ISSN 1519-1397

PHYLLOMEDUSA

Journal of Herpetology

Volume 23 Number 2 December 2024

PHYLLOMEDUSA
Journal of Herpetology

ISSN 1519-1397 (print) / ISSN 2316-9079 (online)

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Cover: Newly hatched tadpoles of *Cycloramphus boraceiensis*
from southeastern Brazil.
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ISSN 1519-1397 (print)
ISSN 2316-9079 (online)

PHYLLOMEDUSA

Journal of Herpetology

VOLUME 23 - NUMBER 2
JULY–DECEMBER 2024

Phyllomedusa

IS PUBLISHED BY **UNIVERSIDADE DE SÃO PAULO,**
ESCOLA SUPERIOR DE AGRICULTURA “LUIZ DE QUEIROZ”

PIRACICABA ESALQ FEALQ BIANNUAL

CREDENCIAMENTO E APOIO FINANCEIRO:
PROGRAMA DE APOIO ÀS PUBLICAÇÕES CIENTÍFICAS PERIÓDICAS DA USP
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Phyllomedusa	Piracicaba	v.23	n.2	pp. 99–242	Jul–Dec 2024

PHYLLOMEDUSA
Journal of Herpetology

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Phyllomedusa: Journal of Herpetology—Vol. 5, No. 1, 2006—Piracicaba, SP, Brazil: Departamento de Ciências Biológicas, Escola Superior de Agricultura “Luiz de Queiroz”, Universidade de São Paulo.

v.; il

Vol. 1 (2002) to Vol. 3 (2004) published by Melopsittacus Publicações Científicas, Belo Horizonte, MG, Brazil.

Vol. 1 (2002) to Vol. 5 (2006) Phyllomedusa: Journal of Neotropical Herpetology

Biannual

Articles and abstracts in English; additional abstracts in Portuguese, Spanish, French, Italian, or German are optional.

ISSN 1519-1397 (print) ISSN 2316-9079 (online)
1. Herpetology CDU - 598

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Phyllomedusa 23(2):101–109, 2024
© 2024 Universidade de São Paulo - ESALQ
ISSN 1519-1397 (print) / ISSN 2316-9079 (online)
doi: http://dx.doi.org/10.11606/issn.2316-9079.v23i2p101-109

The Black Aesculapian Snake *Zamenis longissimus* (Serpentes: Colubridae): characters and possible origin of the *subgrisea* morphotype

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Abstract

The Black Aesculapian Snake, *Zamenis longissimus* (Serpentes: Colubridae): characters and possible origin of the *subgrisea* morphotype. New data acquired on the biology of the Aesculapian snake *Zamenis longissimus* shed light on a widespread specific morphotype in this species: the *subgrisea* form. Through captive breeding of groups of individuals for several generations, it was established that this morphotype is caused by a recessive mutation. The presence and combination of several traits indicate that this form is not from a single genetic mutation, such as melanoticism or axanthism but from a morphotype more complex than previously considered. The expression of this form in several specimens from different European and Middle Eastern countries suggests that the origin of the morphotype is common and ancient, not random. From genetic analyses, fossil records, and the recent observations of wild individuals, it is probable that this form originated in the Balkan peninsula, where it is more common, and spread to central Europe during the species expansion and recolonization in the late Pleistocene after the last glaciation. *Zamenis longissimus* could therefore be considered a polymorphic species.

Keywords: Balkans, Phenotype, Polymorphism, Recessive gene, Snakes.

A serpente-negra-de-esculápio, *Zamenis longissimus* (Serpentes: Colubridae): caracteres e possível origem do morfotipo *subgrisea*. Novos dados adquiridos sobre a biologia de *Zamenis longissimus* lançam luz sobre um morfotipo específico amplamente difundido nessa espécie: a forma *subgrisea*. Por meio da criação em cativeiro de grupos de indivíduos durante várias gerações, foi estabelecido que esse morfotipo é causado por uma mutação recessiva. A presença e a combinação de vários caracteres indicam que essa forma não provém de uma única mutação genética, como o melanismo ou o axantismo, mas sim de um morfotipo mais complexo do que considerado anteriormente. A expressão dessa forma em vários exemplares de diferentes países da Europa e do Oriente Médio sugere que a origem do morfotipo é comum e antiga, não aleatória. A partir de análises genéticas, registros fósseis e observações recentes de indivíduos selvagens, é provável que essa forma tenha-se originado na península dos Balcãs, onde é mais comum, e se espalhado para a Europa Central durante a expansão e a recolonização da espécie no final do Pleistoceno, após a última glaciação. *Zamenis longissimus* poderia, portanto, ser considerada uma espécie polimórfica.

Palavras-chave: Balcãs, Fenótipo, Gene recessivo, Polimorfismo, Serpentes.

Received 13 May 2024
Accepted 05 August 2024
Distributed December 2024

Introduction

Melanism and melanoticism are conditions in which an individual is characterized by an excess of melanin compared to the normal phenotype of intraspecific individuals (Majerus 1998). This form represents the most common and highly variable mutation among ophidians and can express itself in multiple forms, affecting an individual partially or totally or being congenital or ontogenetic; multiple melanotic forms can be present within the same species (Senczuk *et al*. 2021, Fănaru *et al*. 2022, Jablonski *et al*. 2023, Storniolo *et al*. 2023, Meier *et al.* 2024). In snakes, mutations such as melanoticism, melanism, amelanism, leucism, xanthism/axanthism, and erythrism/anerythrism can randomly occur in any individual of any species in any location (Schulz 1996, Bruni 2017, Meier *et al*. 2021, Jablonski *et al*. 2022), leading to the possibility that characters may become fixed within some populations (Tokunaga and Ono 1991, Andrén 2004). Studies on melanistic/melanotic populations of snakes are abundant in the literature, as are reports of a single or a few individuals (Andrén and Nilson 1981, Kuriyama *et al*. 2013, Bury *et al*. 2020).

In this study, the "melanotic" morphotype of the Aesculapian snake, *Zamenis longissimus* (Laurenti, 1768), was examined. Previously, such dark/black individuals have been treated as "simple" or sporadic chromatic mutations, involving single individuals or small populations described in single reports from several Central European, Balkan, and Middle Eastern countries (Fitzinger 1826, 1832, De Betta 1853, Massalongo 1859). In these works, following Schulz (1996) and others (Edgar and Bird 2006, Kreiner 2007), this form is identified by the name designated by Werner (1897) as "var. *subgrisea.*"

Data from the literature, together with observations published on online platforms, were used to define a spatial map in which this morphotype is found. Published genetic and phylogenetic research about populations of *Zamenis longissimus* was considered to hypothesize the most probable origin of the morphotype and how it spread to the locations where it is currently found. This information was combined with a seven-year project in which individuals of the *subgrisea* phenotype were kept and bred in a controlled environment, and several captive-bred generations were produced to determine the genetic nature of this morphotype.

Materials and Methods

Two pairs of the *subgrisea* form born in captivity were donated to OPHIS, Museo Paleontologico e Centro Erpetologico in 2017 and 2023. The most recently received pair consisted of two adult individuals from Montenegro, while the other consisted of two young specimens born in 2017 from parents originating from two Balkan locations (male from Croatia and female from Montenegro). The specimens remain at OPHIS, where they have reproduced for two generations. The adult specimens were raised in terraria with dimensions of 100 × 60 × 50 cm (length, height, and depth, respectively). Their offspring were housed in terraria appropriate to their size and moved to larger ones during growth.

After being collected and measured, the eggs were placed in an incubator (38 × 58 × 36 cm) that had the temperature regulated by an electronic thermostat and digital probes. Each clutch was positioned in a plastic box with a transparent lid filled with moist vermiculite as a medium.

Photographs were obtained with Sony α6000 and Nikon Coolpix P510 digital cameras. Comparative or geographical data were obtained from the literature and from the GPS data provided in the reports on the iNaturalist.org platform.

Results

During the growth of the *subgrisea* specimens, as in the classic phenotype, the juvenile dorsal pattern fades, replaced by a uniform color ranging from solid black to dark grey with very small white flecks (Figure 1A, B). The scales of both black and grey individuals are iridescent, and blue pigmentation may be present in the contact areas of black and white. In some individuals, four longitudinal dorsal lines may be visible. The ventral coloration is black, with white spots at the lateral margins of the ventral scales. These white spots form a longitudinal white stripe along the lower portion of the flanks. The chin and labial scales are white, and the subocular black spot disappears completely in adults. Scattered yellow spots appear on the ventral, subcaudal, and labial scales of adults (Figure 1A, B).

Juveniles of the *subgrisea* form exhibit a dorsal pattern of spots arranged in four longitudinal rows that distinguish all young *Zamenis longissimus* (Figure 1C, D). Unlike the more widespread phenotype, however, longitudinal rows are dark grey on a lighter grey background. As in adults, the belly is completely black with small white dots located on the lateral edges of the ventral scales. The chin and neck are white ventrally with small symmetrical black spots. The labial scales are white, the fourth and fifth with a black tear-shaped spot below the eye. A black line extends along the postocular and temporal scales. The head shields are dark grey, almost black. The iris is dark; in some individuals, the pupil is difficult to distinguish.

Three clutches were produced by the female born in 2017: in 2019, 2021, and 2022. These clutches consisted of 6, 2 and 6 fertilized eggs (plus an infertile egg), respectively (Figure 2A). The elongate white eggs featured irregular concretions on the shell (Figure 2B). Egg length ranged from 50–59 mm and diameter from 20–24 mm; eggs weighed 9–14 g.

Following an incubation of 51–56 days at temperatures between 26 and 28°C, the clutches hatched completely (Figure 2B). The hatchlings measured 30–33 cm and weighed 12–14 g. In the 2019 clutch, half of the specimens exhibited the *subgrisea* form and the other half the classic phenotype (two males and one female for each phenotype; Figure 2B). In the 2021 clutch, both

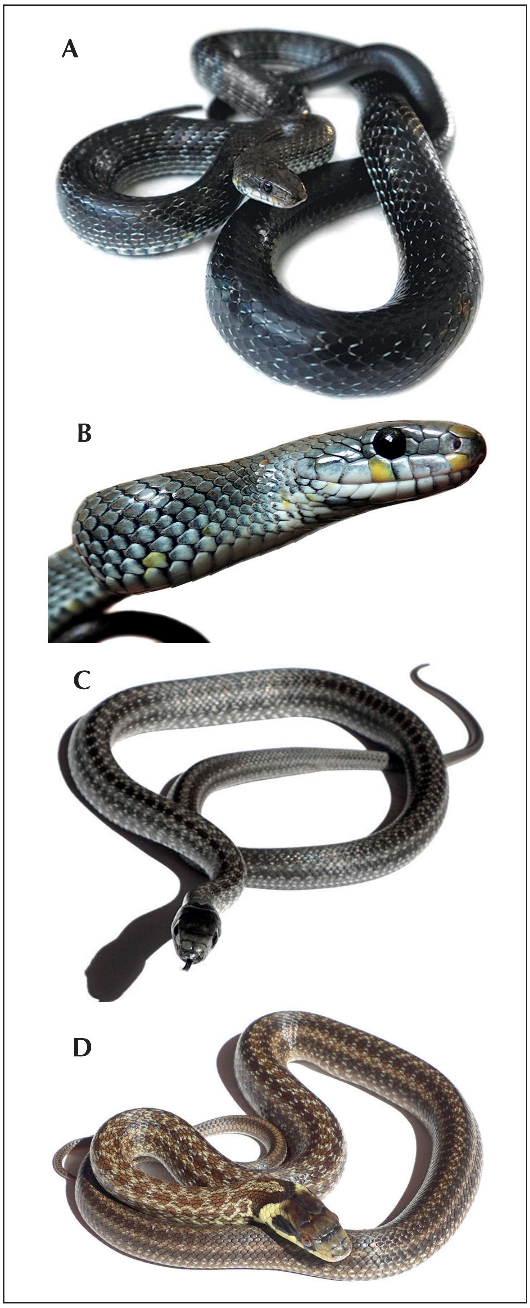

Figure 1. Specimens from the breeding program of *Zamenis longissimus:* **(A)** adult male *subgrisea* from Montenegro; **(B)** adult female *subgrisea*; **(C)** hatchling of the *subgrisea* morphotype; **(D)** hatchling of the dominant morphotype from *subgrisea* parents.

