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Cover: A mating pair of *Anolis biporcatus* from Costa Rica
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First description of the mitogenome of the endangered turtle *Erymnochelys madagascariensis* (Testudines: Podocnemididae) and its implications for conservation

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Abstract

First description of the mitogenome of the endangered turtle *Erymnochelys madagascariensis* (Testudines: Podocnemididae) and its implications for conservation. *Erymnochelys madagascariensis* is ranked first on the EDGE of Existence program's list of priority reptiles. This species is the only living member of the family Podocnemididae found outside of South America. It has a unique evolutionary, as it is endemic to Madagascar. We present the first description of the complete mitogenome of the species. The assembled mitogenome is the third and the smallest described for the Podocnemididae. It has a length of 16,421bp, CG content of 38%, and presents 22 tRNAs, two rRNAs, 13 protein-coding genes (PCGs) and one non-coding region. The gene order and CG content were similar to the mitogenome of the *Podocnemis* species. Selective pressure analysis indicated the PCGs were under purifying selection except for *ATPase 8*. The phylogenetic analysis of PCGs of Pleurodira revealed that *Myuchelys* is a polyphyletic group. Our study demonstrates that the complete mitogenome can be a useful tool to assess genetic diversity via the identification of haplotypes among natural populations and detection of introgression events. This information may have important implications for conservation, especially in designing and implementing protected breeding areas and contributing to programs with restocking purposes.

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Keywords: Madagascar Big-headed Turtle, Mitochondrial DNA, Pleurodira, Purifying selection.

Resumo

Primeira descrição do mitogenoma da espécie ameaçada *Erymnochelys madagascariensis* (Testudines: Podocnemididae) e suas implicações para a conservação. *Erymnochelys madagascariensis* ocupa a primeira posição na lista de prioridades de répteis do programa EDGE of Existence. Esta espécie é a única descrita na família Podocnemididae encontrada fora da América do Sul e possui uma história evolutiva única para a herpetofauna de Madagáscar. Neste trabalho, apresentamos a primeira descrição do mitogenoma completo de *E. madagascariensis*. O mitogenoma montado é o terceiro e o menor mitogenoma descrito para Podocnemididae, com um comprimento de 16.421 pb, conteúdo de CG de 38% e apresenta 22 tRNAs, dois rRNAs, 13 genes codificadores de proteínas e uma região não codificadora. A ordem dos genes e o conteúdo de CG foram semelhantes ao mitogenoma das espécies de *Podocnemis*. A análise de pressão seletiva indicou que os genes codificadores de proteínas estavam sob seleção purificadora, com exceção de *ATPase 8*. A análise filogenética dos genes codificadores de proteínas de Pleurodira revelou que *Myuchelys* é grupo polifilético. Nossos dados demonstram que o mitogenoma pode ser uma ferramenta útil para avaliar a diversidade genética, uma vez que pode permitir a determinação de haplótipos entre populações naturais e detectar eventos de introgressão. Essas informações podem ter importantes implicações para a conservação, especialmente na concepção e implementação de áreas de reprodução protegidas e contribuir para programas com objetivos de repovoamento.

Palavras-chave: DNA mitocondrial, Pleurodira, Seleção purificadora, Tartaruga-de-cabeça-grande-de-madagascar.

Introduction

Madagascar is world-renowned for its unique fauna and flora (Wilmé *et al.* 2006). *Erymnochelys madagascariensis* (Grandidier, 1867), commonly known as the Madagascar Big-headed Turtle or Side-necked Turtle, is one of the most endangered reptile species. It is ranked first on the EDGE of Existence program's list of priority reptiles (EDGE 2023). The IUCN considers it critically endangered (Leuteritz *et al.* 2008).

This endemic freshwater turtle is native to slow-moving rivers, lakes, and swamps in western Madagascar, being the only living species of the family Podocnemididae found outside of South America (Leuteritz *et al.* 2008, Vargas-Ramirez *et al.* 2008), making it a true testament to Madagascar's biological uniqueness. *Erymnochelys* diverged from its closest living relative, *Podocnemis*, around 78 million years ago during the Late Cretaceous period (Vargas-Ramirez *et al.* 2008). Fossils of podocnemidid turtles, closely related to *Erymnochelys* and belonging to the same tribe (Erymnochelyini), have been discovered in Cenozoic deposits in Africa and Europe (Pérez-Garcia 2023). Madagascar is estimated to have separated from Africa around 150 million years ago and from India about 90 million years ago. This long period of isolation has allowed the local fauna and flora to evolve uniquely, leading to a high number of endemic species (Storey 1995), such as *E. madagascariensis.*

With fewer than 10,000 individuals, this species is threatened by human activities such as deforestation, agriculture, and the conversion of lakes to rice fields, resulting in habitat loss (Rakotomanana *et al.* 2013). *Erymnochelys madagascariensis* reaches sexual maturity at a late age (more than 15 years), so extraction of animals for food and medicine frequently results in the removal of individuals before they can contribute to the next generation (Leuteritz *et al.* 2008).

Efforts to conserve and protect the Madagascar big-headed turtle involve various strategies, including habitat preservation, anti-poaching measures, and captive breeding programs. Conservation organizations and researchers are working diligently to ensure the survival of this species and maintain the biodiversity of Madagascar's unique ecosystems.

A captive breeding program was implemented in 1999 to provide stock to be released in locations with depleted populations (Velosoa *et al.* 2013). At that time, no genetic data were available. Recent molecular studies have shown that captive populations have lower genetic diversity compared to native ones, suggesting that new strategies must be implemented to improve the success of the reproductive programs of *E. madagascariensis* (White *et al.* 2022).

According to White *et al.* (2022), the success of the breeding program of *E. madagascariensis* is directly associated with the knowledge of the genetic variation across the wild populations. Velosoa *et al.* (2013) pointed out that it is important to consider collection sites, molecular markers, and samples involved in this assessment. Until now, only a few microsatellite loci and fewer mitochondrial genes were used to estimate the genetic composition of natural and captive populations of Madagascar`s big-head turtle (Rafeliarisoa *et al.* 2006, White *et al.* 2022)

Herein, we present the first description of the complete mitogenome of *E. madagascariensis*. The use of the complete mitogenome offers a more comprehensive and detailed view of genetic variability compared to analyses based on a few mitochondrial genes. For applications in species management and conservation, mitogenomes are more advantageous than other nuclear markers because they allow us to trace the demographic and migratory history of populations. This information may have important implications for conservation, especially in designing and implementing protected areas for unique genetic groups that could contribute to breeding programs for restocking purposes.

Materials and Methods

In this study, we assembled and annotated the mitogenome of *E. madagascariensis* using paired-end sequencing reads (accession: SRR13244422) obtained from the Sequence Read Archive (SRA) of The National Center for Biotechnology Information (NCBI). The assemblage was conducted into the Galaxy Europe platform using the NovoPlasty v4.3.1. tool (Dierckxsens *et al.* 2017). A partial *CytB* sequence for *E. madagascariensis* (OL804198.1) was used as seed. The assembly was conducted by applying options for the mitochondrial genome with the following parameters: read length = 151, insert size = 300, and k-mer = 39.

Subsequently, annotation was performed using MITOS2 (Donath *et al.* 2019) in a module designed to annotate vertebrate mitochondrial genomes. Protein-coding regions (PCGs), Control Region (CR or D-Loop), and rRNA genes were also annotated manually and confirmed by comparison to the mitogenome of *Podocnemis unifilis* Troschel, 1848 (accession: JF802204) and *Podocnemis expansa* (Schweigger, 1812) (accession: MF359933).

The verification and correction of stop codons, nucleotide composition, and the Relative Synonymous Codon Usage (RSCU) were done using MEGA11: Molecular Evolutionary Genetics Analysis version 11 (Tamura *et al.* 2021). Codon usage for each PCG was estimated using the Codon Usage web server (Stothard 2000) with the vertebrate mitochondrial code option.

We analyzed the selective constraints of PCGs using the Codon-based Z-Test of Selection implemented in MEGA11 (Tamura *et al.* 2021), considering the number of nonsynonymous substitutions per nonsynonymous site (dN) and the number of synonymous substitutions per synonymous site (dS). The mitogenome of *P. unifilis* (accession: JF802204) was used for comparisons. Hypothesis tests were performed considering the null hypothesis indicating neutrality (dN = dS) and alternative hypotheses corresponding to Positive Selection (dN > dS) and Purifying Selection (dN < dS). The substitution model of Nei-Gojobori was applied, and gaps and missing data were treated as a pairwise deletion. P-values less than 0.05 were considered significant.

Relying on the propositions of Xiong *et al.* (2010) and Bernacki and Kilpatrick (2020), we sought to identify the primary structure of the CR by examining the presence of termination association sequences (TAS), conserved sequence blocks (CSBs) and the variable number of tandem repeats (VNTR). The number of repeats in the CR was investigated with the Microsatellite Repeats Finder web server (http://insilico.ehu.es/mini_tools/microsatellites/).

The Mfold web server (Zuker 2003), set in default mode with a temperature range of 33 to 40°C, was used to predict the secondary structure of this region. For every sequence analyzed using Mfold, we examined the first and most stable folding patterns generated by the program. We then compared these folding patterns, identifying similarities and differences in their secondary structures.

We conducted an additional step for annotation of tRNAs using ARWEN v1.2 (Laslett and Canbäck 2008) implemented in GeSeq (Tillich *et al.* 2017) with search mode: Metazoan Mitochondrial tRNAs and genetic code: Vertebrate Mitochondrial. The mitogenome of *Chelodina expansa* Gray, 1857 (accession: KY705230) was used as the reference genome. The secondary structures of the tRNAs were visualized using Forna (Kerpedjiev *et al.* 2015) implemented at the ViennaRNA Web Services site.

The graphic map of the circularized mitogenome was drawn using the online software OrganellarGenomeDRAW (Lohse *et al.* 2007).

A phylogenetic analysis was constructed considering the 13 PCGs from mitogenomes recovered from GenBank/RefSeq. For the evolutionary relationships, 40 species of Pleurodira (ingroup) and four species of Cryptodira (outgroup) were used in the analysis. A list of their respective accession numbers is available in Appendix I.

The PCGs were separated into individual datasets and aligned using MAFFT v.7.508 (Katoh and Standley 2013) under the L-INS-i method. We used the Concatenator v0.2.1 (Vences *et al.* 2022) tool for partitioning and concatenating the aligned sequences. Finally, we used IQ-TREE v2.2.2.6 (Minh *et al.* 2020) to infer the best-fit evolutionary models with ModelFinder and to conduct the phylogenetic analysis under the Maximum Likelihood (ML) method. Branch support was accessed with 10,000 replicates of ultrafast bootstrap approximation (UFBoot) (Minh *et al.* 2013). We visualized the resulting tree with iTOL v6 (Letunic and Bork 2021). The best evolutionary models according to the Akaike information criterion (AIC) were TIM+F+I+G4 for *ATPase 6*, HKY+F+I+G4 for *ATPase 8*, GTR+F+I+G4 for *COX I, CytB, ND2, ND4*, and *ND5*, GTR+F+R6 for *COX II*, TIM2+F+I+G4 for *COX III*, GTR+F+R8 for *ND1*, TVM+F+I+G4 for *ND3* and *ND4L*, and TN+F+I+G4 for *ND6*.

Results

The assembled mitogenome of *E. madagascariensis* (Genbank ID BK065183) is the third complete mitogenome described for the turtle family Podocnemididae. It is a closed-circular molecule of 16,421bp in length, CG content of 38%, and presents 22 tRNAs, two rRNAs, 13 PCGs, and one CR (Figure 1). The nucleotide composition is: A = 34.6%, T = 27.12%, C = 25.63%, and G = 12.65%.

One PCG (ND6) and eight tRNAs (tRNA-Glu, tRNA-Pro, tRNA-Gln, tRNA-Ala, tRNA-Asn, tRNA-Cys, tRNA-Tyr, and tRNA-Ser$_2$) were encoded in the light strand. All other genes were encoded in the heavy strand.

Most PCGs used the standard start codon ATN (ATA, ATG, or ATT) except *ATPase8* (TTG). As for the stop codons, TAA (*COXII, COXIII, ATPase6, ND4, ND4L, ND5, CytB* and *ND1*) and TAG (*ND2, ND3*) were predominant. *COXI, ATPase8,* and *ND6* were the only PCGs with AGA, TGA, and AGG as stop codons, respectively.

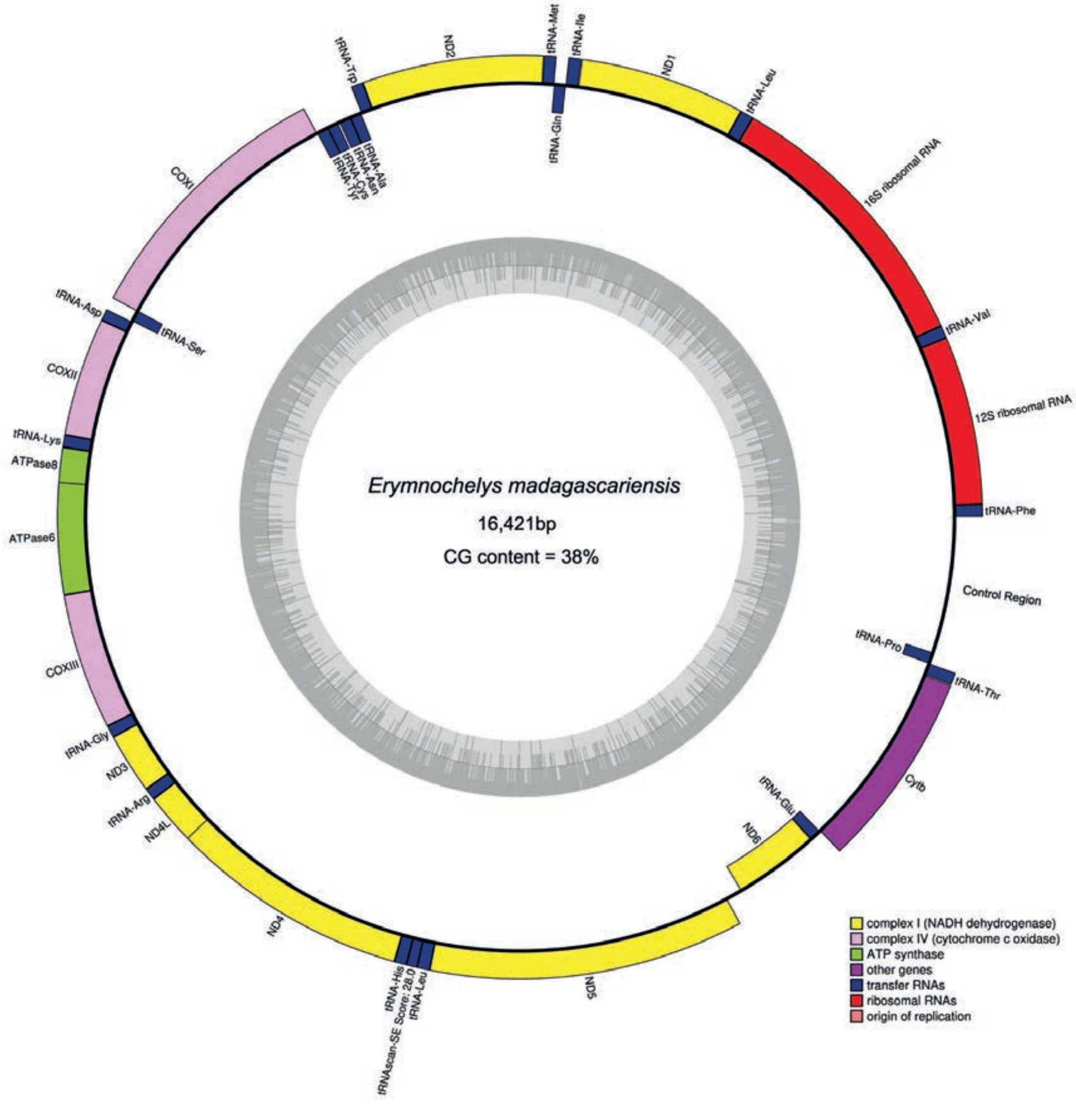

Figure 1. Organization of the mitogenome of *Erymnochelys madagascariensis.*

The RSCU (Relative Synonymous Codon Usage) of PCG codons is summarized in Figure 2.

In the mitochondrial DNA of *E. madagascariensis*, 3703 amino acids were encoded by PCGs. The most frequently used amino acids were Leucine (16.35%), Threonine (8.73%), and Isoleucine (8,51%). Cysteine was the least frequently used amino acid, with less than 0.67%. The analysis of selective constraints of PCGs indicated that most PCGs are evolving under purifying selection, except for *ATPase 8,* which appears to be under no selection (Table 1).

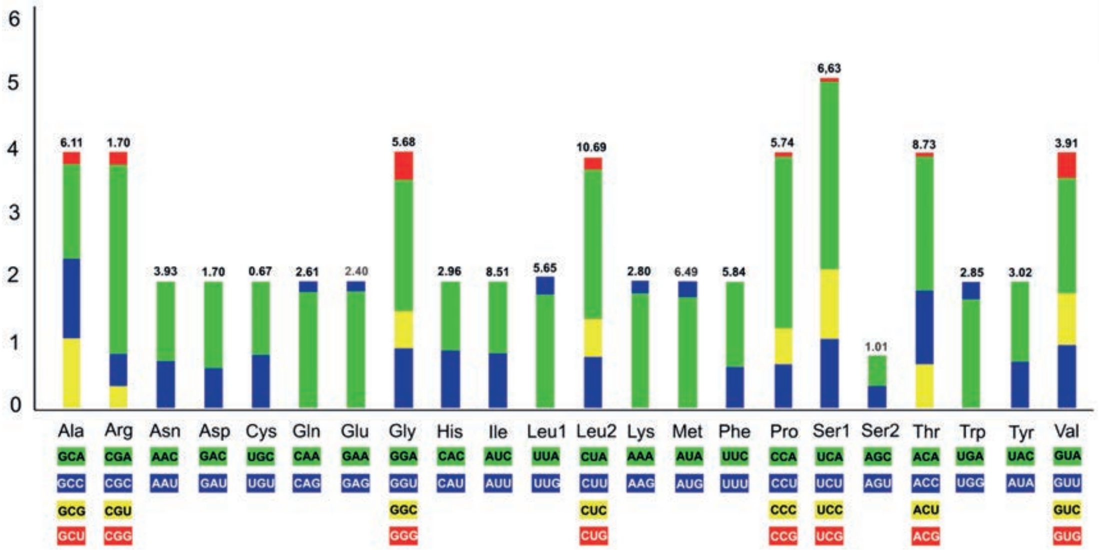

Figure 2. Codon usage of the 13 mitochondrial protein-coding genes of *Erymnochelys madagascariensis*. Codon families are indicated on the X-axis and frequency of RSCU on the Y-axis. The percentage of amino acid usage is indicated above each bar.

Table 1. Codon-based Test of Positive and Purifying Selection for analysis between PCGs sequences. Values of $p < 0.05$ are considered significant and are highlighted.

PCG	Positive selection	Purifying selection
ATP6	1	0.000000016
ATP8	0.0868	1
CytB	1	0
COX1	1	0
COX2	1	0.00000064
COX3	1	0
ND1	1	0.0000000003
ND2	1	0.00000000296
ND3	1	0.000231
ND4	1	0
ND4L	1	0.0000123
ND5	1	0
ND6	1	0.000000173

The lengths of tRNA genes ranged from 62bp (tRNA-Ser_1) to 74bp (tRNA-Ile). The tRNA-Leu and tRNA-Ser were duplicated as expected for vertebrate mitochondrial DNA (mtDNA), and tRNA-Ser1 was the only tRNA missing the dihydorouridinearm (D-arm) (Appendix II). Concerning the rRNAs, 12S rRNA presented 970bp, and 16S rRNA was 1,583bp in length.

The CR was positioned in the heavy strand, between tRNA-Phe and tRNA-Pro, with 830bp in length and GC content of 38.92% (A = 31.2%, C = 23,86%, G = 15.06%, and T = 29.88%). It was possible to identify three distinct regions within CR: (1) the left domain, spanning from the 3´end of tRNA-Pro to the 5´end of CSB-F; (2) the central domain, extending from the 5´end of CSB-F to the 5´end of CSB-1; and (3) the right domain, ranging from the 5´end of CSB-1 to the 5´end of tRNA-Phe. We were able to position five TASs, with two located in the left domain, two in the central domain, and one in the right domain (Figure 3).

Although the search for microsatellite repeats recovered 15 simple sequence repeats for the CR of *E. madagascariensis* (Table 2), we could only differentiate the VNTR2, which was characterized as an AT-rich region at the end of the right domain (Figure 3, Table 2). The most stable secondary structures of CR are presented in Figure 4. Every Mfold output generated comparable models featuring folds in all the domains except for two small loops, that were absent at temperatures of 39 and 40°C. In all models, a conserved stem-loop structure was observed at the 5´end of the left domain and the 3´end of the right domain. No folding loop was formed due to TASs binding.

The relationships among the living families of Pleurodira (Figure 5) received strong bootstrap support, recovering those groups as monophyletic. *Erymnochelys* was recovered as a sister group of *Podocnemis*. However, analysis of our assembled data set suggests the possibility that the genus *Myuchelys* is polyphyletic, as it appears within four separate clades within the Chelidae.

Table 2. Simple sequence repeats found in the Control Region of *Erymnochelys madagascariensis'* mitogenome.

Position	Length of repeats	Number of repeats	Repeats
3	3	2	TTCTTC
57	2	3	CCCCCC
64	9	2	GTGGGGGGGTGGGGGGG
143	3	2	AACAAC
227	4	2	ATCCATCC
268	4	2	GCATGCAT
475	3	2	CATCAT
488	3	2	TAATAA
536	4	2	ACATACAT
623	3	2	ACCACC
648	4	2	ACATACAT
750	2	3	ATATAT
765	2	7	TATATATATATATA
780	2	8	ATATATATATATATAT
814	3	2	TCCTCC

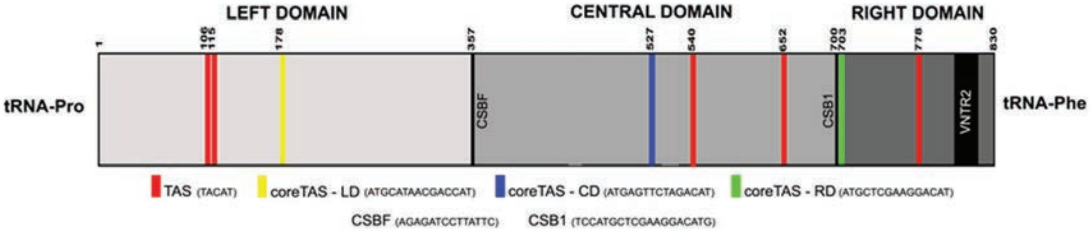

Figure 3. Representation of the primary structure of the Control Region of the mitogenome of *Erymnochelys madagascariensis.*

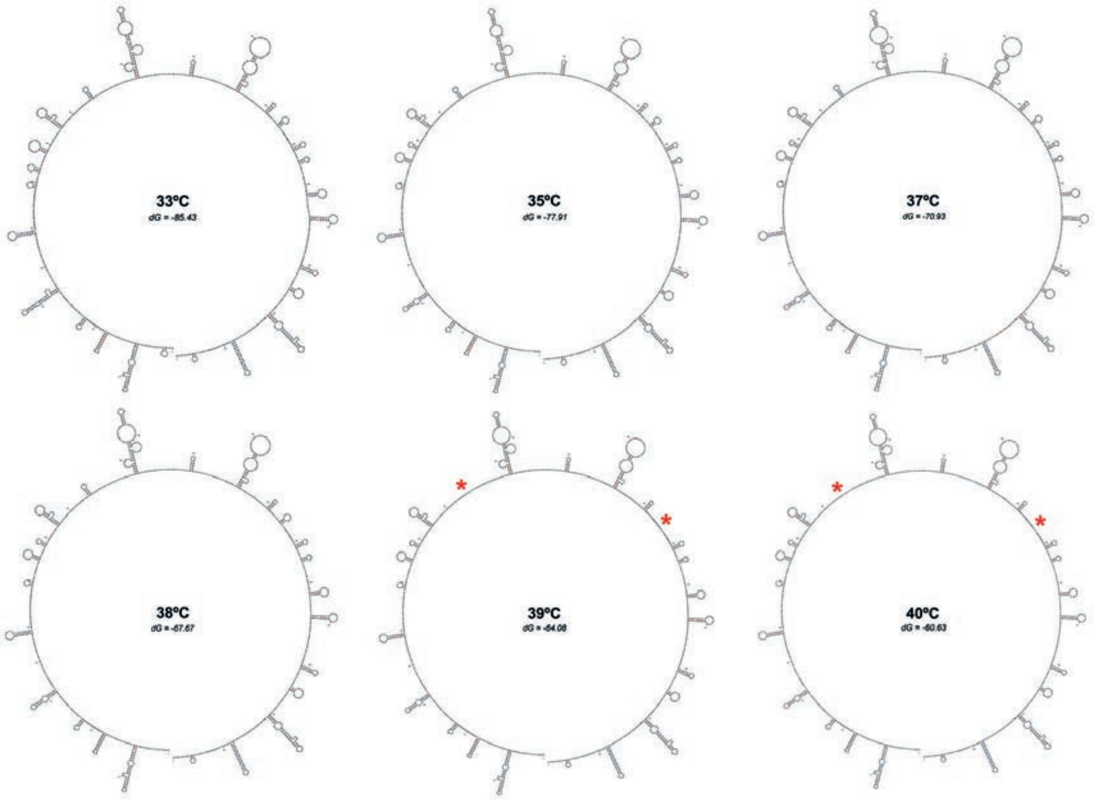

Figure 4. Mitochondrial Control Region possible folding models of *Erymnochelys madagascariensis* under different temperature conditions. The red signs indicate the absence of loops.

