): Linear home ranges: effects of smoothing, sample size, and autocorrelation on Kernel estimates. Ecol. Monogr. **71**: 469-489.

Boerger, L., Dalziel, B.D., Fryxell, J.M. (2008): Are there general mechanisms of animal home range behaviour? A review and prospects for future research. Ecol. Lett. **11**: 637-650.

Burt, W.H. (1943): Territoriality and home range concepts as applied to mammals. J. Mammal. **24**: 346-352.

Cagnacci, F., Boitani, L., Powell, R.A. (2010): Animal ecology meets Gps-based radiotelemetry: A perfect storm of opportunities and challenges. Philos. Trans. R. Soc. B-Biol. Sci. **365**: 2157-2162.

Calenge, C. (2006): The package “Adehabitat” for the r software: A tool for the analysis of space and habitat use by animals. Ecol. Model. **197**: 516-519.

Clement, V.E., Schluckebier, R., Rodder, D. (2022): About lizards and unmanned aerial vehicles: Assessing home range and habitat selection in *Lacerta agilis*. Salamandra **58**: 24-42.

De Solla, S.R., Bonduriansky, R., Brooks, R.J. (1999): eliminating autocorrelation reduces biological relevance of home range estimates. J. Anim. Ecol. **68**: 221-234.

DeNardo, D.F., Sinervo, B. (1994): Effects of corticosterone on activity and home-range size of free-ranging male lizards. Horm. Behav. **28**: 53-65.

Fieberg, J., Kochanny, C.O. (2005): Quantifying home-range overlap: The importance of the utilization distribution. J. Wildl. Manage. **69**: 1346-1359.

Garcia-Rosales, A., Ramirez-Bautista, A., Octavio-Aguilar, P. (2021): Aggressive sexual behaviour and spatial distribution of the polymorphic lizard *Sceloporus minor* (Squamata: Phrynosomatidae) from Central Mexico. Salamandra **57**: 151-161.

Gitzen, R.A., Millspaugh, J.J., Kernohan, B.J. (2006): Bandwidth selection for fixed-kernel analysis of animal utilization distributions. J. Wildl. Manage. **70**: 1334-1344.

Guerrero-Sanchez, S., Majewski, K., Orozco-Terwengel, P. (2022): The effect of oil palm-dominated landscapes on the home range and distribution of a generalist species, the Asian water monitor. Ecol. Evol. **12**: e8531.

Harestad, A.S., Bunnell, F.L. (1979): Home range and body-weight - Re-evaluation. Ecology **60**: 389-402.

Harris, S., Cresswell, W.J., Forde, P.G. (1990): Home-range analysis using radio-tracking data - a review of problems and techniques particularly as applied to the study of mammals. Mammal Rev. **20**: 97-123.

Huey, R.B., Peterson, C.R., Arnold, S.J. (1989): Hot rocks and not-so-hot rocks - Retreat-site selection by garter snakes and its thermal consequences. Ecology **70**: 931-944.

Kearney, M.R., Munns, S.L., Moore, D. (2018): Field tests of a general ectotherm niche model show how water can limit lizard activity and distribution. Ecol. Monogr. **88**: 672-693.

Kie, J.G., Matthiopoulos, J., Fieberg, J. (2010): The home-range concept: Are traditional estimators still relevant with modern telemetry technology? Philosophical Transactions of The Royal Society b-Biological Science, **365**: 2221-2231.

Knapp, C., Owens, A.K. (2005): An effective new radio transmitter attachment technique for lizards. Herpetol. Rev. **36**: 264-266.

Kusaka, C., Valdivia, J. (2021): Methods of estimating lizard space use: A comparison of methods across species, sex, and age classes. Integr. Comp. Biol. **61**: E486-e486.

Laver, P.N., Kelly, M.J. (2008): A critical review of home range studies. J. Wildl. Manage. **72**: 290-298.

Li, W., Song, Y., Shi, L. (2013): Home Range of *Teratoscincus Roborowskii* (Gekkonidae): Influence of sex, season, and body size. Acta Ecol. Sin. **33**: 395-401.

Lira, P.K., Dos Santos Fernandez, F.A. (2009): A comparison of trapping- and radiotelemetry-based estimates of home range of the Neotropical opossum *Philander frenatus*. Mamm. Biol. **74**: 1-8.

Maher, C.R., Lott, D.F. (2000): A review of ecological determinants of territoriality within vertebrate species. Am. Midl. Nat. **143**: 1-29.

Mitchell, M.S., Powell, R.A. (2004): A mechanistic home range model for optimal use of spatially distributed resources. Ecol. Model. **177**: 209-232.

Morrison, S.F., Biciloa, P., Harlow, P.S. (2013): Spatial ecology of the critically endangered Fijian crested iguana, *Brachylophus vitiensis*, in an extremely dense population: Implications for conservation. PLOS One **8**: e73127.

Mysterud, A., Perez-Barberia, F.J., Gordon, I.J. (2001): The effect of season, sex and feeding style on home range area versus body mass scaling in temperate ruminants. Oecologia **127**: 30-39.

Noonan, M.J., Tucker, M.A., Fleming, C.H. (2019): A comprehensive analysis of autocorrelation and bias in home range estimation. Ecol. Monogr. **89**: e01344.

Nunn, C.L., Barton, R.A. (2000): Allometric slopes and independent contrasts: A comparative test of Kleiber's Law in primate ranging patterns. Am. J. Phys. Anthropol. **156**: 519-533.

Payne, E., Spiegel, O., Sinn, D.L. (2022): Intrinsic traits, social context, and local environment shape home range size and fidelity of sleepy lizards. Ecol. Monogr. **92**: e1519.

Perry, G., Garland, T. (2002): Lizard home ranges revisited: Effects of sex, body size, diet, habitat, and phylogeny. Ecology **83**: 1870-1885.