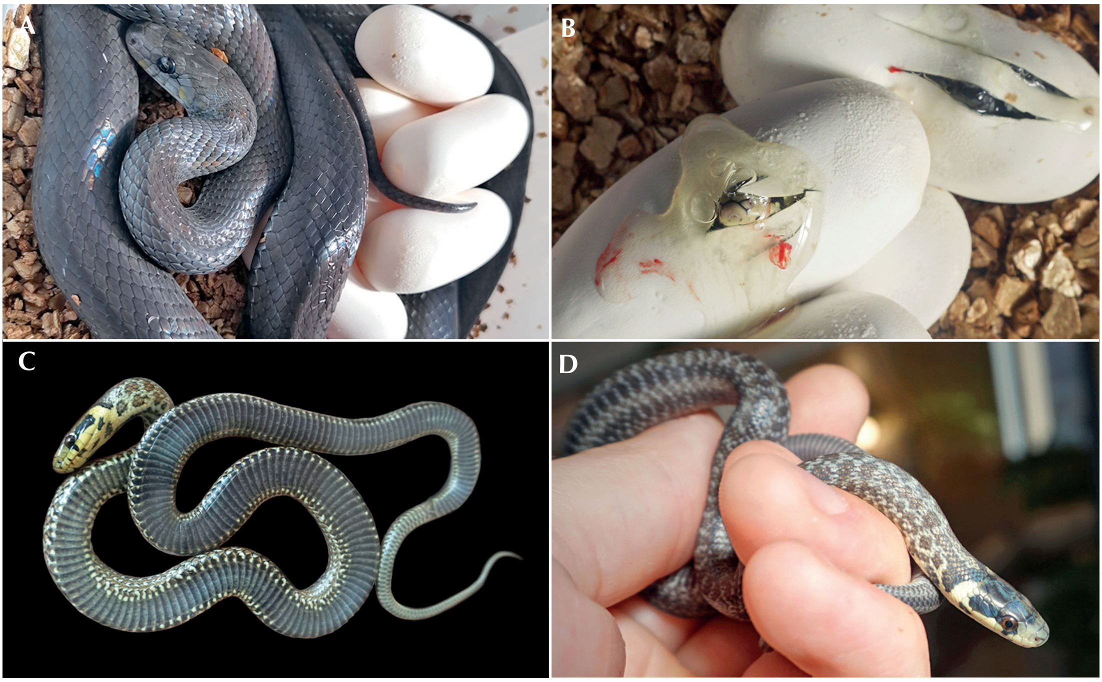

Figure 2. Specimens from the breeding program of *Zamenis longissimus:* **(A)** adult female *subgrisea* with eggs; **(B)** hatchlings from both morphotypes pipping the eggs; **(C)** juvenile female of the dominant morphotype showing black ventral coloration; **(D)** juvenile male showing characters of both dominant and *subgrisea* morphotypes.

hatchlings were of the *subgrisea* form. In 2022, four individuals exhibited the *subgrisea* form, and two were the classic phenotype. At hatching, the individuals of the classic phenotype from the mating of the *subgrisea* specimens exhibited a darker coloration than the hatchlings of *Z. longissimus* from other localities bred by the author and those observed by the author in the wild in Central Europe. The darker coloration becomes paler as the snakes grow. These offspring also exhibit a very bright orange iris (Figure 1D) that contrasts with the dark ground color.

Some chromatic "anomalies" occurred among the captive-bred specimens. One of the snakes born in 2023 (second generation bred in captivity) from heterozygous specimens (first generation in captivity) exhibits typical dorsal coloration but is black ventrally (Figure 2C). A specimen born in 2022 from the original *subgrisea* pair exhibits coloration intermediate between the two morphotypes: hypoxanthistic with darker head shields and a greysh/bluish background dorsal coloration. The black pattern of this individual, if exposed to light, produces the same iridescence typical of the dorsal scales of *subgrisea* individuals (Figure 2D), possibly caused by a variation of concentration of iridophores and xanthophores compared to the classic phenotype.

Discussion

For many years, several authors have reported their observations of dark or "melanistic" individuals of *Zamenis longissimus,* naming this variety/form *nigra,* or *nigrescens* (Fitzinger 1826, 1832, De Betta 1853, Massalongo 1859, Boulenger 1913). The first detailed information for identifying the morphotype, the object of this study, with those described in past literature was by Werner (1897). This author provided precise references to some characters, such as the dark belly and the longitudinal white line generated by the succession of spots located on the external portion of each ventral scale. This particular character was also confirmed by Angel (1946) and Cattaneo (1975), who even specified that this line terminates at the cloaca and is not present on the subcaudal scales.

The *subgrisea* form has been documented in France, Italy, Austria, Hungary, Croatia, Montenegro, Greece, Romania, Bulgaria, and the Caucasus (Werner 1897, Cattaneo 1975, Kreiner 2007, Schulz 1996, 2013). Most reports consist of observations of single individuals, although cases in which this form appears widespread are known (Werner 1897, Cattaneo 2017). This latter situation appears to be more frequent in Balkan localities, where individuals of the classic and *subgrisea* phenotypes share the same habitats. Photos portraying these subjects appear in recent texts (Schulz 1996, 2013, Kreiner 2007), where details and localities are reported. Photographs of the *subgrisea* morphotype of *Z. longissimus* are on online platforms such as iNaturalist, with specific localities of the observations. Both literature and online records point out a high concentration of *subgrisea* sightings in the Balkan peninsula, and the online data confirm the distribution area in literature records (Figure 3). Most of the specimens have been documented in Greece, Bulgaria, Romania, Serbia, and Croatia, with records also in Hungary and Slovakia. The easternmost records reconfirm the presence of such morph in Russia and Turkey, and the westernmost in Italy, France, and Spain (Figure 3).

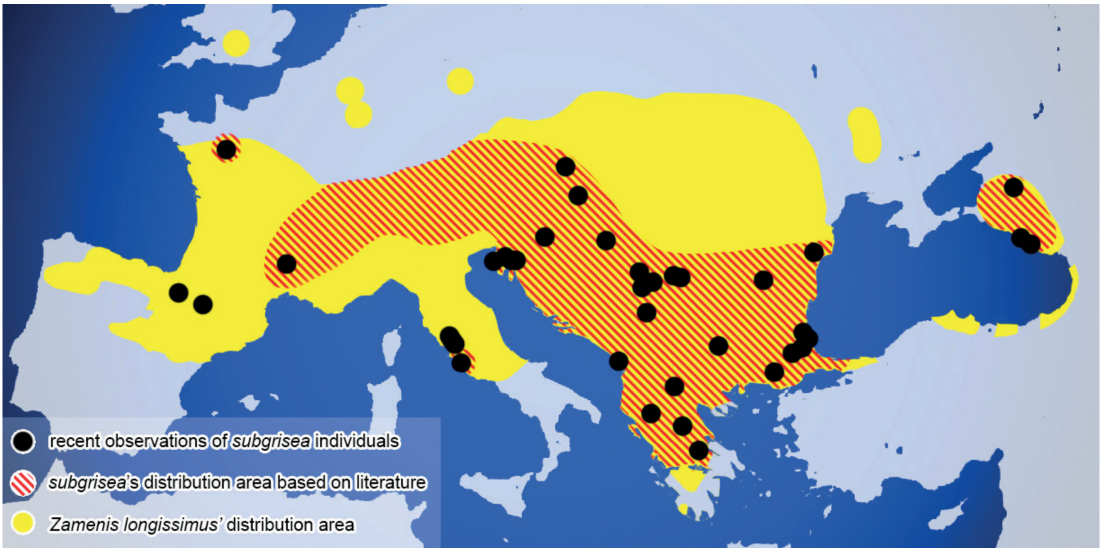

Figure 3. Distribution and observations of individuals of the *subgrisea* form. The yellow area indicates the distribution of *Zamenis longissimus*. Diagonal red lines indicate the predicted area of occurrence of the *subgrisea* form, based on information provided in the literature (Werner 1897, Cattaneo 1975, Kreiner 2007, Schulz 1996, 2013). Black dots correspond to recent observations of individuals of the *subgrisea* form from specific localities (Cattaneo 1975, Kreiner 2007, Schulz 1996, 2013, Zadravec and Lauš 2011) and from the iNaturalist platform (to April 2024).

In the literature, this morphotype has often been treated as melanic or axanthic (Zadravec and Lauš 2011, Cattaneo 2015, 2017). The results obtained by reproduction in captivity highlight the presence of several traits for which this form cannot be considered a single chromatic mutation. A character uniformly present in the *subgrisea* specimens is the black belly. This character is not found in the classic phenotype, in which the chin, belly, and tail are all colored with the same cream/yellowish shade. Pure melanic individuals are completely black, lacking even the small white spots between the dorsal scales, and any axanthistic specimen would have a white or light-grey venter, as in *subgrisea* juveniles, in which labials and the most anterior dorsal scales on the neck are white. Dark grey specimens with uniform white venters have been documented and are present on the iNaturalist platform. The same concept can be verified in the known axanthic/anerythristic forms of other colubroids (Fankhauser and Cumming 2008, Borteiro *et al*. 2021) and in the closely related species *Zamenis lineatus* (Russo *et al*. 2020).

A character not described in the literature is the presence of yellow pigment, which appears in some scales of *subgrisea* subjects. This pigmentation may affect any scale on the body, usually covering part of the scale. The yellow coloration is more evident in larger scales, such as the head shields, ventrals, and subcaudals but is also found on the dorsals. Yellowing scales in adults also occur in other species, such as the sympatric *Elaphe quatuorlineata* Lacépède, 1789 (pers. obs.). The presence of yellow pigmentation contradicts the hypothesis of axanthism and melanism because xanthophores are absent or rare in purely melanic colubrids (Kuriyama *et al*. 2013, 2016). The absence of iridophores was also reported in melanic individuals of the formerly congeneric *Elaphe quadrivirgata* (Boie, 1826) (Kuriyama 2013).

The white line on the external margins of the ventral scales is present in specimens of the classic phenotype. In the typically colored individuals, it is present although barely visible against the light venter.

The data obtained from breeding show that the genetics of this morphotype are recessive. The results shed light on the genetics of the original pair. One of the two *subgrisea* parents that generated the individuals born in 2017 was heterozygous because among the offspring of the second generation (obtained from the mating of the 2017 *subgrisea* specimens) some of the hatchlings exhibit the classic/dominant phenotype. Furthermore, in the third generation produced by individuals of the classic phenotype, all of the hatchlings exhibited the same coloration as the parents. Confirmation of the recessive nature of this morphotype is noted by Cattaneo (1975), where a pregnant *subgrisea* female, captured near Rome in a limited and mixed *subgrisea* colony (possibly now extinct), laid eggs producing offspring of both forms.

Anomalies in the livery appeared in two hatchlings of the classic phenotype (Figure 2 C, D). These anomalies involved typical characteristics of the *subgrisea* form, demonstrating that some characters of this morphotype may appear individually in heterozygous specimens. This may be a case of incomplete dominance of the alleles but would need to be further investigated in future generations.

Conclusion

The results obtained from breeding suggest that the *subgrisea* form is a recessive mutation. Within the groups raised in a controlled environment, specimens manifesting partial traits of such morphotypes were born. An individual born in the third generation, obtained from the mating of recessive heterozygous specimens exhibiting the dominant classic coloration, featured the black belly. This trait is characteristic of the *subgrisea* form, which does not exist in the classic phenotype, as darker iridescent dorsal tones manifested in a second individual. This would likely mean that this morphotype expresses itself in a combination of multiple traits and would be consequently not linked to a single gene, representing a more complex form than a single chromatic mutation.

The fact that such combinate traits can be found in the same form in different sites greatly distant from each other suggests that the observed specimens in the wild may not be the result of individual/occasional mutations, but instead these specimens have an ancient common origin that continues to be actively transmitted by recessive homozygous and heterozygous individuals within populations in which the classic phenotype appears to be the dominant one.

Recent genetic analyses and fossil records could help determine the origins of this form. It is supposed that in the last glacial period, the northern and central European populations of *Z. longissimus* became extinct, and these areas were subsequently repopulated in the Holocene, mainly by populations surviving in Balkan refuges (Musilová *et al*. 2010, Allentoft *et al*. 2018). Most of the observations of individuals or small populations of the *subgrisea* morphotype come from the Balkans. It is conceivable that this form was already present in the Pleistocene of southeastern Europe, and that it has survived to this day in certain central European populations that were recolonized by individuals with the genetics of the eastern clade. Further studies focused on dermal chromatophores could indicate where this form would have been advantaged and more functional during the glacial period (King 1988, Forsman 1995, Trullas *et al*. 2007, Geen and Johnston 2014, Monahan *et al*. 2022). The appearance of the *subgrisea* form could even be backdated because records of this morph come from the Caucasus and Greece (Schulz 1996, 2013, iNaturalist data), of which populations present profound genetic differences from the European and Transcaucasian ones (Musilová *et al.* 2007, 2010), suggesting that these southern-oriental groups remained isolated even longer and did not contribute to the colonizing expansion of Central Europe following the last glacial phase.

The *subgrisea* individuals and populations have been reported from coastal areas in Montenegro, Croatia, Italy, and Russia to mainland forests of the central and eastern European countries (Werner 1897, Cattaneo 1975, Kreiner 2007, Schulz 1996, 2013, Zadravec and Lauš 2011, iNaturalist data). Such differences both in altitudes and biotopes suggest that the presence of this form may not depend exclusively on environmental factors. The occurrence of the *subgrisea* form could not be selected by a switch of an environmental nature but would mainly depend *in primis* on the genetic pool of the micropopulation in question.

Considering the analyzed evidence, the Aesculapian snake could be considered a polymorphic species that expresses (at least) two forms: the dominant classic one and the recessive *subgrisea,* which occurs in several countries within the entire distribution of this colubrid.

Acknowledgments

I thank Rainer Fesser for donating the specimens of *Zamenis longissimus* of the phenotype described here to OPHIS, and all the iNaturalist users who shared their observations about *Z. longissmus*. I thank Ross D. MacCulloch, Jaime Bertoluci, and anonymous reviewers for revising and improving this manuscript.