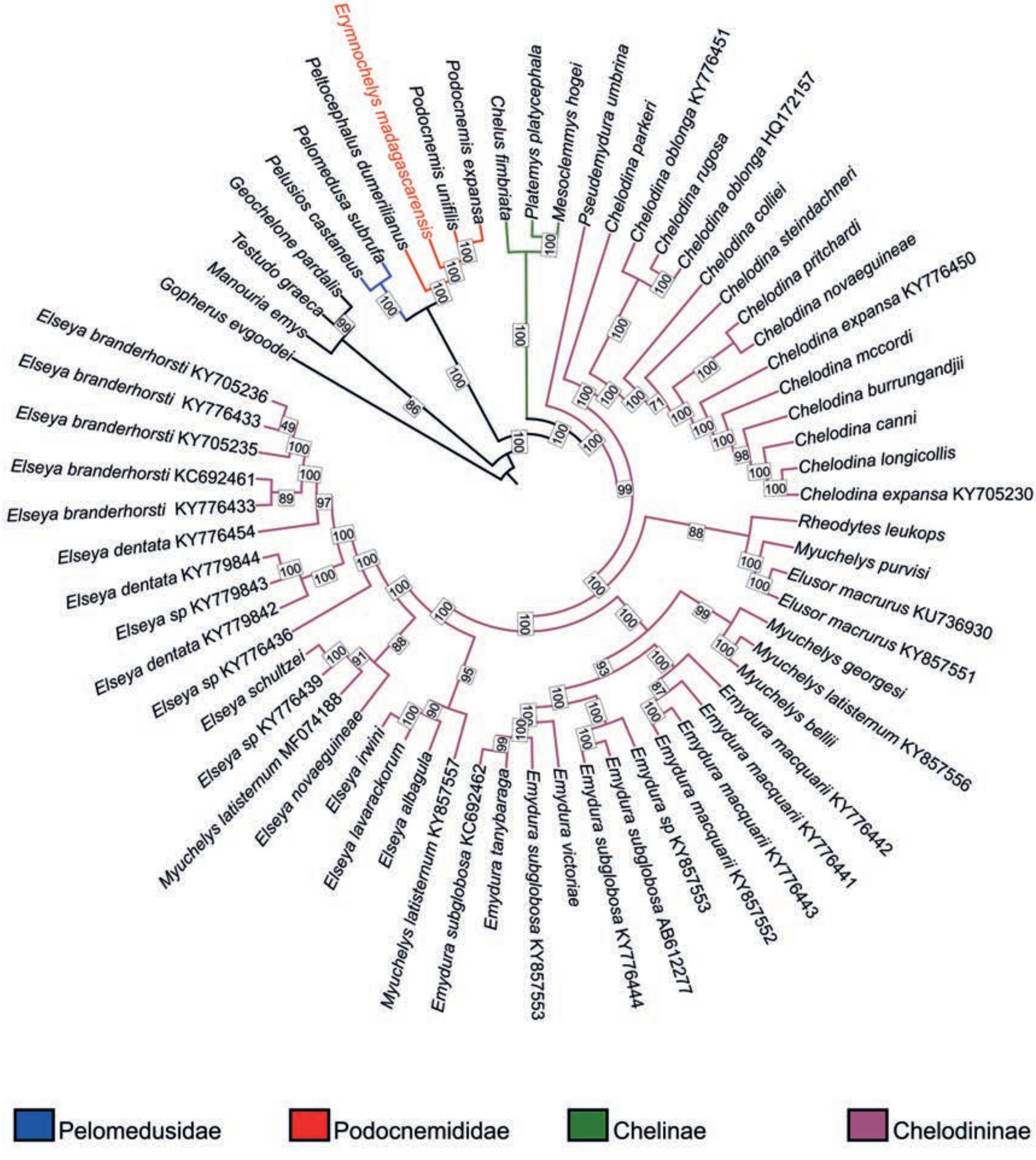

Figure 5. ML phylogenetic tree of Pleurodira inferred in the IQ-TREE employing nucleotide sequences of 13 PCGs. The tree was rooted using *Gopherus evgoodei* (Cryptodira) as outgroup. Ultrafast bootstrap values are indicated at the nodes.

Discussion

Mitochondrial DNA has proven to be a valuable tool for studying various animal groups. This marker allows for the analysis of genetic diversity, the determination of evolutionary relationships, and species identification (Trivedi *et al.* 2016, Georges *et al.* 2018, Carvalho *et al.* 2022).

The unique characteristics of this molecule make it essential for population diversity studies. Due to its non-recombining maternal inheritance pattern, it is possible to identify maternal lineages and introgression events (McDowall 2008, Vogel and Johnson 2008). The high rate of mtDNA substitution enables the identification of single nucleotide polymorphisms (SNPs) and rearrangements (McGuire *et al.* 2023), allowing for the determination of priority haplotypes that can be useful in breeding and conservation programs for threatened groups.

The analysis of *E. madagascariensis'* mitogenome revealed the smallest mitogenome documented among Podocnemididae species. When comparing gene order, CG content, and nucleotide composition, it was found to closely resemble those previously described in the mitogenomes of *P. unifilis* (Zhou *et al.* 2016) and *P. expansa* (Wang *et al.* 2018), reinforcing the evolutionary relationship between *Erymnochelys* and *Podocnemis*.

Within the family Podocnemididae, the CR region spans from 692bp in *P. expansa* (Wang *et al.* 2018) to 985bp in *P. unifilis* (Zhou *et al.* 2016), and this variation is likely attributed to the presence of VNTR. In general, the CR structure and composition in pleurodiran turtles differs considerably from those in Cryptodira. However limited information is available for pleurodiran families, limiting a comprehensive understanding of the CR evolution within this suborder (Bernacki and Kilpatrick 2020).

As the CR is a non-coding region that appears to undergo concerted evolution in Metazoa (Zhang and Hewitt 1997), it is known for its variability in size and composition, even among closely related taxa. Concerning the primary structure of the CR of *E. madagascariensis,* it was possible to identify the presence of TAS regions and the conserved blocks CSB-F and CSB1, which delineate the subdivision of the CR into three domains. These findings corroborate the proposal of Wang *et al.* (2011) and differ from those obtained by Bernacki and Kilpatrick (2020) for the Podocnemididae. While the presence of multiple TASs blocks and the terminal AT-rich VNTR2 appears to be a shared characteristic among different turtle families, our data and that of Bernacki and Kilpatrick (2020) seem to indicate that the absence of CSB2 and CSB3 blocks is a characteristic of pleurodires.

In evolutionary biology, a persisting question centers on the influence of natural selection and environmental factors on the differentiation of mitochondrial genes. Mitochondria play a pivotal role in cellular respiration and aerobic metabolism, imposing significant functional constraints on their genetic material, particularly concerning energy-related processes. A thorough examination of the selective pressures acting upon mitochondrial genes can offer valuable insights into the functional and ecological dimensions of how organisms evolve (Ding *et al.* 2023).

As expected, our analysis demonstrated that most PCGs of *E. madagascariensis* are under purifying selection, except for *ATPase 8,* similar to the results of Escalona *et al.* (2017) for the Cryptodira. Most mitochondrial genes appear to be subject to purifying selection (Shtolz and Mishmar 2019, Ding *et al.* 2023), eliminating harmful mutations and preserving mitochondrial functions. The limited instances of positive selection are often linked to cases of adaptation to new environmental or physiological conditions and are associated with sites of the OXPHOS complex I proteins (Escalona *et al.* 2017, Sahoo *et al.* 2023).

The phylogenetic reconstruction based on the mitochondrial PCGs recovered a sister group relationship between *Erymnochelys* and *Podocnemis*. This differs from the one proposed by Ferreira *et al.* (2018) based on morphological traits, which placed *Peltocephalus* as a sister group of *Erymnochelys*. However, our findings are supported by the studies of Vargas-Ramirez *et al.* (2008) and Thomson *et al.* (2021), which are based on nuclear and/or mitochondrial data.

The coverage of the pleurodiran genera in our analysis is more comprehensive than that of Vargas-Ramirez *et al.* (2008). While our study includes fewer genera than Ferreira *et al.* (2018), it boasts a more robust species sampling. Evolutionary analysis involving numerous taxa using all PCGs relies on a substantial investment in constructing DNA datasets or utilizing available information from databases. This limitation restricted our ability to include all pleurodiran genera in our study.

Polyphyly observed in the genus *Myuchelys* in our study of the mitogenome was also found by other researchers who used both mitochondrial and nuclear genes in their phylogenetic reconstructions (Fielder *et al.* 2012, Le *et al.* 2013, Georges *et al*. 2018). However, *Myuchelys* was considered monophyletic in the study of Thomson *et al*. (2021), based on 15 nuclear genes. Although Thomson *et al.* (2021) included the most extensive coverage of genera used in evolutionary studies of turtles, they only included two samples each of *M. purvisi* (Wells and Wellington, 1985) and *M. latisternun* (Gray, 1867). Therefore, this limited sampling within these species may not have been sufficient to confirm or refute non-monophyly reported by other researchers.

The identification of polyphyly in evolutionary relationships based on mitochondrial data can be attributed to three possible explanations: (1) misidentification of species; (2) cryptic diversity, as suggested by Le *et al.* (2013), Fielder *et al.* (2012), and Kehlmaier *et al.* (2019); or (3) introgression (Kehlmaier *et al.* 2019). Our results indicate that some individuals of *Myuchelys* are genetically related to *Emydura* and others to *Elseya*, Australian genera found in sympatry with *Myuchelys*. Georges *et al.* (2018) have raised the possibility of hybridization between *M. georgesi* (Cann, 1997) and *Emydura macquarii* (Gray, 1830).

The decline of species can result from anthropogenic actions, such as habitat loss, overexploitation, and climate change. In light of this, many species and genetic groups could face extinction even before they are recognized by science (Kehlmair *et al.* 2019). *E. madagascariensis* is currently at risk of extinction due to anthropogenic activities, but we must also consider another risk to maintaining species' integrity: hybridization.

Considering the detrimental aspects of hybridization that can lead a species toward extinction, two noteworthy consequences are outbreeding depression and genetic swamping (Todesco *et al.* 2016). Our phylogenetic analysis has highlighted the possibility of hybridization among sympatric species in the Chelodininae. As instances of ancient introgression have been identified in the evolutionary history of Australasian turtles (Kehlmaier *et al.* 2019), monitoring the mtDNA these turtles enable us to detect potential introgression events.

White *et al.* (2022) employed microsatellites (SSR) and mitochondrial genes (*CytB* and *COXI*) to assess the genetic variability of *E. madagascariensis*. Their data indicate that *COXI* reveals a division of genetic groups based on north-western and south-western distributions, a pattern not recovered by SSR analysis. The mtDNA analysis also facilitated the identification of haplotypes associated with the watershed where these animals were collected, given that females exhibit fidelity to a specific river for breeding. This finding helps explain the genetic structure observed within the species.

This underscores the utility of mitogenomes in evaluating the genetic diversity of *E. madagascariensis* and other endangered Pleurodira. Utilizing the entire mtDNA molecule proves invaluable for identifying introgression events and cryptic diversity, which have significant implications for conservation efforts, especially in establishing and managing protected breeding areas. Determining haplotypes among natural and captive populations is fundamental to the success of programs that restock and preserve these species.

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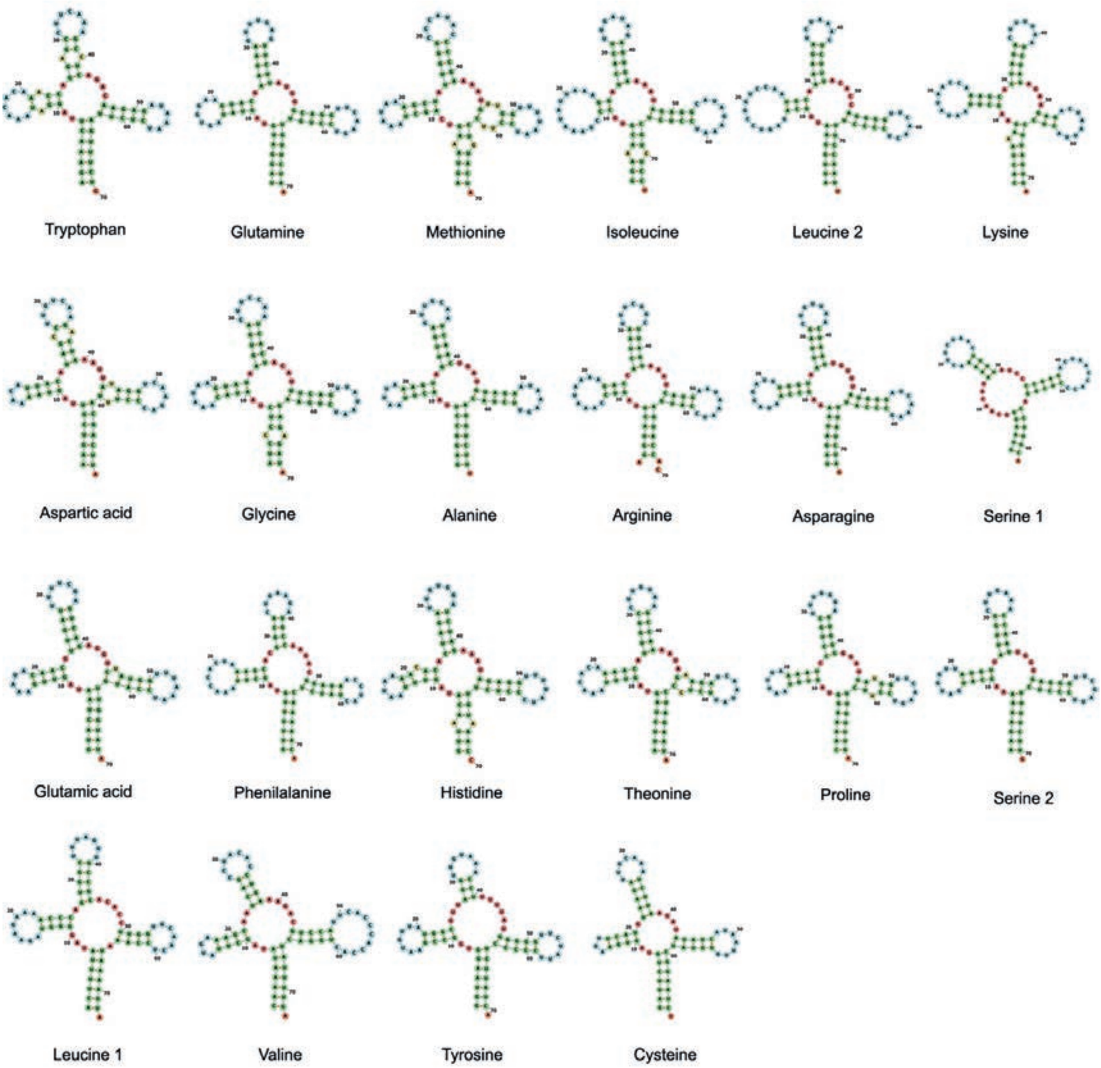
Appendix I. *Taxa included in the phylogenetic analysis of the present study.*

Species	Genbank accession	Family/Subfamily
Chelodina burrungandjii	KY776447.1	Chelidae/Chelodininae
Chelodina canni	KY776448.1	
Chelodina colliei	KY776449.1	
Chelodina expansa	KY705230.1	
Chelodina expansa	KY776450.1	
Chelodina longicollis	KJ713173.1	
Chelodina mccordi	KY705231.1	
Chelodina novaeguineae	KY776446.1	
Chelodina oblonga	KY705234.1	
Chelodina oblonga	KY776451.1	
Chelodina parkeri	KY705232.1	
Chelodina pritchardi	KY705233.1	
Chelodina rugosa	HQ172157.1	
Chelodina steindachneri	KY776452.1	
Elseya albagula	KY776453.1	
Elseya banderhorsti	KC692461.1	
Elseya banderhorsti	KY705235.1	
Elseya banderhorsti	KY705236.1	
Elseya banderhorsti	KY776433.1	
Elseya banderhorsti	KY776434.1	
Elseya dentata	KY776454.1	
Elseya dentata	KY779842.1	
Elseya dentata	KY779844.1	
Elseya irwini	KY776435.1	
Elseya lavarackorum	KY776437.1	
Elseya novaeguineae	KY776438.1	
Elseya schultzei	KY776440.1	
Elseya sp.	KY779843.1	
Elseya sp.	KY776436.1	
Elseya sp.	KY776439.1	
Elusur macrurus	KU736930.1	
Elusur macrurus	KY857551.1	
Emydura sp.	KY857553.1	
Emydura subglobosa	KC692462.1	
Emydura subglobosa	KY776444.1	
Emydura subglobosa	KY776445.1	
Emydura subglobosa	AB612277.1	
Emydura tanybaraga	KY857559.1	

Appendix I. *Continued.*

Species	Genbank accession	Family/Subfamily
Emydura victoriae	KY857554.1	Chelidae/Chelodininae
Emydura macquarii	KY776441.1	
Emydura macquarii	KY776442.1	
Emydura macquarii	KY776443.1	
Emydura macquarii	KY857552.1	
Myuchelys bellii	KY924930.1	
Myuchelys georgesi	KY857555.1	
Myuchelys latisternum	KY857556.1	
Myuchelys latisternum	KY857557.1	
Myuchelys latisternum	MF074188.1	
Myuchelys purvisi	KY883378.1	
Peltocephalus dumerilianus	AB970731.1	
Pseudemydura umbrina	KY486272.1	
Rheodytes leukops	KY857558.1	
Chelus fimbriata	HQ172156.1	Chelidae/Chelinae
Mesoclemmys hogei	MF615513.1	
Platemys platycephala	KC692464.1	
Pelomedusa subrufa	AF039066.1	Pelomedusidae
Pelusios castaneus	KC692463.1	
Podocnemis expansa	MF359933.1	Podocnemididae
Podocnemis unifilis	JF802204.1	
Geochelone pardalis	DQ080041.1	Cryptodira (outgroup)
Gopherus evgoodei	CM017320.1	
Manouria emys	DQ080040.1	
Testudo graeca	DQ080049.1	

Appendix II. *The secondary structure of tRNA genes inferred for the mitogenome of* Erymnochelys madagascariensis.



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Diet, size, and pholidosis of *Crotalus ericsmithi*, and new prey items for *Crotalus lannomi* (Serpentes: Viperidae)

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Abstract

Diet, size, and pholidosis of *Crotalus ericsmithi*, and new prey items for *Crotalus lannomi* (Serpentes: Viperidae). *Crotalus ericsmithi* and *Crotalus lannomi* are two of the least studied rattlesnake species endemic to Mexico. We describe the diet and morphological variation of *C. ericsmithi* and report novel prey items for *C. lannomi* based on data from recent field encounters. The diet of *C. ericsmithi* consists mainly of mammals, followed by reptiles, with no detected ontogenetic shift or sexual differences in prey type. This first detailed study of the diet of *C. ericsmithi* adds six new prey species. *Crotalus ericsmithi* does not show sexual dimorphism in snout–vent length and total length, but males have significantly longer tails and smaller head widths and lengths than females. It also exhibits sexual dimorphism in number of subcaudal scales, dorsal body blotches, and tail bands (higher in males), and in midbody dorsal scale rows and intercanthals (higher in females). The mental scale extends posteriorly, preventing contact between the first pair of infralabials in 75% of the specimens examined; therefore, this scale should not be considered a reliable diagnostic character. We identified two new rodent prey items in the diet of wild *C. lannomi*. Our sample sizes are relatively small, reflecting the rarity of these species, so they must be interpreted with caution. Further research into the natural history of the elusive Long-tailed rattlesnakes and their kin are necessary to enhance conservation efforts.

Keywords: Long-tailed Rattlesnake, Mammals, Morphology, Prey, Sexual dimorphism.

Resumo

Dieta, tamanho e folidose de *Crotalus ericsmithi* e novas presas de *Crotalus lannomi* (Serpentes: Viperidae). *Crotalus ericsmithi* e *Crotalus lannomi* são duas das espécies de cascavéis menos estudadas e endêmicas do México. Descrevemos a dieta e a variação morfológica de *C. ericsmithi* e relatamos novos itens de presas para *C. lannomi* com base em dados de recentes

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encontros de campo. A dieta de *C. ericsmithi* consiste principalmente de mamíferos, seguidos por répteis, sem nenhuma mudança ontogenética detectada ou diferenças sexuais no tipo de presa. Este primeiro estudo detalhado da dieta de *C. ericsmithi* acrescenta seis novas espécies de presas. *Crotalus ericsmithi* não apresenta dimorfismo sexual no comprimento focinho-cloaca e comprimento total, mas os machos têm caudas significativamente mais longas e larguras e comprimentos de cabeça menores do que as fêmeas. Também exibe dimorfismo sexual no número de escamas subcaudais, manchas dorsais do corpo e faixas da cauda (maior nos machos), e nas fileiras de escamas dorsais do meio do corpo e intercantais (maior nas fêmeas). A escala mental estende-se posteriormente impedindo o contato entre o primeiro par de infralabiais em 75% dos espécimes examinados; portanto, esta escala não deve ser considerada um recurso diagnóstico confiável. Identificamos duas novas presas de roedores na dieta de *C. lannomi* selvagem. O tamanho da nossa amostra é relativamente pequeno, refletindo a raridade destas espécies, por isso devem ser interpretadas com cautela. Defendemos mais pesquisas sobre a história natural das esquivas cascavéis de cauda longa e seus parentes para aumentar os esforços de conservação.

Palavras-chave: Cascavel-de-cauda-longa, Dimorfismo sexual, Mamíferos, Morfologia, Presas.

Introduction

Snakes, particularly venomous species like vipers, are one of the least understood and most persecuted groups of animals. If we add that scarce information is available on the biology of many of these species and that viperids possess “slow” life history traits that make them particularly vulnerable to extinction, face threats such as loss of habitat, mortality caused by humans associated with road traffic, and illegal trafficking of species, we understand why their conservation is a challenge (Maritz *et al.* 2016, Fernández-Badillo *et al.* 2021).

Crotalus ericsmithi Campbell and Flores-Villela, 2008 (Guerreran Long-tailed Rattlesnake), a small and slender-bodied snake endemic to the state of Guerrero, Mexico, is a highly secretive species difficult to find in the field. Since its description, it is known only from a few localities, and the only published information concerns its phylogenetic relationships (Reyes-Velasco *et al.* 2013, Alencar *et al.* 2016, Blair and Sánchez-Ramírez 2016), rattle morphology (Meik and Pires-da-Silva 2009), hemipenial morphology (Jadin *et al.* 2010), and venom composition (Neri-Castro *et al.* 2022). To date, the only information about its natural history is that it can be active in the early hours of the night and that it occurs in the ecotone between dry pine forest and tropical deciduous forest (Campbell and Flores-Villela 2008). *Crotalus lannomi* Tanner, 1966 (Manantlán Long-tailed Rattlesnake) is another rare rattlesnake reported from the state of Jalisco. Additional specimens were found at two localities in the state of Colima, providing new information about its morphology, activity, behavior, diet, reproduction, and conservation status (Reyes-Velasco *et al.* 2010).

Crotalus ericsmithi and *C. lannomi* plus *C. stejnegeri* Dunn, 1919 (Sinaloan Long-tailed Rattlesnake) are part of the *Crotalus stejnegeri* group and are known as Long-tailed Rattlesnakes. They are rare species endemic to the foothills of western Mexico, occurring from central Sinaloa to central Guerrero, and were recovered as the sister group to the *Crotalus atrox* plus *Crotalus viridis* groups that branched from the *Crotalus durissus* group (Reyes-Velasco *et al.* 2013, Blair and Sanchez-Ramirez 2016), or sister group to the *C. durissus* group (Alencar *et al.* 2016, Holding *et al.* 2021). Although *C. ericsmithi* was not included in the study by Birskis-Barros *et al.* (2019), it is highly likely that it shares the category of rarest (RT) with its sister species *C. lannomi* and *C. stejnegeri*.

In the case of rare species, growing evidence shows that they play key ecological roles and tend to be more affected by habitat disturbance; therefore, rare vipers are targets of conservation programs (Birskis-Barros *et al.* 2019). Our goal is to contribute to the ecology and conservation of these species by providing information about the diet and morphology of the rare *C. ericsmithi* and to report new data on the diet of *C. lannomi,* based on specimens from recent field surveys.

Materials and Methods

Data Collection

We obtained dietary and morphological data from 21 specimens of *Crotalus ericsmithi,* of which 20 were recently found dead (killed by local residents or road-killed) in the vicinity of the type locality (carretera La Laguna–Bajitos de La Laguna, Guerrero, Mexico; 17.55330° N, 100.77472° W, WGS84; elevation 1037 m a.s.l.), between 2016–2021 and deposited in the herpetological collection of Universidad Autónoma de Aguascalientes (UAAREP-796, 811–827, 981, 1063). We obtained morphological data from the holotype (UTAR-55372) reported by Campbell and Flores-Villela (2008). Snakes were examined for stomach and intestinal contents through a mid-ventral incision.

We obtained scats by palpation of the last third of the body of two *C. lannomi* found 50 km SE from the type locality, which is 2.9 km west of the pass, Puerto Los Mazos, on Mexican Highway 80, Jalisco, Mexico (19.694871° N, 104.414092° W, WGS84; elevation 1151 m a.s.l.) (Reyes-Velasco *et al.* 2010), and released at the same collection site after obtaining morphometric data. A photographic voucher of one *C. lannomi* was deposited at the University of Texas at Arlington (UTADC 9713), and the scat of the second specimen was deposited at Universidad Autónoma de Aguascalientes (UAAREP-1019).

We measured snout–vent length (SVL) and total length (TTL) to the nearest 1 mm with a measuring tape. Tail length (TL) was obtained by subtracting the SVL from the TTL; head length (HL) and head width (HW) were measured to the nearest 0.1 mm with a digital caliper. We calculated the tail percentage of total length with the following formula %TL = TL.100/TTL. Ontogeny was roughly estimated by examining the rattle structure for near parallelism of successive rattle segments, indicating that growth rates were asymptotic at the time of preservation (Klauber 1940, Meik *et al.* 2010). We determined sex by cloacal probing or by everting hemipenes. We recorded date, locality, number, and identity of prey items. Mass was not included because of the inaccuracy of weight measurements associated with preserved specimens. We calculated the weight ratio (WR) for one *C. ericsmithi* specimen (UAAREP-823) that contained a partially digested prey.

From *Crotalus ericsmithi* we obtained data for 18 pholidosis characters: number of preventral scales (PVEN) and number of ventral scales (VEN) following Dowling (1951), subcaudal scales (SBC), midbody dorsal scale rows (MDR), dorsal body blotches (DBB), tail bands (TB), number of intercanthals (ICS), number of supralabial scales (SLS), number of infralabial scales (ILS), number of prefoveal scales (PFO), number of intersupraoculars at midlevel of supraoculars (ISM), presence of mental scale (MEN) produced posteriorly to mostly preclude contact between first pair of infralabials, presence of postrostral scale (PRT), number of internasal scales (INS), number of canthal scales (CAS), number of loreal scales (LOR), presence vs. absence of postloreal scale (PLO), and lacunal–supralabial contact vs. no contact (LAC-SLS) following Campbell and Flores-Villela (2008). For bilateral characters, we usually examined the left side, or the right side only when damage to the specimen prevented us from recording the former. For PRT, PLO, LAC-SLS, we considered it when at least in one side was present. In the results, we present these data as mean ± one standard deviation.

Identification of Prey Items

Prey remains found in the stomach and intestines of specimens of *Crotalus ericsmithi* and in scats of two *Crotalus lannomi* were stored individually in 70% ethanol. To identify mammal prey, dorsal guard hairs were mounted on glass slides and allowed to set for at least 12 h using a glass coverslip (see Moore *et al.* 1974). These hairs were then compared with those of small mammals (shrews, rodents, skunks, and marsupials) obtained from the mastozoological collection of El Colegio de la Frontera Sur (ECO-SC-M) and from Universidad Autónoma de Aguascalientes (UAA-MA) and with keys to dorsal guard hairs (e.g., Baca-Ibarra and Sánchez-Cordero 2004, Pech-Canché *et al.* 2009). Sometimes, bones, molars, claws, hind limbs, and tails were recovered and used in multiple diagnostics (see Whorley 2000). Reptile scales were identified by comparison with a reference collection of the reptiles from the UAA-REP. Additionally, we used the known distribution of possible prey species as a criterion to reach species-level identification. The data reported for both snakes and their prey follow the suggestions of Maritz *et al.* (2021).

Analyses

Raw morphometric variables of *Crotalus ericsmithi* were tested for normality and homoscedasticity using Shapiro-Wilk and Levene tests. We tested for differences in SVL and TTL of all specimens between sexes using Mann-Whitney U-tests. We calculated the degree of sexual size dimorphism in SVL following Gibbons and Lovich (1990) and Shine (1994). We used Analyses of Covariance (with SVL as the covariate) to detect differences in TL, HL, and HW of all specimens between sexes after we log transformed all variables to meet assumptions of parametric testing, and we tested the assumption of homogeneity of regression slopes. We calculated the WR for only one individual, by dividing the mass of the prey by the mass of the snake. To detect ontogenetic shift in diet we tested differences between the SVL of snakes and prey taxon using Mann-Whitney U-tests. We performed non-parametric Fisher´s Exact Test to compare proportions of specimens containing food items between males and females; the same test was used to compare consumed prey classes (Mammalia and Reptilia) between sexes. We performed Mann-Whitney U-tests for ordinal scale characters, and Fisher´s Exact Test for nominal scale characters to compare between sexes and age classes. We performed all statistical analyses in Statistica (StatSoft Inc. 2005), and considered results to be statistically significant when $p \leq 0.05$.