Powell, G.L., Russell, A.P. (1985): Growth and sexual size dimorphism in Alberta populations of the eastern short-horned lizard, *Phrynosoma-douglassi-brevirostre*. Can. J. Zool. **63**: 139-154.

Powell, R.A. (2000): Animal home ranges and territories and home range estimators. Res. Tech. Anim. Ecol. **442**: 65-110.

Powell, R.A., Mitchell, M.S. (2012): What is a home range? J. Mammal. **93**: 948-958.

Renet, J., Dokhelar, T., Thirion, F. (2022): Spatial pattern and shelter distribution of the ocellated lizard (*Timon lepidus*) in two distinct Mediterranean habitats. Amphib. Reptil. **43**: 263-276.

Ryberg, W.A., Garrett, T.B., Adams, C.S. (2019): Life in the thornscrub: Movement, home range, and territorialityof the reticulate collared lizard (*Crotaphytus reticulatus*). J. Nat. Hist. **53**: 1707-1719.

Salido, C.A., Vicente, N.S. (2019): Sex and refuge distance influence escape decision in a *Liolaemus* lizard when it is approached by a terrestrial predator. Behaviour **156**: 909-925.

Schoener, T.W. (1968): Sizes of feeding territories among birds. Ecology **49**: 123.

Seaman, D.E., Millspaugh, J.J., Kernohan, B.J. (1999): Effects of sample size on Kernel home range estimates. J. Wildl. Manage. **63**: 739-747.

Seaman, D.E., Powell, R.A. (1996): An evaluation of the accuracy of Kernel density estimators for home range analysis. Ecology **77**: 2075-2085.

Sillero, N., Dos Santos, T.A.C. (2021): Ecological niche models improve home range estimations. J. Zool. **313**: 145-157.

Silverman, B.W. (1986): Density estimation for statistics and data analysis. Chapman & Hall, London.

Stamps, J.A., Krishnan, V.V. (1994): Territory acquisition in lizards. 1. First encounters. Anim. Behav. **47**: 1375-1385.

Team, R.C. (2019): A language and environment for statistical computing. r foundation for statistical computing. Vienna, Austria.

Tisell, H.B., Degrassi, A.L., Stephens, R.B., (2019): Influence of field technique, density, and sex on home range and overlap of the Southern red-backed vole (*Myodes Gapper*i). Can. J. Zool. **97**: 1101-1108.

Turner, F.B., Jennrich, R.I., Weintraub, J.D. (1969): Home ranges and body size of lizards. Ecology **50**: 1076.

Utsumi, K., Staley, C., Nunez, H. (2022): The social system of the lava lizard, *Microlophus atacamensis*: The interplay between social structure and social organization. Rev. Chil. Hist. Nat. **95**: 2-13.

Ventura, S., Vaclav, A., Pinheiro, L. (2022): Habitat suitability or female availability? What influences males' home-range size in a Neotropical montane lizard? Can. J. Zool. **101**: 57-64

Verwaijen, D., Van Darnme, R. (2008): Wide home ranges for widely foraging lizards. Zoology **111**: 37-47.

Warner, D.A., Shine, R. (2008): Maternal nest-site choice in a lizard with temperature-dependent sex determination. Anim. Behav. **75**: 861-870.

Williams, H.J., Taylor, L.A., Benhamou, S. (2020): Optimizing the use of biologgers for movement ecology Research. J. Anim. Ecol. **89**: 186-206.

Worton, B.J. (1995): Using Monte-Carlo simulation to evaluate Kernel-based home-range estimators. J. Wildl. Manage. **59**: 794-800.

Zhao, X.L., Yu, W., Zhu, Z.Y. (2022): Factors influencing home ranges of the Qinghai toad-headed lizard (*Phrynocephalus vlangal*ii) on the Dangjin Mountain, Gansu. Asian Herpetol. Res. **13**: 137-144.

Zhong, Y.X., Chen, C.W., Wang, Y.P. (2021): Biological and extrinsic correlates of extinction risk in Chinese lizards. Curr. Zool. **68**: 285-293.

Acta Herpetologica 20(1): 93-95, 2025
DOI: 10.36253/a_h-17768

Land leech (*Haemadipsa zeylanica*) parasitizing *Indosylvirana indica* (Indian golden-backed frog) in Agumbe, India

Mahesh Bilaskar[1,*], Surendra Walke[2]

[1] *Department of Environmental Sciences, Savitribai Phule Pune University, Ganeshkhind Road, Pune, 411007, Maharashtra, India*
[2] *Survey No. 112/2/2/2, Opposite Hemgiri Apartment, Baner, Pune -411045, Maharashtra, India*
**Corresponding author.* Email: rishi08sep@gmail.com

Submitted on: 2025, 29th April; revised on: 2025, 1st May; accepted on: 2025, 14th May
Editor: Enrico Lunghi

Abstract. This study documents the first recorded instance of ectoparasitism of the Indian golden-backed frog (*Indosylvirana indica*) by the land leech (*Haemadipsa zeylanica*) in Agumbe, India. Observation indicates that a specimen of *I. indica* was infested by *H. zeylanica*, with the leech attached to the frog's tympanum. While leeches are known ectoparasites of amphibians and can disrupt their health by serving as vectors for pathogens, this association had not been previously documented for *I. indica*. Our findings highlight the need for further research into leech-anuran interactions to better understand ecological dynamics and their implications on amphibian health.

Keywords. Anura, brown leech, endemic, Haemadipsidae, jawed land leech, predation, Ranidae, type leech (Cochin).