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Editor: Ross D. MacCulloch

Phyllomedusa 23(2):111–124, 2024
© 2024 Universidade de São Paulo - ESALQ
ISSN 1519-1397 (print) / ISSN 2316-9079 (online)
doi: http://dx.doi.org/10.11606/issn.2316-9079.v23i2p111-124

Analyzing and comparing the buccal anatomy of European colubroid snakes: A reassessment of dentition models

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Abstract

Analyzing and comparing the buccal anatomy of European colubroid snakes: A reassessment of dentition models. Anatomical analyses reveal the presence of two rear-fanged models among European colubroids, categorized by different types of venom glands and rear maxillary fangs and differences in the features of the maxillary bones. The first model, opisthoglyphous, is characterized by the presence of purely serous venom glands, from which the secretions produced flow in the grooved posterior maxillary fangs. The posterior fangs are separated from the anterior ones by an alveolar diastema and a deviation of the maxillary bone. The second model, blade-fanged, has a venom gland composed of seromucous cells, anatomically positioned in a more recessed position compared to the opisthoglyphous model. In this model the posterior maxillary fangs are enlarged but have distal and mesial keels instead of grooves, but are not separated from the anterior teeth by diastemas or deviations of the maxillary bone. The European group of colubroids, previously composed almost exclusively of snakes considered "aglyphous," must be reconsidered in light of a system that consists mostly of opisthoglyphous snakes (predominantly the large group of Whip snakes, in addition to some species of Colubridae and Lamprophiidae), "aglyphous" snakes (exclusively Colubridae, including Ratsnakes and Dwarf snakes), and blade-fanged snakes (Natricidae, including Grass snakes and Water snakes). This reassessment is justified and supported by a comparative analysis of various European and non-European species, which confirms that "aglyphous" colubrids are residual forms of a rear-fanged model.

Keywords: Blade-fanged, Opisthoglyphous, Rear-fanged, Serpentes, Venom glands.

Resumo

Analisando e comparando a anatomia bucal de serpentes colubróides europeias: uma reavaliação dos modelos de dentição. Análises anatômicas revelam a presença de dois modelos com presas posteriores entre os colubróides europeus, categorizados por diferentes tipos de glândulas de veneno e presas maxilares posteriores e diferenças nas características dos ossos maxilares. O primeiro modelo, opistóglifo, é caracterizado pela presença de glândulas de veneno puramente serosas, das quais as secreções produzidas fluem nas presas maxilares posteriores sulcadas. As presas

Received 10 July 2024
Accepted 05 November 2024
Distributed December 2024

posteriores são separadas das anteriores por um diastema alveolar e um desvio do osso maxilar. O segundo modelo, presas em forma lâminas, apresenta uma glândula de veneno composta de células seromucosas, anatomicamente posicionadas em uma posição mais recuada em comparação ao modelo opistóglifo. Neste modelo, as presas maxilares posteriores são aumentadas, mas têm quilhas distais e mesiais em vez de sulcos, mas não são separadas dos dentes anteriores por diastemas ou desvios do osso maxilar. O grupo europeu de colubróides, anteriormente composto quase exclusivamente por serpentes consideradas “áglifas”, deve ser reconsiderado à luz de um sistema que consiste principalmente de serpentes opistóglifas (predominantemente o grande grupo de cobras-chicote, além de algumas espécies de Colubridae and Lamprophiidae), serpentes “áglifas» (exclusivamente Colubridae, incluindo Cobras-rato e Cobras-anãs) e serpentes com presas em forma de lâminas (Natricidae, incluindo cobras-da-grama e cobras-d’água). Esta reavaliação é justificada e apoiada por uma análise comparativa de várias espécies europeias e não-europeias, o que confirma que os Colubridae «áglifos» são formas residuais de um modelo com presas posteriores.

Palavras-chave: Glândulas de veneno, Opistóglifa, Presas em forma de lâmina, Presas posteriores, Serpentes.

Introduction

To date, most of the European colubroid species have been considered “aglyphous,” that is, equipped with non-grooved teeth (aglyphous = without glyphs), and consequently lacking structures suitable for the inoculation of venom during a bite (Kreiner 2007, Sindaco *et al.* 2013). The remaining small number of these snakes (5 of at least 37 species of colubroids) is considered “opistoglyphous,” i.e. having furrowed or grooved teeth suitable for conveying mixed oral secretions. As indicated by the word itself (opisthoglyphous = glyphs behind), these traits occur mainly in the posterior area of the maxillae of these snakes, and in some cases in a more advanced position, but never in the anterior area, as happens in proteroglyphous (Elapidae) and solenoglyphous (Viperidae) snakes (Weinstein *et al*. 2011). The grooved teeth alone, however, are not sufficient to determine the opisthoglyphous nature of a species. In fact, snakes that share this condition have a venom gland located beneath the supralabial scales, known as “Duvernoy’s gland.” This gland is named after the French anatomist George Louis Duvernoy, who first described it in 1832 (Taub 1966, 1967). Within this category of ophidians, the morphology of the teeth, the posterior maxillary fangs, and the structure of the maxillary bones vary in size and composition. In some species these traits can barely be seen, whereas in others the structures are more defined and developed, leading to the possibility that the bite of certain species can cause clinical consequences in humans that may vary from mild localized symptoms to death (Weinstein *et al*. 2011).

Recent studies have demonstrated that a group of snakes, including whip snakes and racers of the western Palearctic region, that have been considered aglyphous are actually opisthoglyphous (Paterna 2023, Paterna and Grano 2024). This study investigated the buccal anatomy of various European colubroids currently considered aglyphous and opisthoglyphous and other species considered opisthoglyphous to verify whether these conditions are still attributable to these species.

Materials and Methods

An adult male *Hierophis viridiflavus carbonarius* (Lacépède, 1789), an adult male *Zamenis longissimus* (Laurenti, 1768), and two adult females *Natrix helvetica* (Linnaeus, 1758), all recent road-killed specimens, were collected in the province of Teramo, Abruzzo, Italy. The four specimens, all in excellent condition, were transported to OPHIS Museo Paleontologico e Centro Erpetologico, where dissection and specimen preparation were carried out. Dissection was performed with surgical tools, and specimen preparation used sodium hypochlorite. During the different steps of each phase, photographs were taken using a Nikon Coolpix P510 camera. The prepared bones were subjected to microscopy at the laboratories of the Faculty of Veterinary Medicine of the University of Teramo, using a Nikon SMZ1500 stereomicroscope. The anatomy of the palatomaxillary arches of different colubroid species and genera was examined in vivo and specimens were compared with each other. Photographic details of the buccal anatomy of the examined individuals were acquired with the same camera model previously reported. *Elaphe quatuorlineata* (Bonnaterre, 1790), *Hemorrhois hippocrepis* (Linnaeus, 1758), *Hierophis viridiflavus, Natrix helvetica, Thamnophis marcianus* (Baird and Girard, 1853), *Zamenis longissimus,* and *Zamenis scalaris* (Schinz, 1822) were photographed at OPHIS, while specimens of *Boiga dendrophila divergens* (Boie, 1827), *Coronella austriaca* (Laurenti, 1768), *Dolichophis caspius* (Gmelin, 1789), *Hemorrhois ravergieri* (Ménétries, 1832), *Heterodon nasicus* (Baird and Girard, 1852), *Philodryas baroni* (Berg, 1895), and *Platyceps najadum* (Eichwald, 1831) were photographed in private collections.

Results

Anatomical Analysis

In *Hierophis viridiflavus* the venom gland is clearly distinguished from the supralabial salivary gland (Figure 1A). The venom gland consists of small pinkish lobules and occurs laterally from the posterior portion of the fifth supralabial to the entire seventh. Dorsally this gland reaches the upper border of the supralabial scales, while ventrally it reaches the lower limit of such scales only in the region of the enlarged posterior maxillary teeth. This point corresponds to the lowest point ventrally reached by the lower edge of the supralabial scales (Figure 1A). The venom gland is located immediately beneath the supralabial scales and the wall of connective tissue that covers it. Ventromedially the gland is in contact with the cuff/sheath that covers the posterior maxillary teeth (Paterna 2023). Medially, the gland is in contact with the lateral wall of the maxillary bone, where, during one of the sample preparation steps, it was possible to preserve the venom gland to observe its medial extension (Figure 1B). The posterior extension reaches the posterior limit of the maxillary bone and culminates at the adductor *mandibulae externus profondus.* Anteriorly, the most voluminous portion of the gland culminates at the alveolar diastema and slightly more anteriorly with the lateral nutritive foramen, while the most anterior limit reaches the space between the last and the penultimate anterior tooth. As previously described (Paterna 2023), in addition to the posterior teeth, these anterior teeth also have grooves (Figure 1B, C). The traits observed agree with the histological analysis of Taub (1967) in which, regarding the group in which the former genus *Coluber* (actually *Hemorrhois*) is included, it is stated: “the supralabial and Duvernoy’s glands are easily distinguishable in section, as they are composed of two markedly different types of cells. Duvernoy’s glands are encased in a capsule of connective tissue and are composed primarily of serous cells. There is a small lumen in the center of each tubule or cord, a lumen that generally leads into a secondary collecting duct. These secondary ducts join to form a collecting or primary duct, which is lined with a mucous epithelium and leads into the mucous epithelial sheath of the posterior maxillary teeth.”

In *Natrix helvetica* the venom gland is relatively larger than that present in *H. viridiflavus.* This gland is developed laterally and formed of white/cream lobules (Figure 2A), analogous to that described in *Natrix tessellata* (Akat *et al*. 2011). Compared to *H. viridiflavus,* this gland is less distinguishable from the

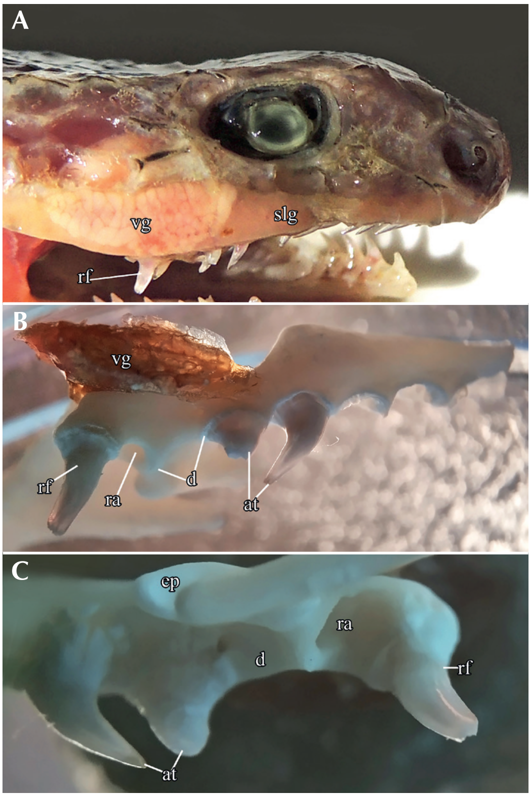

Figure 1. Adult male *Hierophis viridiflavus carbonarius* from Abruzzo, Italy. (**A**) Dissected head in right lateral view. (**B**) Prepared right maxilla in lateral view. (**C**) Prepared right maxilla in medial view. Abbreviations: at, anterior maxillary teeth; d, diastema; ep, ectopterygoid process; slg, supralabial salivary gland; ra, rear alveolus; rf, rear fang; vg, venom gland.

anterior supralabial salivary glands, which have a lobular structure of similar color. The venom gland is located below the sixth, seventh, and eighth supralabials, which delimit the gland extension both anteroposteriorly and dorsalventrally. In contrast to *H. viridiflavus,* the center of this gland is not found concomitantly with the enlarged posterior maxillary teeth, but these occur in the most anterior part of the gland (Figure 2B). These teeth do not have grooves, but they have a distal carina and a smaller mesial carina, which forms a blade shape. The anterior maxillary teeth are undulated, suitable for providing grip in holding viscous prey (Figure 2B; Paterna 2023). The venom gland is therefore located further back in this species and extends far beyond the posterior end of the maxillary bone and the adductor *mandibulae externus profondus*. Different from that observed in whip snakes, in *N. helvetica* the cuffs/sheaths of the posterior maxillary teeth are not distinct in coloration from the surrounding mucosa. The lateral wall of these cuffs/sheaths is hemispherical, and clearly distinguishable due to a depression produced by the medial contact with the venom gland. The orifices for the rear fangs are visible.

As a comparison species, similarly to the previous study (Paterna 2023), a colubrid was chosen that is sympatric with European whip snakes and grass snakes, the Aesculapian Snake *Zamenis longissimus.* In lateral view of the prepared specimen, the venom gland is absent and the supralabial salivary glands are present; these glands track the size and extension of the maxillary bone (Figure 2C). At the posterior extremity of the maxilla, behind the last maxillary tooth, the salivary gland is more dorsoventrally compressed and a different color and consistency compared to the more anterior portion. This could conceivably be a remnant of a possible Duvernoy's gland/venom gland. The last trait is identifiable in a deeper stage of preparation than the one illustrated, although it is visible in Figure 2C. This eventual remnant is positioned just below the Harderian gland, which is located posterior to the postorbital bone and adjacent to the temporal scales (Figure 2C). The supralabial salivary glands, together with the infralabial ones, have the function of lubricating the prey during swallowing through the secretion of mucus (Kochva 1978). In this species ("aglyphous") the reverse condition of the opisthoglyphous model is notable, whereby the anterior maxillary teeth are longer than the posterior ones (Figure 2C; Paterna 2023).