Results

Diet of Crotalus ericsmithi

Of the 20 *Crotalus ericsmithi* specimens examined, 12 (60%) contained prey items, for a total of 13 items, 12 in the hindgut, and one in the stomach and hindgut. A total of six prey species were identified; only one specimen contained two types of prey. The class Mammalia represented 84.6%, and class Reptilia represented 15.4% of all samples (Table 1). Regarding mammals, three fecal contents were identified only to Order as unidentified rodents, and one sample to family level as an unidentified Soricidae. In the Order Rodentia and family Cricetidae one individual contained remains of a Deer mouse (*Peromyscus* sp.), and another of a Cotton rat (*Sigmodon* sp.). In the family Heteromyidae, one individual contained remains of the Painted spiny pocket mouse (*Heteromys pictus* Thomas, 1893). In the Order Soricomorpha, family Soricidae, four prey samples represented remains of Goldman's small-eared shrew, *Cryptotis goldmani* (Merriam, 1895). Regarding reptiles, one individual contained remains of a Spiny lizard (*Sceloporus* sp., family Phrynosomatidae), while one individual exhibited cannibalism, containing remains of another *C. ericsmithi* (Table 1). We obtained the weight ratio (WR) of an adult male *C. ericsmithi* (SVL = 520 mm, TL = 68 mm, TTL = 588 mm, mass = 50 g; UAA-REP 823) that contained a *Peromyscus* sp. (mass = 40 g), yielding a WR = 0.8.

Dietary items were obtained from specimens of SVL 225–550 mm. The SVL of the snakes that fed on mammals ranged from 225–550 mm, mean 379.2 ±23.2 mm (*N* = 10); snakes that fed on reptiles measured 270–284 mm, mean 277 ± 9.9 mm (*N* = 2). No differences in SVL were found between the snakes that consumed mammals or reptiles (Z = 1.07, *p* = 0.28; $N_{\text{juveniles}}$ = 8, N_{adults} = 4; Figure 1), implying no appreciable ontogenetic shift, although our sample size is small. Males (60% of *N* = 10) and females (60% of *N* = 10) were equally likely to contain prey items (Fisher's Exact Test, *p* = 0.67). There was no significant difference in prey class (Mammalia and Reptilia) consumed between sexes (Fisher's Exact Test, *p* = 0.77).

Size and Pholidosis of Crotalus ericsmithi

The largest male in our sample measured 588 mm TTL (SVL = 520 mm, TL = 68 mm; UAAREP-823), the largest female 689 mm TTL (SVL = 625 mm, TL = 64 mm; UAAREP-822). Snout–vent length of all specimens ranged between 225–625 mm (394.9 ± 118.7; *N* = 21) and TTL between 251–689 mm (446 ± 136.2; *N* = 21). SVL of juveniles ranged between 225–364 mm (293.8 ± 42.1; *N* = 11) and TTL between 251–419 mm (328 ± 50.8; *N* = 11). SVL of adults ranged between 450–625 mm (506 ± 56.1; *N* = 10) and TTL between 521–689 mm (575.9 ± 50.9; *N* = 10). SVL of males (400.8 ± 85.5; *N* = 11) was 3.11% larger than that of females (388.3 ± 152; *N* = 10), and did not vary significantly (Z = 0.49, *p* = 0.62). The degree of sexual size dimorphism in SVL of all specimens was -0.03. We found no significant difference in TTL (Z = 0.56, *p* = 0.57), between males (460.9 ± 103.6; *N* = 11) and females (429.7 ± 169.5; *N* = 10), but males were generally longer. The TL of juvenile males ranged between 33–55 mm (41.6 ± 9.8; *N* = 5) and juvenile females between 23–34 mm

Table 1. Frequency and percentage of occurrence of the taxa that comprise the diet of *Crotalus ericsmithi* from specimens analyzed in this study.

Prey taxon	Frequency	%
MAMMALIA	11	84.6
SORICOMORPHA		
Soricidae		
Unidentified shrew	1	7.7
Cryptotis goldmani	4	30.8
RODENTIA		
Unidentified rodent	3	23
Cricetidae		
Peromyscus sp.	1	7.7
Sigmodon sp.	1	7.7
Heteromyidae		
Heteromys pictus	1	7.7
REPTILIA	2	15.4
SQUAMATA		
Phrynosomatidae		
Sceloporus sp.	1	7.7
Viperidae		
Crotalus ericsmithi	1	7.7
Total	**13**	**100**

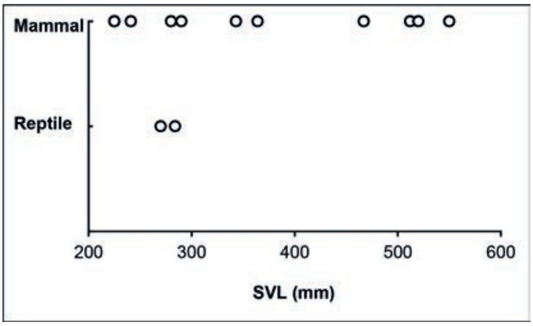

Figure 1. Relationship between prey types (mammals and reptiles) and snake snout–vent length (SVL) of individuals of *Crotalus ericsmithi*.

(28 ± 3.8; $N = 6$). TL of adult males ranged between 68–86 mm (75.5 ± 6.3; $N = 6$) and adult females between 54–73 mm (61.5 ± 8.9; $N = 4$). The TL of males (60.1 ± 19.3 mm; 13% of TTL; $N = 11$) was longer than that of females (41.4 ± 18.3 mm; 9.6% of TTL; $N = 10$) of all specimens, as was the %TL of males (Table 2). Females had significantly shorter tails than males ($F_{2,18} = 250.79$, $p < 0.0001$).

The HL of juvenile males ranged between 17–21.4 mm (19.3 ± 1.9; $N = 5$) and juvenile females between 16–21 mm (18 ± 1.8; $N = 6$). HL of adult males ranged between 23–27 mm (25.5 ± 1.9; $N = 6$) and adult females between 28–33 (30.5 ± 2.4; $N = 4$). We found significant difference between female HL ($N = 10$) and males HL ($N = 11$) of all specimens ($F_{2,18} = 208.68$, $p = 0.0001$), with females having longer heads (Table 2). The HW of juvenile males ranged between 8–13.1 mm (11 ± 2.4; $N = 5$) and juvenile females between 8–13 mm (10.2 ± 2.3; $N = 5$). HW of adult males ranged between 13.5–14.8 mm (13.8 ± 0.8; $N = 6$) and adult females between 17–24 mm (19 ± 3.4; $N = 4$). We found significant difference between female HW ($N = 9$) and males HW ($N = 11$) of all

Table 2. Sample sizes (*N*), mean (± standard deviation), and range of snout–vent length (SVL, mm), tail length (TL, mm), percentage of tail length (%TL), total length (TTL, mm), head length (HL, mm), head width (HW, mm), and scale counts of preventrals (PVEN), ventrals (VEN), subcaudals (SBC), midbody dorsal scale rows (MDR), dorsal body blotches (DBB), tail bands (TB), intercanthals (ICS), supralabials (SLS), infralabials (ILS), prefoveal (PFO) and intersupraoculars at midlevel of supraoculars (ISM) of both sexes of *Crotalus ericsmithi* analyzed in this study.

	Males			**Females**		
	N	Mean ± SD	Range	*N*	Mean ± SD	Range
SVL	11	400.8 ± 85.5	284–520	10	388.3 ± 152	754–1519
TL	11	60.1 ± 19.3	33–86	10	41.4 ± 18.3	23–73
%TL	11	12.8 ± 1.8	10.4–15.9	10	9.5 ± 1.0	7.8–11.8
TTL	11	460.9 ± 103.6	317–588	10	429.7 ± 169.5	251–689
HL	11	22.5 ± 3.5	17–27	10	23.0 ± 6.7	16–33
HW	11	12.6 ± 2.2	8–14.8	10	14.1 ± 5.3	8–24
PVEN	11	2.7 ± 0.5	2–3	10	2.8 ± 0.8	3–4
VEN	11	174.0 ± 2.4	172–179	10	173.6 ± 3.0	169–180
SBC	11	42.7 ± 1.3	41–45	10	34.3 ± 0.9	33–36
MDR	11	25.5 ± 0.8	25–27	10	26.8 ± 1.4	25–29
DBB	11	37.5 ± 2.3	34–41	10	35.3 ± 1.3	33–37
TB	11	14.5 ± 2.1	12–18	10	12.3 ± 1.9	10–16
ICS	10	1.7 ± 1.1	1–4	10	3.4 ± 1.3	2–6
SLS	10	13.4 ± 0.7	12–14	9	14.0 ± 0.5	13–15
ILS	10	14.5 ± 0.5	14–15	10	14.8 ± 0.6	14–16
PFO	10	4.7 ± 0.9	3–6	9	4.9 ± 1.1	4–6
ISM	10	4.6 ± 0.5	4–5	10	4.5 ± 0.7	3–5

specimens ($F_{2,1}$ = 44.25, *p* = 0.0001) with females having wider heads.

MEN was present in 15 of 20 *Crotalus ericsmithi* (75%). In the five specimens where the first supralabials are in partial contact, the mental scale is similar to the shape of that of *C. lannomi* (Figure 2). No significant differences were found between sexes (N_{males} = 8 of 10; N_{females} = 7 of 10; Fisher's Exact Test, *p* = 0.5) and age classes ($N_{\text{juveniles}}$ = 7 of 10; N_{adults} = 8 of 10; Fisher's Exact Test, *p* = 0.5), when comparing the presence of MEN. The postrostral scale (PRT) was absent in all specimens, INS was paired in all specimens, except one having one internasal on the left side and two on the right side, and CAS was paired in all specimens. We found two loreal scales (LOR) (one upper and one lower loreal) on each side in all specimens. The lacunal-supralabial (LAC-SLS) contact was absent in all specimens, except in one on the left side of the head.

Males were significantly different from females in SBC (Z = 3.87, *p* < 0.0001), DBB (Z = 2.25, *p* = 0.02), TB (Z = 2.35, *p* = 0.01) being higher in males, and in MDR (Z = -2.0, *p* = 0.04), which was higher in females. We found no significant differences between sexes in PVEN (Z = -0.07, *p* = 0.94), VEN (Z = 0.14, *p* = 0.88), ISM (Z = 0.15, *p* = 0.87), PFO (Z = -0.28, *p* = 0.77), SLS (Z = -1.67, *p* = 0.09) or ILS (Z = -0.94, *p* = 0.34; Table 2). The number of ICS varies significantly (Z = -2.68, *p* = 0.007) between males (1–2 scales anteriorly and 0–2 scales posteriorly) and females (0–3 scales anteriorly and 1–3 posteriorly), being higher in females. We found no significant differences between sexes in the presence of PLO (N_{males} = 5 of 10; N_{females} = 8 of 10; Fisher's Exact Test, *p* = 0.17; present in 65% of all specimens, *N* = 20).

Diet of Crotalus lannomi

Two scats of *Crotalus lannomi* were obtained. The scat obtained from an adult female *C. lannomi* (SVL = 470 mm, TL = 50 mm, TTL = 520 mm; UTADC 9713) contained hairs and

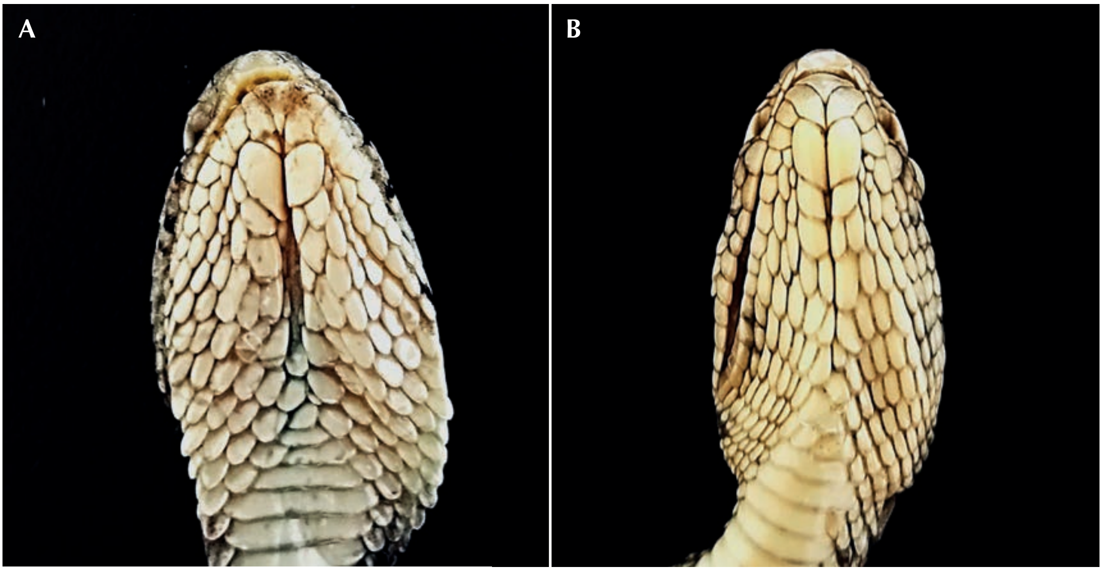

Figure 2. (**A**) Adult female *Crotalus ericsmithi* (UAAREP-827) with the first pair of infralabials mostly separated by the mental. (**B**) Adult male *Crotalus ericsmithi* (UAAREP-823) with mental produced anteriorly and the first pair of infralabials in broad contact.

molars that were identified as a Cotton Rat (*Sigmodon* sp.). The scat of a juvenile male (SVL = 320 mm, TL = 44 mm, TTL = 364 mm, HL = 21 mm; UAA-REP 1019) also contained remains of a Cotton Rat (*Sigmodon* sp.), as well as remains of insects; the latter are usually considered secondary ingestion.

Discussion

Diet Variation in Crotalus ericsmithi

Due to the scarcity of information about *Crotalus ericsmithi,* any documented prey has value. Furthermore, little is known about its biology, natural history, and morphology (Campbell and Flores-Villela 2008, Meik and Pires-da-Silva 2009, Jadin *et al.* 2010). For natural history, the related species *C. stejnegeri* can exhibit arboreal behavior (Van Der Heiden 2021, Aguirre-Zazueta *et al.* 2022), although it is not known for what purpose they climb vegetation (e.g., thermoregulation, foraging, or to avoid predation). It is likely that this behavior is also present in *C. lannomi* and *C. ericsmithi.* Diet information for *C. lannomi* and *C. stejnegeri* is scarce (Klauber 1972, Reyes-Velasco *et al.* 2010, Aguirre-Zazueta *et al.* 2022).

Our results revealed that *Crotalus ericsmithi* most likely specializes in consuming mammalian prey (84.6%) and to a lesser extent, reptiles. Among mammals, the main prey were shrews [*Cryptotis goldmani* (Merriam, 1895) and an unidentified shrew], followed by rodents, *Peromyscus* sp., *Sigmodon* sp., and *H. pictus*. The frequent consumption of shrews may be because they are small and easy to swallow and are abundant in pine-oak forest, the type of vegetation occurring in the area inhabited by *C. ericsmithi*. Rodents in the family Cricetidae reported in this study correspond to findings for *C. lannomi* and *C. stejnegeri* (Klauber 1972, Aguirre-Zazueta *et al.* 2022). More information on the diet of *C. lannomi* and *C. stejnegeri* and an increase the number of samples for *C. ericsmithi* will allow a better comparison of diet among these species.

Specialization on mammals by *Crotalus ericsmithi* coincides with a similar mammal diet of related species like *Crotalus horridus* Linnaeus, 1758 (Timber Rattlesnake) (Clark 2002, Reinert *et al.* 2011), as well as in members of the *C. durissus* group, such as *Crotalus durissus* Linnaeus, 1758 (Neotropical Rattlesnake) (Salomão *et al.* 1995, Sant'Anna and Abe 2007, Hoyos and Almeida-Santos 2016), *Crotalus tzabcan* Klauber, 1952 (Tzabcan Rattlesnake) (Carbajal-Márquez *et al.* 2020a), the *C. atrox* group, such as *Crotalus atrox* Baird and Girard, 1853 (Western Diamond-backed Rattlesnake) (Reynolds and Scott 1982, Pisani and Stephenson 1991, Spencer 2003), and *Crotalus ruber* Cope, 1892 (Red Diamond Rattlesnake) (Dugan and Hayes 2012).

Reptiles accounted for 15.4% of prey items in the diet of *C. ericsmithi.* A Spiny Lizard (*Sceloporus* sp.) and a snake (*C. ericsmithi*) were consumed by newborn specimens. Rattlesnake species closely related to *C. ericsmithi,* such as *C. lannomi,* are known to consume reptiles (Reyes-Velasco *et al.* 2010); however, related medium- to large-bodied species with large young consume mainly mammals from birth but rarely consume reptiles (Clark 2002, Spencer 2003, Reinert *et al.* 2011, Hoyos and Almeida-Santos 2016). Lizards are common prey of newborn and juvenile small-bodied rattlesnakes, although they may also be consumed by adults (Keenlyne and Beer 1973, Campbell and Armstrong 1979, Holycross *et al.* 2002, Prival and Schroff 2012, Güizado-Rodríguez *et al.* 2016, Mociño-Deloya 2016).

It is not surprising that a hatchling of *Crotalus ericsmithi* fed on a conspecific individual. Members of the *C. stejnegeri* group consume other snakes (Reyes-Velasco *et al.* 2010). Cannibalism has been reported in wild, mostly adult individuals of *Crotalus aquilus* Klauber, 1952 (Queretaran Dusky Rattlesnake) (Mociño-Deloya 2016), *C. atrox* (Myers 2015), *C. cerastes* Hallowell, 1854 (Sidewinder) (Funk 1965), *C. triseriatus* Wagler, 1830 (Western Dusky Rattlesnake) (Mociño-Deloya and Setser 2009), *C. polystictus* (Cope, 1865) (Mexican Lancehead Rattlesnake) (Mociño-Deloya *et al.* 2009, Mociño-Deloya 2016), *C. pricei* Van Denburgh, 1895 (Twin Spotted Rattlesnake) (Prival and Schroff 2012), *C. viridis* (Rafinesque, 1818) (Prairie Rattlesnake) (Gloyd 1933, Genter 1984), and *Sistrurus catenatus* (Rafinesque, 1818) (Massasauga) (Ruthven 1911, Hallock 1991).

Juveniles of *Crotalus ericsmithi* feed on both mammals and reptiles, and adults apparently only mammals. We did not find significant differences between the size of the snakes regarding the type of prey consumed, indicating that *C. ericsmithi* feeds on mammals at all stages of life, although the sample size is small. Because of the advanced degree of digestion of prey recovered from specimens of *C. ericsmithi,* we could not obtain the size of most prey. The WR estimated for adult male *C. ericsmithi* and its *Peromyscus* sp. prey was fairly high and is a consistent pattern for vipers, since they occasionally consume large prey with a high WR, even higher than WR = 1.0 (Greene 1983, 1992, Loughran *et al.* 2013).

Size and Pholidosis Variation of Crotalus ericsmithi

Males and females of *Crotalus ericsmithi* have are similar in body size. With the few data available, it appears that *C. lannomi* (SVL = 569 mm, TTL = 638 mm; Reyes-Velasco *et al.* 2010) is similar in size as *C. ericsmithi,* and *C. stejnegeri* is longer (TTL = 790 mm; Van Der Heiden and Flores-Villela 2013). Both species appear to have males and females of similar sizes. Although *C. durissus* and C. *tzabcan* do not show sexual dimorphism in SVL and TTL (Santa'Anna and Abe 2007, Barros *et al.* 2012, Carbajal-Márquez *et al.* 2020a,b), dimorphism occurs in other related species as *C. horridus* (Gibbons 1972), *C. atrox* (Spencer 2003), *C. catalinensis* Cliff, 1954 (Avila-Villegas *et al.* 2007), and *C. ruber* (Dugan and Hayes 2012).

Crotalus ericsmithi is sexually dimorphic in TL (males have longer tails). The greater %TL in males of *C. ericsmithi* is similar to the greater %TL reported for males of *C. lannomi* (Reyes-Velasco *et al.* 2010) and *C. stejnegeri* (Van Der Heiden and Flores-Villela 2013). The maximum %TL reached by *C. ericsmithi* (15.9%) is greater than that reported for *C. lannomi* (14.4%) and *C. stejnegeri* (14.5%). The related species *C. durissus* and *C. tzabcan* are sexually dimorphic in TL, and due to the presence of hemipenes, most rattlesnakes are sexually dimorphic (Barros *et al.* 2012, Carbajal-Márquez *et al.* 2020a,b).

The smaller HL and HW in males of *Crotalus ericsmithi* suggest that sexual differences may influence the size of prey they consume; however, we could not determine the size of most prey, and we found no sex differences in the prey classes consumed. Although SVL is not significantly different between sexes, females have greater HL and HW and may possibly feed on larger prey items. Forsman and Lindell (1993) found that swallowing performance varied among snakes of the same SVL because of differences in HL. Sexual differences in HL and HW may also be due to phenotypic plasticity in response to environmental factors (Meik *et al.* 2012). Data to compare HL and HW in the closely related species *C. lannomi* and *C. stejnegeri* is insufficient (Reyes-Velasco *et al.* 2010, Van Der Heiden and Flores-Villela 2013). Other species related to *C. ericsmithi* show no sex differences in prey type consumed as *C. atrox* (Spencer 2003), *C. catalinensis* (Avila-Villegas *et al.* 2007), *C. durissus* (Santa'Anna and Abe 2007), *C. horridus* (Reinert *et al.* 2011, Wittenberg 2012), prey mass as *C. ruber* (Dugan and Hayes 2012), or prey type and mass in *C. tzabcan* (Carbajal-Márquez *et al.* 2020a).

MEN was present in 75% of specimens of *Crotalus ericsmithi.* This separation is apparently not dependent on sex or age. The shape of the mental scale in the five specimens examined does not prevent contact of the first supralabials and is similar to the shape in *C. lannomi* (Campbell and Flores-Villela 2008). This character is not reliably diagnostic.

Crotalus ericsmithi is sexually dimorphic in MDR and ICS (greater number in females). Because of the small number of specimens, no data are available to verify if this pattern is shared with *C. lannomi* (MDR = 25–29; ICS male = 0–4; ICS female = 2–3) and *C. stejnegeri* (MDR = 25–27; ICS = 2–12) (Reves-Velasco *et al.* 2010, Van Der Heiden and Flores-Villela 2013). *C. ericsmithi* is sexually dimorphic in SBC, DBB, and TB (greater number in males). This trend is apparently present in *C. lannomi* SBC (male = 49; female = 35–37), *C. stejnegeri* SBC (male = 42–45; female = 36–37) and *C. lannomi* DBB (male = 35; female = 31–33) but not in *C. stejnegeri* DBB (male = 32–43; female = 34–43). Sexual dimorphism is apparently present in *C. lannomi* TB (male = 12–17; female = 12–15), but data are insufficient for C. *stejnegeri* TB (13–16).

Sexual dimorphism was not significant in other characters of pholidosis of *Crotalus ericsmithi,* similar to *C. lannomi* VEN (male = 171–175; female = 168–176) and *C. stejnegeri* VEN (male = 172–178; female = 171–176), where the number of VEN overlap between sexes (Reyes-Velasco *et al.* 2010, Van Der Heiden and Flores-Villela 2013). The paired INS found in all specimens of *C. ericsmithi* (except for one specimen with one internasal on the left side and two on the right side) is comparable to *C. lannomi,* which has relatively narrow and commonly paired INS, except for two specimens that have two internasals on the left side and one on the right side (UTADC 4003, 4006; see Reyes-Velasco *et al.* 2010). *Crotalus stejnegeri* has broad triangular and commonly paired INS (Van Der Heiden and Flores-Villela 2013). The absence of sexual differences in ISM seems to be present in *C. lannomi* (male = 3–4; female = 4–5) and *C. stejnegeri* (5–8). Data are insufficient to compare *C. lannomi* PFO (4) and *C. stejnegeri* PFO (7–8) (Campbell and Lamar 2004, Reyes-Velasco *et al.* 2010, Van Der Heiden and Flores-Villela 2013).

Crotalus ericsmithi is a slender rattlesnake that is not sexually dimorphic in body length. Females of *C. ericsmithi* have a greater number of MDR, possibly because increased maternal body size allows larger clutches (Shine 2000). Greater MDR in females may also be related to their larger HL and HW, allowing them to consume larger prey items, thus sequestering more energy available for growth, storage, and reproduction (Forsman and Lindell 1993, Shine 2000). This greater girth in females is also present in *C. lannomi* (Reyes-Velasco *et al.* 2010). Additionally, the greater number of ICS in females may be related to a larger HL and HW than in males. The greater number of SBC and TB in males has been previously reported for *C. stejnegeri* and for related species such as *C. durissus*, *C. tzabcan*, and most species in the genus, possibly because of the presence of the hemipenes (Klauber 1952, Campbell and Lamar, 2004, Carbajal-Márquez *et al.* 2020b).

Diet of Crotalus lannomi

Both prey items of *Crotalus lannomi* obtained in this study are mammals. Only two diet records, both mammals, are known for *C. stejnegeri* (Klauber 1972, Aguirre-Zazueta *et al.* 2022). Reyes-Velasco *et al.* (2010) observed that a juvenile specimen of *C. lannomi* found in July 2008 contained scales from a lizard (*Sceloporus* sp.), arthropod remains, plant matter, and an unidentified snake jaw. It is likely that *C. lannomi* and *C. stejnegeri* have feeding habits similar to *C. ericsmithi,* which may be a shared pattern in Long-tailed rattlesnakes.

Our study provides baseline data on the diet and morphology of specimens of wild *Crotalus ericsmithi,* and adds new diet data for *C. lannomi.* Both species are secretive and difficult to encounter and study in the field; thus, any new information is valuable. *Crotalus ericsmithi* is likely a specialized mammal predator, with habits similar to its related species, members of the *C. atrox* and *C. durissus* groups. Our sample size is relatively small and must be interpreted with caution, but it reflects the difficulty of accessing areas where these rattlesnakes live, the rarity of the species, the difficulty of finding specimens that have recently eaten, and the infrequent collection of individuals. Snakes face several threats to their survival (e.g., climate change, habitat loss, illegal species trafficking; see Fernández-Badillo *et al.* 2021). The diet and morphological data provided here contribute to our understanding of the biology and ecology of *C. ericsmithi* and *C. lannomi,* and how these data relate to that of other rattlesnakes, leading to better decision-making regarding conservation. Additional data must be collected to further our knowledge of the natural history and distribution of these enigmatic rattlesnakes.

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An experimental evaluation of substrate type and color selection by the endangered salamander *Ambystoma altamirani* (Caudata: Ambystomatidae)

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Abstract

An experimental evaluation of substrate type and color selection by the endangered salamander *Ambystoma altamirani* (Caudata: Ambystomatidae). The stream habitats of Mexican *Ambystoma* are being degraded by human activities, which can have detrimental effects on their populations. A better understanding of the microhabitat and habitat use of these amphibians is therefore important. We used preference experiments to examine the selection of substrate type (gravel, mud, sand, and stone) and color (dark brown, brown, light brown, and gray) by the endangered, endemic salamander *Ambystoma altamirani*. *Ambystoma altamirani* used mud substrates more than the other substrate types. Overall, *A. altamirani* showed no preference for substrate color; however, females used light brown substrates significantly more often and gray substrates less often than males and juveniles did. These experimental results are consistent with the observed substrate type use of *A. altamirani* in the field, but the lack of a substrate color preference differed from the observed preference for dark brown substrates in the field.

Keywords: Arroyo los Axolotes, Behavior, Mexico, Microhabitat, Mountain Stream Siredon.