Empirical evidence of anuran–leech parasitic associations suggests that anurans represent a principal host group for leeches (Berven and Boltz, 2001; Merilä and Sterner, 2002). Leeches are extensively documented as ectoparasites of amphibians, exhibiting parasitic interactions across multiple life stages, including ovum, embryonic (Burgin and Schell, 2005), and adult phases (Merilä and Sterner, 2002). These hematophagous organisms not only inflict direct physiological stress through blood-feeding and tissue damage but also play a potential role as mechanical vectors in pathogen transmission. Notably, leeches have been implicated in the dissemination of protozoan and fungal pathogens, including *Ichthyophonus* spp. and *Trypanosoma* spp., thereby contributing to the epidemiology of infectious diseases within amphibian populations (Delima et al., 2024).

In India, the ecological and parasitic associations between leeches and anurans remain insufficiently investigated and represent a relatively underexplored area of research. Numerous field observations have been reported by herpetologists; however, the majority of these accounts remain undocumented in peer-reviewed literature. To date, there are no published accounts documenting leech–anuran interactions involving species of the genus *Indosylvirana*. Nevertheless, a previously published record reports the presence of leeches affixed to vocalizing male rhacophorid frogs (*Raorchestes* sp.) within Silent Valley National Park, situated in the Western Ghats of India (Zachariah et al., 2016).

Indosylvirana indica, the Indian golden-backed frog, is a species of frog endemic to Western Ghats, north of the Palghat Gap in the states of Karnataka and Kerala (Biju et al., 2014; Frost, 2019). This species was formerly considered conspecific with *Indosylvirana temporalis*, but was subsequently recognized as a distinct species based on morphological and molecular evidence (Biju et al., 2014). On 06 June 2024, at 12.43 PM in the Agumbe Forest, Shimoga district, Karnataka, India (13.5087°N, 75.0959°E; elevation 660 m asl), we observed an individual of *I. indica* being parasitized by a leech (Fig. 1). The

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

www.fupress.com/ah

Fig. 1. Land leech (*Haemadipsa zeylanica*) attached to the tympanum of *Indosylvirana indica.*

posterior end of the leech was attached to the frog's tympanum and anterior was towards the narrower end of the frog's mouth. The species of leech was identified as *Haemadipsa zeylanica cochiniana*, which is considered a land leech (Fogden and Proctor, 1985). This leech is endemic to India, having type locality Cochin (Chandra, 1982; Mandal, 2004). Land leeches (*Haemadipsa* spp.), known for biting humans and cattle's (Chandra, 1982), are widely distributed throughout rainforests from Madagascar and India to Southeast Asia, the western Pacific, and Australia. During the 1977-1978 joint expedition to Gunung Mulu National Park, Sarawak, conducted by the Sarawak Forest Department and the Royal Geographical Society, researchers studied leech species in the area. Two of the three *Haemadipsa* species known from Borneo – *Haemadipsa zeylanica* and *H. picta* – were recorded in the park. *H. zeylanica* was found to be the more abundant and widespread species, primarily occurring at ground level, while the rarer *H. picta* was often observed climbing up to two metres into the undergrowth. Both species attacked humans, but only a single instance of non-human predation by *Haemadipsa* species – specifically *H. zeylanica* Moore and *H. picta* Moore – on a frog has been reported (Fogden and Proctor, 1985).

However, land leeches, particularly species like *Batracobdella algira*, have been documented parasitizing various amphibians, including the endemic Sardinian *Speleomantes* salamanders (Ben Ahmed et al., 2015; Manenti et al., 2016). These leeches feed on amphibians in both aquatic and terrestrial environments, often attaching to their hosts, consuming their blood, and sometimes acting as vectors for micro-parasites such as *Trypanosoma* and *Lankesterella* (Jiménez Sánchez, 1997). Despite the limited number of documented cases, the parasitic relationship between land leeches and amphibians, including potential impacts on body condition, warrants further study, as some reports suggest negative effects on the hosts' health (Elliot and Dobson, 2015). While leech predation on amphibians has generally been seen as opportunistic without causing immediate death, the role of environmental factors, such as stable microclimates in terrestrial habitats, may influence the extent of this parasitism and its long-term consequences for amphibian populations (Rocha et al., 2012). The study performed by Lunghi et al. (2018) also revealed a strong association between environmental features, such as high humidity and water hardness in karst cave systems, and leech prevalence. While *B. algira* did not significantly reduce the Body Condition Index (BCI) of its salamander hosts overall, individuals carrying heavier parasite loads exhibited a decline in BCI, suggesting cumulative physiological costs (Lunghi et al., 2018). This pattern parallels the potential, yet undocumented, fitness effects that *H. zeylanica* may have on *I. indica* when attachment duration or infestation intensity is high. These findings together highlight that leech–amphibian associations, though often overlooked, can be influenced by shared ecological variables and may bear sublethal consequences worthy of further investigation. To the best of our knowledge, this represents the first documented instance of ectoparasitism by a land leech in *I. indica*, thereby expanding our understanding of host-parasite interactions within this species. While prior observations have reported mosquitoes (*Uranotaenia* sp.) engaging in hematophagy on *I. indica* in the Western Ghats (Kalki et al., 2020), there are no published records detailing leech parasitism in this amphibian. This novel finding underscores the need for further research into the ecological dynamics and potential health implications of such parasitic relationships in amphibian populations.

ACKNOWLEDGMENTS

We thank Deepak Deshpande and Amatya Sharma for their assistance in confirming the identification of the species and Virendra Gaikwad for helping us with photo editing.

REFERENCES

Ben Ahmed, R., Romdhane, Y., Tekaya, S. (2015): Checklist and distribution of marine and freshwater leeches (Annelida, Clitellata, Hirudinea) in Tunisia with identification key. Ecol. Monogr. **2**: 3-19.