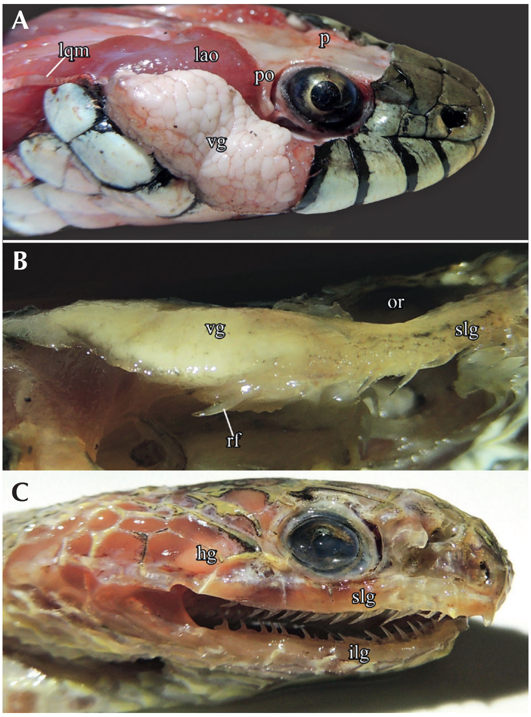

Figure 2. Adult specimens of *Natrix helvetica* and *Zamenis longissimus* from Abruzzo, Italy. (**A**) *Natrix helvetica* dissected head in right lateral view. (**B**) *Natrix helvetica* dissected head in ventrolateral view. (**C**) *Zamenis longissimus* dissected head in right lateral view. Abbreviations: hg, Harderian gland; ilg infralabial salivary gland; lao, levator anguli oris; lqm, ligamentum quadrato-maxillare; or, orbit; p, parietal bone; po, postorbital bone; rf, rear fang; slg, supralabial salivary gland; vg, venom gland.

Palatomaxillary Arches

Of the three species discussed, the palatomaxillary arches were examined in both dead and in vivo specimens, with the aim of comparing them with other species of European and non-European aglyphous and rear-fanged colubroids (Figure 3). In the material examined, in the palatomaxillary arch of *H. viridiflavus* (Figure 3A) the cuffs/sheaths that medially extend from the venom glands and cover the posterior maxillary teeth are easily visible and distinguishable (Figure 3B). This trait is also observable in other inspected opistoglyphous species, such as the European whip snakes *Platyceps najadum, Hemorrhois hippocrepis, Hemorrhois ravergieri,* and *Dolichophis caspius* (Figure 3E–H), and the extra-European opistoglyphous *Boiga dendrophila, Philodryas baroni,* and the rear-fanged *Heterodon nasicus* (Figure I–K). Cuffs/sheaths covering the enlarged maxillary posterior teeth are present in *N. helvetica* (Figure 3C), but less visible than the ones found in whip snakes (Figure 3D) and in the American natricid *Thamnophis marcianus,* where instead these are more distinguishable (Figure 3L). Consequently, the palatomaxillary arches of this European natricid appear similar, at first sight, to those of the sympatric aglyphous colubrids such as *Elaphe quatuorlineata, Z. longissimus,* and *Zamenis scalaris* (Figure 3M–O), as no color variations highlight the cuffs/sheaths. The substantial difference is that in the natricids the lateromedial space between the walls of the supralabials and the maxillary bones is filled by the venom gland (Figure 3), while in ratsnakes this is hollow, generating a large longitudinal canal that occurs between the maxilla and the posterior supralabial scales (Figure 3M–O). Furthermore, in aglyphous colubrids, the posterior maxillary teeth are uncovered and visible, lacking the structures present in the other analyzed groups (Figure 3 M–O).

In the analysis of the palatal arches of another European colubrid, *Coronella austriaca,* the presence of enlarged posterior maxillary teeth covered by cuffs/sheaths that morphologically resemble those previously described is observable. Analogous to *N. helvetica,* these structures show the same color as the surrounding mucosa, and the openings from which the posterior maxillary teeth unsheathe are observable (Figure 3P).

As control tests, other colubrids from the Old World, including *Elaphe anomala, Elaphe*

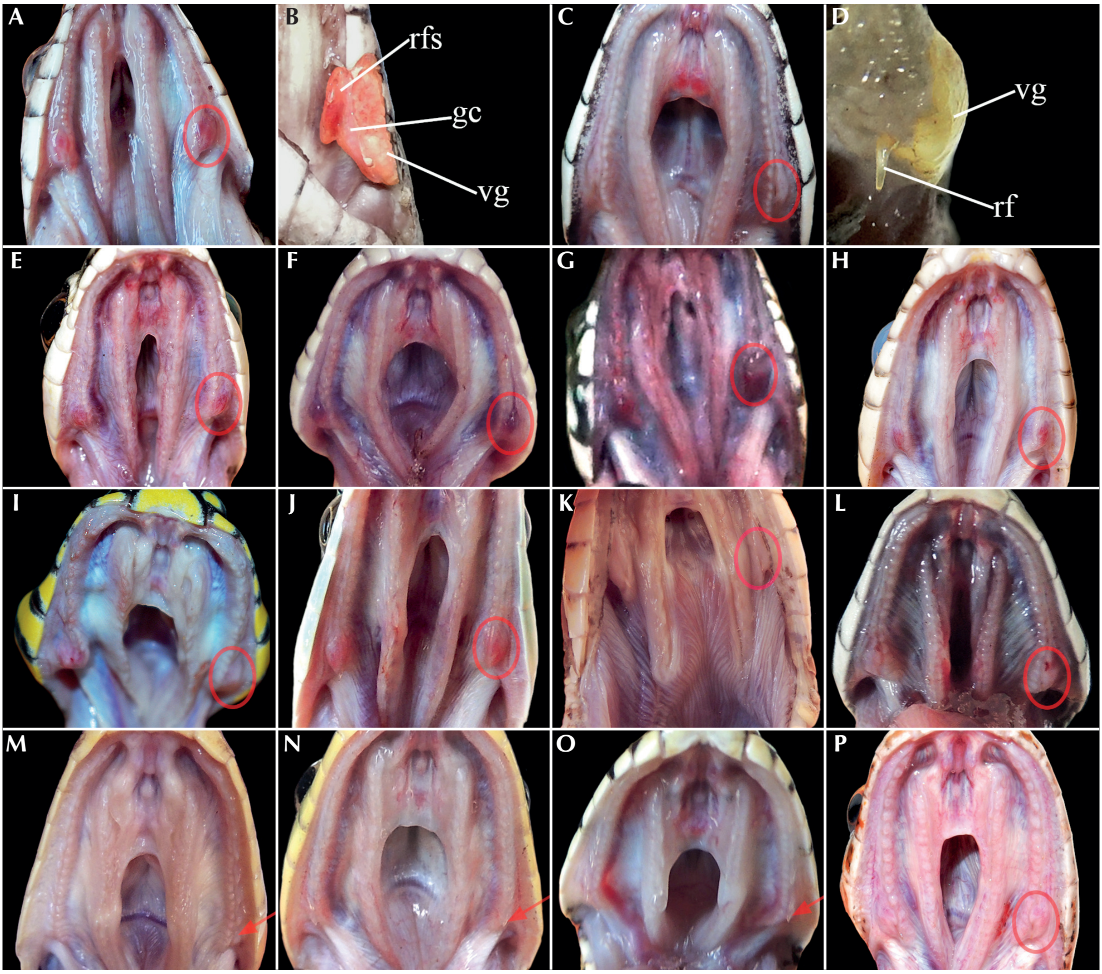

Figure 3. Analogies and differences among the palatomaxillary arches of aglyphous, opisthoglyphous, and blade-fanged colubroids. From the top, first horizontal row: (**A**) *Hierophis viridiflavus carbonarius*, (**B**) *Hierophis viridiflavus carbonarius*, dissected right maxilla in ventral view, (**C**) *Natrix helvetica*, (**D**) *Natrix helvetica*, dissected right maxilla in ventral view. Second row, European whipsnakes: (**E**) *Platyceps najadum*, (**F**) *Hemorrhois hippocrepis*, (**G**) *Hemorrhois ravergieri*, (**H**) *Dolichophis cascpius*. Third row, extra-European rear-fanged snakes: (**I**) *Boiga dendrophila divergens*, (**J**) *Philodryas baroni*, (**K**), *Heterodon nasicus,* (**L**) *Thamnophis marcianus*. Fourth row, ratsnakes and the smooth snake: (**M**) *Elaphe quatuorlineata*, (**N**) *Zamenis longissimus*, (**O**) *Zamenis scalaris*, (**P**) *Coronella austriaca*. Red circles delimitate the sheaths of rear fangs, red arrows indicate the last (naked) maxillary tooth. Abbreviations: gc, connective tissue capsule of the gland; rf, rear fang; rfs, sheath of rear fangs; vg, venom gland.

bimaculata, Elaphe carinata, Elaphe dione, Orthriophis taeniurus friesi, Zamenis persicus, and from the New World, including *Pantherophis guttatus* and *Pituophis catenifer sayi,* were examined. In the palatomaxillary arches of the Old World species, observed in *E. quatuorlineata, Z. longissimus,* and *Z. scalaris,* there are no cuffs/sheaths in relation to the posterior maxillary teeth, much less the lateromedial thickening of the supralabial walls, while in the two New World species small cuffs/sheaths are present in the posterior margins of the maxillae, although less well defined than the whip snakes, grass snakes, and the other rear-fanged snakes analyzed.

Discussion

In addition to the presence of Duvernoy's glands/venom glands, several traits may be completely or partially shared in opisthoglyphous species. These are: 1) enlarged posterior maxillary teeth; 2) grooved posterior maxillary teeth; 3) blade-shaped posterior maxillary teeth; 4) alveolar diastema separating the posterior maxillary teeth from the anterior ones; 5) mediolateral or/and dorsoventral deviation of the maxillary bone corresponding to the alveolar diastema; and 6) presence of cuffs/sheaths covering the posterior maxillary teeth in the palatomaxillary arch (Table 1). A seventh trait, which does not affect European colubrids, is the extreme reduction of the jaw in which the number of teeth is considerably reduced, as in the case of *Dispholidus* (Kardong 1982). Deviations of the maxillae and diastemas that separate the posterior teeth from the anterior ones, as in the case of *Hierophis viridiflavus* and *Dolichophis* (Paterna 2023, Paterna and Grano 2024), are traits shared between opistoglyphous species (Weinstein *et al*. 2011) and not present in the aglyphous colubroid species, or in those in which only enlarged posterior maxillary teeth are present. Such a condition is documented in *Elaphe xiphodonta* (Qi *et al*. 2021) and in other species of Asian colubrids (Cundall and Irish 2008, Qi *et al*. 2021), many of which predominantly prey on anurans (Knox and Jackson 2010). The separation of the posterior maxillary teeth from the anterior ones is instead present in opisthoglyphous colubrids as in the case of *Boiga irregularis, Thrasops flavigularis, Thrasops jacksoni,* and *Philodryas baroni* (Broadley and Wallach 2002, Weinstein *et al*. 2011). The presence of grooves on the enlarged maxillary posterior teeth further justifies the role they have in the conveyance of the secretions of the venom glands. This trait does not seem to be a constant within the ophidians currently considered opisthoglyphous, where it may even be absent, as in the case of *Heterodon platirhinos, Rhabdophis subminiatus, Rhabdophis tigrinus, Helicops modestus,* and *Alsophis cantherigerus* (Mittleman and Goris 1974, Jackson 2003, Weinstein *et al*. 2011, Oliveira *et al*. 2016), where the term "rear-fanged" better fits these species. A further trait that does not represent a constant in opisthoglyphous snakes is the deviation of the maxillary bone, which repositions the enlarged and/or modified posterior teeth on a different plane than the anterior ones. This is the case of *Hydrodynastes gigas, Liophis breviceps, Helicops leopardinius,* and *Homalopsis buccata* (Knox and Jackson 2010). The occurrence of visible cuffs/sheaths in the posterior ends of the maxillae, as observed in the European whip snake/racers (*Hierophis, Hemorrhois, Platyceps,* and *Dolichophis*), it is a trait present in many species of opisthoglyphous colubroids such as the sympatric *Malpolon monspessulanus* and the extra-European species analyzed here: *Boiga dendrophila divergens, Philodryas baroni,* and *Heterodon nasicus* (Figure 3). The presence of this trait, obvious in whip snakes, and the absence of this trait in purely constrictor colubrids (Figure 3) represents yet another marked feature that firmly places these animals within the group of opisthoglyphous snakes. Together with the presence of these cuffs/sheaths, the presence of the venom gland/ Duvernoy's gland is notable, which fills the space occurring between these and the supralabial

Table 1. Variation of the presence of the opisthoglyphous traits within the three European colubroids examined and three allopatric opisthoglyphous snakes.

Species	Duvernoy's glands	Enlarged rear teeth	Grooved rear teeth	Blade rear teeth	Alveolar diastema	Deviation of the maxilla	Rear fangs' cuffs/sheats
Hierophis viridiflavus	•	•	•		•	•	•
Natrix helvetica	•	•		•			•
Zamenis longissimus							
Boiga dendrophila	•	•	•		•		•
Heterodon nasicus	•	•			•	•	•
Philodryas baroni	•	•	•		•	•	•

scales (Figure 3). In *Natrix helvetica* and *Coronella austriaca* the cuffs/sheaths are different and less evident from those of the whip snakes, in which the color is homogeneous with the surrounding mucosa, even if the bulging at the posterior margin of the maxillary bone and the bulking of the venom gland are distinguishable (Figure 3C, D, P).