Resumo

Uma avaliação experimental do tipo de substrato e da seleção de cores pela salamandra ameaçada de extinção *Ambystoma altamirani* (Caudata: Ambystomatidae). Os habitats de riacho das salamandras do gênero *Ambystoma* no México estão sendo degradados pelas atividades humanas, o que pode ter efeitos prejudiciais sobre suas populações. Assim, é importante compreender melhor o uso de habitas e micro-habitatas por parte desses anfíbios. Utilizamos experimentos de preferência para examinar a seleção do tipo (cascalho, lama, areia e pedra) e da cor (castanho-escuro, castanho, castanho-claro e cinzento) do substrato pela salamandra *A. altamirani*, uma espécie endêmica e ameaçada de extinção. *Ambystoma altamirani* utilizou mais os substratos de lama do que os outros

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tipos. De modo geral, *A. altamirani* não mostrou preferência pela cor do substrato; no entanto, as fêmeas usaram substratos castanhos claros com mais frequência e substratos cinzentos com menos frequência do que os machos e os juvenis. Esses resultados experimentais são consistentes com o uso do tipo de substrato observado em *A. altamirani* no campo, mas a ausência de preferência pela cor do substrato difere da preferência por substratos marrons escuros observada no campo.

Palavras-chave: Arroyo los Axolotes, Comportamento, México, Micro-habitat, Siredon-de-riachos-de-montanha.

Introduction

The quality of freshwater habitats is frequently negatively affected by humans (Carpenter *et al.* 2011, Oberdorff 2022). In particular, a substantial percentage of streams and rivers are degraded by human activities (Kaufmann *et al.* 2022b, McManamay *et al.* 2022). Indeed, higher human population densities are correlated with a loss of biodiversity in streams (Urban *et al.* 2006). Anthropogenic impacts on streams include sedimentation, changes in vegetation within or surrounding the stream, or changes in habitat complexity (Kaufmann *et al.* 2022a), water flow and hydrology (Poff *et al.* 2006, McManamay *et al.* 2022), and water quality (e.g., chemistry, temperature; Poole and Berman 2001, Ferreira *et al.* 2017, Vázquez *et al.* 2023).

Several species of salamanders use streams, either as places to breed or for their entire life cycle (Wells 2007). As such, the characteristics of stream habitats can be important in determining their distribution and abundance. Often, aspects of the stream substrate are important factors driving salamander abundance or distribution. In particular, substrate composition (e.g., gravel, sand, silt, mud, etc.) has been shown to determine the abundance or distribution of salamanders along streams (e.g., Bowles *et al.* 2006, Miller *et al.* 2007, Kroll *et al.* 2008, Rizzo *et al.* 2016). Because of the importance of substrate composition in determining the abundance and distribution of stream salamanders, the anthropogenic activities that influence the amount or composition of stream substrates can negatively affect populations of stream salamanders. In particular, increased sedimentation, often associated with alterations in water flow or changes in land use near streams, has been shown to negatively impact stream salamander populations, primarily by increasing sediment embeddedness (e.g., Lowe and Bolger 2002, Lowe *et al.* 2004, Moseley *et al.* 2008).

The degradation of stream habitats due to human activities—such as deforestation, livestock, and agriculture—is detrimental to populations of Mexican *Ambystoma* Tschudi, 1838 due to changes in water chemistry and water flow, the introduction of nonnative species, and increased substrate embeddedness (e.g., Estrella Zamora *et al.* 2018, Guerrero de la Paz 2020, Piñon-Flores *et al.* 2021). Many populations and species of Mexican *Ambystoma* are threatened (Heredia-Bobadilla and Sunny 2021). It is therefore important to better understand their microhabitat and habitat use in the streams and lakes they inhabit.

In field studies of the substrate use of Mexican *Ambystoma*, several species have been found to frequently use mud substrates (*A. rivulare*: Bille 2009, Lemos-Espinal *et al.* 2015; *A. leorae:* Lemos-Espinal *et al.* 2017). Other species of Mexican *Ambystoma* use multiple substrate types, including rock, sand, and mud substrates (*A. leorae:* Sunny *et al.* 2014, Monroy-Vilchis *et al.* 2015), but others do not (*A. rosaceum* Taylor, 1941: Anderson 1961).

Ambystoma altamirani Dugès, 1895 is an endangered, endemic salamander found in streams in the mountains near Mexico City (Lemos-Espinal *et al.* 1999, Woolrich-Piña *et al.*

2017). *Ambystoma altamirani* lives most, if not all, of its life in streams or immediately adjacent to streams (< 5 m; Lemos-Espinal *et al.* 1999). The dorsal color of *A. altamirani* is usually olive-green with black or yellow markings or solid black (Villarreal Hernández *et al.* 2020b). In-stream characteristics appear to be more important than terrestrial characteristics (e.g., presence of livestock, distance to forest) in determining the abundance of *A. altamirani* along a stream (Gómez-Franco *et al.* 2022). *Ambystoma altamirani* in the Arroyo Los Axolotes selected sites with mud and avoided sites with gravel or bedrock (Lemos-Espinal *et al.* 2016, Villarreal Hernández *et al.* 2020a). In nature, *A. altamirani* was found on dark brown substrates more than brown, light brown, or gray substrates (Villarreal Hernández *et al.* 2020a; see also Lemos-Espinal *et al.* 2016), and this selection did not differ with the color of the salamander (Villarreal Hernández *et al.* 2020b). Despite these field studies, there have been no experimental investigations of substrate selection in *A. altamirani*. Such experiments would help inform our understanding of the basis for the field observations (i.e., Is it a true preference or simply a reflection of availability?), which can inform our knowledge of what aspects of a stream, especially its substrates, are needed for the persistence of these endangered salamanders.

Here, we report on the results of experiments designed to determine the preferences of *A. altamirani* for substrate type and color. We also determined whether these preferences differed among adult males, adult females, and juveniles.

Materials and Methods

We collected 135 individuals of *A. altamirani* from multiple sites along a 1 km section of the Arroyo del Axolotes, Isidro Fabela municipality, Mexico, using a dipnet and transported them to a nearby facility for the preference experiments. We collected salamanders and conducted experiments in December 2021 (*N* = 2 salamanders), February 2022 (*N* = 4), March 2022 (*N* = 13), April 2022 (*N* = 32), May 2022 (*N* = 19), June 2022 (*N* = 35), July 2022 (*N* = 8), August 2022 (*N* = 10), September 2022 (*N* = 5), October 2022 (*N* = 4), and November 2022 (*N* = 3). We categorized individuals as adult males, adult females, or juveniles. We used the presence of a bulge on the tail near the cloaca to identify adult males, whereas adult females lack this bulge (Brandon and Altig 1973). We classified a salamander as a juvenile if it possessed gills (see Villarreal Hernández *et al.* 2020a).

We conducted two preference experiments: one for substrate type and one for substrate color (see Mushinsky 1976, Rittenhouse *et al.* 2004, Martin *et al.* 2012, and Sánchez-Sánchez *et al.* 2023 for similar experiments). The experiments were performed one to four hours after capture. We kept the salamanders in plastic containers between capture and experimentation. For each experiment, we established multiple test arenas using 36 L aquaria (40 × 30 × 30 cm) with each aquarium divided into sections as described below. At the start of each trial, we placed a single *A. altamirani* in the center of an aquarium and allowed it to acclimate for 5 min prior to data collection. During the acclimation period, the salamanders were allowed to move about the aquarium. After the acclimation period, we recorded the substrate type or color on which the salamander was located every minute for 15 minutes. Each salamander was tested only once in each experiment.

To test for substrate type preferences, we established four equal sections on the bottom of the aquaria with mud, sand, gravel, and rock using material collected from the stream (Figure 1). The mud substrate was dark brown in color, the sand was brown, the gravel was grey, the rock was light brown or light grey. We used small rectangular plastic trays to keep the substrate types separate. Since color and substrate type were confounded in the substrate type experiment, we conducted a second experiment to specifically study the effect of substrate color. For the substrate color experiment, we divided the aquarium floor into four equal sections with colored paper under the clear bottom of the tank. We chose colors to approximate the four most common substrate colors in the Arroyo Los Axolotes using the COMEX color palette (brown color family): dark brown, brown, light brown, and grey (Villarreal Hernández *et al.* 2020a,b). We replaced the water in the aquaria between trials and the orientations of the experimental aquaria were arbitrarily established prior to each set of experiments (i.e., each monthly trial).

We used two-way repeated measures ANOVAs to analyze substrate type and color preferences, with sex/stage (male, female, juvenile) and season (wet, dry) as the independent variables and substrate type or color as the repeated measures. We analyzed the number of observations of each individual found in a substrate or color. We used Wilcoxon signed-rank tests to compare means for significant terms.

Results

The results of the two-way repeated measures ANOVA for the substrate type and for the substrate color experiments are given in Table 1. The only significant effect was that mud was used more than the other substrate types (Figure 2A). There was a significant interaction between color used and the sex/stage of the salamander, with females using light brown substrate more and gray substrates less than males and juveniles (Figure 2B). No other terms were significant.

Discussion

Ambystoma altamirani individuals used mud substrates more often than stone, gravel, or sand substrates. This pattern did not differ among males, females, and juveniles or between the wet and dry seasons. Our experimental results are generally consistent with field studies on substrate use by *A. altamirani* which found that

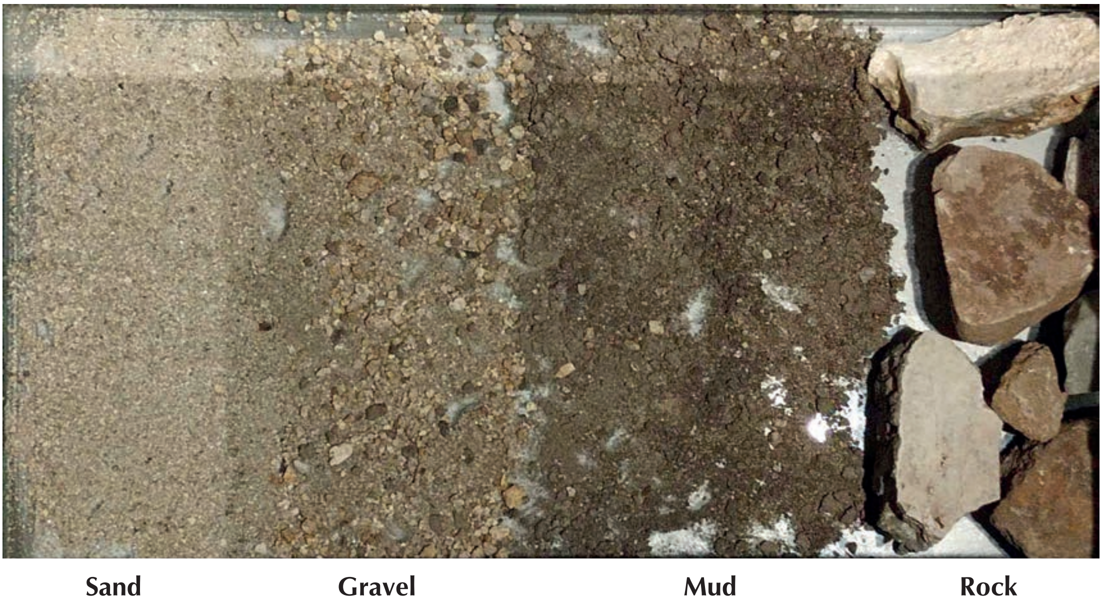

Figure 1. A photograph showing the different substrate types used in the experiment.

Table 1. Results of a two-way repeated measures analysis of variance on substrate type/color preference of *Ambystoma altamirani*, with sex/stage (male, female, juvenile) and season (wet, dry) as independent variables and substrate type/color as the repeated measure.

	Substrate type			**Substrate color**		
	dfs	*F*	*p*	dfs	*F*	*p*
Sex/stage	2, 129	1.86	0.16	2, 129	0.87	0.42
Season	1, 129	2.83	0.10	1, 129	1.00	0.32
Sex/stage * season	2, 129	0.85	0.43	2, 129	0.28	0.76
Substrate type	3, 387	3.35	0.019	3, 387	0.80	0.49
Substrate type * sex/stage	6, 387	0.90	0.49	6, 387	2.52	0.022
Substrate type * season	3, 387	0.85	0.47	3, 387	2.18	0.10
Substrate type * sex/stage * season	6, 387	1.08	0.38	6, 387	1.19	0.26

sections of streams with mud were used more often than sections of streams with other substrates were (Lemos-Espinal *et al.* 2016, Villarreal Hernández *et al.* 2020a). Mud is also a commonly used substrate for other Mexican *Ambystoma*, including *A. rivulare* (Bille 2009, Lemos-Espinal *et al.* 2015) and *A. leorae* (Sunny *et al.* 2014, Lemos-Espinal *et al.* 2017). All of these results suggest that mud substrates have some quality that is attractive or beneficial to these salamanders. For example, mud substrates may provide salamanders with refugia from predators or other disturbances by allowing them to bury themselves (see Bille 2009, Lemos-Espinal *et al.* 2015).

In our experiments, *A. altamirani* did not show any overall preference for substrate color. However, females differed from males and juveniles in their use of substrate colors, with females using light brown substrates more and gray substrates less than males and juveniles. It is unclear why such a difference might exist. Previous field studies have shown that *A. altamirani* uses darker substrates, such as dark brown or black, more than lighter substrates, such as grey (Lemos-Espinal *et al.* 2016, Villarreal Hernández *et al.* 2020a,b). These previous field studies also did not distinguish between males and females (Lemos-Espinal *et al.* 2016, Villarreal Hernández *et al.* 2020a,b). It is possible that the differences in the color of substrates used between our experimental study and the previous field results may reflect differences in salamander color or salamander perception of risk, or perhaps reflect differences in the sex or stage of the individuals observed in the field.

In other *Ambystoma*, there can be a relationship between individual color and substrate color, where individuals physiologically adjust their color to match the substrate color (Garcia and Sih 2003) or choose substrates to match their color (Garcia *et al.* 2003). In addition, the substrate color selected can depend on the presence of predator cues (Garcia and Sih 2003). There is also some evidence that substrate color choice can be conditioned by early experience (Garcia and Sih 2003). However, in at least one field study, *A. altamirani* showed no evidence of substrate color matching (Villarreal Hernández *et al.* 2020b). Future experiments that examine substrate color choice by *A. altamirani* in the presence of predator cues or after conditioning on particular substrate colors might help elucidate the factors driving substrate color selection in this endangered salamander. In addition, manipulating the color of the mud while retaining other characteristics could also

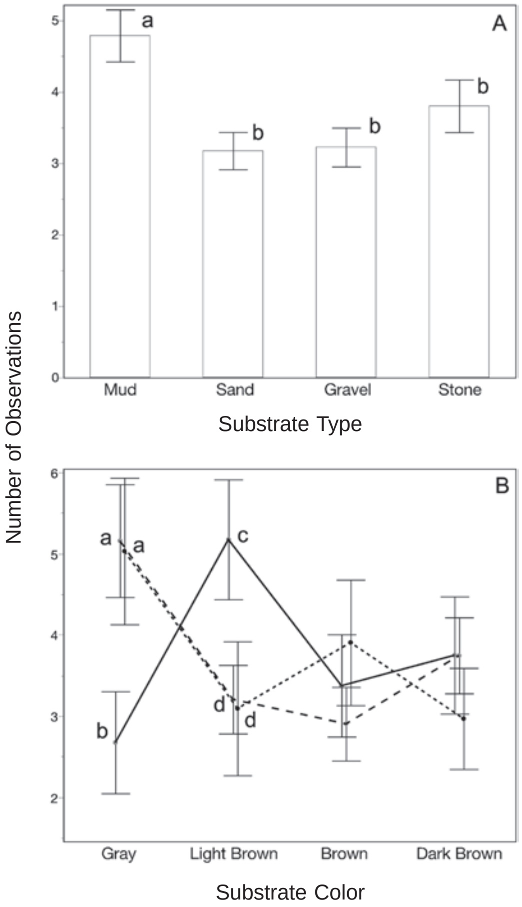

Figure 2. The mean number of times a individual *Ambystoma altamirani* used (**A**) a particular substrate type and (**B**) male (dotted line), female (solid line), and juvenile (dashed line) *A. altamirani* used a particular substrate color in the dry and wet seasons. Means sharing a letter are not significantly different (Wilcoxon signed rank test, $p > 0.05$). For (B) post-hoc tests were applied to each substrate color separately.

elucidate whether *A. altamirani* prefers the color or the mud.

Overall, our experiment results and similar results from field observations suggest that mud substrate is an important characteristic of streams used by the endangered salamander, *A. altamirani* (Lemos-Espinal *et al.* 2016, Villarreal Hernández *et al.* 2020). Taken together, these results indicate that preventing the loss of mud substrates is important when establishing conservation or management plans for these streams. In particular, this means maintaining stream flows that prevent the scouring of mud substrates, replacing mud substrates with other types of sediments (e.g., sand or other sediments with different particle sizes), or channelizing streams. Similar future experiments should focus on other aspects of the stream environment that have been shown to be related to the distribution and abundance of *A. altamirani,* such as dissolved oxygen, water velocity, water depth, refuse and garbage, and water temperature (Lemos-Espinal *et al.* 2016, Villanueva Camacho *et al.* 2020, Gómez Franco *et al.* 2022). In addition, experiments that use individuals from more than one stream would allow for greater generalization of the results since our study only included individuals of *A. altamirani* from a single study stream.

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Plasticity of metamorphic traits of tadpoles of *Rana chensinensis* (Anura: Ranidae): interactive effects of food level and water exchange

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Abstract

Plasticity of metamorphic traits of tadpoles of *Rana chensinensis* (Anura: Ranidae): interactive effects of food level and water exchange. In nature, ambient conditions may strongly affect morphological variation, especially in animals with complex life cycles, such as amphibians. Although food level and water exchange have a strong effect on the metamorphic traits of larvae, little is known about effects of interaction between both factors on length of larval period and size at metamorphosis. In this study, we evaluated plasticity of metamorphic traits of the Chinese brown frog (*Rana chensinensis*) under different combinations of food levels and water exchange. Age and mass at metamorphosis were susceptible to food level and varied with water exchange. High food levels could lead to shorter larval periods except for weekly water changes. Further, at low food levels, tadpoles with frequent water exchange (renewed every 2 days) attained a larger size than those at other treatments of water changes. The effects of water changes were dependent on food level. Our results also demonstrated that frequent water exchange and high food levels have positive effects on survival of tadpoles of Chinese brown frogs. We found a significant interaction between food level and water exchange, suggesting that this novel mechanism is selected for when frequent water exchange is likely to prove profitable when food is insufficient or environmental stresses are present.

Keywords: Amphibians, Chinese Brown Frog, Food availability, Growth rate, Mass at metamorphosis, Water exchange period.

Resumo

Plasticidade das características metamórficas dos girinos de *Rana chensinensis* (Anura: Ranidae): efeitos interativos do nível de alimento e das trocas de água. Na natureza, as condições ambientais podem afetar fortemente a variação morfológica, especialmente em animais com ciclos de vida complexos, como os anfíbios. Embora o nível de alimento e a troca de água tenham um forte efeito nas características metamórficas das larvas, pouco se sabe sobre os efeitos da interação entre

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ambos os fatores na duração do período larval e no tamanho na metamorfose. Neste estudo, avaliamos a plasticidade das características metamórficas da rã-castanha-chinesa (*Rana chensinensis*) sob diferentes combinações de níveis de alimento e troca de água. A idade e a massa na metamorfose foram susceptíveis ao nível de alimento e variaram com a troca de água. Níveis elevados de alimento podem levar a períodos larvais mais curtos, exceto com trocas de água semanais. Além disso, com níveis baixos de alimento, os girinos com trocas frequentes de água (renovada cada 2 dias) atingiram um tamanho maior do que os girinos com outros tratamentos de trocas de água. Os efeitos das mudanças de água dependeram do nível de alimento. Nossos resultados também demonstraram que a troca frequente de água e níveis elevados de alimento têm efeitos positivos sobre a sobrevivência dos girinos da rã-castanha-chinesa. Encontramos uma interação significativa entre o nível de alimento e a troca de água, o que sugere que este novo mecanismo é selecionado quando a troca frequente de água é suscetível de ser proveitosa quando o alimento é insuficiente ou quando há estresse ambiental.

Palavras-chave: Anfíbios, Disponibilidade de alimento, Massa na metamorfose, Período de troca de água, Rã-castanha-chinesa, Taxa de crescimento.

Introduction

In nature, length of larval period and size at metamorphosis are important fitness components (Arnold and Wassersug 1978, Wilbur 1980), especially for animals with complex life cycles, such as amphibians. Larval amphibians are more likely to experience variation in food availability owing to the variety of aquatic spawning sites (Morey and Reznick 2004, Skelly 2004). Besides energy absorption, food supply has been regarded as an essential proximal cause of plasticity of metamorphic traits (Newman 1998, reviewed by Álvarez and Nicieza 2002, Castano *et al.* 2010).

Generally, adequate food supply enhances growth and development of larvae, thus allowing them either to maximize size at metamorphosis or minimize larval period (Pandian and Marian 1985, Arendt and Hoang 2005, Yu *et al.* 2015, 2016a, b, c, Yu and Han 2020). In contrast, low food levels, high larval density, or both in combination usually result in food limitation, thus negatively weighing on metamorphosis. For example, tadpoles prolong larval period to meet the minimum threshold of size at metamorphosis because growth rate is low when food limitation leads to poor conditions (reviewed by Wilbur and Collins 1973). Burraco *et al.* (2021) confirmed that when conditions are favorable (such as warm temperature and abundant food), larvae completely offset the delay in hatching without any negative impact on their body mass. When conditions are unfavorable (such as cold temperature and limited food), these compensatory responses are hindered, and if the hatching delay is prolonged in such adverse conditions, it completely disrupts the ability to compensate. Beyts *et al.* (2023) found that under familiar conditions, there was an increase in individual variance in plasticity and predictability in the high food treatment, while in an unfamiliar context, there was an increase in individual variance in personality only in the low food treatment.

Other factors can also affect metamorphic time and growth rate (Rose 2005). Intraspecific competition is a widespread phenomenon in nature, acting through interference and exploitative mechanisms (Steinwascher 1978). For example, some anuran larvae can employ chemical cues to inhibit the growth of small individuals in intraspecific competition (e.g., Rose and Rose 1961, Stepanova 1974, Rot-Nikcevic *et al.* 2005, 2006). In particular, interference mechanisms embrace direct interactions between individuals of the same species (Hettyey *et al.* 2014, Zewe and Booth 2014), usually occurring at low food levels

(Richards 1962). Carbon dioxide, oxygen, nitrite, and heavy metals present in water have an impact on the growth and development of aquatic organisms (e.g., Ishimatsu *et al.* 2004, Hong *et al.* 2020, Verberk *et al.* 2020, Edwards *et al.* 2023). For example, higher oxygen tension or decreasing carbon dioxide tension seems to prevail in frequent water exchange (Adolph 1931). It is generally known that low population density and frequent water change accelerate growth of aquatic organisms (Richards 1958, Hailey *et al.* 2006).

Few studies have investigated how water exchange and food level are interrelated to influence metamorphic traits of anuran larvae. In this study, we evaluated whether food level and water exchange affect metamorphic time and size at metamorphosis of the Chinese brown frog (*Rana chensinensis* David, 1875). Specifically, we hypothesized that tadpoles fed on high food levels and reared at frequent water changes should have higher growth and survival rates. We also hypothesized that tadpoles should have larger size at metamorphosis when they were reared with frequent water changes because of lower chemical waste.

Materials and Methods

Study Species

Female frogs are the larger sex and are widely distributed in regions north of the Yangtze River in China (Yu *et al.* 2015). These frogs are explosive breeders because they have a relatively short breeding season (8–16 days; Wells 2007, Yu *et al.* 2015). The frogs prefer to select small and medium quiet water bodies as spawning habitats and lay eggs along edges of ponds. The beginning of the breeding period for *R. chensinensis* tends to be later at higher latitudes. Tadpoles have a longer development time to reach metamorphosis (80–90 days) in natural ponds because water temperature is colder during the larval stage (mean temperature less than 20°C; Yu pers. obs.). The oral structure of tadpoles is highly unique, characterized by semi-circular grinding teeth that can crush both animal and plant food into a paste, enabling them to be classified as scraper species (Feng *et al.* 2003). Rich *Spirogyra* Link and Nees and *Potamogeton crispus* L. are considered as the natural food of tadpoles of *R. chensinensis* (Cao *et al.* 2002).

Field and Laboratory Procedures

From 11 to 19 February 2021, ten fresh egg masses of *R. chensinensis* were collected in Xinyang (114°06' E, 32°12' N; 22–100 m a.s.l.), Henan, China. We selected 100 eggs from each of the egg masses and put them into a 2–l plastic container filled with a depth of approximately 15 cm of fresh water, where they were allowed to hatch. The experiment commenced on 28 February 2021 and concluded on 24 June 2021, spanning a duration of 116 days. We conducted this research in the laboratory located 0.5 km from the spawning site, thus avoiding confounding environmental effects and predation pressure.

Experiment Design

We used a 2 × 4 factorial design to evaluate the effects of food level and water exchange on metamorphic traits of tadpoles of *R. chensinensis* (Figure 1). A total of 320 tadpoles were randomly allocated into eight experimental treatments. To estimate the effects of food level, half of the tadpoles in all treatments were placed at low mass-specific food level (6% of tadpole mass per day, LFL) and half on a high food regimen (12% of tadpole mass per day, HFL) based on previous studies on this species (Zhang *et al.* 2007). For each food regimen, four treatments of water exchange were used during the growing period: once every two days, once every four days, once a week, and once every two weeks. In this experiment, an opaque round plastic bowl with dimensions of 15 cm upper diameter, 10.5 cm lower diameter, and 7.3 cm height, each of which is 0.5 l, was used to house two tadpoles; thus, 20 bowls (40 tadpoles) were used in each experimental treatment. During the water exchange process, a large bucket with a capacity of 100 L was filled with tap water and exposed to chlorine for three days prior to use. Subsequently, all water in the containers was replaced simultaneously.