Berven, K.A., Boltz, R.S. (2001): Interactive effects of leech (*Desserobdella picta*) infection on wood frog (*Rana sylvatica*) tadpole fitness traits. Copeia **4**: 907-915.

Biju, S.D., Garg, S., Mahony, S., Wijayathilaka, N., Senevirathne, G., Meegaskumbura, M. (2014): DNA barcoding, phylogeny and systematics of Golden-backed frogs (*Hylarana*, Ranidae) of the Western Ghats-Sri Lanka biodiversity hotspot, with the description of seven new species. Contr. Zool. **83**: 269-335.

Burgin, S., Schell, C.B. (2005): Frog eggs: Unique food source for leech *Bassianobdella fusca*. Acta Zool. Sin. **51**: 349-353.

Chandra, M. (1982): A Check-List of Leeches of India. Rec. Zool. Surv. India **80**: 265-290.

Delima-Baron, E.M., Tagoon, M.D., Bosabica, Z.C., Vitor-Maluya, K.C. (2024): First account of a leech attached to *Philautus acutirostris* (Peters, 1867) in southern Mindanao, Philippines. Herpetol. Notes **17**: 499-501.

Elliot, J.M., Dobson, M. (2015): Freshwater leeches of Britain and Ireland: keys to the Hirudinea and a review of their ecology. The Freshwater Biological Association, Ambleside.

Fogden, S. C. L., Proctor, J. (1985): Notes on the Feeding of Land Leeches (*Haemadipsa zeylanica Moore* and *H. picta Moore*) in Gunung Mulu National Park, Sarawak. Biotropica **17**: 172-174.

Frost, D.R. (2019): "*Indosylvirana indica* (Biju, Garg, Mahony, Wijayathilaka, Senevirathne, and Meegaskumbura, 2014)": Amphibian Species of the World: An Online Reference. Version 6.0. American Museum of Natural History.

Jiménez Sánchez, M.S. (1997): Contribución al conocimiento de la parasitofauna de la *Rana perezi* Seoane, 1885 (Amphibia: Ranidae) de la provincia de Ávila. Parasitología. Universidad Complutense de Madrid- Facultad de Farmacia, Madrid.

Kalki, Y., Huizinga, R., Weiss, M. (2020): Mosquitoes (*Uranotaenia sp.*) biting an Indian Golden-backed Frog (*Indosylvirana indica*). Reptil. Amphib. **27**: 454-455.

Lunghi, E., Ficetola, G.F., Mulargia, M., Cogoni, R., Veith, M., Corti, C., Manenti, R. (2018): *Batracobdella* leeches, environmental features and *Hydromantes* salamanders.vInt. J. Parasitol-Par. **7**: 48-53.

Mandal, C.K. (2004): Checklist of the Hirudinea (Leeches) of India. Rec. Zool. Surv. India **102**: 41-46.

Manenti, R., Lunghi, E., Canedoli, C., Bonaccorsi, M., Ficetola, G.F. (2016): Parasitism of the leech, *Batracobdella algira* (Moquin-Tandon, 1846), on Sardinian cave salamanders (genus *Hydromantes*) (Caudata: plethodontidae). Herpetozoa **29**: 27-35.

Merilä, J., Sterner, M. (2002): Medicinal leeches (*Hirudo medicinalis*) attacking and killing adult amphibians. Ann. Zool. Fenn. **34**: 343-346.

Rocha, R., Borda, E., Andreone, F., Rosa, G.M. (2012): First reports of leech parasitism in Malagasy anurans. Comp. Parasitol. **79**: 352-356.

Zachariah, A., Cyriac, V.P., Chandramohan, B., Ansil, B.R., Matthew, J.K., Raju, D.V. Abraham, R.K. (2016): Two new species of *Raorchestes* (Anura: *Rhacophoridae*) from the Silent Valley National Park in the Nilgiri hills of the Western Ghats, India. Salamandra **52**: 63-76.

Acta Herpetologica 20(1): 97-101, 2025
DOI: 10.36253/a_h-16272

Screening for *Batrachochytrium salamandrivorans* in a population of Golden Alpine Salamanders at the edge of their distribution range

Antonio Romano[1,2,*], Emma Centomo[2], Lorenzo Dondero[3], Elena Grasselli[3], Paolo Pedrini[2], Luca Roner[2,*]

[1] *Consiglio Nazionale delle Ricerche - Istituto per la BioEconomia, Via dei Taurini 19, I-00185 Roma, Italy*
[2] *Ambito Biologia della Conservazione, Ufficio Ricerca e Collezioni, MUSE—Museo delle Scienze, Corso del Lavoro e della Scienza 3, I-38122 Trento, Italy*
[3] *Department of Earth, Environment and Life Sciences (DISTAV), University of Genova, Corso Europa 26, I-16132 Genova, Italy*
**Corresponding authors.* Email: antonio.romano@cnr.it; luca.roner@muse.it

Submitted on: 2024, 20th June; revised on: 2024, 20th June; accepted on: 2024, 28th November
Editor: Enrico Lunghi

Abstract. Amphibian populations worldwide are experiencing significant declines, highlighting a critical aspect of the broader biodiversity crisis. Approximately 43% of all amphibian species are facing extinction due to factors such as habitat loss, pollution, climate change, and emerging diseases. The chytrid fungus *Batrachochytrium salamandrivorans* (Bsal) represents one of the major threats, because it is particularly dangerous for European salamanders. Southern Europe is especially vulnerable due to the presence of numerous endemic salamander species. Despite the risks, few studies have screened Italian salamanders for Bsal. We conducted a Bsal screening on 44 Golden Alpine Salamanders (*Salamandra atra aurorae*) from the Vezzena plateau in the Trentino-Alto Adige region (Northern Italy). Our molecular analysis of skin swabs revealed no presence of Bsal in any of the 44 specimens examined. Additionally, no macroscopic signs of Bsal-related skin damage were observed. The absence of Bsal in our samples is encouraging, suggesting that the investigated sites are currently unaffected by this pathogen. This finding aligns with other studies reporting no evidence of Bsal in Italy. Future research should explore the factors contributing to the absence of Bsal and the effectiveness of current conservation practices. While our findings are reassuring, the threat of Bsal remains a critical concern. Continued vigilance and enhanced conservation efforts are essential to protect salamander diversity and ensure the long-term survival of these important amphibian populations.