No less worthy of the same consideration are the traits observed in *N. helvetica.* Unlike *H. viridiflavus* and the Western Palearctic clade of whip snake/racers, this species possesses enlarged posterior maxillary teeth, but these are characterized by large distal and a smaller mesial carinae, which confer a blade-like shape to the enlarged rear fangs (Paterna 2023). Although the maxillae of *N. helvetica* do not have many of the features typical of the opisthoglyphous colubroids (Table 1), such as alveolar diastemas and deviations (Paterna 2023), the large venom glands and the blade-like teeth still play a role in predation and the inoculation of mixed oral secretions (Jackson *et al*. 2016). In the past, some authors argued that in the species having a specialized diet of anurans, these teeth functioned to pierce the tegument of the prey to neutralize the anti-predatory mechanism by which many amphibians inflate to resist swallowing (Pope 1947, Edgren 1955, McAlister 1963). Today we know instead that these function to lacerate the prey's tegument and to introduce buccal secretions (Weinstein and Kardong 1994, Averill-Murray 2006). In defense of this last thesis, many natricids are today considered "mid-venomous" or "low toxic" (Weinstein *et al*. 2011). Some are also highly venomous to humans, as in the case of the genus *Rhabdophis,* in which the bite can have serious and even fatal consequences in humans (Nakayama *et al*. 1973, Ogawa and Sawai 1986, Kikuchi *et al*. 1987). In our case, analogous to the Old World whip snakes/racers (Paterna and Grano 2024), there are reports in the literature in which the clinical consequences following the bite of *Natrix natrix* (*sensu lato*) in humans (Gardner-Thorpe 1967, Satora 2004, Gläßer-Trobisch and Trobisch 2008) and in anurans are described (Delisle 1981). Similar cases are reported in the related American natricids of the genus *Thamnophis* (Vest 1981, Hayes and Hayes 1985, Gomez *et al*. 1994, Grenard 1994). Perry *et al*. (2018) discussed how the genes that encode protein composition of the venom of *Thamnophis sirtalis* "find their evolutionary origins in a rear-fanged venomous colubrid species," while other studies have been conducted on the mechanical action involving the emptying of the glands of Duvernoy present in this genus of snakes (Jansen and Foehring 1983, Kardong and Luchtel 1986).

Regarding the presence of Duvernoy's glands/venom glands in both *N. helvetica* and *H. viridiflavus,* information is provided in the previous literature. Taub (1967) analyzed 180 species from 120 genera of colubroids, which he divided into groups. One group consisted of colubrids that lack Duvernoy's gland, generally in the genera *Boaedon, Elaphe* (*sensu lato*), and *Lampropeltis*. The Aesculapian Snake *Z. longissimus* is included in this group. A second group contains colubroids that possess a Duvernoy's gland composed of mucous cells intermingled with serous cells. This group include the natricids *Thamnophis* and *Nerodia* (genera related to *Natrix*), and *Rhabdophis*. A third group consists of colubroids that have a purely serous Duvernoy's gland. Among the species examined are the European *Hemorrhois ravergieri* (Whip Snake of the same clade as *H. viridiflavus*), *Malpolon monspessulanus,* and *Telescopus fallax,* together with other non-European opisthoglyphous species of the genera *Boiga, Spalerosophis, Philodryas,* and *Psammophis* (more species belonging to this last group have been chosen to be part of the actual research). Together with the "officially considered" opisthoglyphous species, the smooth snake, *Coronella austriaca,* is included in this group (Taub 1967). The inclusion of the European species examined by Taub in this last group confirms the morphological and anatomical analogies observed in this study (Figure 1, 3) and the consideration of the opisthoglyphous model in whip snakes discussed in previous studies (Paterna 2023, Paterna and Grano 2024). Subsequently, many studies have analyzed these types of glands and their secretions in different species (Hill and Mackessy 2000, Mackessy 2002). Some of these studies provide photographs, for example Jackson *et al*. (2016), in which it is noted that the large serous-secreting venom gland of *M. monspessulanus* is morphologically similar to that observed in *H. viridiflavus* (Figure 1). The Montpellier snake *M. monspessulanus* has been considered the most venomous colubroid among European opisthoglyphous species. In addition, the cuffs/sheaths covering the posterior maxillary teeth in this species are similar to those found in *H. viridiflavus*. The morphology of the supralabial and infralabial glands of *H. viridiflavus* is described (Baccari *et al*. 2002), but no further references are made to other glands.

Regarding the genus *Natrix,* many authors state that the anatomy of these ophidians is unclear; in fact, Phisalix (1922) described the presence of Duvernoy's glands in *N. natrix,* while Smith and Bellairs (1947) reported the opposite. Akat *et al*. (2011) reconfirmed Taub's description, stating that the venom glands of *Natrix tessellata* consists of many small lobules mainly composed of mucous and seromucous acini. The authors also noted that Duvernoy's gland secretions of *Natrix tessellata* have toxic effects on prey. The glands were composed mainly of seromucous cells. and the authors compare them to those of known venomous ophidians, suggesting that further research on the serum secreted by these glands could reveal medical and pharmacological properties (Akat *et al*. 2011).

The smooth snake, *Coronella austriaca,* possesses venom glands of the same type as the opisthoglyphous snakes (Taub 1967, Kochva, 1978), and has enlarged posterior maxillary teeth covered by cuffs/sheaths visible in its palatomaxillary arch. This snake, together with the congeneric *C. girondica,* requires further anatomical investigation to establish whether both share this condition, and in which sub-group of the rear-fanged snakes these species belong. The genus *Coronella* separated from the macro-group of Old World "aglyphous" rat snakes (Taub 1967, Nagy *et al*. 2004, Figueroa *et al*. 2016), followed by New World rat snakes, of which *Pantehrophis guttatus* and *Pituophis catenifer sayi* have small cuffs/sheaths at the posterior end of the maxillae. Jackson *et al*. (2016) reported the presence of a small venom gland in *P. guttatus.* Although Old World rat snakes appear to lack modified fangs and venom glands, they have residual traits that can be traced back to these adaptations. The four-lined rat snake, *Elaphe quatuorlineata,* features a longitudinal groove and depressions in the posterior maxillary teeth (Paterna 2023), and *Z. longissimus* appears to have a small residual gland that may differ from the supralabial salivary gland. These features are unusual in a fully aglyphous model because they represent relics of a previous opisthoglyphous model. This would explain the different levels of adaptation phylogenetically related to colubrine snakes, in which the rear-fanged model is more conserved in *Coronella,* scarcely conserved in *Pantherophis* and *Pituophis,* and completely lost in *Elaphe* and *Zamenis.* Another case of loss of the opisthoglyphous model within European colubrids is that of the genus *Eirenis,* a group of dwarf snakes descended from whip snakes, and which, following miniaturization and a specialized diet, developed a type of aglyphous dentition suitable for the predation of arthropods (Çiçek and Mermer 2007, Rajabizadeh 2019, Paterna and Grano 2024). The "loss" of the opistglyphous model across entire genera within rear-fanged colubrids is not limited to the two cases just discussed. Examining the phylogenetic trees of this family (Figueroa *et al*. 2016) another striking case is that of the genus *Dasypeltis.* This genus of snakes is equipped with small rudimentary teeth, an adaptation to their specialized diet consisting of bird eggs, which derived from the opisthoglyphous snakes of the genera *Toxicodryas* and *Boiga.* The latter are highly developed rear-fanged snakes whose bite can cause clinical consequences in humans.

Conclusions

From the data and evidence obtained, it is clear how European whipsnake/racers came to share traits of the opisthoglyphous model. In some cases, these species even have more traits than other snakes currently considered to be opisthoglyphous. From anatomical analyses of the species examined, it is possible to generate guidelines for identification of the opisthoglyphous state of certain ophidians, even when this has not yet been verified or is currently poorly identified. The most explicit features are observable using dissection, bone preparation, and microscopy, but a preliminary evaluation can also be obtained without resorting to these practices. An initial identification of a possible opisthoglyphous model can be obtained by inspecting the head and jaws of a particular colubroid. Confirmed by examination of the palatomaxillary arches, the presence of cuffs/sheaths at the posterior margin of the maxillae is a trait that indicates the opistoglyphus model. The color and morphology of the mucosa covering the posterior maxillary teeth are easily distinguishable from the surrounding mucosa. A second feature that can be observed in the open jaws of opisthoglyphous colubroids is the thickness that occurs lateromedially between the posterior portion of the maxilla and the supralabial scales. In many opisthoglyphous species, the presence of the venom gland indicates a larger or more filled space than in the aglyphous species, where this transverse space is generally hollow (Figure 3). A third factor is not diagnostic on its own, but in some species could represent further confirmation following the observation of the first two traits, is the shape of the ventral margin of the supralabials. As observed in European whipsnakes/racers and non-European opisthoglyphous species, a distinctly wavy silhouette in the posterior supralabials (e.g., *Hemorrhois, Spalerosophis*) could correspond to the dorsoventral deviation of the maxillary bones, the presence of enlarged posterior teeth, and the venom gland. A fourth consideration, and not at all obvious, is whether cases of envenomation or clinical reactions in humans or animals occur following a snakebite.

Particular attention should be paid to colubroids such as *Natrix helvetica,* which presents fewer opistoglyphous traits than whip snakes/racers, but which nevertheless may have structures for the production and inoculation of toxins. Both by definition and for anatomical reasons, it would be wrong to consider these snakes opisthoglyphous, but because they are capable of inflicting toxic bites, they can be grouped into a different category of colubroids that could be called "blade-fanged." The distinction of this new category from the opisthoglyphous group might seem counterintuitive because the term "opisthoglyphous" is rarely used. Snakes in which these dentition models are present are generally referred to as "non-front-fanged" snakes (Weinstein *et al*. 2011, Jackson *et al*. 2016, Weinstein 2017). Furthermore, the use of the name "Duvernoy's glands" is not recommended because these structures are homologous to the venom glands of elapids and viperids (Kochva and Gans 1970, Weinstein and Kardong 1994, Fry *et al*. 2008). Because the toxic oral secretions produced by rear-fanged snakes are intended to subdue prey, these secretions are considered "venom" in all respects (Jackson *et al*. 2016), and "it is most incorrect to view colubrid venoms as imperfect venoms" (Mackessy 2002). The use of the terms "opisthoglyphous" for whip snakes/racers, and "blade-fanged" for natricids is, however, necessary in this work to distinguish the different morphology and biology of the taxa taken into consideration. Within the "non-front-fanged" snakes several variations of the "rear-fanged" model occur, including some cases in which the enlarged teeth are not positioned at the posterior end of the maxillae and the "aglyphous" snakes.

In light of this study and in the two previous works (Paterna 2023, Paterna and Grano 2024), we can group European colubroids into three main categories corresponding to the different dentiferous models: "opisthoglyphous, blade-fanged, and aglyphous." Contrary to the past, current estimates indicate that the group comprising the largest number of European species is that of the opisthoglypha, consisting of taxa of Colubridae and Lamprophiidae, followed by the "aglypha" composed exclusively of colubrids, and finally the blade-fanged group, composed of natricids (Table 2). It is interesting and at the same time an inevitable consequence to see how within the aglypha, the medium- to large-sized colubrids all belong to *Elaphe* and *Zamenis,* are all powerful constrictors, and are all phylogenetically related. In the past, all were placed within the former genus *Elaphe* (Fitzinger) (Schulz 1996). The term "aglyphous" has almost always been reported in quotation marks because

Table 2. Dentition models within European colubroids. Within "rear-fanged" are indicated those genera that need further investigation to determine in which sub-group these should be placed.

Group	Genus	Type	Past consideration
Whip snakes	*Hierophis*	opisthoglyphous	aglyphous
	Hemorrhois	opisthoglyphous	aglyphous
	Platyceps	opisthoglyphous	aglyphous
	Dolichophis	opisthoglyphous	aglyphous
Dwarf snakes	*Eirenis*	aglyphous	aglyphous
Smooth snakes	*Coronella*	rear-fanged	aglyphous
False smooth snakes	*Macroprotodon*	rear-fanged	opisthoglyphous
Catsnakes	*Telescopus*	opisthoglyphous	opisthoglyphous
Ratsnakes	*Elaphe*	aglyphous	aglyphous
	Zamenis	aglyphous	aglyphous
Grass/water snakes	*Natrix*	blade-fanged	aglyphous
Lamprophiids	*Malpolon*	opisthoglyphous	opisthoglyphous

following what has been discussed, its application to colubrids would become technically incorrect, since this word describes the dentiferous model of ophidians of other families (e.g., Boidae and Pythonidae), and no longer refers to European colubrid genera such as *Eirenis, Elaphe,* and *Zamenis,* which were part of an earlier opisthoglyphous model. Obviously, as happened for the ophidians analyzed in this study, in-depth analyses are necessary to verify certain conditions in species that are still unsuspected or little studied to date. Discovery of such conditions could lead to a further reclassification of these, and determine if a certain species is in the process of development or suppression of a rear-fanged model. Additionally, the evolution of venom glands is not parallel with the evolution of certain types of posterior maxillary fangs, and of the number and arrangement of these (Taub 1967). Within the species analyzed in this study, the "adaptation" to the different types of rear-fanged models present profound variations even within the same group or genus (e.g., Whipsnakes).