Tadpoles were carefully chosen before being used in each treatment. Initially, any large or small tadpoles were visually identified and removed. A total of 30 tadpoles, similar in size, was placed in a circular basin measuring 40 cm in diameter and filled with 2 cm of clean water. A caliper was positioned at the center, and photographs were taken. The *tpsdig2* computer

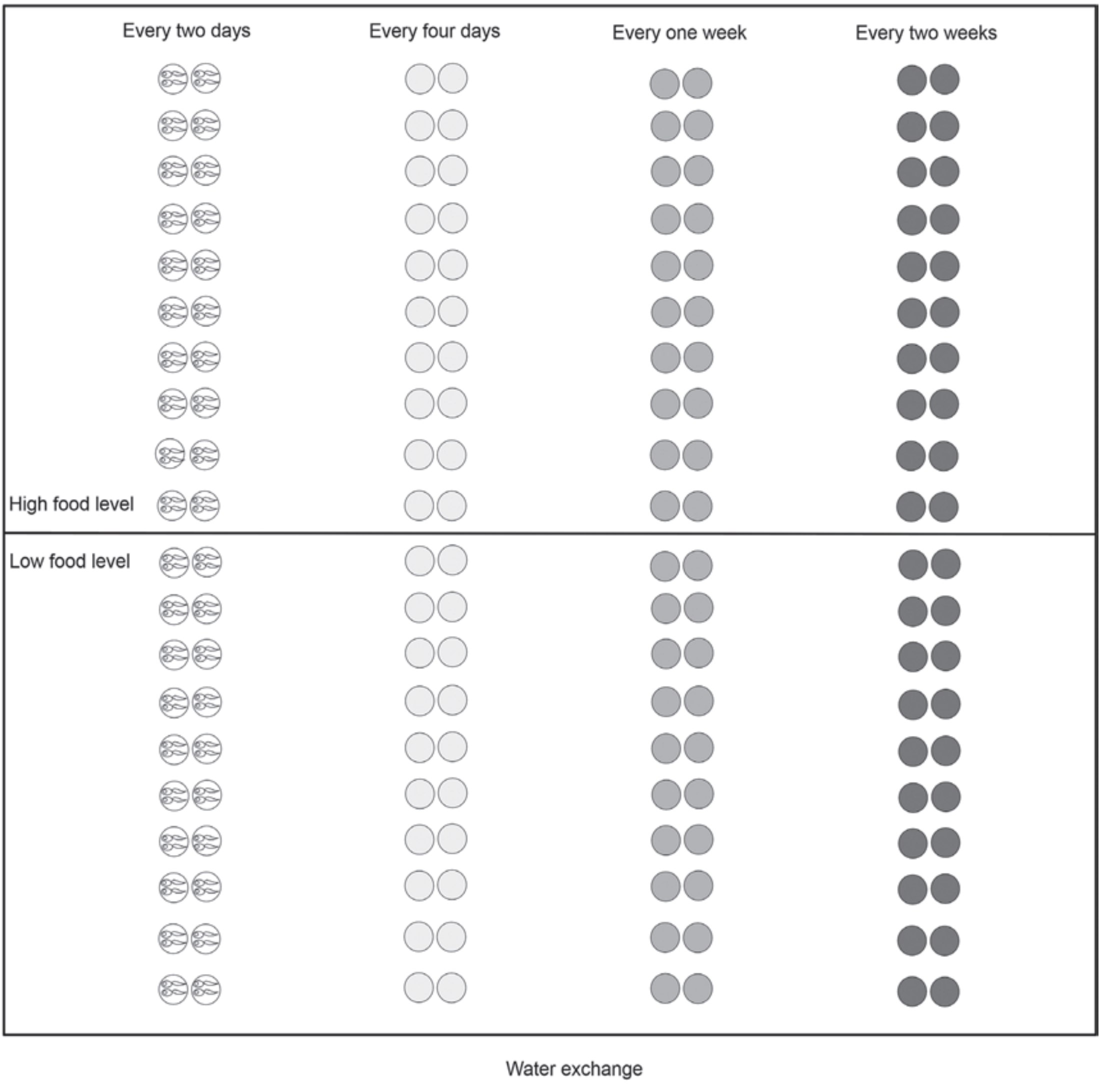

Figure 1. Experimental set-up (above the horizontal line, high food level; below the horizontal line, low food level).

software was used to calculate the average body length. Throughout the experiment, tadpoles with a body length exceeding or falling below the mean by 1 mm were excluded, guaranteeing that individuals of the same size were used at the beginning of an experiment. We randomly chose one tadpole from each clutch to put into each treatment so that the same number of tadpoles from each family was used in the experiment, thus avoiding parental or genetic effects and intraspecific competition. At 10:00 h every morning, a designated individual fed the tadpoles with commercial fish food (30% protein, 10% lipids, 18% algae, 10% fiber, 15% ash, 10% moisture) or changed the water. Tadpoles were reared at ambient temperature (17.3 ± 1.33°C) and a photoperiod of 13L:11D.

Once the first metamorph (29 April 2021, defined as the emergence of at least one forelimb, Gosner Stage 42) was discovered, we surveyed 160 bowls at least once a day until all metamorphs were found (24 June 2021). Four variables were measured: (1) length of larval period was calculated as number of days from hatching until metamorphosis; (2) body mass was measured using an electric balance (to the nearest 0.001 g); (3) growth rate was calculated as the mass at metamorphosis divided by the larval period (Laurila 2000); and (4) survivorship was classified on a three-point scale based on measurements of tadpoles in a plastic bowl: 100% = two tadpoles survived until metamorphosis; 50% = one of two tadpoles survived until metamorphosis; 0 = no tadpoles survived until metamorphosis.

Data Analysis

We used a generalized linear model (GLM) with type III mean squares to analyze the effects of water exchange, food level, and their interaction on the length of larval period, mass at metamorphosis, growth rate, and survivorship. If the overall GLM results were significant, we used ANOVAs with post-hoc multiple comparisons (Fisher's LSD) to assess differences between food levels or between water exchanges. All the analyses were done with SPSS 20.0 (SPSS Inc., Chicago, Illinois, USA).

Results

Effects of Food Level and Water Exchange on Length of Larval Period and Mass at Metamorphosis

The effect of food level on length of the larval period was significant ($F_{1,\ 208} = 25.522$, $p < 0.001$, Table 1, Figure 2A). Frequent water exchanges tended to reduce the larval period but was not significant ($F_{3,\ 208} = 2.148$, $p = 0.095$). A significant interaction between food level and water exchange ($F_{3,\ 208} = 3.038$, $p = 0.030$) revealed that high food availability resulted in faster growth, leading to shorter larval periods when tadpoles were reared at three treatments of water exchange (all $p < 0.05$), after once a week ($p = 0.529$).

Mass at metamorphosis was affected by food level ($F_{1,\ 209} = 136.043$; Table 1, Figure 2B), but the effect of water exchange was not statistically significant ($F_{3,\ 209} = 1.841$, $p = 0.141$). The interaction between food level and water exchange was significant ($F_{3,\ 209} = 3.970$, $p = 0.009$), revealing that LFL tadpoles reared at water exchange once every two days were larger than those reared at water exchanges once a week and once every two weeks (both $p < 0.020$), but the latter was similar ($p = 0.869$). HFL tadpoles had a larger body mass than LFL tadpoles independent of water exchange (all $p < 0.001$).

Effects of Food Level and Water Exchange on Growth Rate and Survivorship

The effect of food level on growth rate was significant ($F_{1,\ 208} = 30.272$, $p < 0.001$), while water exchange had no effect on growth rate ($F_{3,\ 208} = 2.425$, $p = 0.067$; Table 1, Figure 2C). The interaction between food level and water exchange was not significant ($F_{3,\ 208} = 2.438$, *p* = 0.066); however, LFL tadpoles reared at frequent water exchange (once every two days) were larger than those reared at other treatments of water exchange (once every four days or two weeks, both $p < 0.042$), but there was no difference between once every two days and once a week ($p = 0.088$). HFL tadpoles had faster growth than LFL tadpoles independent of water exchange (all $p < 0.001$).

The effect of food level on survivorship was significant ($F_{1, 208} = 119.434$, $p < 0.001$, Table 1, Figure 2D), indicative of high survival with high food quantity. Water exchange had an effect on survivorship ($F_{3, 151} = 2.958$, $p = 0.034$), revealing that tadpoles reared at frequent water exchange have higher survivorship to metamorphosis than those reared at other treatments of water exchange (both $p < 0.034$), but water exchange

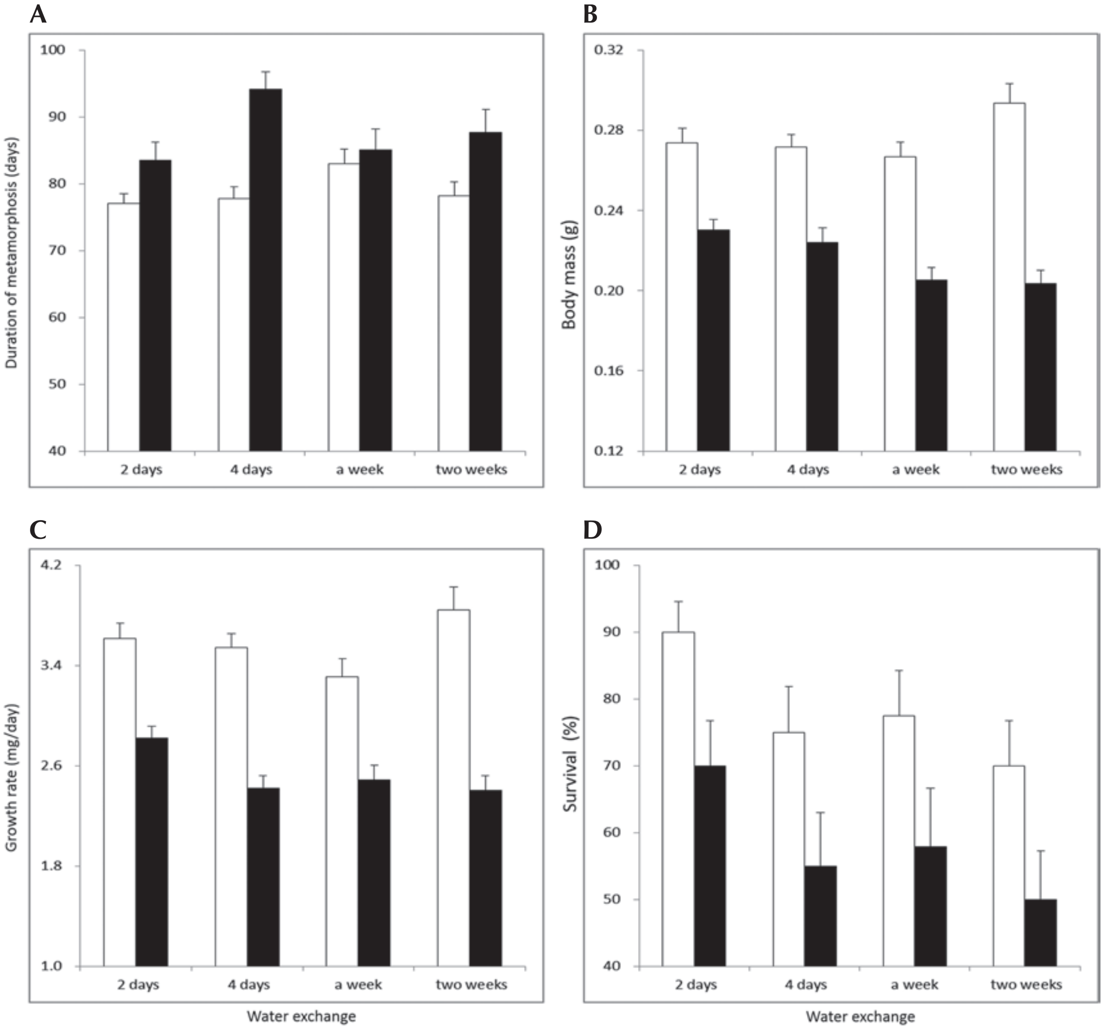

Figure 2. Influence of water exchange and food level on age at metamorphosis (**A**), body mass (**B**), growth rate (**C**), and survival (**D**) of the Chinese brown frog, *Rana chensinensis* at forelimb emergence (Gosner stage 42; open columns, high food level; black columns, low food level).

Table 1. The generalized linear model for the effects of water changes and food level on metamorphic traits in a population of *Rana chensinensis.*

Response variable	**Source of variation**	***df***	**MS**	***F*-value**	***p*-value**
Length of larval period	Water exchanges	3	323.015	2.148	0.095
	Food level	1	3837.183	25.522	< 0.001
	Water exchanges × Food level	3	456.764	3.038	0.030
	Error	208	150.348		
Body mass	Water exchanges	3	0.003	1.841	0.141
	Food level	1	0.193	136.043	< 0.001
	Water exchanges × Food level	3	0.006	3.970	0.009
	Error	209	0.001		
Growth rate	Water exchanges	3	1.152	2.425	0.067
	Food level	1	56.746	119.434	< 0.001
	Water exchanges × Food level	3	1.159	2.438	0.066
	Error	208	0.475		
Survival	Water exchanges	3	0.289	2.958	0.034
	Food level	1	1.574	16.092	< 0.001
	Water exchanges × Food level	3	0.00004	< 0.001	1.000
	Error	151	0.098		

once every two days and once a week was similar ($p = 0.089$). The interaction of food level and water exchange was not significant ($F_{3,\,151} < 0.001$, $p = 1.000$).

Discussion

Many environmental factors, especially temperature, food source, and predation pressure, may affect metamorphic traits of larval amphibians (reviewed by Laurila *et al.* 2001, Pacheco *et al.* 2019, Borah *et al.* 2022, Grott *et al.* 2022). In most cases, high quality environmental conditions often lead to faster development (reviewed by Álvarez and Nicieza 2002). Several experimental studies have demonstrated that high food level with a large proportion of protein can lead to double effects, accelerating both growth and development (Nathan and James 1972, Steinwascher and Travis 1983, Pandian and Marian 1985, Leips and Travis 1994). Our results revealed that food level affects metamorphic time, mass at metamorphosis, growth rate, and survivorship, suggesting that high food availability plays an important part in accelerating both growth and development of tadpoles of Chinese brown frogs.

Bilski (1921) first found that frequent water exchange or crowding would retard the growth rate of tadpoles of *Bufo* Garsault, 1764 and *Pelophylax lessonae* (Camerano, 1882). In this case, frequent water exchange was considered to be an interference, which stimulated a decrease of body fat stores and resulted in inhibition of growth (Meier *et al.* 1973). Further analysis found that interference must reach a certain kind and amount before growth can be stopped. Previous studies confirmed that flowing water and the severity and amount of agitation could inhibit growth. For example, tadpoles were put into unfolded cheese cloth bags, which were lifted up and down at rates of 6 to 12 strokes per minute in beakers of water (Adolph 1931). Our results indicate positive effects of frequent water exchange on the growth of tadpoles. Results of Hailey *et al.* (2006) were consistent with ours, indicating that changing water (once every 2–3 days) has a positive effect on growth and development of tadpoles of *Engystomops pustulosus* (Cope, 1864) compared to the control (once every 7 days). Frequent water exchange (e.g., once every two days) in our study not only failed to inhibit growth of tadpoles of Chinese brown frogs but also promoted their growth.

This finding may have two possible explanations. First, frequent water exchange seems to reduce intraspecific competition. When food is scarce, a significant interaction between food level and water exchange revealed that LFL tadpoles reared at frequent water exchange were larger at metamorphosis than those reared at middle and low water exchange. Previous studies have demonstrated that growth-inhibiting alga or chemicals were detected in the faeces or old medium of anuran larvae (Griffiths *et al.* 1993, Bardsley and Beebee 2001). Intraspecific competition can be mediated by the production of chemical waste or growth-inhibiting cells released into the spawning sites by anuran larvae (Schoener 1983, Griffiths 1991, Griffiths *et al.* 1991). Morin and Johnson (1988) found that growth inhibitors operate in natural ponds, suggesting that competition mechanisms based on food limitation are probably most important (Petranka 1989). Frequent water exchange can minimize the accumulation of chemical waste or inhibitory cells that have impacts on growth. Second, in the current study, a single individual had a surface water area of 36.2 sq. cm per liter, which was much lower than that of an optimal surface water area (133 sq. cm) for growth at the same rate (Adolph 1931). In this case, frequent water exchange is beneficial to growth because this increases oxygen tension or decreases carbon dioxide tension. Additionally, water exchanges occurring early in the daily photoperiod stimulated larval growth, whereas water exchanges in the middle of the daily photoperiod stimulated metamorphosis in Bullfrog tadpoles, *Aquarana catesbeiana* (Shaw, 1802) (Horseman *et al.* 1976). Our results indicated that frequent water exchanges have positive effects on tadpole growth.

We found a significant interaction between food level and water exchange, indicating that LHL tadpoles with frequent water exchange (once every two days) had significantly faster growth than those in any other treatment of water exchange. The underlying mechanism for this result may be the effect of water exchange on social behavior (Griffiths and Foster 1998). Tadpoles in low food treatments could grow faster from frequent water exchange because it helps to minimize intraspecific competition and increase oxygen tension, which may result in elevating metabolic rate (reviewed by Beck and Congdon 2000).

In conclusion, we found evidence that both frequent water exchange and high food level have positive effects on growth and development of tadpoles of Chinese brown frogs. Moreover, we found a significant interaction between food level and water exchange, suggesting that this novel mechanism is selected for when frequent water exchange is likely to prove profitable when food is insufficient or the environment is stressful.

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Behavioral responses of tadpoles of *Sphaerotheca breviceps* (Dicroglossidae), *Duttaphrynus melanostictus* (Bufonidae), and *Polypedates maculatus* (Rhacophoridae) to kairomones and dietary cues of a rare insect predator, *Ranatra elongata* (Nepidae)

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Abstract

Behavioral responses of tadpoles of *Sphaerotheca breviceps* (Dicroglossidae), *Duttaphrynus melanostictus* (Bufonidae), and *Polypedates maculatus* (Rhacophoridae) to kairomones and dietary cues of a rare insect predator, *Ranatra elongata* (Nepidae). Tadpoles of three sympatric anuran species, *Sphaerotheca breviceps*, *Duttaphrynus melanostictus*, and *Polypedates maculatus*, use chemoreception to detect kairomonal cues and excretory metabolites from active predatory anuran tadpoles (*Hoplobatrachus tigerinus*) that consume them. Herein, we describe the behavioral responses of tadpoles of these three species to a rare sit-and-wait predatory insect, the nepid *Ranatra elongata*. The predator's kairomones (water conditioned by a starved predator) or kairomones + diet-derived metabolites (dietary cues released in excreta of the predator after consumption of conspecific prey tadpoles) were used to simulate predation risk. Tadpoles of the three species had no behavioral responses to predator kairomones. However, all three species drastically reduced swimming movements and overall time spent swimming and had a higher burst speed/swimming velocity in response to water-borne cues released from the excreta of predators fed conspecific prey items. Because the presence of *R. elongata* does not elicit defense behaviors in tadpoles of these three prey species, additional information related to digestion of conspecifics is required to elicit defensive behaviors, suggesting that the presence of kairomones is not sufficient to elicit defense behavior.

Keywords: Anuran tadpoles, Chemoreception, Defense behavior, Predation threat, Predator-prey interactions, Reduced activity, Sit-and-wait predators.

Resumo

Respostas comportamentais de girinos de *Sphaerotheca breviceps* (Dicroglossidae), *Duttaphrynus melanostictus* (Bufonidae) e *Polypedates maculatus* (Rhacophoridae) a cairomônios e sinais dietéticos de um inseto predador raro, *Ranatra elongata* (Nepidae). Os girinos de três espécies

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simpátricas de anuros, *Sphaerotheca breviceps, Duttaphrynus melanostictus* e *Polypedates maculatus,* usam a quimiorrecepção para detectar sinais de cairomônios e metabólitos excretórios de girinos predadores ativos de anuros (*Hoplobatrachus tigerinus*) que os consomem. Neste trabalho, descrevemos as respostas comportamentais dos girinos dessas três espécies a um raro inseto predador do tipo senta-e-espera, o nepídeo *Ranatra elongata.* Os cairomônios do predador (água condicionada por um predador faminto) ou cairomônios + metabólitos derivados da dieta (sinais dietéticos liberados na excreta do predador após o consumo de presas coespecíficas) foram usados para simular o risco de predação. Os girinos das três espécies não apresentaram respostas comportamentais aos cairomônios do predador. No entanto, todas as três espécies reduziram drasticamente os movimentos de natação e o tempo total gasto nadando e tiveram uma maior velocidade de explosão/velocidade de natação em resposta a sinais transportados pela água e liberados da excreta de predadores alimentados com presas coespecíficas. Como a presença de *R. elongata* não provoca comportamentos de defesa nos girinos dessas três espécies de presas, informação adicional relacionada à digestão de coespecíficos é necessária para eliciar comportamentos defensivos, sugerindo que a presença de cairomônios não é suficiente para provocar comportamentos de defesa.

Palavras-chave: Atividade reduzida, Comportamento defensivo, Girinos, Interações predador-presa, Predadores senta-e-espera, Quimiorrecepção, Risco de predação.

Introduction

In nature, predation is a major selective force that acts on prey to drive the evolution of strategies for assessment of predation risk and the development of antipredator defense strategies in order to optimize survival and fitness. Detection of predators before an encounter can give prey the opportunity to respond behaviorally and reduce the probability of being detected, and hence eaten (Lima and Dill 1990). Inducing anticipatory antipredator behavior has direct energetic costs, in addition to costs associated with reduced opportunity to feed, that affect growth and development of prey (Lima and Dill 1990, Ferrari *et al.* 2010). The majority of aquatic animals including anuran larvae assess predation risk by using chemosensory mechanisms before responding with defense behaviors (Kats and Dill 1998, Saidapur *et al.* 2009, Ferrari *et al.* 2010, Mogali *et al.* 2012, 2015, Mogali 2018). Anuran larvae specifically perceive either alarm cues released by damaged prey tadpoles or kairomones of predators (chemicals originating from the body of predators) or dietary cues of predators (excretory metabolites or substances of predators fed conspecific prey) to alter behavior to avoid or escape predation (Wisenden 2000, Kiesecker *et al.* 2002, Schoeppner and Relyea 2009a, b, Mogali *et al.* 2011, Carlson *et al.* 2015, Scherer and Smee 2016). Previous studies have revealed that anuran larvae utilize a variety of antipredatory behaviors in response to chemical cues of predators. Those responses include increased activity or swimming speed to escape from predators (Hews 1988, Van Buskirk and McCollum 2000, Dayton *et al.* 2005, Mogali *et al.* 2021), reduction of activity (Skelly and Werner 1990, Mogali *et al.* 2012), aggregation (Spieler and Linsenmair 1999), and increased use of refuge sites (Hossie and Murray 2010, Mogali *et al.* 2019, 2022, 2023c).

In South India, most anurans, including the present study species, *Sphaerotheca breviceps* (Schneider, 1799), *Duttaphrynus melanostictus* (Schneider, 1799), and *Polypedates maculatus* (Gray, 1830), breed in ephemeral ponds following the Southwest monsoon rains. Tadpoles of several sympatric species coexist (Saidapur 2001, Mogali *et al.* 2017, Mogali 2018). Some of these are carnivorous and even cannibalistic, devouring coexisting tadpoles. For example, tadpoles of the Indian bullfrog

Hoplobatrachus tigerinus (Daudin, 1802) are voracious predators that hunt actively; they also exhibit cannibalism (Saidapur 2001, Rajput *et al.* 2011). The ponds in this region harbour many predatory aquatic invertebrates such as beetles (e.g., *Dineutus* sp., *Eretes sticticus*), dragonfly larvae (e.g., *Pantala flavescens*, *Bradinopyga geminata*), damselfly larvae (e.g., *Ceriagrion cerinorubellum*), backswimmers (e.g., *Notonecta* sp.), crabs (e.g., *Barytelphusa* sp.), giant water bugs (e.g., *Belostoma* sp.) and water scorpions (e.g., *Ranatra elongata*). Of these, water scorpions, *Ranatra elongata* (Hemiptera; Nepidae), are interesting and rare tadpole predators that climb among aquatic vegetation, slowly moving near the surface or hiding unnoticed in debris or leaf litter (pers. obs.).

Water scorpions are ambush or sit-and-wait non-gape-limited predators with excellent vision. They are inconspicuous or camouflaged within the vegetation and ambush unsuspecting prey (e.g., tadpoles, water boatmen, and other insects) with a quick grasping action of the forelegs (pers. obs.). They use a segmented beak to pierce prey and suck the body fluids. They capture small to large tadpoles by quickly grabbing and immobilizing them using the front pair of raptorial legs. Tadpoles of *S. breviceps, D. melanostictus,* and *P. maculatus* detect kairomones of tadpoles of *H. tigerinus*, as well as dietary metabolites or substances from predators that have fed on conspecific tadpoles (Saidapur *et al.* 2009, Mogali *et al.* 2011, 2023a, b, d, 2024). The present study examines antipredator behavioral responses of tadpoles of these three species in the presence of sit-and-wait individuals of *R. elongata*. Such studies shed light on predator–prey relations and the diversity of antipredator behavioral responses in tadpoles.

Materials and Methods

Tadpoles of *Sphaerotheca breviceps* [Gosner stages 26–27; $N = 100$; 21.30 ± 0.54 mm total length (mean ± SE); Gosner 1960], *Duttaphrynus melanostictus* (stages 27–28; $N = 100$; 21.27 ± 0.49 mm total length), and *Polypedates maculatus* (stages 26–27; $N = 100$; 21.33 ± 0.58 mm total length) were collected from the same temporary ponds on the Karnatak University Campus, Dharwad (15.44° N, 74.98° E), Karnataka State, India. In the laboratory, tadpoles of each species were placed in separate glass aquaria (75 × 45 × 15 cm) containing 20 L of aged tap water and used as stock for experiments. Tadpoles of the three species are herbivores and were fed boiled spinach. Individuals of *Ranatra elongata* (mean length 75.20 ± 0.68 mm, width 4.5 ± 0.22 mm, and weight 280.0 ± 3.85 mg; $N = 30$) were obtained from the same site as the tadpoles. Predators were reared individually to avoid cannibalism. They were housed in small plastic tubs (19 cm diameter and 7 cm deep) filled with 0.5 L of aged tap water. They were fed with tadpoles of either *S. breviceps, D. melanostictus,* or *P. maculatus*.

Behavioral responses of tadpoles of each prey species were studied by exposing them to stimulus solutions of either predator kairomones alone or kairomones + dietary cues (dietary metabolites of predators fed conspecific tadpoles).

Preparation of Kairomones

Individuals of *R. elongata* were placed in separate plastic tubs ($N = 25$ tubs; 19 cm diameter and 7 cm depth) containing 200 mL of aged tap water without food for 96 h to eliminate diet-derived excretory metabolites from the stimulus solution, resulting in a stimulus solution with only kairomones (Mogali *et al.* 2012, 2020). After 96 h of starvation, predators were removed from the tubs, and the stimulus solutions were further filtered using fine cheese cloth to completely remove any fecal matter. The solutions, containing only kairomones, were used immediately for experimental trials.

Preparation of Dietary Cues of Conspecific Origin and of a Predator Fed Tadpoles of S. breviceps

A single *R. elongata* was placed in a plastic tub (*N* = 8 tubs; 19 cm diameter and 7 cm depth) with 200 mL of aged tap water with four tadpoles of *S. breviceps* in Gosner stages 26–27 (around 08:30 h). *Ranatra elongata* consumed all tadpoles provided to them by evening (18:30 h). The following day between 09:30 h and 11:30 h, predators were removed and water from the tubs was filtered using fine cheese cloth. The filtrate served as the stimulus solution containing the diet-derived excretory metabolites of predators fed conspecific prey, and may contain some extent of kairomones of predators, and are unlikely to have contained the alarm cues of prey. Prey alarm cues are known to be labile in nature (Peacor 2006, Ferrari *et al.* 2008, Wisenden *et al.* 2009, Chivers *et al.* 2013). It is unlikely that prey alarm cues were present in the stimulus solution because all prey were consumed more than 15 h before the solution was collected. The same protocol was followed for dietary cues of the other two species.

Behavioral Responses of Prey Species to the Predator's Kairomones

Behavioral responses of tadpoles of different species to kairomones (water conditioned with starved predators) were recorded by placing one test tadpole (either *S. breviceps, D. melanostictus, P. maculatus*) in a rectangular glass tank (28 × 15 × 15 cm) containing 600 mL of aged tap water. A video camera (Sony, DCR-SR300/E) was fixed above the tank to record tadpoles in the entire tank. The camera was connected to a computer with the Ethovision Video Tracking System (Noldus Information Technology, The Netherlands) to track movements of the tadpole before and after addition of the stimulus solution to the test tank. The Ethovision system was used to record maximum swimming speed (V_{max}), distance traversed by the tadpole, number of swimming bouts, and time spent swimming during an entire trial.

For each trial, one test tadpole was first introduced into the tank and left undisturbed for 5 min. A burette was placed ~1 cm above the water level, and 50 mL of aged tap water (chemical blank solution) was added at the rate of ~1 mL/s. The burette was removed. Movement of the test tadpole was recorded for 5 min using Ethovision to record its baseline activity in the absence of any cues. After tracking baseline activity, 50 mL of stimulus solution containing kairomones was added as described above. Movement of the tadpole was recorded for another 5 min to determine the activity pattern after exposure to kairomones. For tadpoles of each species, 25 trials were conducted (3 species × 25 trials = 75 trials in total). Data on the behavioral responses of tadpoles of different species before and after addition of the stimulus solution (kairomones) were compared separately by using the Paired-Samples t-test (SPSS v. 16.0).