Keywords. Amphibians, Chytridiomycosis, conservation, endemic taxon, threats.

INTRODUCTION

Amphibian populations worldwide are experiencing a significant decline, underscoring a critical aspect of the broader biodiversity crisis. Approximately 43% of all amphibian species face extinction due to factors like habitat loss, pollution, climate change, and emerging diseases (Luedtke et al., 2023). Two chytrid fungi, *Batrachochytrium dendrobatidis* (Bd) and *B. salamandrivorans* (Bsal), pose major threats to amphibians' conservation. They are capable of causing mass die-offs likely due to pathogen pollution (caused by global animal trade for food, collecting, etc.) and the susceptibility of species naïve to new chytrid lineages (McKenzie et al., 2012; Martel et al., 2013; Rosa et al., 2013; O'Hanlon et al., 2018). Although our understanding of the immune response is still limited, Bsal is especially dangerous for European salamanders due to their susceptibility and the virulence factors of this fungal pathogen, which can lead to immune system compromise, tissue erosion, and impaired respiratory and rehydration functions (Martel et al., 2014; Stegen et al., 2017; Grogan et al., 2020).

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

www.fupress.com/ah

Southern Europe, including the Italian and Iberian peninsulas, is particularly vulnerable due to numerous endemic salamander species. Italy, for instance, hosts 19 species of urodeles, many of which are endemic (Sindaco and Razzetti, 2021). Despite the risks, only in recent years few studies have screened Italian salamanders for Bsal (Grasselli et al., 2019; Grasselli et al., 2021; Bernabo et al., 2024; Böning et al., 2024). Although Bsal has not yet been reported in Italian salamander populations, the entire urodeles fauna remains at risk from potential pathogen pollution. Therefore, proactive monitoring of Bsal in wild amphibian populations is crucial.

The Alpine salamander, *Salamandra atra* Laurenti 1758, has been found to be highly susceptible to Bsal in captivity (Fitzpatrick et al., 2018), raising significant conservation concerns. This species, restricted to the European and Dinaric Alps, includes several intraspecific lineages, with some recognized as subspecies (Bonato et al., 2018). The Italian endemic subspecies, *S. a. aurorae* Trevisan 1982, and *S. a. pasubiensis* Bonato and Steinfartz 2005, inhabit areas of less than 100 km² each. In a recent study involving 90 individuals of *S. atra*, including 28 *S. a. aurorae*, the Golden Alpine Salamander, no presence of Bsal was found (Böning et al., 2024). Here, we report the results of a Bsal screening conducted on a population of the Golden Alpine Salamander from Trentino province. This locality differs from those sampled in Böning et al. (2024) and is located at the edge of the distribution range of this taxon.

MATERIALS AND METHODS

Salamander sampling

The study area (Fig. 1) encompasses a small segment of the Golden Alpine Salamander' range, situated on the Vezzena plateau in the Trentino Alto Adige region (45°57′10″N, 11°22′25″E) at an elevation of approximately 1450 meters above sea level. The Vezzena plateau is characteristic of the Alpine Mountain region's general climatic conditions. Forty-four Golden Alpine salamanders (10 females, 31 males, 3 juveniles) were captured on summer 2020, measured (total length, weight) and sexed. All skin swabs were collected using a standardized protocol (Blooi et al., 2013), specifically following the procedure established in previous Bd and Bsal studies on Italian salamanders (Grasselli et al., 2019; Costa et al., 2021; Grasselli et al., 2021). Sterile cotton swabs were rubbed 30 times on various parts of the salamander's body and stored in individual sterile plastic tubes at 4 °C until extraction (Dondero et al., 2023). Additionally, all individuals were visually inspected for any physical abnormalities and signs of chytridiomycosis like skin lesions and ulcerations (Martel et al., 2013).

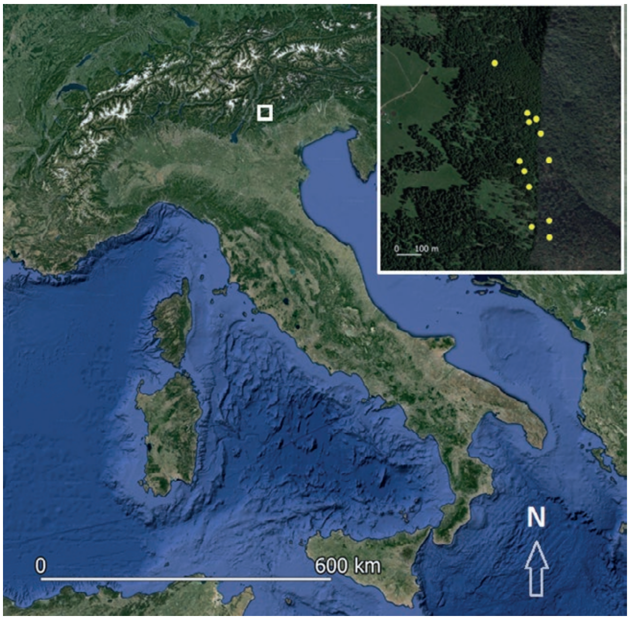

Figure 1. Map showing sampling area of the Golden Alpine salamander on the Vezzena plateau (Trentino-Alto Adige, northern Italy) for *Batrachochytrium salamandrivorans* screening. The box shows the detail of the salamander sampling points.