A final consideration is that being opisthoglyphous, blade-fanged, or rear-fanged (to indicate other intermediate groups) does not mean being toxic or harmful to humans. An opistoglyphous species does not necessarily have to cause effects in humans to be considered as such, but it must present certain anatomical characteristics. The structures seen in these reptiles have developed in colubroids as adaptations and specializations derived from the diet and the ecology of the latter (Modahl *et al.* 2016) in relation to predation and digestion of prey, as well as in proteroglyphous and solenoglyphous snakes. The case of European colubroids confirms the thesis that the rear fangs (venom fangs) did not evolve independently multiple times but find their origin in common ancestors (Palci *et al*. 2021), and loss of fangs is a recurring trend within this superfamily (Westeen *et al*. 2020). Fangs and venom glands developed prior to the appearance of colubroids (Fry *et al*. 2009, Figuero *et al*. 2016, Streicher and Ruane 2018, Zaher *et al*. 2019), whose most basal species alive today are opisthoglyphous, which has subsequently differentiated within the several groups that have preserved, developed, or lost various traits. The key model used in this study can be applied to reclassify colubroids in other regions of the world that have a similar ophidiofauna.

Acknowledgments

I deeply thank my friends and colleagues who supported me in this study by providing the samples analyzed herein, the specimens observed in vivo, and the photographic material: Alessio Martella, Andrea Fioretti, Bruno De Ruvo, Francesco Di Carlo, Grégoire Meier, Luca Fioretti, Luca Palazzese, Marco Di Feliciantonio, Matteo Meraviglia, Samuele Valeani, Simone Di Feliciantonio, Tomaz Jagar. I thank Jaime Bertoluci and Janalee Caldwell for their work on my manuscript, and the anonymous reviewers for their comments.

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Editor: Jaime Bertoluci

Phyllomedusa 23(2):125–135, 2024
© 2024 Universidade de São Paulo - ESALQ
ISSN 1519-1397 (print) / ISSN 2316-9079 (online)
doi: http://dx.doi.org/10.11606/issn.2316-9079.v23i2p125-135

Dorsal polymorphism and perch height use by *Liolaemus bellii* (Squamata: Liolaemidae) in central Chile

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Abstract

Dorsal polymorphism and perch height use by *Liolaemus bellii* (Squamata: Liolaemidae) in central Chile. Dorsal polymorphism is a trait broadly studied in different animal species, and polymorphism within a population is common. It is presumed that individuals with a given color pattern will have an ecological or social advantage over conspecifics without it. In several lizard species, dorsal pattern polymorphism has been related to perch use, which may be determined by a lizard's social status. Here, we studied dorsal polymorphism and perch height use by *Liolaemus bellii,* a high-elevation, diurnal, saxicolous, viviparous lizard in central Chile. Discernable sexual dimorphism is absent in the species. Males and females show two morphs, a patternless one and an irregular, colored dorsal pattern characterized by olive or yellowish-brown color, flecked with yellow, black, and brown, with 8 to 10 blackish crossbars broken backward over the vertebral field (chevrons), forming an open V-like angle. We aimed to determine if lizards with this distinct chevron dorsal pattern perch higher than patternless conspecifics and if so, if conspecific social pressures might be responsible. Also, we measured eight morphological variables and evaluated their effect on perch height as covariables when lizards were grouped by sex, age class, and morph. None of the studied variables significantly influenced the presence of any morph. However, the patternless morph was found significantly more often in older lizards. Naturally broken tails increase in older lizards, although lack of a complete tail is not related to dorsal pattern. Bigger lizards with longer tails perched significantly higher, and therefore size may play a social role in perch use in *L. bellii*. Dorsal polymorphism in *L. bellii* is a conspicuous trait that has been overlooked in scientific literature concerning the species.

Keywords: Behavior, Lizard, Reptile, Saxicolous, Sociality.

Received 26 September 2023
Accepted 23 May 2024
Distributed December 2024

Resumo

Polimorfismo dorsal e uso da altura de poleiro por *Liolaemus bellii* (Squamata: Liolaemidae) no centro do Chile. O polimorfismo dorsal é uma característica amplamente estudada em diferentes espécies animais, e o polimorfismo intrapopulacional é comum. Presume-se que os indivíduos com um determinado padrão de coloração terão uma vantagem ecológica ou social sobre os indivíduos da mesma espécie sem o padrão. Em diversas espécies de lagartos, o polimorfismo do padrão dorsal tem sido relacionado ao uso de poleiros, que pode ser determinado pelo status social do lagarto. Estudamos aqui o polimorfismo dorsal e o uso da altura do poleiro por *Liolaemus bellii,* um lagarto vivíparo, diurno, saxícola e de grandes altitudes do centro do Chile. O dimorfismo sexual discernível está ausente na espécie. Machos e fêmeas apresentam duas formas, uma sem padrão e outra com um padrão dorsal colorido irregular, caracterizado pela cor verde-oliva ou marrom-amarelada, salpicado de amarelo, preto e marrom, com 8 a 10 barras transversais enegrecidas dobradas para trás sobre o campo vertebral (divisas), formando um ângulo aberto em forma de V. Nosso objetivo foi determinar se lagartos com esse padrão dorsal distinto de divisas empoleiram-se em maiores alturas do que os da mesma espécie sem padrão e, em caso afirmativo, se as pressões sociais coespecíficas podem ser responsáveis. Além disso, medimos oito variáveis morfológicas e avaliamos seu efeito na altura do poleiro como covariáveis quando os lagartos foram agrupados por sexo, classe etária e forma. Nenhuma das variáveis estudadas influenciou significativamente a presença de qualquer forma. No entanto, a forma sem padrão foi encontrada significativamente com mais frequência em lagartos mais velhos. Caudas quebradas naturalmente aumentam em lagartos mais velhos, embora a falta de uma cauda completa não esteja relacionada ao padrão dorsal. Lagartos maiores com caudas mais longas empoleiraram-se em alturas significativamente maiores e, portanto, o tamanho pode desempenhar um papel social no uso de poleiros em *L. bellii.* O polimorfismo dorsal em *L. bellii* é uma característica cosnpícua que tem sido negligenciada na literatura científica relativa à espécie.

Palavras-chave: Comportamento, Lagarto, Réptil, Saxícola, Socialidade.

Introduction

Dorsal polymorphism has been studied in many insect, arachnid, bird, crustacean, mammal, and herpetofauna species (Mayr 1963, Oxford and Gillespie 1998, Hoffman and Blouin 2000, Steffen 2010). The polymorphism may be expressed as color or as pattern variation between populations and within individuals of the same population (Eifler and Fogarty 2006, Santos *et al*. 2018, Utsumi *et al*. 2021). Dorsal polymorphism may be sex-restricted, or age-associated, but in both scenarios, the polymorphism may have different ecological functions (Mayr 1963, Eifler and Fogarty 2006, Steffen 2010, Ortega *et al*. 2014). In squamate reptiles, dorsal polymorphism is associated with background matching, antipredator defense and escape, habitat use, resource availability, mating opportunities, sexual selection, social structure, and perch use (Mayr 1963, Zucker 1986, Eifler and Fogarty 2006, Ortega *et al*. 2014, Medina *et al*. 2017). Thus, it is expected that individuals with a certain dorsal color or pattern will have some sort of advantage over conspecifics without the trait (Farallo and Forstner 2012). These traits may influence perch selection.

Perching is a common behavior in numerous arboreal and saxicolous lizard species (Zucker 1986, Núñez 1996, Smith and Ballinger 2001, Losos 2009). This behavior may be influenced by ecological selective forces (Losos 1990, Baird and Sloan 2003, Radder *et al*. 2006, Ortega *et al*. 2014, Medina *et al*. 2017). Both perch type and location may affect the lizard's thermoregulation, visibility, mating opportunities, and opportunities to escape from predators (Scott 1976, Avery 1991, Eifler and Fogarty 2006, Vicenzi *et al*. 2021). For example, after the reproductive season, female *Crotaphytus collaris* (Say, 1822), a saxicolous diurnal lizard, decreased predation risk primarily by diurnal snakes by perching on high rocks close to a refuge and distant from vegetation that might conceal a snake (Eifler and Fogarty 2006). In different *Liolaemus* lizard species, tail loss has been correlated with the frequency of observations on elevated perches (Jaksić and Fuentes 1980). Noteworthy, perch height selection by a lizard may vary throughout the year to increase its feeding opportunities while adjusting its diet as shown in *Gonatodes humeralis* (Guichenot, 1855) (Miranda and Andrade 2003).

Perch height occupation may also result from social relations among conspecifics (Zucker 1986, Fox and Shipman 2003, Deodhar and Isvaran 2018). For example, in *Agama, Anolis, Crotaphytus, Psammophilus, Sceloporus,* and *Urosaurus* lizards, males and older individuals perch higher than females and younger conspecifics (Pounds and Jackson 1983, Zucker 1986, Anibaldi *et al*. 1998, Ramírez-Bautista and Benabib 2001, Baird and Sloan 2003, Losos 2009). During the breeding season, territorial males of *Psammophilus dorsalis* (Gray, 1831) display from prominent rock perches (Deodhar and Isvaran 2018). Although males use high perches year-round, bigger males perch on higher perches compared to smaller lizards (Radder *et al*. 2006).

Thus, use of higher perches may relate to lizard sociality: higher perches might offer better opportunities for individuals to establish a dominance hierarchy, defend their territory, advertise their presence to conspecifics, and perform courtship displays (especially in polygamous species) (Zucker 1986, Ramírez-Bautista and Benabib 2001, Radder *et al*. 2006). It is expected that saxicolous lizard species will inhabit suitable rocky habitats; however, high perches may not be in abundance and may be considered a limited resource occupied primarily by bigger, older, dominant individuals (Radder *et al*. 2006, Deodhar and Isvaran 2018).

With 289 recognized species, *Liolaemus* is the world's second most speciose genus of lizards and the most diverse in South America (Pincheira-Donoso 2012, Stellateli e*t al*. 2016, Abdala *et al*. 2020). As with many other lizards around the world (Losos 2009), little is known about the ecology of most of them (Utsumi *et al*. 2021). Thanks to recent studies, our knowledge of the natural history, sociality, and ecology of various *Liolaemus* species is on the rise (Fox and Shipman 2003, Pincheira-Donoso 2012, Santoyo-Brito *et al*. 2021).

Liolaemus bellii (Gray, 1985) is a high-elevation, diurnal, viviparous lizard. Individuals inhabit open rocky mountainous areas in central Chile (Donoso-Barros 1966, Pincheira-Donoso and Núñez 2005). The species is abundant and conspecific tolerance suggests a social structure of dominance hierarchy (Fox and Shipman 2003). Discernable sexual dimorphism is absent in the species although males have two precloacal pores that females lack (Pincheira-Donoso and Núñez 2005). Adult *L. bellii* are medium-sized and stocky, and both sexes show irregular dorsal patterning, characterized by olive or yellowish-brown color, flecked with yellow, black, and brown (Figure 1A), sometimes with 8–10 blackish crossbars broken backward over the vertebral field (chevrons), forming an open V-like angle (Pincheira-Donoso and Núñez 2005) (Figure 1B), and sometimes without this chevron pattern (Figure 1C).

Although dorsal polymorphism in *L. bellii* is evident (Donoso-Barros 1966, Pincheira-Donoso and Núñez 2005), the influence that dorsal pattern may have on habitat use, social status, and fitness of individuals has not been tested. In this sense, *L. bellii* is an excellent model to address behavior questions and evaluate some variables that may affect perch use by conspecifics of different age classes, sexes, and dorsal pattern morphs.

In this study, we collected data from *L. bellii* in a high-elevation population in central Chile. Our objectives were to determine if lizards with a distinct chevron dorsal pattern perch higher than patternless conspecifics and, if so, if conspecific social pressures might be responsible. At the same time, we measured eight morphological variables and evaluated their effect on perch height as covariables when lizards were grouped by sex, age class, and morph.