Behavioral Responses of Prey Species to Dietary Cues of Conspecific Origin (Kairomones + Dietary Cues)

In this experiment, the stimulus solution contained chemical cues from excretory metabolites or substances of *R. elongata* fed with conspecifics (kairomones + dietary cues) i.e., either with tadpoles of *S. breviceps, D. melanostictus,* or *P. maculatus* instead of only kairomones of the predator. The behavioral responses of tadpoles of each species were recorded separately as described above, before and after the addition of stimulus solutions that included their respective conspecific prey (kairomones + dietary cues). For each species, 25 trials were conducted. The data on the behavioral responses of tadpoles of different species, before and after addition of the stimulus solution (dietary cues of predators fed with conspecifics) were compared separately using the Paired-Samples t-test (SPSS v. 16.0). All experimental trials were run using a new healthy test tadpole each time. The test tank was cleaned thoroughly and replenished with aged tap water between trials.

Results

Behavioral Responses of Prey Species to Predator's Kairomones

V_{max}, frequency of swimming bouts, time spent swimming, and total distance traversed by tadpoles of *S. breviceps* (Figure 1), *D. melanostictus* (Figure 2), and *P. maculatus* (Figure 3) exposed to chemical blank water were similar to those exposed to kairomones.

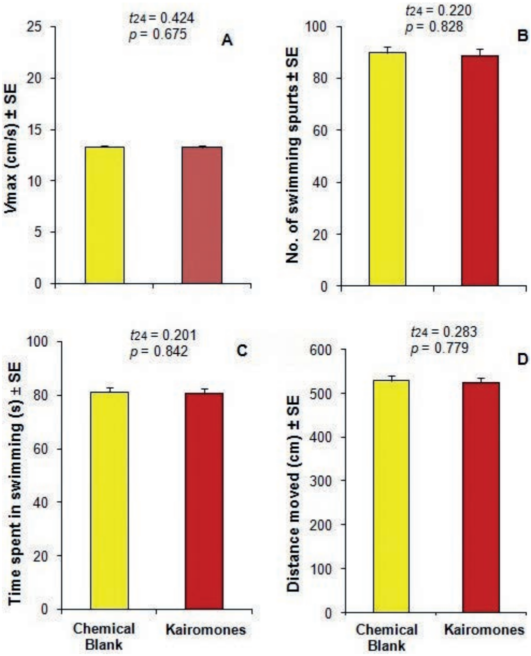


Figure 1. Maximum swimming speed (V_{max}) (**A**), swimming bouts (**B**), time spent swimming (**C**), and distance moved (**D**) by tadpoles of *Sphaerotheca breviceps* exposed to chemical blank water (aged tap water) or a stimulus solution of a starved predator (kairomones), *Ranatra elongata*. Data are represented as mean ± SE; *N* = 25 trials; data analyzed by Paired-Samples t-test.

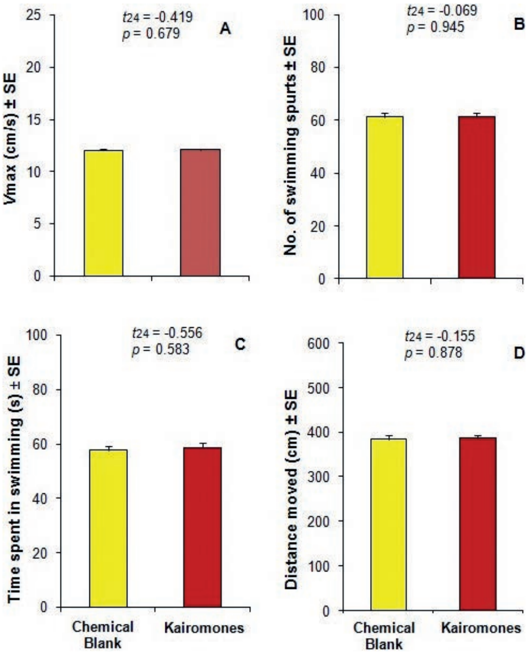


Figure 2. Maximum swimming speed (V_{max}) (**A**), swimming bouts (**B**), time spent swimming (**C**), and distance moved (**D**) by tadpoles of *Duttaphrynus melanostictus* exposed to chemical blank water (aged tap water) or a stimulus solution of a starved predator (kairomones), *Ranatra elongata*. Data are represented as mean ± SE; *N* = 25 trials; data analyzed by Paired-Samples t-test.

Behavioral Responses of Prey Species to Dietary Cues of Conspecific Origin (Kairomones + Dietary Cues)

Upon exposure to the predator' diet-derived metabolites following consumption of conspecific prey (kairomones + dietary cues), tadpoles of *S. breviceps* (Figure 4), *D. melanostictus* (Figure 5), and *P. maculatus* (Figure 6) showed a significant increase in V_{max} and significant declines in the number of swimming bouts, time spent swimming, and total distance moved when compared to their baseline activities in stimulus-free water.

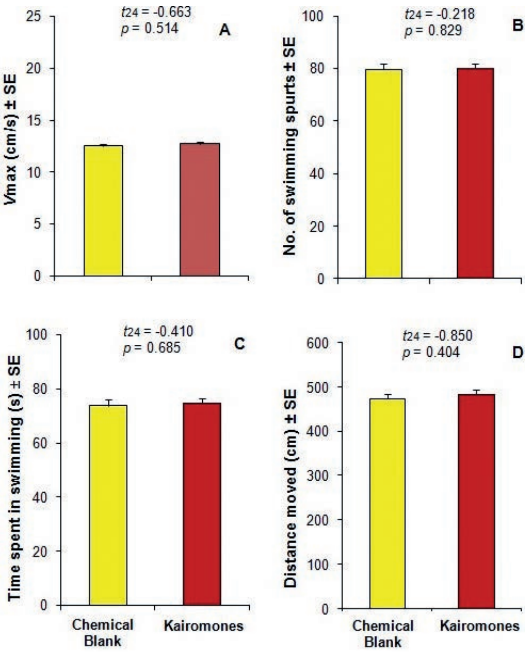


Figure 3. Maximum swimming speed (V_{max}) (**A**), swimming bouts (**B**), time spent swimming (**C**), and distance moved (**D**) by tadpoles of *Polypedates maculatus* exposed to chemical blank water (aged tap water) or a stimulus solution of a starved predator (kairomones), *Ranatra elongata*. Data are represented as mean ± SE; *N* = 25 trials; data analyzed by Paired-Samples t-test.

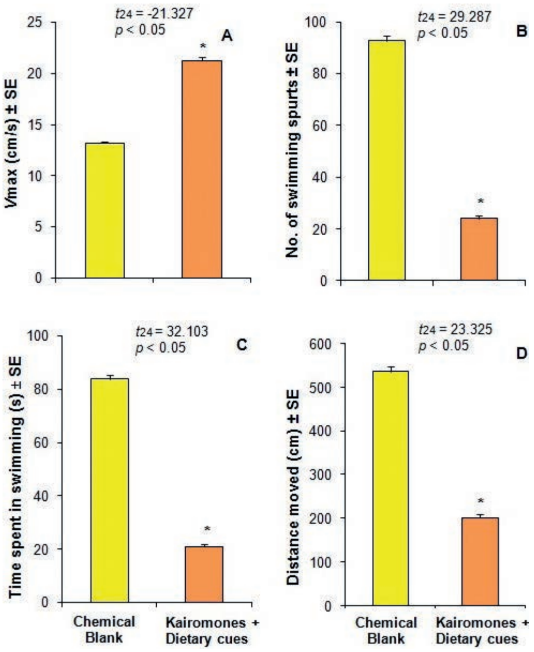
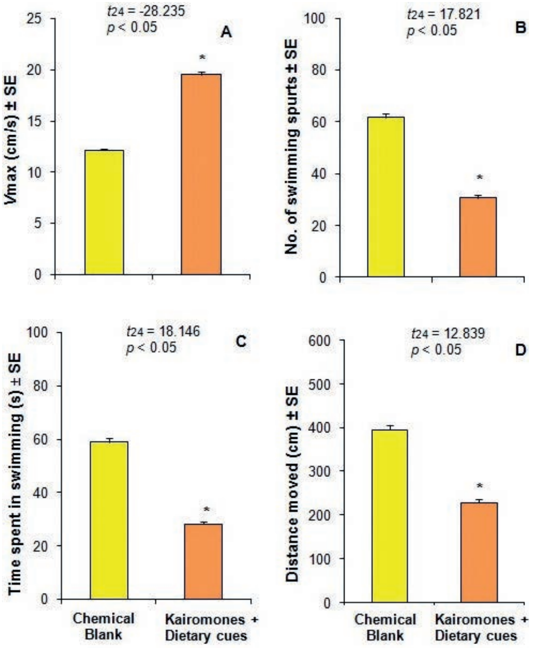


Figure 4. Maximum swimming speed (V_{max}) (**A**), swimming bouts (**B**), time spent swimming (**C**), and distance moved (**D**) by tadpoles of *Sphaerotheca breviceps* exposed to chemical blank water (aged tap water) or a stimulus solution (kairomones + dietary cues) of a nepid predator, *Ranatra elongata,* fed with conspecific tadpoles. Data are represented as mean ± SE; *N* = 25 trials; data analyzed by Paired-Samples t-test. Asterisks over the bars indicate significant differences between the treatment groups.

Discussion

In aquatic ecosystems, most prey organisms, including anuran tadpoles, are at risk of predation (Lima and Dill 1990, Kats and Dill 1998). In such systems, various types of chemical cues (e.g., kairomones, alarm, dietary, and disturbance cues) affect the behavioral responses of tadpoles (Wilson and Lefcort 1993, Schoeppner and Relyea 2005, Scherer and Smee 2016, Rivera-Harnández *et al.* 2022). Anuran larvae are able to discriminate different chemical cues and exhibit differential antipredator behavioral responses according to perceived levels of threat (Mogali *et al.* 2011, 2023b).

The present study shows that tadpoles of three prey species (*S. breviceps*, *D. melanostictus*, and *P. maculatus*) do not exhibit behavioral changes in response to kairomones of the relatively rare nepid predator, *R. elongata*. This result suggests that they do not perceive cues from *R. elongata* as a predation threat sufficient to elicit the behavioral responses we tested. Similar results were reported for tadpoles of *Indosylvirana temporalis* (Mogali *et al.* 2012), *D. melanostictus* (Mogali *et al.* 2020), and *Clinotarsus curtipes* (Mogali *et al.* 2023d).



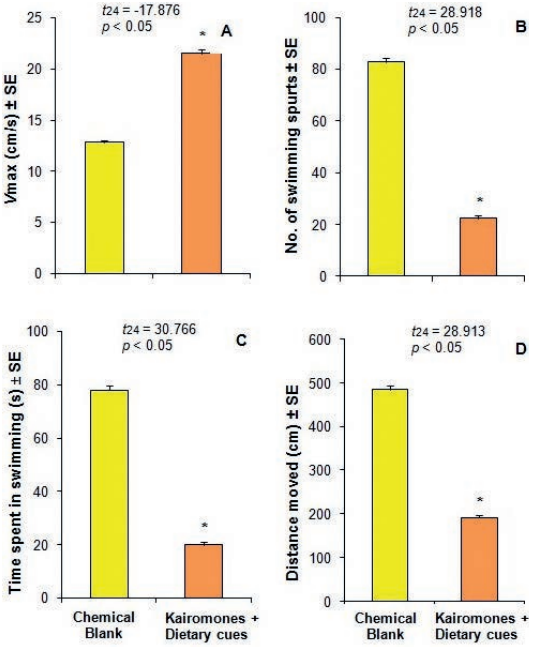


Figure 5. Maximum swimming speed (V_{max}) (**A**), swimming bouts (**B**), time spent swimming (**C**), and distance moved (**D**) by tadpoles of *Duttaphrynus melanostictus* exposed to chemical blank water (aged tap water) or a stimulus solution (kairomones + dietary cues) of a nepid predator, *Ranatra elongata,* fed with conspecific tadpoles. Data are represented as mean ± SE; *N* = 25 trials; data analyzed by Paired-Samples t-test. Asterisks over the bars indicate significant differences between the treatment groups.

Figure 6. Maximum swimming speed (V_{max}) (**A**), swimming bouts (**B**), time spent swimming (**C**), and distance moved (**D**) by tadpoles of *Polypedates maculatus* exposed to chemical blank water (aged tap water) or a stimulus solution (kairomones + dietary cues) of a nepid predator, *Ranatra elongata,* fed with conspecific tadpoles. Data are represented as mean ± SE; *N* = 25 trials; data analyzed by Paired-Samples t-test. Asterisks over the bars indicate significant differences between the treatment groups.

In contrast, tadpoles of the three species (*S. breviceps*, *D. melanostictus,* and *P. maculatus*) exhibit strong behavioral changes (i.e., drastically reduced swimming movements and high burst speed) when exposed to kairomones of predatory sympatric tadpoles of *H. tigerinus* (Mogali *et al.* 2011, 2023a, 2024). Tadpoles of *H. tigerinus* locate prey by means of both visual and chemical senses (Saidapur *et al.* 2009). They are active predators, and prey on co-occurring sympatric anuran tadpoles (Saidapur 2001, Saidapur *et al.* 2009). Species such as *H. tigerinus* thus pose a serious predation threat. A long ecological coexistence of tadpoles of these three species with sympatric carnivorous tadpoles such as *H. tigerinus* may have led to the evolution of antipredator defense strategies in response to kairomones of these predators. In contrast, the nepid *R. elongata* is a sit-and-wait predator that moves slowly and waits for prey to come near before attacking (Miller *et al.* 2014). It is possible that *R. elongata* poses less predation risk to these three species than *H. tigerinus*, or that other behavioral responses not measured here are used to evade predation from this different attack strategy. Sit-and-wait predators in general are under intense selection pressure to suppress chemical (e.g., kairomones) evidence of their presence (Miller *et al.* 2015), because they need prey to closely approach them. Also, it is possible that predation pressure by *R. elongata* on these species of tadpoles may be low. If so, tadpoles of these species might be better off conserving their energy by not engaging in antipredator defenses in response to kairomones, if any, of *R. elongata*. On the other hand, water-soluble substances in the excreta of *R. elongata* following consumption of conspecific prey (kairomones + dietary cues) seems to indicate intense predation threat and elicited strong behavioral changes in the three tadpole species in the form of overall reduced swimming movements and high burst speed. Evidence of recent predation on conspecific members of the group indicates a high predation risk for tadpoles in the vicinity. Our results are in agreement with those reported for tadpoles of *Hyla versicolor* (Schoeppner and Relyea 2009b).

Some previous evidence indicates that alarm cues can quickly degrade in nature (Peacor 2006, Ferrari *et al.* 2008, Wisenden *et al.* 2009, Chivers *et al.* 2013). In the present study, given the 15 h time window, it is unlikely. This idea has not been tested in this system, but in other systems involving fish, the effect of cues in eliciting defensive behavior lasted for 6 h (Wisenden *et al.* 2009), whereas in larval woodforgs, the cues lasted 2 h (Ferrari *et al.* 2008). The antipredator defense behavior of the three tadpole species in the present study was specifically in response to diet-derived excretory metabolites or substances released by *R. elongata* that have consumed conspecific prey items. In conclusion, kairomones are not sufficient to induce defensive behavior but require the addition of diet-derived metabolites. The three tadpole species in this study can exhibit different behavioral responses to different predator-related cues.

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Abundance and microhabitat association of *Barbourula busuangensis* (Anura: Bombinatoridae) in Busuanga Island, Philippines

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Abstract

Abundance and microhabitat association of *Barbourula busuangensis* (Anura: Bombinatoridae) in Busuanga Island, Philippines. *Barbourula busuangensis,* commonly known as the Philippine Flat-Headed Frog, is a near-threatened species endemic to Palawan, Philippines. Despite its distinct ecological niche and unique characteristics, including a flat head and body to support its fully aquatic lifestyle, limited studies have been conducted to understand its habitat requirements and population status. We conducted visual encounter surveys and hand captures along 30–100 m belt transects in two Barangays, Cheey and New Busuanga. A total of 270 individuals were recorded and microhabitat variables were compared between the two sites. However, the abundance of *B. busuangensis* was significantly higher in Cheey compared to New Busuanga. We found significant differences in six key microhabitat variables (relative humidity, temperature, stream/river depth and width, rocks percentage, water flow and turbidity) between the two locations, suggesting diverse environmental conditions impacting frog populations. Additionally, 14% of the captured frogs (23 individuals) exhibited morphological deformities, though the cause remains undetermined. Our findings underscore the urgent need for further research and targeted conservation efforts to ensure the survival of this species. This study provides essential data on the distribution and habitat preferences of *B. busuangensis,* informing conservation strategies to mitigate population declines and address environmental challenges affecting this unique amphibian.

Keywords: Amphibians, Endemic species, Palawan, Stream, Watershed.

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Resumo

Abundância e associação de micro-habitats de *Barbourula busuangensis* (Anura: Bombinatoridae) na ilha de Busuanga, Filipinas. *Barbourula busuangensis*, popularmente conhecida como rã-de-cabeça-chata-das-filipinas, é uma espécie quase ameaçada e endêmica de Palau, Filipinas. Apesar de seu nicho ecológico distinto e características únicas, como a cabeça e o corpo achatados que suportam seu estilo de vida totalmente aquático, são limitados os estudos efetuados para compreender seus requisitos de habitat e status populacional. Realizamos levantamentos visuais e capturas manuais ao longo de transectos de 30–100 m em dois distritos, Cheey e New Busuanga. Um total de 270 indivíduos foi registado, e as variáveis de micro-habitat foram comparadas entre os dois locais. A abundância de *B. busuangensis* foi significativamente maior em Cheey do que em New Busuanga. Encontramos diferenças significativas em seis variáveis-chave do micro-habitat (umidade relativa, temperatura, profundidade e largura do riacho/rio, percentagem de rochas, fluxo e turbidez da água) entre os dois locais, sugerindo condições ambientais diversas com impacto nas populações. Além disso, 14% das rãs capturadas (23 indivíduos) apresentavam deformações morfológicas, embora a causa permaneça indeterminada. Nossas descobertas ressaltam a necessidade urgente de mais pesquisas e esforços de conservação direcionados a garantir a sobrevivência dessa espécie. Este estudo fornece dados essenciais sobre a distribuição e as preferências de habitat de *B. busuangensis*, sugerindo estratégias de conservação para mitigar o declínio da população e abordar os desafios ambientais que afetam esse anfíbio único.

Palavras-chave: Anfíbios, Bacia hidrográfica, Espécie endêmica, Palawan, Riacho.

Introduction

The Philippine archipelago is notably considered as one of the most important centres of amphibian and reptile diversity in Southeast Asia (Brown and Alcala 1970, Diesmos *et al.* 2002). This accounts for 116 species of amphibians, with 99 (85%) being endemic (Diesmos *et al*. 2015) now known in the country. With its rich archipelago, one of its unique biodiversity islands is the Palawan which is also home to one of the country’s evolutionary distinct frog species—the *Barbourula busuangensis* Taylor and Noble, 1924. This is one of the ancient frog genera *Barbourula* that has only two known species worldwide including the Philippine Flat-headed Frog, *B. busuangensis*, and the Bornean Flat-headed Frog, *B. kalimantanensis* Iskandar, 1978. Notably, both are considered threatened by the International Union for Conservation of Nature (IUCN 2019).

Barbourula busuangensis was first described in Busuanga Island and was later known to occur in Northern Palawan (Myers 1943, Brown and Alcala 1983). There remains to be a question on the validity of the record in the town of Culion (IUCN 2018). It is highly aquatic and requires clean and cool mountain streams (Alcala and Brown 1987, Alcala and Brown 1998). It usually hides in crevices or under boulders in the stream bed during the day (Schoppe and Cervancia 2009) and emerges at dusk to forage. Individuals can easily be detected since their eyes reflect the light of a torch or a flashlight. There is few published information available on the ecology and conservation status of the species and most were conducted in mainland Palawan and very little information is available on the Calamian Islands including Busuanga Island from which it gets its name.

The Calamian Islands constitute the easternmost extension of the “Palawan Faunal Region”; a globally important center of species biodiversity and endemism, partially connecting two of the world’s major biogeographic regions. The island group comprises a total of about 95 isles and islets, covering a total area of 1,554 km^2, including the four largest islands of Busuanga, Culion, Coron and Calauit, which are included amongst the 117 Philippine ‘Important Bird

Areas (IBAs)', and 206 'Key Biodiversity Areas (KBA's)' identified in the 2002 Philippines Biodiversity Conservation Priority Setting and Action Plan.

The Municipality of Busuanga has a total freshwater area of 136.87 ha. A total of 12 streams and rivers were surveyed by Afuang and Cielo in 2009, three rivers and streams by Paguntalan *et al.* (2012) and two rivers and two streams by Paguntalan *et al.* (2015) and in the current study one sampled stream, the Chinibayan overlap with the past studies of Afuang and Cielo (2009) and Paguntalan *et al.* (2012). The rivers and streams in Calamian Islands are still unclassified according to DENR-EMB (2019). Given the limited data on *B. busuangensis* in the Calamian Islands and the pressing need to understand its ecology for conservation efforts, this study aims to present ecological information on the species and the conservation status of its habitat in Busuanga Island. Our research is significant for filling the knowledge gaps regarding the species' distribution, population dynamics, and habitat requirements, ultimately contributing to the development of effective conservation strategies in the area.

Materials and Methods

Site Description

Busuanga is the largest island in the Calamian group of islands (12°08'42.00" N, 120°05'40.92" E) and is divided into two municipalities: 'Busuanga' in the west and 'Coron' in the east. Its highest peak is Mount Kilien located in the South-eastern section of the Island. The mountain is listed amongst the 206 Key Biodiversity Areas (KBA's) identified in the 2002 Philippines Biodiversity Conservation Priority Setting and Action Plan. It spans at 392.9 km² mosaic of habitats including pastureland, agricultural areas, and karst forest (Figure 1). Most of the remaining forests in Busuanga Island were established as a Pastureland Reserve under Proclamation 1387 issued by President Ferdinand Marcos on 13 February 1975.

A total of three rivers and three streams were surveyed in Busuanga Island from 04–10 July 2017. Rivers are defined as relatively large lotic waterbody of >5 m wide, while streams are relatively small lotic waterbodies of < 5 m wide (Davies *et al.* 2008). Chinabayan River (12°13'33.9924" N, 119°56'24.1440" E). Located in Datay Mountain Range in New Busuanga with an elevation of 43 m a.s.l. The river expands up to 20 m wide and up to 126 cm deep. Deep pools with cloudy appearance interspersed with large boulders and rock crevices characterized the area near waterfalls. The canopy coverage on the riverbanks was at 60% and understory coverage was at 50% maximum. The sampling area comprises old-growth (containing large, mature trees, a multi-layered canopy, and diverse understory vegetation) and secondary forest (containing younger, rapidly growing tree species) with a closed canopy and understory filled with shrubs and tree ferns. The Chinabayan River is continuously flowing throughout the year. We established and surveyed a total of seven transects with 100 m length in the area (Figure 2).

The first stream feeding into Chinabayan River (stream 1: 12°13'16.3344" N, 119°56'29.1768" E) has an elevation of 48 m a.s.l. The width and depth of the stream were measured, and it expands of up to 4.5 m wide and 18 cm deep. The water was very clear even the submerged features in stream like rocks and sand particles were visible. We visually measured canopy coverage of the stream, which was at 90% and understory coverage was at 70% maximum. Epiphytes, shrubs, and fern trees were present with few *Rattanus* sp. observed. In this stream, we established surveyed at least two transects with a 100-m length.

The second tributary that also feeds Chinabayan river (stream 2: 12°13'14.4840" N, 119°56'25.6200" E) has an elevation of 74 m a.s.l. and expands up to 3.5 m wide and reaches 38 cm deep. We visually measured canopy

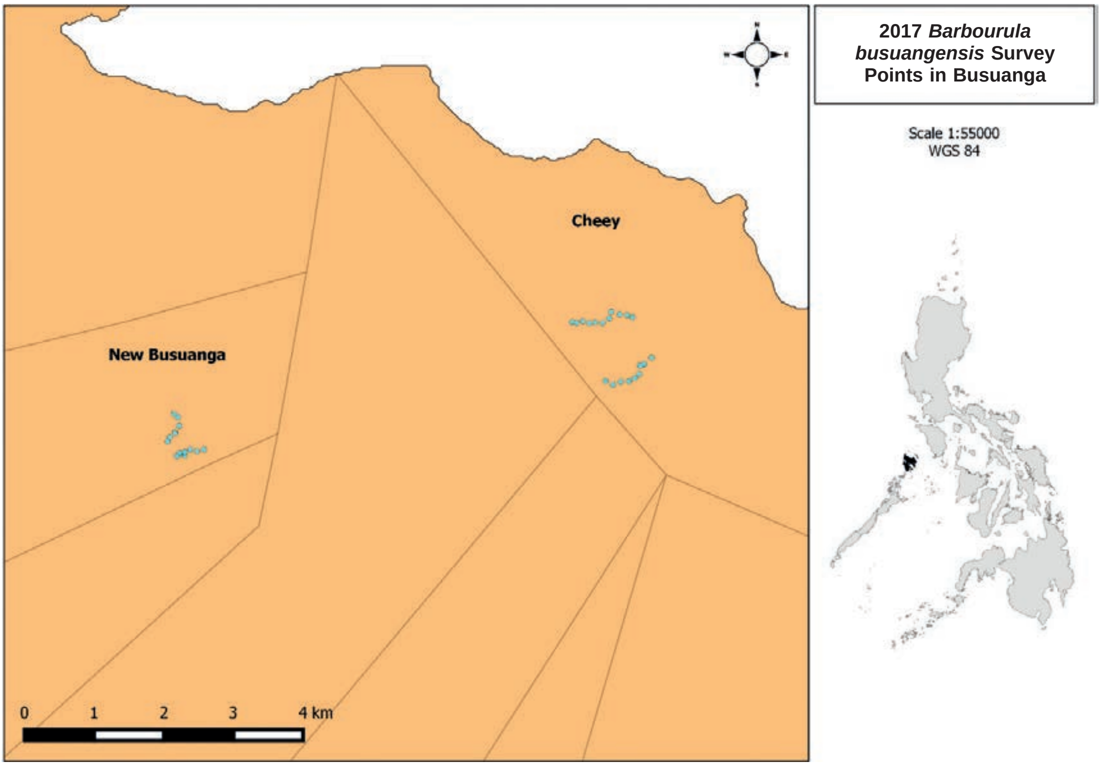


Figure 1. Location of study site (dot) in Busuanga Island.

coverage of the stream along stream banks which was at 90% and understory coverage was at 40%. Epiphytes, shrubs and fern trees were present with few *Rattanus* sp. observed. We established and surveyed at least four transect lines measuring up to 100 m.

The third tributary in Chinabayan river (stream 3: 12°13'25.2480" N, 119°56'24.5760" E) was at an elevation of 40 m a.s.l., width expanding to 4.8 m wide and depth at 20 cm. Like the first and second tributaries, water was very clear where submerged particles were visible. We visually measured canopy coverage of the river which was at 85% and understory coverage was at 60%. Epiphytes, shrubs and bamboo species were present with few *Rattanus* sp. observed. In this stream, we established and surveyed at least two transect lines measuring up to 100 m.

The forest in Cheey serves as the watershed of the barangay and covers less than 100 hectares with an elevation of 74 m a.s.l. The Malapinggan River (12°14'21.4800" N, 119°59'51.8640" E) is the largest river traversing the mountings and has a width of 9.6 m and depth of up to 219.5 cm. with clear water. We visually measured canopy coverage of the river which was at 90% and understory coverage was at 50% maximum. Presence of bamboo, Narra and Ipil—*Intsia bijuga* (Colebr.) Kuntze—dominate the surrounding forests with *Ficus* species scattered within the forests. The understory is mostly dominated by palms, rattan and wildlings of various kinds. We established and surveyed at least eight transect lines measuring up to 100 m (Figure 3A).