Laboratory protocols for Bd and Bsal detection

Nucleic acid extraction from skin swabs for quantitative PCR (qPCR) was performed according to the method described by Boyle et al. (2004). In brief, nucleic acids were extracted using 200 µL of PrepMan Ultra - Sample Preparation Reagent (Thermo-Fisher Scientific Technologies, Monza, Italy) and 0.03-0.04 g of Zirconium/silica beads (Biospec Products). The extraction involved two rounds of incubation in a Bead Beater (MM200 - Retsch GmbH, Hann, Germany) for 1 minute at the highest frequency (25 Hz), followed by centrifugation at 13,000 g for 1 minute. This was followed by incubation at 100 °C for 10 minutes in a Dry Block Thermostat, and a subsequent centrifugation at 13,000 g for 3 minutes. The supernatant was then recovered and stored at -20 °C until Real-Time PCR analysis was performed. Samples were analyzed in at least duplicate for the presence of Bsal DNA using a SYBR Green- based Real-Time PCR assay targeting the 5.8S rRNA gene of Bsal, as described by Blooi et al. (2013), without the employ of the probe, as in the original assay. The Bsal SYBR Green assay was conducted on a CFX96 real-time system (Bio-Rad Laboratories, Hercules, CA) with a reaction mixture comprising 12.5 µL iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA), 300 nM of each primer (Bsal fwd primer: SterF - 5′-AGCCAAGAGATCCGTTGTCAAA-3′; Bsal rev primer: SterR - 5′-TGAACGCACATTGCACTCTAC-3’), 5 µL of template, and RNase/DNase-free water to a total volume of 25 µL per reaction. To quantify Bsal DNA, ten-fold serial dilutions of Bsal DNA were prepared, achieving concentrations ranging from 1,000 to 0.1 genomic equivalents (GEs) of zoospores per reaction mixture, as per Thomas et al. (2018). Bsal DNA standards for qPCR were kindly provided by Prof. An Martel and Frank Pasmans (University of Ghent, Belgium). The amplification conditions were set at 10 minutes at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and 15 seconds at 62 °C. A melting curve from 60 to 95 °C was obtained, with readings at every 0.5°C increment (Blooi et al., 2013).

It is noteworthy that our results are derived from a modification of the Blooi et al. 2013 method, as we did not include the probe in our assay. The absence of positive samples within the salamander population is accompanied by a positive amplification of the standard curve.

RESULTS

The males were on average 12.6 cm long (range: 10.7-14.9 cm; SD = 0.95), the females 13.0 cm (range: 12.0-13.9 cm; SD = 0.67), the juveniles 10.3 cm (range: 6.6-12.6 cm; SD = 2.07), and weighed 7.9 g (range: 5.3-9.7 g; SD = 1.08), 9.6 g (range: 7.4-13.9 g; SD = 1.82) and 5.0 g (range: 3.9-6.2; SD = 1.13) respectively. Our molecular analysis of skin swabs revealed the absence of Bsal in all 44 specimens examined in this study. Additionally, no Bsal-typic macroscopic skin damage was observed during our surveys.

DISCUSSION

Bsal infection poses a major threat to salamander diversity in Central Europe and Spain (Martel et al., 2014; Bosch et al., 2021) and, in future projections, particularly to Italian amphibians (Luedtke et al., 2023; but see the critique in Canessa et al., 2024). However, none of the qPCR samples from our study sites tested positive for Bsal infection. The relatively high number of salamanders we sampled from the same population should ensure accurate screening, capable of detecting infections even at low prevalence rates. The absence of Bsal infection in the qPCR samples collected from our investigated sites is an encouraging finding. This result suggests that, at least for the moment, the sites under study remain unaffected by this pathogen. Our finding align with the study by Dondero et al. (2023), Bernabo et al. (2024) and Böning et al. (2024), who also reported no evidence of Bsal presence in Italy.

Several factors may contribute to the absence of Bsal in our sampled areas. Simulation studies, employing Species Distribution Models (SDMs) to anticipate the spread of invasive species, have considered bioclimatic suitability, salamander species richness, and salamander imports (Katz and Zellmer, 2018). These studies suggest that, although most Italian salamanders are highly susceptible to chytrid fungus (Beukema et al., 2018; Dondero et al., 2023), the ecological and climatic conditions in Italy are not optimal for Bsal. Consequently, the risk of pathogen spread in Italy seems to be relatively low. However, the specific area we studied is nationally recognized as having the highest probability (refer to Fig. 3 in Katz and Zellmer, 2018).

One possibility is that the geographic or environmental conditions in these regions are not conducive to the survival or spread of Bsal. Additionally, local salamander populations might possess some level of resistance or immunity to the pathogen, which could prevent its establishment.

The current absence of Bsal in the study area provides a valuable opportunity to implement proactive preventive measures. As highlighted by Thomas et al. (2019), effective mitigation strategies in Bsal-free areas include establishing early warning systems, continuous surveillance, and stringent biosecurity protocols. The surveyed area has traditionally been subject to forest management, ungulate hunting, and regulated mushroom picking, with more recent and limited recreational activities, such as outdoor walking. Completely restricting access for biosecurity reasons, however, is both impractical and potentially ineffective, particularly within the study area in Trentino. This is because the Trentino populations of the salamanders is territorially contiguous with the area in Veneto occupied by the rest of the population of this taxon. Nevertheless, other targeted measures are both feasible and necessary. These include regular (e.g., biennial) screening for Bsal infection, educational initiatives emphasizing the importance of practices like shoe disinfection, and managing human access if recreational use significantly increases. It is particularly important to emphasize the need for health precautions for herpetologists conducting studies in the area. These researchers must continue adhering to the health protocols established by the *Societas Herpetologica Italica* (http://www-9.unipv.it/webshi/conserv/monitanf.htm), which they have so far complied with. This is crucial, as the health impact of herpetological research may not be negligible (Razzetti and Bonini, 2001). The relative isolation of the habitat and the currently low intensity of human activity are favourable factors, potentially reducing the risk of pathogen introduction. However, these conditions should not lead to complacency, and ongoing surveillance is essential to ensure the area remains pathogen-free. This aligns with broader efforts in Italy, where continuous surveillance for herpetofaunal diseases, as outlined by Marini et al. (2023), has proven effective. Regular monitoring of salamander populations and their habitats can help detect early signs of Bsal infection, enabling swift responses to prevent the pathogen's establishment and spread.