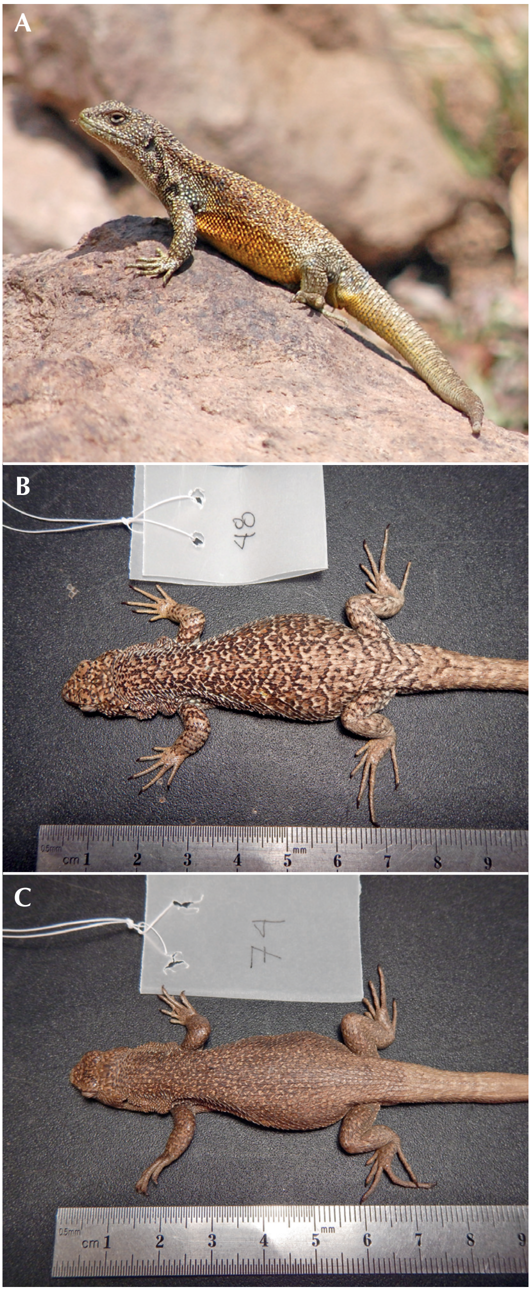

Figure 1. Three adults of *Liolaemus bellii* observed or collected in 2017 at our field site at 2960 m a.s.l. nearby the village of El Colorado, central Chile. The two vouchered adult female specimens (**B** and **C**) show the dorsal pattern polymorphism of the species.

Materials and Methods

We collected 69 *L. bellii* from 23 to 25 February 2017 (austral Summer) at our study site (2960 m a.s.l.; 33°20' S, 70°16' W) near the village of El Colorado, Metropolitan Region, in central Chile. This site is characterized by bare rocks aligned in straight-line formations (Figure 2). The rock lines are likely human-made since our site is located on the slope and open-ground ski lines of a popular ski resort. Vegetation at the site is predominantly spiny and shrub-like (*Berberis empetrifolia* Lam., *Chuquiraga oppositifolia* D. Don, and *Azorella* sp.).

We used binoculars to scan the area and locate lizards from a distance; all specimens were caught by noosing (Ferner 2007, Cooper 2009, Santoyo-Brito *et al*. 2018) from 09:00 to 18:00 h, when lizards are active and displaying normal behavior. The body temperature (BT) readings were obtained via a quick-reading Miller & Webber (0–50°C) cloacal bulb thermometer within 30 seconds of capture. Snout–vent and tail length (SVL and TL, respectively) were measured with a ruler (error: ± 1 mm). Lizards were assigned to one of two age classes as per SVL measurements as modified from Labra *et al*. (2003): juveniles = 4.4–6.7 cm; adults > 6.7 cm). Tail break status was recorded (i.e., intact tail or naturally broken tail [i.e., result of a predation attempt or an agonistic encounter]). Mass was measured with a 30-g Pesola spring scale. Left forearm length (LFAL), front left third digit length (FLDL), left hind leg length (LHLL), and hind left fourth digit length (HLDL) were measured with digital calipers. All variables were measured by one person, ES-B.

Perch height from the ground (PH in cm) was measured with a flexible measuring tape positioned close to the base of the perch type. The categorical dorsal pattern (i.e., chevron *vs.* patternless; Figure 1 B and C) of each lizard was determined at the point of capture by ES-B and corroborated by HN. No intermediate pattern categories were considered in this study. Data

Figure 2. View of our study site (2960 m a.s.l.) nearby the village of El Colorado, central Chile.

normality was tested using the Kolmogorov-Smirnov test. We used the Pearson Correlation test to estimate the correlation between body-size variables (LFAL, FLDL, LHLL, HLDL, mass, and SVL). We used a Factor Analysis to reduce the dimensionality of highly correlated morphological variables. Statistical differences of PH and BT between age groups (adults *vs* juveniles), and of PH, BT, BODY, and TL between sex (males *vs* females), and morph (pattern vs. patternless) were determined by the Student's t-test. We tested for homogeneity of variances using Levene's test. If variances were not homogeneous, we used the Welch's t-test. We first conducted an exploratory analysis, a full General Lineal Model (GLM) with PH as the dependent variable and the main factors (age, sex, and morph), all two-way interactions, and the one three-way interaction, plus the covariates BODY and TL as the independent variables. Subsequently, we subsetted the data and conducted separate one-way ANCOVAs to more closely examine the individual effects of explanatory variables age, sex, and morph on PH. Interactions were evaluated in the respective model with BODY and TL as covariables. The Pearson Chi-Square test estimated the association between age and morph, sex and morph, age and natural tail break, and sex and natural tail break in contingency tables. All statistical analyses were performed using SPSSv21 (IBM 2012).

Results

The One-Sample Kolmogorov-Smirnov Test indicated that all variables were normally distributed. The Pearson correlation matrix indicated a high correlation between LFAL, FLDL, LHLL, HLDL, mass, and SVL. Because correlation values among all six body-size indicators were highly significant, we used Factor Analysis to reduce the dimensionality (Johnson and Wichern 1998) of body variables into a new variable, BODY, in which larger values indicated a larger overall body and appendage size.

Results of the full GLM with PH as the dependent variable showed a significant ($F_{9,59} = 2.320$; $p = 0.026$) full model explaining PH (but explaining only 26% of its variation) and no explanatory variable was significant except for BODY ($F_{1,59} = 8.22$; $p = 0.006$). In other words, the interaction age*sex, age*morph, sex*morph, and age*sex*morph played no significant role in the determination of PH.

Statistical Analyses by Age Class

The independent sample t-test and Welch's t-test indicated no statistical difference in PH or BT between adults (47) and juveniles (22) (both $p > 0.05$; descriptive statistics in Table 1).

Table 1. Perch height and body temperature [mean ± SD (range)] statistics of 69 *Liolaemus bellii* collected at 2960 m a.s.l. nearby the village of El Colorado, central Chile. Lizards are grouped by age. Abbreviations: *N* = number of individuals, PH = perch height, and BT = body temperature.

Age	PH (cm)	BT (°C)
Adults (*N* = 47)	23.21 ± 12.97 (0–62.00)	31.7 ± 2.68 (24.00–36.20)
Juveniles (*N* = 22)	18.00 ± 16.60 (0–50.00)	31.61 ± 1.66 (29.90–36.00)

Setting Age as the independent variable, BODY as the covariate, and PH as the dependent variable, the one-way ANCOVA showed a statistical difference in perch height use in relation to BODY ($F_{1,65} = 10.02$; $p = 0.002$), but not Age. The interaction Age*BODY was not significant. In a second analysis setting Age as the independent variable, TL as the covariate, and PH as the dependent variable, the one-way ANCOVA showed a statistical difference in perch height use in relation to TL ($F_{1,65} = 6.30$; $p = 0.015$), but not Age. The interaction Age*TL also was not significant. Larger lizards (i.e., bigger BODY) and those with longer tails occupied significantly higher perches. The lack of interactions indicates that the effect of BODY and TL was the same for juveniles and adults.

Statistical Analyses by Sex

The independent sample t-test indicated no statistical difference in PH, BT, BODY, or TL between males (32) and females (37) (all $p > 0.05$; descriptive statistics in Table 2).

Setting sex as the independent variable, BODY as the covariate, and PH as the dependent variable, the one-way ANCOVA showed a statistical difference in perch height use in relation to BODY ($F_{1,65} = 10.98$; $p = 0.002$), but not sex. The interaction sex*BODY also was not significant. In a second analysis setting sex as the independent variable, TL as the covariate, and PH as the dependent variable, the one-way ANCOVA showed a statistical difference in perch height use in relation to TL ($F_{1,65} = 6.98$; $p = 0.010$), but not sex. The interaction between sex*TL was not significant. Larger lizards (i.e., bigger BODY) and those with longer tails occupied significantly higher perches. The lack of interactions indicates that the effect of BODY and TL was the same for males and females.

Statistical Analyses by Morph

The independent sample t-test indicated no statistical difference in PH, BT, BODY, or TL between patternless (19) and chevron patterned morphs (50) (all $p > 0.05$; descriptive statistics in Table 3).

Setting morph as the independent variable, BODY as the covariate, and PH as the dependent variable, the one-way ANCOVA showed a statistical difference in perch height use in relation to BODY ($F_{1,65} = 9.45$; $p = 0.003$), but not morph. The interaction morph*BODY also was not significant. In a second analysis setting morph as the independent variable, TL as the covariate, and PH as the dependent variable, the one-way ANCOVA showed a statistical difference in perch height use in relation to TL ($F_{1,65} = 6.12$; $p = 0.016$), but not morph. The interaction between morph*TL was not significant. Larger lizards (i.e., bigger BODY) and those with longer tails occupied significantly

Table 2. Descriptive morphometrics, body temperature, and perch height [mean ± SD (range)] statistics of 69 *Liolaemus bellii* collected at 2960 m a.s.l. nearby the village of El Colorado, central Chile. Lizards are grouped by sex. Abbreviations: *N* = number of individuals, PH = perch height, BT = body temperature, BODY = reduced dimensionality of body variables, and TL = tail length.

Sex	PH (cm)	BT (°C)	BODY (no unit)	TL (mm)
Males (*N* = 32)	20.71 ± 13.43	31.54 ± 2.20	0.11 ± 1.06	97.28 ± 20.24
	(0–50.00)	(26.00–36.20)	(-2.10–1.93)	(55.00–132.00)
Females (*N* = 37)	22.27 ± 15.19	31.78 ± 2.56	-0.10 ± 0.94	94.83 ± 18.95
	(0–62.00)	(24.00–35.20)	(-2.58–1.25)	(67.00–139.00)

Table 3. Descriptive morphometrics, body temperature and perch height [mean ± SD (range)] statistics of 69 specimens of *Liolaemus bellii* collected at 2960 m a.s.l. nearby the village of El Colorado, central Chile. Lizards are grouped by morph. Abbreviations: *N* = number of individuals, PH = perch height, BT = body temperature, BODY = reduced dimensionality of body variables, and TL = tail length.

Morph	PH (cm)	BT (°C)	BODY (no unit)	TL (mm)
Patternless (*N* = 19)	21.36 ± 12.28	31.20 ± 2.22	0.27 ± 0.79	96.23 ± 21.40
	(0–42.00)	(26.00–35.30)	(-1.95–1.44)	(64.00–139.00)
Chevron (*N* = 50)	21.62 ± 15.13	31.85 ± 2.45	-0.10 ± 1.05	95.86 ± 18.89
	(0–62.00)	(24.00–36.20)	(-2.58–1.93)	(55.00–134.00)

higher perches. The lack of interactions indicates that the effect of BODY and TL was the same for patternless and lizards with pattern.

Pearson Chi-square results indicated there was a significant relationship between age and morph. The patternless morph becomes more prevalent as lizards age (Pearson Chi-square = 5.51, *df* = 1, *p* = 0.019) (Table 4). The dorsal pattern does not appear to be a sexual dimorphism. Sex did not significantly relate to morph in individuals of *L. bellii* (Pearson Chi-square = 0.42, *df* = 1, *p* = 0.521).

Interestingly, 40 of the 69 lizards had a naturally broken tail (Table 4). Of those, 7 were juveniles and 33 were adults, 20 were males and 20 were females, and 13 were patternless and 27 had chevron patterns. Natural tail break was statistically different only between age classes (Pearson Chi-square = 9.07, *df* = 1, *p* = 0.003), with adults having proportionately more broken tails than juveniles (Table 5).

Discussion

Dorsal polymorphism in *L. bellii* is evident (Donoso-Barros 1966, Pincheira-Donoso and Núñez 2005). In our preliminary full model GLM analysis, contrary to our expectations, dorsal pattern morph did not relate to perch height (PH), but BODY did. Interestingly, the interaction age*sex, age*morph, sex*morph, and age*sex*morph played no significant role in the determination of PH. However, when the data were subsetted and analyzed by age, sex, or morph, we found that both BODY and TL significantly influenced PH. Thus, regardless of their age, sex, and morph, lizards with a bigger BODY and longer tail occupied higher perches, suggesting that the combination of both variables may play a social role in *L. bellii*.

How TL influences PH is not clear. Tail loss (i.e., decreased regenerated tail length) has been negatively associated with dominance in side-

Table 4. Tail status (i.e., intact, and naturally broken tail) of 69 specimens of *Liolaemus bellii*, grouped by age, sex, and morph collected at 2960 m a.s.l. nearby the village of El Colorado, central Chile. *N* = number of individuals.

Age	Sex	Morph	*N*	Intact tail	%	Naturally broken tail	%
Adult	Male	Patternless	9	2	22.2	7	77.8
		Chevron	10	1	10.0	9	90.0
	Female	Patternless	8	3	37.5	5	62.5
		Chevron	20	8	40.0	12	60.0
Juvenile	Male	Patternless	1	0	0.0	1	100.0
		Chevron	12	9	75.0	3	25.0
	Female	Patternless	1	1	100.0	0	0.0
		Chevron	8	5	62.5	3	37.5

Table 5. Observed vs expected counts of 69 *Liolaemus bellii* collected at 2960 masl nearby the village of El Colorado, central Chile. Pearson Chi-square = 9.07, *df* = 1, *p* = 0.003.