Lungon River (12°14'00.1320" N, 120°00' 11.1600" E) is in Barangay Cheey with an

Figure 2. Sampling stations including Chinabayan River (**A**), tributary 1 (**B**), tributary 2 (**C**), and tributary 3 (**D**) in Brgy, New Busuanga.

elevation of 32 m a.s.l. The width and depth of the river were measured, and it expands up to 5.5 m wide and 40.7 cm deep. The water was slightly turbid. Canopy coverage was also at 90% and understory coverage was at 60% maximum. The surrounding area includes a mixed plantation of bananas, coconut trees, jackfruit, citrus, etc. Dipterocarps were also observed interspersed in some areas surrounding the stream and a few species of shrubs dominated the understory. Ferns and mosses were very rare. We established and surveyed at least 10 transect lines measuring up to 100 m (Figure 3B).

Frog Detection Transect

Frog composition was determined using belt transect method which measured 100 m each and were lined continuously along the streams and rivers of Barangays Cheey and New Busuanga from 04–10 July 2017. Juveniles were identified based on body size and based on Alcala and Brown's (1998) description that colored morph indicates that the frog is juvenile whereas dark morph indicates that the frog is an adult. Females are significantly larger than males in most of the frog species to accommodate mating (i.e.

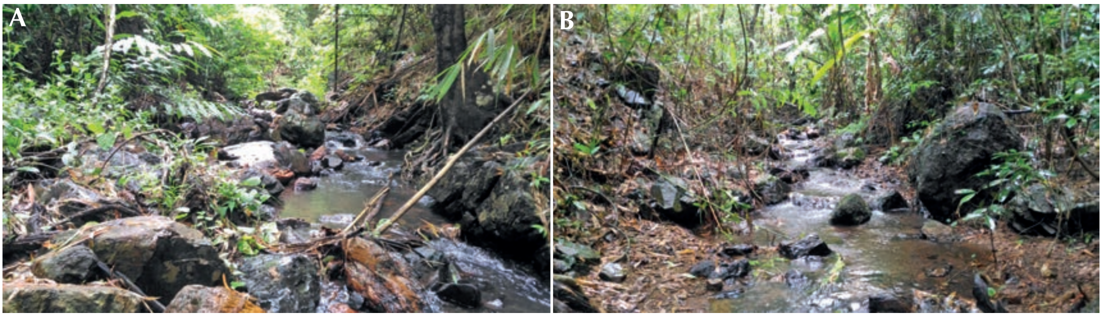


Figure 3. Malapinggan River (**A**) and Lungon River (**B**) in Cheey.

amplexus) and females are the ones responsible in laying eggs. Larger females tend to produce more eggs than average-sized females. We surveyed a total of thirty transects with at least one hundred meters in length established on streams. Visual Encounter Survey (VES) and hand capture were conducted. We conducted the surveys from 1800 to 2100 hours where *B. busuangensis* were active.

Stream Microhabitat Assessment Characteristics

Microhabitat characteristics such as rate of water flow, turbidity, stream width and depth, percentage of rocks, relative humidity (day and night), and the temperatures of water, air and soil (day and night) were determined using 10 × 10 m^2 plots (Table 1). These variables were measured to understand the specific environmental conditions that influence the distribution of and survival of species within a habitat (Dunlop *et al.* 2005, Goldstein *et al.* 2017). A total of 36 plots were sampled following the modified stream visual assessment protocol by Magbanua *et al.* (2008). The description of each microhabitat variable and the methods used for their measurement are provided in Table 1.

Data Analysis

We recorded the biometrics, age and sex of captured individuals in standard data sheets. Morphometric measurements such as snout–vent length (SVL) and weight were measured. We used Mann-Whitney U test to compare two sample means of each microhabitat variables from the same population. The Mann-Whitney U test is a non-parametric test, making it suitable for comparing two independent groups when the data do not necessarily follow a normal distribution. We used the Mann-Whitney U, given that our microhabitat data did not meet the assumptions of normality and homoscedasticity required for parametric tests.

Results

Species Abundance

A total of 270 individuals of *Barbourula busuangensis* was recorded in rivers and streams in New Busuanga and in Cheey, of which 20.37% were juveniles and 70.63% were adult frogs with the majority represented by females with a relative abundance of 64.07%, while only 15.56% were males (Figure 4). Juveniles where mostly seen and captured in streams in puddles and in slow-moving waters. We saw and captured individuals of *B. busuangensis* in rivers of Malapinggan and Lungon, wherein most of which were adults. We also observed the presence of eggs in some female frogs. Possible nesting sites were not discovered during our study, so the opportunity to understand the mode

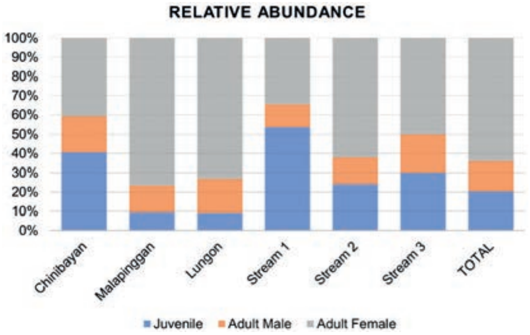


Figure 4. Comparison of recorded individuals in rivers and streams in Brgy. Cheey and Brgy. New Busuanga.

of reproduction of *B. busuangensis* remains unknown. We identified at least seventy-five gravid females (indicated by the presence of large yellow eggs in their bellies). Females ranged in weight from 18–149.9 g and SVL from 50.7–99.7 mm, while males ranged in weight from 13–87 g and SVL from 47.7–89.5 mm (Table 2).

We observed a few individuals of *B. busuangensis* with morphological abnormalities (e.g., missing hindlimbs, forelimbs and toe phalanges). These deformities were observed on individuals captured in Malapinggan river in Barangay Cheey but not in New Busuanga. Out of the 163 individuals 23 or 14% (15 females and 8 males) had this condition (Figure 6).

Microhabitat Characteristics Assessment

We used Mann-Whitney U test to compare microhabitat variables between downstream and upstream with the presence of *B. busuangensis*. We observed significant difference in night soil temperature with a difference of 1.55°C (*p*-value = 0.04). Mean soil temperatures downstream during the night was 25.9°C while upper stream was 24.3°C. Also, we noted a significant difference of 6.18 cm depth in the upper stream. The widest recorded width was 9.6 m in Malapinggan river while Chinabayan river was the deepest. Apart from this, the percentage of rocks and the water flow velocity were greater in areas downstream. Shallow, cold, and clear waters were mostly observed in the upper stream. Canopy cover and understory cover also thicken going upstream (Table 3).

At least six microhabitat variables such as night relative humidity, air temperature, day soil temperature, day water temperature, night soil temperature, and night water temperature presented significant differences across sites. Also, Chinibayan river in New Busuanga was significantly deeper than that of Cheey's. In New Busuanga, water in stream tributaries were clearer and had a lesser velocity than the Chinibayan river. However, in Cheey, Malapinggan river was clearer than that of Lungon's (Table 4).

Table 1. Stream and river characteristics, unit and methods used.

Stream characteristics	Method of acquisition
Width (m)	Visual Estimate
Depth (cm)	Steel Tape/Meter Stick
Rocks (%)	Visual Estimate
Water, Air and Soil Temperatures (°C)	Digital Thermometer
Relative Humidity (%)	Psychrometer
Rate of Water Flow/Velocity (scoring)	Visual Estimate
Turbidity (scoring)	Visual Estimate

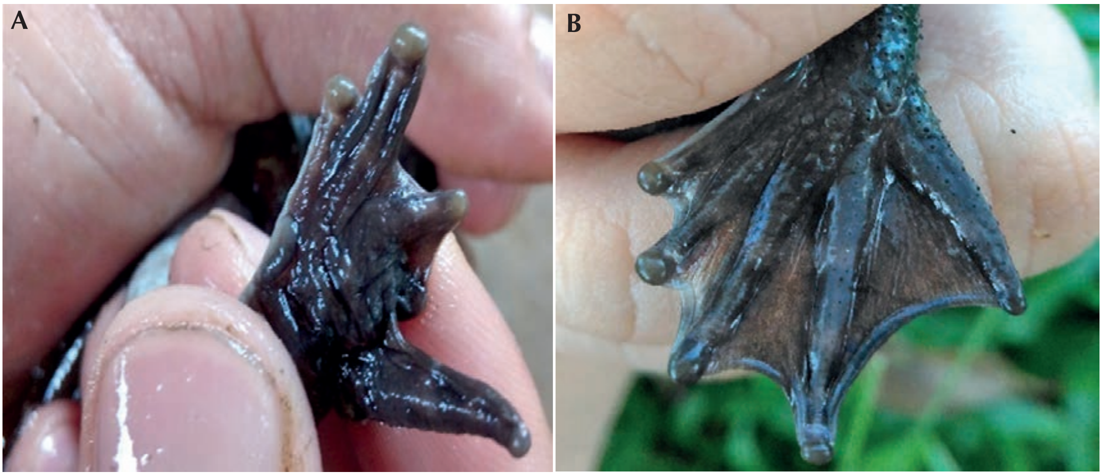
Figures 5. Photos of *Barbourula busuangensis* recorded in Barangays New Busuanga and Cheey. Juveniles (**A–B**), adult (**C**).

Table 2. Comparison of weight and SVL of captured females and male individuals in Brgy. Cheey and Brgy. New Busuanga.

	Weight (g)		Snout–vent length (mm)	
	Range	Mean	Range	Mean
Females (*N* = 115)	18–149.9	60.98	50.7–99.7	76.50
Males (*N* = 20)	13–87	35.85	47.7–89.5	86.88

Table 3. Results of Mann-Whitney U Test between stream habitat variables of downstream (DS) and upstream (US). *Significant at 95% confidence interval.

Microhabitat characteristics	DS Mean	US Mean	Difference	*p-value*
Day Relative Humidity (%)	86.777778	87.94444	-1.1666667	0.21288109
Night Relative Humidity (%)	85.888889	86.66667	-0.7777778	0.59604419
Air Temperature Day (°C)	26.244444	26.04444	0.2000000	0.61009404
Soil Temperature Day (°C)	26.038889	25.92778	0.1111111	0.84830844
Water Temperature Day (°C)	26.166667	25.86667	0.3000000	0.35446707
Air Temperature Night (°C)	25.583333	25.28333	0.3000000	0.48342756
Soil Temperature Night (°C)	25.872222	24.32222	1.5500000	0.03925102*
Water Temperature Night (°C)	25.822222	24.95000	0.8722222	0.12011229
Stream Width (m)	4.770556	4.51000	0.2605556	0.81232551
Stream Depth (cm)	32.533333	38.71111	-6.1777778	0.97475214
Rocks Percentage (%)	45.083333	45.83333	-0.7500000	0.96184032
Rate of Water Flow (scoring)	13.555556	14.22222	-0.6666667	0.64428892
Turbidity (scoring)	15.777778	15.11111	0.6666667	0.54388968



Figures 6. Abnormal deformities (absence of 2nd toe on the left) vs. normal (right) phalanges of *Barbourula busuangensis.*

Table 4. Results of Mann-Whitney U Test between river habitat variables of Cheey and New Busuanga. *Significant at 95% confidence interval.

Microhabitat characteristics	Cheey mean	New Busuanga mean	Difference	*p-value*
Day Relative Humidity (%)	86.700	88.187500	-1.487500	0.114
Night Relative Humidity (%)	85.350	87.437500	-2.087500	0.002*
Air Temperature Day (°C)	25.250	27.262500	-2.012500	0.000001*
Soil Temperature Day (°C)	25.305	26.831250	-1.526250	0.0000009*
Water Temperature Day (°C)	25.465	26.706250	-1.241250	0.0000005*
Air Temperature Night (°C)	25.300	25.600000	-0.300000	0.378
Soil Temperature Night (°C)	24.450	25.906250	-1.456250	0.037
Water Temperature Night (°C)	24.925	25.962500	-1.037500	0.006
Stream Width (m)	3.955	5.496875	-1.541875	0.239
Stream Depth (cm)	32.615	39.381250	-6.766250	0.192
Rocks Percentage (%)	44.075	47.187500	-3.112500	0.677
Rate of Water Flow (scoring)	14.100	13.625000	0.475000	0.988
Turbidity (scoring)	15.350	15.562500	-0.212500	0.552

Discussion

Our observations indicate distinct habitat preferences between adult and juvenile frogs. Adult frogs predominantly inhabit rivers, while juveniles prefer streams or small channels. In New Busuanga, the prevalence of juveniles in streams is likely due to the presence of three tributaries in the area. Notably, a significant proportion of the captured females were gravid, easily identified by their large yellow eggs. It is also evident that females are generally larger and heavier than males. This size difference is likely an adaptation to accommodate the reproductive demands of amphibian mating, as larger females can produce and lay more eggs. However, potential nesting sites were not observed during our study, and the mode of reproduction of *B. busuangensis* remains unknown. Additional research and field monitoring of *B. busuangensis* is required to understand their habitat requirement for potential nesting sites, behavioral observation of the species and microhabitat analysis. According to the studies of Wells (2010), Crump (2015), and Glime and Boelema (2017), additional steps include field observations, studying eggs and larvae, conducting histological examinations of gonadal tissues, acoustic monitoring, and genetic investigation to understand mating systems and implementing long-term monitoring to observe seasonal as well as annual variations in reproductive activity (Figure 5).

Considering the two barangays studied, Cheey recorded the highest number of *B. busuangensis.* This variation in population density may be attributed to several factors including habitat quality and availability, water quality, temperature, and anthropogenic factors (Collins and Storfer 2003, Cushman 2006, Stuart *et al*. 2004, Blaustein *et al.* 2010). Out of the thirteen microhabitat characteristics tested using Mann-Whitney U test, soil temperature at night was the only variable to show a significant difference between downstream and upstream sites. This indicates that soil temperature at night significantly differs between these two areas, suggesting it may play a crucial role in habitat selection for these frogs. Additionally, six microhabitat variables showed significant differences between Cheey and New Busuanga. Night relative humidity significantly differed between the two sites. Relative humidity is essential for amphibians as they are highly sensitive to desiccation, and high humidity levels are crucial for their skin respiration and hydration (Wells 2010). Daytime air temperature had a strong significant difference between the two locations. Air temperature affects the metabolic rates and activity levels of amphibians, and cooler daytime temperatures may be preferable for *B. busuangensis,* aligning with their need for a cooler habitat (Infante *et al.* 2002). Soil temperature also showed significant differences both during the day and at night. Soil temperature can influence the thermal environment of frogs, affecting their behavior, physiology, and microhabitat use. Optimal soil temperatures are crucial for maintaining the body temperature of these ectothermic animals. Water temperature also had significant differences during the day and at night. Water temperature is a critical factor for amphibians, particularly for those like *B. busuangensis* that inhabit streams. Cooler water temperatures are typically associated with higher dissolved oxygen levels, which are beneficial for both the frogs and their prey (Phochayavanich *et al.* 2010).

Additionally, based on the study of Wells (2010), the abundance of this frog species can also be influenced by various environmental factors, crucial for their survival given the sensitivity of anurans to changes in the environment. According to Infante *et al.* (2002), *B. busuangensis* inhabit cool and clean mountain streams, making their homes beneath and between rocks. These frogs often hide underwater or in crevices during the day to avoid predators, preferring clean, cool waters and soil/sand surfaces. At night, they are active, hunting for food and resurfacing within the vicinity of rivers and streams with a good environment thus provides a suitable habitat for these frogs. Stream width and depth also play significant roles in the abundance of adult and juvenile frogs. Streams in the studied areas vary greatly in habitat characteristics, influencing the presence of frog species. Research by Porter (2010) and Phochayavanich *et al.* (2010) indicate that stream-breeding frogs require streams large enough to hold water for extended periods, ensuring tadpole survival. Larger streams provide suitable breeding habitats for a greater variety of frog species due to the extended water presence and greater diversity of microhabitats and food resources. Our results also suggest that microhabitat characteristics significantly influence the presence and distribution of *B. busuangensis.* The significant differences in soil and water temperatures, as well as relative humidity, highlight the species' preference for specific environmental conditions. These findings underscore the importance of preserving the unique microhabitats that support the survival of this species, particularly in the face of environmental changes and habitat disturbances (Hopkins 2007). Understanding these microhabitat preferences is crucial for developing effective conservation strategies. By maintaining optimal environmental conditions, such as appropriate humidity levels and cooler temperatures, we can support the continued survival of *B. busuangensis* and other sensitive amphibian species in these areas.

In our selected sites, we observed that streams with specific characteristics tended to support different life stages of *B. busuangensis.* For instance, in New Busuanga, the tributaries are located deep inside the forest with nearly 100% canopy cover, slow to moderate water flow, clear turbidity, and narrower, shallower streams compared to the Chinibayan stream. Juveniles, typically less than one inch, were predominantly found in these tributaries and small puddles near the stream, while none were observed in the wider, deeper, and faster-flowing Chinibayan stream. In Cheey, the Malapinggan stream, which has the clearest water based on turbidity rates and slow to moderate flow, supported a higher presence of *B. busuangensis.* Conversely, the Lungon stream, the most disturbed site, recorded a very low presence of *B. busuangensis,* with only 26 individuals seen and captured despite the established transect points.

Prior to the implementation of active enforcement measures in Cheey, the use of pesticides by locals for fishing and catching freshwater crabs and shrimps was widespread (Paguntalan 2015). Additionally, farmers near the river applied herbicides to their agricultural lands. Research by Bishop and Haas (2009) indicates that the rampant use of chemicals in agriculture significantly affects the survival of both tadpoles and adult frogs. This can lead to morphological abnormalities, such as the presence of extra or missing legs or limbs, a phenomenon also observed in the Philippines (Shuman-Goodier *et al.* 2017, Propper *et al.* 2020). The use of these chemicals has detrimental effects on the frog population. Pesticides and herbicides can contaminate water bodies, leading to toxic environments for aquatic life. For frogs, which have permeable skin and rely on clean water for reproduction and development, exposure to these chemicals can be particularly harmful. Tadpoles are highly susceptible to these toxins, which can interfere with their growth and lead to deformities. These deformities not only affect individual frogs but can also reduce the overall fitness and survival of frog populations.

After engaging with the community, it is evident that the local communities in these areas are aware of the importance of biodiversity, especially due to their firsthand experiences of declining water levels and the drying up of streams. Despite the awareness, the size of these forests is decreasing due to deforestation and conversion to various land uses, driven by human consumption and activities. The ongoing reduction in forest size poses a significant threat to the habitat and survival of *B. busuangensis* and other local species. Understanding the value of the remaining forests, their direct relationship with water quality, and the importance of wildlife in preserving the forest ecosystem is crucial for effective conservation. The forests in Cheey and New Busuanga serve as watersheds, maintaining clean water supplies essential for both human and wildlife communities. Educating local communities about the ecological roles of species like the Philippine Flat-Headed Frog and promoting sustainable practices can help protect these habitats. Engaging communities in conservation efforts and demonstrating the long-term benefits of preserving natural resources are key steps towards sustainable environmental stewardship. Fragmentation of forests habitat and threats to survival of single-island endemic species remains a major concern. The loss of forests is happening due to insufficient local governance mechanisms towards effective biodiversity conservation and the lack of technical capacity of local government units to manage these unique ecosystems. Added to this, the continuing land conversions, road development, river/stream poisoning, low appreciation on the uniqueness of the biodiversity of Busuanga contributed to the low support for biodiversity conservation. Although Chinibayan river is within the jurisdiction of Busuanga Pasture Reserve at the time and Malapinggan river as source of drinkable water to Barangay. On-going habitat changes brought by human activities pose a threat to *Barbourula busuangensis* 's population and to other species. The most common occurrences observed in these areas are the conversion of its surrounding forests into agricultural lands, streams and rivers as local destination and using of chemicals that poison rivers and streams to capture freshwater fishes and shrimps according to the local guides. With these threats occurring, it is deemed necessary that immediate and appropriate attention and efforts be placed for the establishment of further effective protection for the preservation of *B. busuangensis* and other wildlife species thriving in the same areas.

There are several local organizations advocated for the conservation of wildlife and its habitats. Many activities were conducted to raise biodiversity awareness within schools and local communities. Philippines Biodiversity Conservation Foundation Inc. (PhilBio) and C3 Philippines (Coastal Conservation and Education Foundation, Inc.) which are Non-Government Organizations (NGOs) that play crucial roles in protecting the Philippines' rich biodiversity this is through different aspects of conservation including terrestrial to marine environments, involving local communities. These organizations had assisted in the creation and sustaining two locally managed conservation areas in the Municipality of Busuanga (Cheey and Bogtong) while one more (New Busuanga) was in the process of declaration through barangay resolution. Barangay Quezon was recently declared as a Local Conservation Area under barangay resolution, while majority of the remaining forests in Busuanga Island falls within the jurisdiction of Busuanga Pasture Reserve Management Office of Forest Management Bureau (FMB) at the time of the survey.

Enforcement activities have been mobilized and strengthened by locals, Busuanga Pasture Reserve (BPR), Local Government Unit (LGU) and Palawan Council for Sustainable Development (PCSDS) through foot patrolling monitoring for the success of protection in Busuanga. Hukbong Pangkalikasan in Barangay Cheey conducted evidence-based monitoring survey and the results of were incorporated in their regular report. Comprehensive Land Use Plan for Busuanga municipality was updated for 2015–2017. It aimed to contribute to the meaningful development of the Municipality of Busuanga, Province of Palawan by providing sound and comprehensive land use plan that will guide the Municipality's actions in the next nine years to ensure and enable the future generations of Busuanga to enjoy the rich and diverse natural resources of the island.

Recommendations

The Municipality of Busuanga had numerous rivers and streams that are still clean or less polluted and has a continuous waterflow throughout the year. It is precisely for these same reasons that the *Barbourula busuangensis* and other endemic species chose to inhabit these rivers and streams (Infante *et al.* 2002, Bosch *et al.* 2023). River developments should planned carefully to avoid altering the natural river flow or reducing the surface water of rivers and streams. Income generated from these water systems should also allocate budget for watershed protection. Replanting of native trees on the island should be prioritized within clearings between patches of forests, watersheds and along both sides of rivers and streams banks. The remaining forests and watersheds within the Municipality of Busuanga and other neighboring areas that hold population of the endemic *B. busuangensis* should be prioritized and declared as areas for local conservation. Most of the locals people are not aware of the importance of the endemic wildlife in the area and the importance of protecting the forests (Paguntalan 2015). Community-based biodiversity conservation education activities (ecological camps etc.) must be conducted to raise awareness and foster appreciation in the region.

Initial discussions have already taken place with local governments, communities, and water resources board regarding the use of agro-pollutants such as herbicides and pesticides on agricultural areas near rivers and streams. A follow-up discussion to develop and support river/stream conservation policy is needed to establish a foundation for longer-term sustainability. Additionally, conducting a follow-up survey on remaining priority sites is crucial. Palawan is a vast area with several sites that have not yet been surveyed and areas that require updated information. Given its unique forest habitat, additional surveys should be conducted on clean rivers and streams to update the population status of *Barbourula busuangensis* and to investigate its reproductive mode, habitat requirements, diet, and status of other species of importance in the region.

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SHORT COMMUNICATION

Maximum egg mass size of *Ambystoma altamirani* (Caudata: Ambystomatidae)

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Palavras-chave: Axolote-de-riacho, História natural, Ovipostura, Ovos, Salamandra-de-riacho.

Salamanders of the genus *Ambystoma* Tschudi, 1838, commonly known in Mexico as axolotls or achoques, are a group of amphibians represented by 25 species distributed from southern Canada to central Mexico (Frost 2024). Axolotls have a robust, elongated body, a compressed tail, smooth skin, and three pairs of external gills (Canseco-Márquez and Gutiérrez-Mayén 2010, Stebbins and McGinnis 2018). These amphibians live in lentic and lotic aquatic ecosystems and hide among aquatic plants and within openings formed by rocks and walls. They breed in water and are polygamous (Feder and Lynch 1982, Stebbins and McGinnis 2018, Ávila-Akerberg *et al.* 2021).

Of the 25 species that comprise the genus *Ambystoma*, 11 are found in Mexico and 10 are endemic to this country (Everson *et al.* 2021, Balderas-Valdivia and González-Hernández 2024, Frost 2024). Axolotls can be divided into two groups depending on their habitat: those that live in lagoons and dams and those that live in high mountain streams (Casas-Andreu *et al.* 2003). *Ambystoma altamirani* Dugès, 1895 belongs to the latter group and was previously considered to be three species: *A. altamirani,* found in the Lagunas de Zempoala and the Sierra de las Cruces, in Morelos, Mexico City and Mexico state; *A. leorae* Brandon, 1989, found in the Sierra Nevada in Puebla and Mexico state; and *A. rivulare* Frost, 2004, found in the Sierra de Taxco in Guerrero and Sierra Chincua in Michoacan and Mexico State (Everson *et al.* 2021). It is one of the two river axolotls found in the Trans-Mexican Volcanic Belt, between 2720 and 3479 m a.s.l. (Lemos-Espinal *et al.* 1999, Woolrich-Piña *et al.* 2017, Lemos-Espinal and Smith 2020, Sánchez-Sánchez *et al.* 2022). Although the species is apparently widespread, its populations are restricted to small patches within its habitat. Populations of *A. altamirani* are surrounded by some of the most highly urbanized areas of central Mexico; thus, the species faces serious environmental problems due to anthropogenic pressure, even within protected areas (Heredia-Bobadilla and Sunny 2021).

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The relatively few studies of *A. altamirani* are in contrast to other species such as *Ambystoma mexicanum* (Shaw and Nodder, 1798). Some of these studies on *A. altamirani* include field observations describing the eggs, larvae morphology, and size (Campbell and Simmons 1962, Lemos-Espinal and Ballinger 1994), larval diet (Lemos-Espinal *et al.* 2015), behavior and physiology (Sánchez-Sánchez *et al.* 2022), health condition, parasites, and deformities (Barriga-Vallejo *et al.* 2015, Sánchez-Manjarrez *et al.* 2022, Hernández-Luría *et al.* 2023), relationships between coloration and use of the substrate (Villarreal-Hernández *et al.* 2020), analysis of genetic variability, population size and structure (Heredia-Bobadilla *et al.* 2016, 2017), and evaluations of the impact of some habitat characteristics and the presence of livestock on their abundance (Gómez-Franco *et al.* 2022).

Adult males of *A. altamirani* reach a snout–vent length (SVL) ranging from 77 ± 1.6 mm to 95 ± 6.03 mm, while females range in size from 76 ± 1.4 mm to 99 ± 5.6 mm (Lemos-Espinal *et al.* 2017, Sánchez-Manjarrez 2017). The dorsal coloration of this species is blackish to olive green, and the color is less intense on the sides. The tail and belly are blackish gray and yellow or black spots may occur on the back (Taylor 1940). Only about six records of the egg mass size of *A. altamirani* have been reported. The number of eggs varies between 1 and 463, which have been found in masses or isolated on different elements of the habitat, such as roots, vegetation, rocks, and pine needles (Brandon and Altig 1973, Bille 2009, Legorreta-Balbuena *et al.* 2014, Sunny *et al.* 2014, Monroy-Vilchis *et al.* 2015, Lemos-Espinal *et al.* 2016, 2017). Herein we report an egg mass of *A. altamirani* that represents the maximum size known to date. Characteristics of the oviposition site and hatching time are described.