ACKNOWLEDGEMENTS

Captured animals were handled with the authorisation of the Italian Ministry of Ecological Transition (PNM-2018-0006709). Thanks to Giulia Bombieri, Claudia Pellegrini and Aurora Colangelo for the support in the field. The municipality of Levico Terme (TN) provided logistical facilities. The Autonomous Province of Trento supported the research activity.

REFERENCES

Bernabò, I., Cittadino, V., Iannella, M., Corapi, A., Tiberti, R., Dondero, L., Grasselli, E., Romano, A. (2024): A taxon under siege from multiple threats: results of analysis of chytrid fungi on the Calabrian alpine newt. Amphib.-Reptil. **46**: 1-12.

Beukema, W., Martel, A., Thien Nguyen, T.T, Goka, K, Schmeller, D.S., Yuan, Z., Laking, A.E., Quang Nguyen, T., Lin, C-F., Shelton, J., Loyau, A., Pasmans, F. (2018): Environmental context and differences between native and invasive observed niches of *Batrachochytrium salamandrivorans* affect invasion risk assessments in the Western Palaearctic. Divers. Distrib. **24**: 1788-1801.

Blooi, M., Pasmans, F., Longcore, J.E., Spitzen-van der Sluijs, A., Vercammen, F., Martel, A. (2013): Duplex Real-Time PCR for Rapid Simultaneous Detection of *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* in Amphibian Samples. J. Clin. Microbiol. **54**: 246-246.

Bonato, L., Corbetta, A., Giovine, G., Romanazzi, E., Sunje, E., Vernesi, C., Crestanello, B. (2018): Diversity among peripheral populations: genetic and evolutionary differentiation of *Salamandra atra* at the southern edge of the Alps. J. Zool. Syst. Evol. Res. **56**: 533-548.

Böning, P., Lötters, S., Barzaghi, B., Bock, M., Bok, B., Bonato, L., Ficetola, G.F., Glaser, F., Griese, J., Grabher, M., Leroux, C., Munimanda, G., Manenti, R., Ludwig, G., Preininger, D., Rödel, M.O., Seibold, S., Smith, S., Tiemann, L., Thein, J., Veith, M., Plewnia, A. (2024): Alpine salamanders at risk? The current status of an emerging fungal pathogen. PLOS One **19**: e0298591.

Bosch, J., Martel, A., Sopniewski, J., Thumsova, B., Ayres, C., Scheele, B.C., Guillermo Velo-Anton, G., Pasmans, F. (2021): *Batrachochytrium salamandrivorans* threat to the Iberian urodele hotspot. J. Fungi **7**: 644.

Boyle, D.G., Boyle, D.B., Olsen, V., Morgan, J.A.T., Hyatt, A.D. (2004): Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Dis. Aquat. Organ. **60**: 141-148.

Canessa, S., Biancolini, D., Iannella, M., Bernabò, I., Salvi, D., Vignoli, L., Lunghi, E., Costa,E.,Razzetti, E., Ficetola, G.F., Romano,A. (2024): Uncertain future and uncertain projections: assessing extinction risks in European salamanders from projected chytrid fungus invasion using IUCN Criterion E. Acta Herpetol. **19**: 155-159.

Costa, A., Dondero, L., Allaria, G., Morales Sanchez, B.N., Rosa, G., Salvidio, S., Grasselli, E. (2021): Modelling the amphibian chytrid fungus spread by connectivity analysis: towards a national monitoring network in Italy. Biodivers. Conserv. **30**: 2807-2825.

Dondero, L., Allaria, G., Rosa, G., Ficetola, G. F., Cogoni, R., Grasselli, E., Salvidio, S. (2023): Threats of the emerging pathogen *Batrachochytrium salamandrivorans* (Bsal) to Italian wild salamander populations. Acta Herpetol. **18**: 3-9.

Fitzpatrick, L.D., Pasmans, F., Martel, A., Cunningham, A.A. (2018): Epidemiological tracing of *Batrachochytrium salamandrivorans* identifies widespread infection and associated mortalities in private amphibian collections. Sci. Rep. **8**: 13845.

Grasselli, E., Bianchi, G., Dondero, L., Marchiano, V., Carafa, M., Perrone, M., Salvidio, S. (2019): First screening of *Batracochytrium salamandrivorans* (Bsal) in wild and captive salamanders from Italy. Salamandra **55**: 124-126.

Grasselli, E., Bianchi, G., Salvidio, S. (2021): *Batrachochytrium salamandrivorans* in Europe: the Italian contribution to the international mitigation project. In: Atti del XII Congresso Nazionale della Societas Herpetologica Italica (Rende, Cosenza), pp. 214-217. Tripepi, S., Ed, Ventura Edizioni, Senigallia.

Grogan, L.F., Humphries, J.E., Robert, J., Lanctot, C.M., Nock, C.J., Newell, D.A., McCallum, H.I. (2020):

Immunological aspects of chytridiomycosis. J. Fungi **6**: 234.