On 21 March 2022, an egg mass of *A. altamirani* (previously considered *A. rivulare*) was observed in San Francisco Oxtotilpan, municipality of Temascaltepec, state of Mexico, (19.161723° N, 99.903279° W at 2633 m a.s.l.). The location is within two protected areas: the Nevado de Toluca Flora and Fauna Protection Area and the Natural Resources Protection Area in the catchment of the rivers Valle de Bravo, Malacatepec, Tilostoc and Temascaltepec (CONANP 2023; Figure 1). It is a high mountain ecosystem with a temperate subhumid to semi-cold climate composed of fir (*Abies* spp.), pines (*Pinus* spp.), oaks (*Quercus* spp.) and aile (*Alnus acuminata* Kunth) (CONANP 2014). Numerous watersheds provide aquatic habitats for a diversity of organisms such as axolotls.

To estimate the size of the egg mass, the number of eggs was estimated from a sample of 93 eggs, which accounted for about 10% of the total mass; thus, the egg mass was estimated to contain between 930 and 1000 eggs (Figure 2). The eggs were black and had diameters of approximately 6 mm. They were located at the edge of a stream in a single mass attached to rocks and roots. In addition, more than half of the egg mass was covered by floating aquatic vegetation (*Lemna minor* L.) and small pieces of bark. The stream depth was 16 cm and its width was 36 cm, water temperature was 14°C, and the bottom had a sandy–muddy substrate. After observation, the collected sample was deposited in the same place together with the rest of the mass and observed periodically. All eggs hatched between 336 and 360 hours.

The size of the egg mass of *A. altamirani* in this observation was more than twice as large as previously reported egg masses, where between 1 and 463 eggs were observed, either isolated or in a single mass (Brandon and Altig 1973, Bille 2009, Legorreta-Balbuena *et al.* 2014, Sunny *et al.* 2014, Lemos-Espinal *et al.* 2016, 2017). The number of eggs per mass varies greatly in *A. altamirani;* nonetheless, such variation is not surprising considering that this species belongs to the *A. tigrinum* complex, whose species show enormous variation in their life history due to proximal factors (temperature, predation, competition, etc.) and genetic factors (Collins 1981, Collins *et al.* 1993, Sorci *et al.* 1996,

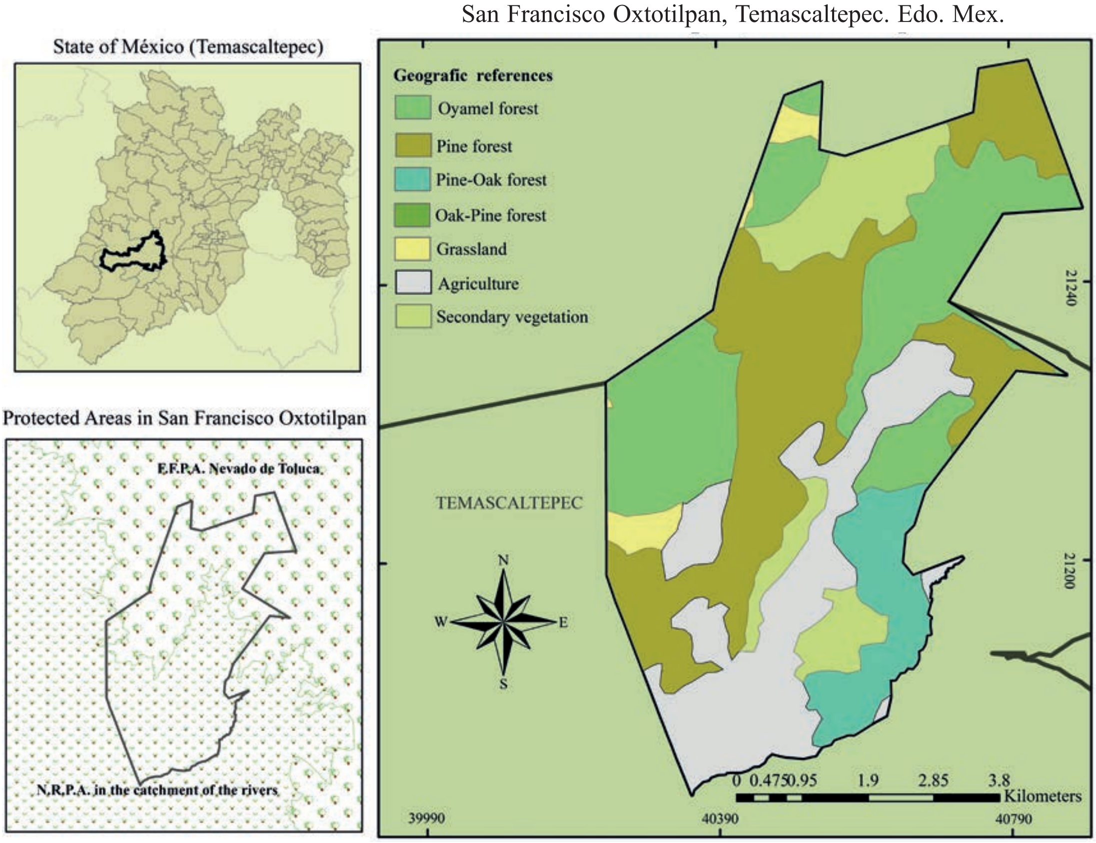

Figure 1. Map showing location of the egg mass of *Ambystoma altamirani.* The center of the image shows the location of San Francisco Oxtotilpan and the left side of the image shows its location within the Natural Areas of the zone.

Everson *et al.* 2021). Large egg masses such as that observed may be due to a proportional relationship between the size and age of the female and the number of eggs per mass (Parker and Begon 1986, Flemming 1994). Larger females and young females tend to lay a greater number of smaller eggs (Kuramoto 1975, Morrison and Héroe 2003).

The observed egg mass was attached to rocks and roots covered with bark and aquatic plants on a sandy–muddy bottom. Bille (2009) stated that *A. altamirani* seems to randomly select the places for egg deposition. For example, Brandon and Altig (1973) found an egg mass on roots and under a rock; Bille (2009) found numerous isolated eggs and egg masses on submerged pine branches on a rocky–muddy bottom, while Sunny *et al.* (2014) and Lemos-Espinal *et al.* (2016, 2017) found isolated eggs or egg masses on aquatic plants on a muddy bottom. Axolotls also appear to select sites that have structures that provide protection and sufficient support for egg deposition (Martínez 2007), such as vegetation, rocks, or roots. The sandy–muddy substrate in which the egg mass in this study was found coincides with other records of the characteristics of the habitat chosen by the species (SEMARNAT 2018).

Figure 2. (**A**) Egg mass of *Ambystoma altamirani* within a small stream. (**B**) Sample of 93 eggs from the clutch deposited by *A. altamirani.*

The depth of the stream in which the egg mass was located was 16 cm with a width of 36 cm and a velocity of 0.36 m/s. The velocity was estimated based on the time it takes for a 10-gram ball to travel a distance of one meter above the water surface of the stream. We used this technique because we did not have a flow meter at hand; thus, the measure must be taken with caution. The recorded depth is within the range of 10 cm to 57 ± 7.4 cm reported in other observations, while the width is close to the 40 and 69 ± 4.8 cm previously reported by other authors (Lemos-Espinal *et al.* 2016, 2017). The current velocity is also within the previously reported 0.3 to 0.4 m/s (SEMARNAT 2018), which is consistent with habitat characteristics of *A. altamirani,* which lives in small high mountain streams with low water flow.

The time from sighting to hatching of eggs in the field was between 336 and 360 hours at a temperature of 14°C with 100% of the eggs hatching, which is a longer period than that reported by Legorreta-Balbuena *et al.* (2014). Those authors reported that eggs in captivity hatched from 288 to 312 hours at a temperature of 18°C, with 86% of the eggs hatching. Temperature has been shown to strongly influence the growth and development of embryos and larvae (Brown 1976, Pepin 1991). In *Ambystoma* in general, eggs hatch relatively quickly at high temperatures but at a lower percentage, which is consistent with our observations and other literature reports (Light and Bogart 1989).

Our observations synthesize and add to what is known about some aspects of reproduction in *A. altamirani* in the central region of Mexico. We emphasize the need to conduct further research to gain a better understanding of the natural history of the species in the states of Guerrero, Michoacán, Morelos, Mexico City, and Puebla.

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SHORT COMMUNICATION

Notes on feeding and mating behavior of *Anolis biporcatus* (Squamata: Dactyloidae) in Costa Rica

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Anoles are small to midsize tree-dwelling lizards that inhabit a broad range of microhabitats along the vertical strata of Neotropical forests, ranging from those that are highly terrestrial or that occupy lower levels of the forest canopy to species that thrive in higher levels within the canopy (Losos 1994, Irschick *et al*. 1997). Natural history information, particularly for species that live at canopy levels like *Anolis biporcatus* (Wiegmann, 1834), remains scarce. This diurnal lizard can reach a total length of approximately 70–107 mm and ranges from southern Mexico to western Ecuador at elevations from near sea level to about 1220 m. a.s.l. (Savage 2002, Armstead *et al*. 2017). Individuals are typically green but can exhibit significant color changes (Savage 2002). Males have dewlaps with a blue center and a white base bordered by a red margin, while females possess white dewlaps without the distinctive coloration seen in males (Armstead *et al*. 2017).

Anolis biporcatus lays a few eggs in moss-covered branches and tends to be active during the rainy season (Fitch 1975, Leenders 2019). It also perches on trunks at heights between 20 cm to 18 m (Perez-Martinez *et al.* 2021) and consumes small to mid-size arthropods such as beetles, ants, and spiders (Savage 2002). Individuals are keratophagous (Rojas-Carranza and Anderson 2021) and consume small lizards as vertebrate prey (Taylor 1956). Due to its arboreal behavior, information about its diet and reproductive biology remain limited because individuals seldom descend from the canopy (but see Armstead *et al*. 2017, Esquivel and Vargas-Acuña 2017, Perez-Martinez *et al*. 2021, Rojas-Carranza and Anderson 2021). To better understand the natural history of this species, we present a series of field observations on adults of *A. biporcatus* at Veragua Rainforest in Las Brisas de Veragua, Limón Province, Costa Rica (9°55'21" N, 83°10'2" W, 420 m. a.s.l.).

On 19 February 2023 at approximately 13:00 h, we observed an adult female with a mid-size dragonfly in its mouth, clinging head down from the upper side of a leaf on a *Syzygium malaccense* (L.) Merr. and L. M. Perry tree at a height of

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about 2 m along an open trail (Figure 1). The lizard held its prey by the abdomen and using its jaw continually chewed its prey for about 2 minutes, holding on to the leaf using only its toes and claws. Though we did not witness the entire consumption process, given the small size of the prey relative to the lizard's body size, we expect that the lizard ingested it completely.

We classified the dragonfly as a member of the genus *Gynacantha* (Odonata: Anisoptera: Aeshnidae), based on the width of the base of the posterior wings and the absence of a triangular and elongated cell in this region (Esquivel 1995), thus adding adult odonates to the known diet of *Anolis biporcatus*. Color change has been reported several times during feeding events of *A. biporcatus*. Esquivel and Vargas-Acuña (2017) reported individuals transitioning from dull brown when perched to uniform green while capturing and ingesting stingless bees. Rojas-Carranza and Anderson (2021) witnessed changes from dull brown to light green on the head and upper body of an adult male while performing keratophagy of its shed skin. In contrast, Perez-Martinez *et al*. (2021) documented opposite color changes in *A. biporcatus,* shifting from a uniform green to prominent lateral stripes of contrasting dark green and yellow-green while feeding on a grasshopper. We noted that *A. biporcatus* was uniformly green while capturing and consuming the dragonfly; however, we did not witness any color change during consumption.

On 11 May 2023 at 13:42 h, we observed a breeding pair of *Anolis biporcatus* in a vertical, head-down position on a tree trunk at a height of 70 cm (Figure 2A). At the time of observation, both individuals were uniformly green, although the female's dorsum was darker than the male's. The male held its tail under the female's tail, with its arms and legs positioned across her back, facilitating cloacal contact while holding vertically to the tree trunk. These observations lasted about two minutes, during which no other courtship displays were witnessed. The lizards remained practically motionless, with their brown-colored, curved tails positioned away from the tree trunk and their heads pointing upward in opposite directions. The male adhered to the trunk using only one of its arms and one of its legs, while the other arm held the female by the back, and the other leg surrounded the female's back.

Figure 1. An adult female *Anolis biporcatus* eating a dragonfly of the genus *Gynacantha* (Odonata: Anisoptera: Aeshnidae) in Veragua Rainforest, Costa Rica. Photo credit to Kenneth González Nájera.

On 15 October 2023 at 13:45 h, we observed a male *Anolis biporcatus* approaching a female that was vertically perched facing downward on a *Protium glabrum* (Rose) Engl. tree at a height of approximately 3 m (Figure 3). The male was light green, while the female looked darker than the male (Figure 3A). The male positioned itself

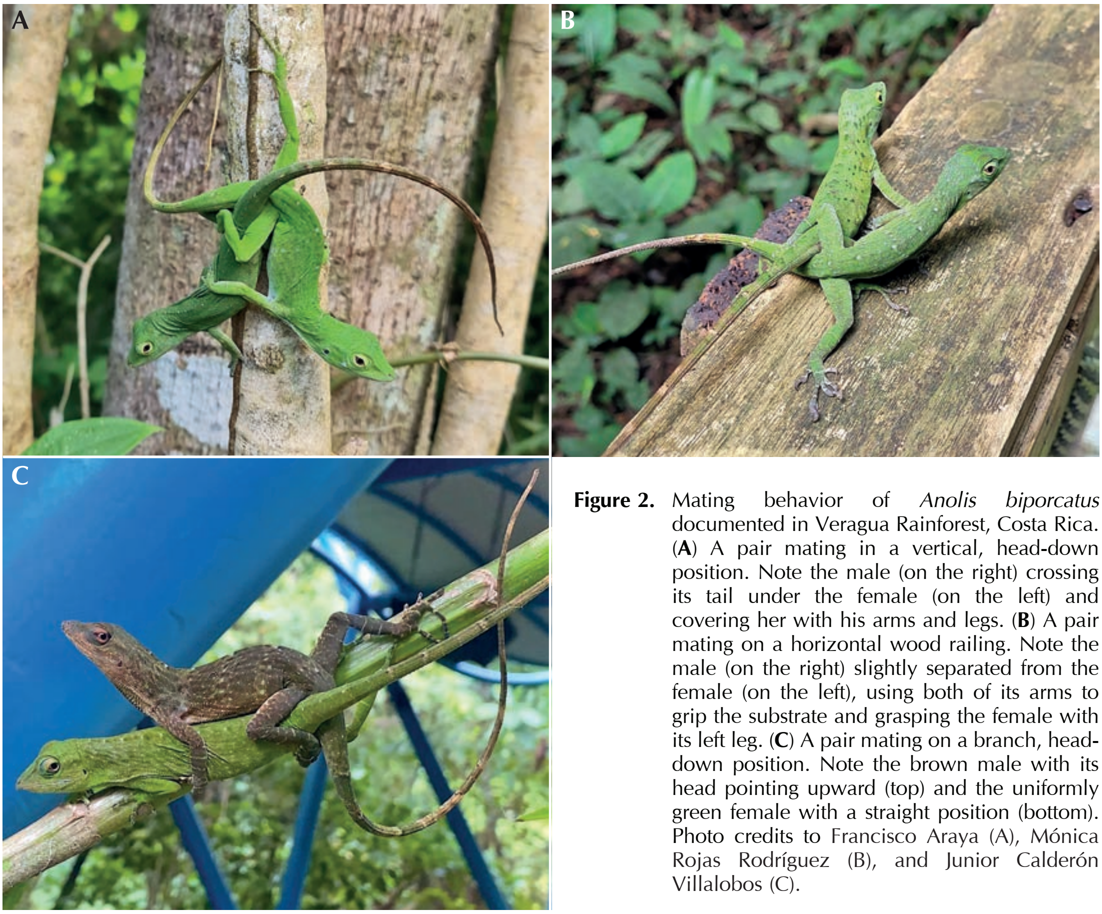

Figure 2. Mating behavior of *Anolis biporcatus* documented in Veragua Rainforest, Costa Rica. (**A**) A pair mating in a vertical, head-down position. Note the male (on the right) crossing its tail under the female (on the left) and covering her with his arms and legs. (**B**) A pair mating on a horizontal wood railing. Note the male (on the right) slightly separated from the female (on the left), using both of its arms to grip the substrate and grasping the female with its left leg. (**C**) A pair mating on a branch, head-down position. Note the brown male with its head pointing upward (top) and the uniformly green female with a straight position (bottom). Photo credits to Francisco Araya (A), Mónica Rojas Rodríguez (B), and Junior Calderón Villalobos (C).

beside the female and held her at midbody with its arm, positioning its left leg around her inguinal region, and engaged in several dewlap displays (Figure 3B). After 10 seconds, the male proceeded to bite the female's neck for approximately one minute (Figure 3C). Subsequently, within seconds, the male changed from uniform green to green with distinct dark markings (Figure 3D) before reverting to its uniform green after approximately 30 s. The female then descended the trunk with the male on her back. After two minutes, the male began tail movements and initiated cloacal contact, maintaining this position for approximately five minutes. Both individuals repeatedly displayed their dewlaps for about 40 min (Figure 3E–F). Next, the male and female began moving their legs, increasing cloacal contact for the next 15 minutes and continued displaying their dewlaps several times. At that moment, heavy rain began and we could not continue with the observations.

On 17 October 2023 at 14:13 h, a third party observed another breeding pair of *Anolis biporcatus* perched over a wood railing at a height of approximately 1 m, located 5 m horizontally from the previous observation site (Figure 2B). The male's dorsum was green with white markings, while the female's dorsum was lighter with faint dark green markings. The tails

Figure 3. Courtship repertoire of a pair of *Anolis biporcatus* breeding in Veragua Rainforest, Costa Rica. (**A**) Breeding pair's position on the tree trunk, with the light green male on top and the darker green female on bottom. (**B**) Male using its left arm to hold onto the female, with its left leg positioned around her inguinal region. (**C**) Male biting the back of the female. (**D**) Color change by the male, shifting to green with distinct dark markings. (**E**) Male displaying an expanded dewlap. (**F**) Female with an expanded dewlap.

of both individuals were brown. The pair engaged in mating behavior, with the male positioned on top of the female, holding her midbody with its arm and left leg around her inguinal region for approximately 5 minutes. The lizards oriented their heads in the same direction, and, although the male's tail was aligned with that of the female, it was curved due to cloacal contact. The pair remained motionless and no additional courtship behavior was observed on this occasion.

On 14 January 2024 at 14:38 h, we observed another breeding pair of *Anolis biporcatus* on a branch of a plant in the family Melastomataceae at about 2.5 m height (Figure 2C), close to an area of frequent human activity. The male was dark brown with clear white markings, while the female was uniformly green with faint white markings. The male positioned himself on top of the female, holding her midbody with its arm and left leg around her inguinal region for approximately 10 minutes. The male's head was positioned in the same direction as the female's but was tilted upward. The female's tail remained straight and green, while the male's tail curved downward after cloacal contact, extending toward the female's tail before curving upward again to pass under the medial section of the female's tail.

Considering the limited information available on the reproductive biology of *Anolis biporcatus*, our observations of its behavior provide new insights into its ecology and reproductive biology, revealing details of the courtship and copulatory behavior. After analyzing the four breeding events, we noted that both sexes of *A. biporcatus* use dewlap displays and have color variants (color intensity, patterns, and distribution of markings) during mating. The breeding pairs showed differences in perching angles, mating in vertical (Figure 2A), horizontal (Figure 2C), and diagonal (Figure 2C) profiles. The most common breeding position seems to be with both individuals facing downward from a vertical substrate (Figure 2A, Figure 3). These variations suggest potential individual or contextual differences in mating behavior among individuals of *A. biporcatus*, further underscoring the complexity of mating behaviors and sexual selection processes in this species.

Most anoles exhibit territoriality, wherein males defend an exclusive area containing female territories, suggesting that males may mate with multiple females, while females mate exclusively with the male residing in their territory (Kamath and Losos 2018). Whether territoriality occurs in *Anolis biporcatus* is unknown due to the limited exploration of its arboreal habits and reproductive biology. Based on parental investment, Trivers (1976) proposed that differences in male and female reproductive strategies could influence courtship behaviors and territoriality patterns in *A. garmani* Stejneger, 1899, a green Jamaican anole related to *A. biporcatus*. Further exploration is warranted on *A. biporcatus*, particularly considering its shared characteristics with *A. garmani*, Stejneger, 1899, including their canopy habitat, arboreal behavior, medium size, and distinctive color change and dewlap displays (Trivers 1976). A deeper understanding of these aspects will not only enhance our understanding of the reproductive biology of this species but also shed light on the evolution of mating behaviors in arboreal anoles.

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SHORT COMMUNICATION

On the clutch size of *Geophis omiltemanus* (Squamata: Dipsadidae) in the Sierra Madre del Sur, Mexico

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Geophis omiltemanus Günther, 1893 is a Mexican endemic snake restricted to the Sierra Madre del Sur, known from central Guerrero, and a disjunct record from western Oaxaca, near San Andrés Chicahuaxtla in an elevation range of 2153–2413 m a.s.l. (AMNH 97962; Campbell 1988, Heimes 2016). Despite being described over a century ago and despite its type locality, "Omilteme", being one of the most extensively collected sites of the Sierra Madre del Sur (Flores-Villela and Muñoz-Alonso 1993), almost nothing is known of its natural history. Additionally, recollections beyond a ca. 20 km radius from its type locality have not been reported after Campbell's record (Campbell 1988). Here we report the first data known on the clutch size of this species and compare it to existing published reports in the genus.

During fieldwork conducted at the El Llano, Llanos de Tepoxtepec, municipality of Chilpancingo de los Bravo, Guerrero, Mexico (17.45711°N, -99.53738°W; 2413 m a.s.l.) on 22 June 2019 three specimens of *G. omiltemanus* were found inside rotting logs. The specimens were collected, humanely euthanized, fixed in 10% formalin, transferred to 70% ethanol for permanent storage, and accessioned at the herpetological collection of the Museo de Zoología "Alfonso L. Herrera", Facultad de Ciencias, Universidad Nacional Autónoma de México under a collecting permit issued by the Dirección General de Vida Silvestre, Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT). The specimens included an adult male (MZFC 35735) and two adult females (MZFC 35736–35737), of which one (MZFC 35736, Figure 1) was pregnant. The female measured 408 mm snout–vent length, 63 mm tail length, with 158 ventral scales, and 37 subcaudals. Upon dissection we found five

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oviductal eggs on the left ovary; the eggs measured (mean ± SD) 7.4–11.1 mm in length ($\bar{x}$ = 9.3 ± 1.373) and 4.3–5.6 mm in width ($\bar{x}$ = 4.7 ± 0.503). Measurements of each egg are in Table 1.

According to the published literature, clutches have been recorded in other *Geophis* every month except December, with most records being from June to October (Table 2). The number of eggs per clutch varies from one to six in species with available data (Table 2), and an annual reproductive cycle has been suggested in most cases. However, the phylogenetic relationships of *Geophis* remain unclear, hindering comparisons among closely related taxa. Phylogenetic hypothesis by Sheehy (2012) and Grünwald *et al.* (2021) place *G. omiltemanus* and members of the *G. chalybeus* group closer to large-bodied taxa formerly included in *Tropidodipsas* [i.e., *G. annuliferus* (Boulenger, 1894) and *G. sartorii* (Cope, 1863)] and *Sibon* [*G. sanniolus* (Cope, 1866)].

Geophis sartorii is reported to have a clutch size of 3 to 6 eggs in examined specimens from Veracruz and the Yucatán Peninsula, although without exact dates of reproductive activity (Campbell 1998, Goldberg 2017). *Geophis sanniolus* is reported to have seasonal and annual reproductive activity, with clutches of 2–5 eggs deposited between June and September on the Yucatán Peninsula (Kofron 1983). Our data on *G. omiltemanus* is similar to these reported clutch sizes, and dates of pregnancy suggest a seasonal, probably annual, reproductive cycle, as reported in *G. sanniolus* (Kofron 1983).

It is worth noting that several authors (e.g., Sheehy 2012, Figueroa *et al.* 2016, Zaher *et al.* 2019, Grünwald *et al.* 2021, Arteaga and Batista 2023) have found *Geophis* to be non-monophyletic, and the relationships of many of the supraspecific groups defined by Downs (1967) remain ambiguous. We believe that more information on representatives, as well as better phylogenetic sampling, is needed before properly comparing data on reproduction and other interesting life-history traits of *Geophis* and other Neotropical snake genera.

Figure 1. Gravid female *Geophis omiltemanus* (MZFC 35736) in life. Photo by RPA.

Table 1. Individual measurements of the eggs found in *Geophis omiltemanus* (MZFC 35736).

Egg number	Length (mm)	Width (mm)
1	8.9	4.5
2	10	5.6
3	7.4	4.3
4	9.1	4.6
5	11.1	4.7

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Table 2. Compiled information on clutch size, reproduction date, and location of the genus *Geophis*.

Species	Species group	Number of eggs		Month of observation	Location	References
		Min.	Max.			
G. godmani	*championi*	-	6	Not provided	Costa Rica	Solórzano 2004
G. anocularis	*dubius*	Ovarian folicles		April	Oaxaca, Mexico	Campbell *et al.* 1983
G. dubius	*dubius*	3	4	August	Oaxaca, Mexico	Bogert and Porter 1966
G. mutitorques	*latifrontalis*	3	6	March	Hidalgo, Mexico	Cruz-Elizalde *et al.* 2012
G. omiltemanus	*omiltemanus*	-	5	June	Guerrero, Mexico	This study
G. sanniolus	*sanniolus*	2	5	June–September	Yucatán Peninsula	Kofron 1983
G. sartorii	*sartorii*	3	6	Not provided	Yucatán Peninsula/Veracruz, Mexico	Campbell 1988, Goldberg 2017
G. semidoliatus	*semidoliatus*	1	3	July–October	Veracruz, Mexico	Goldberg 2006b
G. bellus	*sieboldii*	1	1	September	Panamá	Elizondo-Lara *et al.* 2015
G. brachycephallus	*sieboldii*	3	6	July–February	Costa Rica	Sasa 1993
G. hoffmani	*sieboldii*	2	5	June–November	Costa Rica	Goldberg 2006a, Solórzano 2004
G. laticollaris	*sieboldii*	-	3	September	Guerrero, Mexico	Palacios-Aguilar *et al.* 2022

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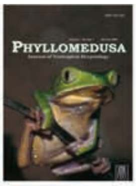
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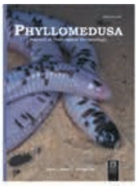
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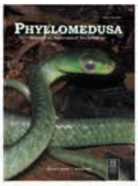
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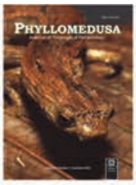
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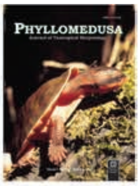
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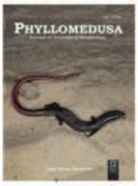
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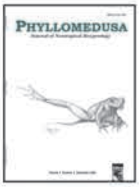
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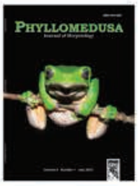
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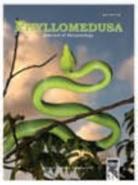
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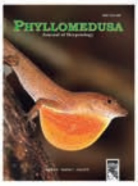
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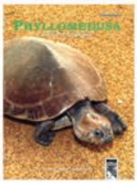
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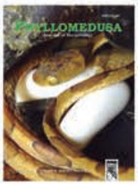
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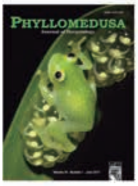
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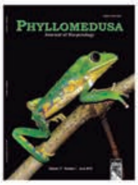
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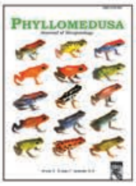
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