Katz, T.S., Zellmer, A.J. (2018): Comparison of model selection technique performance in predicting the spread of newly invasive species: a case study with *Batrachochytrium salamandrivorans*. Biol. Invasions **20**: 2107-2119.

Luedtke, J. A., Chanson, J., Neam, K., Hobin, L., Maciel, A. O., Catenazzi, A., Borzée, A., Hamidy, A., Aowphol, A., Jean, A., Sosa-Bartuano, Á., Fong G, A., de Silva, A., Fouquet, A., Angulo, A., Kidov, A. A., Muñoz Saravia, A., Diesmos, A. C., Tominaga, A., Shrestha, B., ... Stuart, S. N. (2023): Ongoing declines for the world's amphibians in the face of emerging threats. Nature **622**: 308-314.

Marini, D., Filippi, E., Montinaro, G., Origgi, F.C., (2023): Screening of *Ophidiomyces ophidiicola* in the free-ranging snake community annually harvested for the popular ritual of San Domenico e dei Serpari (Cocullo, AQ, Italy). Acta Herpetol. **18**: 45-52.

Martel, A., Blooi, M., Adriaensen, C., Van Rooij, P., Beukema, W., Fisher, M.C., Farrer, R.A., Schmidt, B.R., Tobler, U., Goka, K., Lips, K.R., Muletz, C., Zamudio, K., Bosch, J., Lotters, S., Wombwell, E., Garner, T.W.J., Cunningham, A.A., Spitzen-van der Sluijs, A., Salvidio, S., Ducatelle, R., Nishikawa, K., Nguyen, T.T., Kolby, J.E., Van Bocxlaer, I., Bossuyt, F., Pasmans, F. (2014): Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. Science **346**: 630-631.

Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M.C., Woeltjes, A., Bosman, W., Chiers, K., Bossuyt, F. (2013): *Batrachochytrium salamandrivorans* sp nov causes lethal chytridiomycosis in amphibians. Proc. Natl. Acad. Sci. USA **110**: 15325-15329.

McKenzie, J.V., Peterson A.C. (2012): Pathogen pollution and the emergence of deadly amphibian pathogen. Mol. Ecol. **21**: 5151-5154.

O'Hanlon, S.J., Rieux, A., Farrer, R.A., Rosa, G.M., Waldman, B., Bataille, A., Kosch, T.A., Murray, K.A., Brankovics, B., Fumagalli, M., Martin, M.D., Wales, N., Alvarado-Rybak, M., Bates, K.A., Berger, L., Boll, S., Brookes, L., Clare, F., Courtois, E.A., Cunningham, A.A., Doherty-Bone, T.M., Ghosh, P., Gower, D.J., Hintz, W.E., Hoglund, J., Jenkinson, T.S., Lin, C-F., Laurila, A., Loyau, A., Martel, A., Meurling, S., Miaud, C., Minting, P., Pasmans, F., Schmeller, D.S., Schmidt, B.R., Shelton, J.M.G., Skerratt, L.F., Smith, F., Soto-Azat, C., Spagnoletti, M., Tessa, G., Toledo, L.P., Valenzuela-Sanchez, A., Verster, R., Voros, J., Webb, R.J., Wierzbicki, C., Wombwell, E., Zamudio, K.E., Aanensen, D.M., James, T.Y., Thomas, M., Gilbert, P., Weldon, C., Bosch, J., Balloux, F., Garner, T.M.J., Fisher, M.C. (2018): Recent Asian origin of chytrid fungi causing global amphibian declines. Science **360**: 621-627.

Razzetti, E., Bonini, L. (2001): Infezioni e parassitosi negli Anfibi: il possibile impatto delle ricerche erpetologiche. Atti Soc. It. Sc. Nat. **14**: 97-102.

Rosa, G. M., Anza, I., Moreira, P. L., Conde, J., Martins, F., Fisher, M. C., Bosch, J. (2013): Evidence of chytrid-mediated population declines in common midwife toad in Serra da Estrela, Portugal. Anim. Conserv. **16**: 306-315.

Sindaco, R., Razzetti, E. (2021): An updated check-list of Italian amphibians and reptiles. Nat. Hist. Sci. **8**: 35-46.

Stegen, G., Pasmans, F., Schmidt, B.R., Rouffaer, L.O., Van Praet. S., Schlaub, M., Canessa, S., Laudelout, A., Kinet, T., Adriaensen, C., Haesebrouck, F., Bert, W., Bossuyt, F., Martel, A. (2017): Drivers of salamander extirpation mediated by *Batrachochytrium salamandrivorans*. Nature **544**: 353-356.

Thomas, V., Blooi, M., Van Rooij, P., Van Praet, S., Verbrugghe, E., Grasselli, E., Lukac, M., Smith, S., Pasmans, F., Martel, A. (2018): Recommendations on diagnostic tools for *Batrachochytrium salamandrivorans*. Transbound. Emerg. Dis. **65**: e478-e488.

Thomas, V., Wang, Y., Van Rooij, P., Verbrugghe, E., Baláž, V., Bosch, J., Cunningham, A.A., Fisher, M.C., Garner, T.W.J.,Gilbert, M.J., Grasselli, E., Kinet, T., Laudelout, A., Lötters, S., Loyau, A., Miaud, C., Salvidio, S., Schmeller, D.K., Schmidt, B.R., Spitzen-van der Sluijs, A., Steinfartz, S., Veith, M., Vences, M., Wagner, N., Canessa, S., Martel, A., Pasmans, F. (2019): Mitigating *Batrachochytrium salamandrivorans* in Europe. Amphib.-Reptil. **40**: 265-290.

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

Cover: *Speleomantes italicus*, adult male, photographed in north Tuscany, Italy (photo by Andrea Settesoldi).

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

CONTENTS

June **2025** Vol. **20** – N. **1**