		Intact tail	Naturally broken tail	Total
Adult	Observed	14.0	33.0	47.0
	Expected	19.8	27.2	47.0
Juvenile	Observed	15.0	7.0	22.0
	Expected	9.2	12.8	22.0

blotched lizards (Fox *et al*. 1990). According to Fox and Shipman (2003) *L. bellii* is not territorial. Although the specific social organization of the population we studied has not yet been determined, its high density suggests hierarchical dominance. Nevertheless, during visits to our field site and surrounding areas, we never observed aggression between sexes or between individuals of the same or a different age class. From our field observations and results, one can infer that bigger *L. bellii* (controlling for age or sex) with longer tails (controlling for age or sex) may be dominant individuals occupying the less abundant taller rocks, which offer higher perches.

Our results indicate that the patternless morph is significantly more prevalent in older individuals, indicating an ontogenetic loss of dorsal pattern in *L. bellii*. Even though a rather high frequency of tail breaks occurred in the population, there was no suggestion that dorsal pattern was related to tail loss. Not surprisingly, adults suffered significantly more tail losses than juveniles. Tail loss in different *Liolaemus* species has been interpreted as the result of failed predation events (Jaksić and Fuentes 1980, Medel *et al*. 1988, Utsumi *et al*. 2021). It seems reasonable that adults who have lived longer would have more evidence of tail breaks than juveniles (Tinkle and Ballinger 1972, Núñez and Yánez 1984, Bateman and Fleming 2009). At the high elevation of our study site, visually oriented bird predators that prey on lizards are two abundant species of shrike-tyrants, *Agriornis montanus* (d'Orbigny and Lafresnaye, 1837) and *Agriornis lividus* (Kittlitz, 1835); the less abundant American Kestrel, *Falco sparverius* Linnaeus, 1758 (Ridgely and Tudor 1994), and the very common Rufous-banded Miner, *Geositta rufipennis* (Burmeister, 1860) (Santoyo-Brito *et al*. 2014). During March–April, *A. montanus, A. lividus,* and *G. rufipennis* are abundant in the area. This abundance of visual predators might explain the high frequency of natural tail loss in our population.

In conclusion, morph is not sex-related nor related to perch height, but still might be involved in social relations among individuals since bigger lizards with longer tails perched significantly higher, and the patternless morph was found significantly more often in older lizards. Morph was related to age but not to the frequency of broken tails.

Acknowledgments

This research was made possible due to financial funding by The National Geographic Society, Delta Foundation, and Oklahoma State University. Research was conducted according to ACUP AS-15-14 approved by Oklahoma State University IACUC, and followed the protocol approved by SAG (Resolución Exenta N° 3/2016).

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Editor: Jaime Bertoluci

Phyllomedusa 23(2):137–152, 2024
© 2024 Universidade de São Paulo - ESALQ
ISSN 1519-1397 (print) / ISSN 2316-9079 (online)
doi: http://dx.doi.org/10.11606/issn.2316-9079.v23i2p137-152

Habitat and microhabitat use by *Crossodactylus schmidti* (Anura: Hylodidae) in Atlantic Rainforest streams

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Abstract

Habitat and microhabitat use by *Crossodactylus schmidti* (Anura: Hylodidae) in Atlantic Rainforest streams. A combination of biotic and abiotic factors influences the habitat and microhabitat selection by anurans. To analyze the habitat and microhabitat use by adults of *Crossodactylus schmidti,* we sampled 13 streams in the Atlantic Rainforest of Iguaçu National Park, Southern Brazil, between November 2014 and August 2015. We recorded abundance data and measured structural environmental variables of the streams related to habitat selection on three occasions in each stream. Data collection occurred along a 50-meter transect in each stream. Additionally, two samplings were conducted specifically to obtain microhabitat usage data, sampling only the same three streams on each sampling. We employed Generalized Least Squares analysis to assess spatial variation in abundance as a function of environmental structural variables, considering models with and without spatial structuring. We applied the Multinomial Goodness-of-Fit Test to elucidate the types of microhabitats most utilized by the frogs. Our analysis revealed that considering or not the spatial autocorrelation in the variation in *C. schmidti* abundance, none of the predictor variables was significant. The results obtained demonstrate that the analyzed environmental variables are not important for habitat selection of *C. schmidti,* and the spatial configuration and distance among streams are not determinants for abundance distribution. At the habitat scale, this species exhibits a random distribution independent of spatial and environmental features. Regarding microhabitats, individuals were predominantly found inhabiting streams in pool areas characterized by rocky substrate or organic matter accumulation. Conservation strategies for this species should encompass the scale-dependent relationships.

Keywords: Brazil, Distribution, Frogs, Habitat selection, Spatial dynamics.

Received 31 July 2024
Accepted 05 November 2024
Distributed December 2024

Resumo

Uso do habitat e micro-habitat por *Crossodactylus schmidti* (Anura: Hylodidae) em riachos da Mata Atlântica. Uma combinação de fatores bióticos e abióticos influencia a seleção de habitat e micro-habitat por anuros. Para analisar o uso de habitat e micro-habitat por adultos de *Crossodactylus schmidti*, amostramos 13 riachos na Mata Atlântica no Parque Nacional do Iguaçu, sul do Brasil, entre novembro de 2014 e agosto de 2015. Registramos dados de abundância e medimos variáveis ambientais estruturais dos riachos relacionadas à seleção de habitat pelos anuros em três ocasiões em cada riacho. A coleta de dados ocorreu ao longo de um transecto de 50 m em cada riacho. Além disso, foram realizadas duas coletas especificamente para a obtenção de dados do uso de micro-habitat, amostrando somente os mesmos três riachos em cada amostragem. Nós empregamos a análise Generalized Least Squares para avaliar a variação espacial na abundância em função das variáveis ambientais estruturais dos riachos, considerando modelos com e sem estruturação espacial. Nós aplicamos o Multinomial Goodness-of-Fit Test para elucidar os tipos de micro-habitat mais utilizados pelas rãs. Nossa análise revelou que, considerando ou não a autocorrelação espacial na variação da abundância de *C. schmidti*, nenhuma das variáveis preditoras foi significativa. Os resultados obtidos demonstram que as variáveis ambientais analisadas não são importantes para a seleção de habitat de *C. schmidti*, e a configuração espacial e a distância entre os riachos não são determinantes para a distribuição da abundância. Portanto, na escala do habitat, esta espécie exibe uma distribuição aleatória independente das características ambientais e espaciais. Em relação aos micro-habitats, os indivíduos foram encontrados predominantemente ocorrendo em áreas de remanso dos riachos, contendo substrato rochoso ou com acúmulo de matéria orgânica. Dada a variação na seleção de habitat entre as escalas avaliadas, as estratégias de conservação para esta espécie devem levar em conta as relações dependentes da escala.

Palavras-chave: Brasil, Dinâmica espacial, Distribuição, Rãs, Seleção de hábitat.

Introduction

Habitats and microhabitats exhibit variation in conditions and quality within natural ecosystems. Individuals are expected to optimize their survival and reproductive success by selecting suitable habitats and, within these environments, favorable microhabitats (Arlt and Pärt 2007). The selection of habitat and microhabitat is influenced by a combination of biotic and abiotic factors, as well as individual organism characteristics (Rosenzweig 1981, Morris 2003). Biotic factors influencing habitat selection include the presence of predators, parasites, and competitors (Binckley and Resetarits 2005, Tolvanen *et al.* 2017, Rushing *et al*. 2021), as well as the quantity and quality of food resources (e.g., Nielsen *et al.* 2010, Rushing *et al*. 2021). Regarding abiotic factors, conditions (e.g., temperature), habitat structure, and surrounding landscape also play significant roles in habitat selection (Pelinson *et al.* 2016, Marques *et al.* 2019). Individual characteristics such as sex (Chinchilla-Lemus *et al.* 2020), life stage (Wells 2007), and individual behavior (e.g., prior experience) (Liford and Cecala 2017) can influence habitat selection. Habitat selection represents a complex behavioral decision-making process directly impacting individuals' fitness (Freitas *et al*. 2016) and spatial distribution (Morris 2003).

In amphibians, habitat and microhabitat selection play a crucial role because reproductive success and population maintenance of this group are directly linked to the surrounding environment (Murphy 2003). Among abiotic factors influencing habitat selection by adult anurans, structural features of the habitat appear to be paramount (Watson *et al*. 2003, Carisio *et al*. 2014), as they directly impact the selection of calling, courtship, and spawning sites (Parris 2001, Touchon and Warkentin 2008, Buxton and Sperry 2017). In lotic environments utilized by anurans for reproduction, width and depth serve as indicators of available habitat for individuals. In streams, a greater abundance of adult anurans is anticipated in water bodies of intermediate sizes, as these habitats are expected to exhibit greater microhabitat heterogeneity (Hopey and Petranka 1994, Parris 2001). Canopy cover is another significant variable that is associated with species adaptation to forested or open areas (Van Buskirk 2005). Light penetration directly affects primary productivity and the nutritional quality of food ingested by tadpoles (Schiesari 2006). Water flow speed is another critical variable in lotic environments because higher speeds create a noisier environment, thereby interfering more with intraspecific acoustic communication (Vielliard and Cardoso 1996) and potentially carrying away tadpoles and adults. Success in occupying lotic environments is associated with, among other factors, the need for behavioral and/or acoustic adaptations that promote intraspecific communication (Heyer *et al*. 1990, Goutte *et al*. 2013) and local permanence (Wells 2007). Lotic environments exhibit structural variation, and the selection of appropriate habitat for reproduction is expected to confer specific advantages to males in acquiring a mate and subsequently maintaining the local population.

Amphibians typically select specific locations within their habitat, known as microhabitats, to occupy, vocalize, and spawn (Buxton and Sperry 2017, Melo *et al*. 2018, Flores *et al*. 2024). The selection of microhabitats by anurans is of paramount importance for their reproductive success and overall ecological fitness. Similar to habitat selection, anurans exhibit discerning preferences for particular microhabitats based on several environmental features, including water flow speed, depth, substrate type, and vegetation structure (Eterovick 2003, Melo *et al*. 2018, Flores *et al.* 2024). The ability of anurans to selectively utilize microhabitats that best suit their ecological requirements is crucial for maximizing fitness and ensuring population persistence in heterogeneous environments (Buxton and Sperry 2017).

The family Hylodidae comprises 48 described species (Frost 2024) of diurnal frogs commonly found in forest streams (Jordão-Nogueira *et al.* 2006, Caldart *et al*. 2010, but see Caldart *et al.* 2011a). One of these species is *Crossodactylus schmidti* Gallardo, 1961, a frog occurring in southern Brazil, southern Paraguay, and northern Argentina (Caldart *et al.* 2010, Lucas and Garcia 2011, Bastiani *et al.* 2012, Frost 2024). The species is currently classified as "Least Concern" by IUCN (2023), but its population trend is declining mainly due to habitat loss, modification, and fragmentation, and pollution (IUCN 2023). *Crossodactylus schmidti* has a snout–vent length of 28 mm and 2.5 g of body mass, and it is cryptically colored. It inhabits forest streams and is considered a habitat specialist, with individuals strongly associated with well-preserved habitats (Bastiani *et al*. 2012). Males vocalize throughout the year, with monthly variation positively influenced by accumulated precipitation and air temperature (Caldart *et al.* 2016). The species occupies rocky stream habitats wherein males prefer to utilize exposed rocks for acoustic and visual communication (Caldart *et al.* 2011b, 2016). *Crossodactylus schmidti* preys on arthropods, primarily ants, beetles, and dipterans (Caldart *et al.* 2012). Conversely, it serves as prey for spiders and snakes with both diurnal and nocturnal habits (Caldart *et al*. 2011). Despite being a species with considerable ecology and natural history information available (e.g., Caldart *et al.* 2010, 2011a, b, 2012, 2016, Bastiani *et al.* 2012), studies are lacking that evaluate the relationship between habitat and microhabitat features and the occurrence of individuals of *C. schmidti*. This study addresses two questions: (1) What is the relative influence of stream environmental features on the spatial variation of abundance in *C. schmidti*? (2) Is there differential utilization of existing microhabitats within streams by adult *C. schmidti*? Understanding distribution patterns among and within habitats is crucial for establishing effective conservation measures.

Materials and Methods

Study Area

The study was conducted in Iguaçu National Park (INP), a conservation unit located in the western region of the state of Paraná, southern Brazil (25°05'–25°41' S, 53°40'–54°38' W; Figure 1). The INP covers 185,265 ha across five municipalities: Céu Azul, Foz do Iguaçu, Matelândia, São Miguel do Iguaçu, and Serranópolis do Iguaçu. The vegetation comprises Semideciduous Seasonal Forests, Mixed Ombrophilous Forests, and Alluvial Pioneer Formations of the Atlantic Rainforest (Souza *et al.* 2017). Specifically, in the area where the present study was conducted, the vegetation is predominantly Mixed Ombrophilous Forest. The climate in the region is classified as Cfa (temperate and humid) according to Köppen's classification (Alvares *et al.* 2013), with four well-marked seasons. Precipitation is evenly distributed throughout the year, with no dry season, and a higher volume of rainfall in the summer, resulting in an annual precipitation total of around 1800 mm (Nitsche *et al.* 2019). The average annual temperature varies between 20°C and 22°C, with summer averaging 26°C and winter averaging 17°C (Nitsche *et al.* 2019).

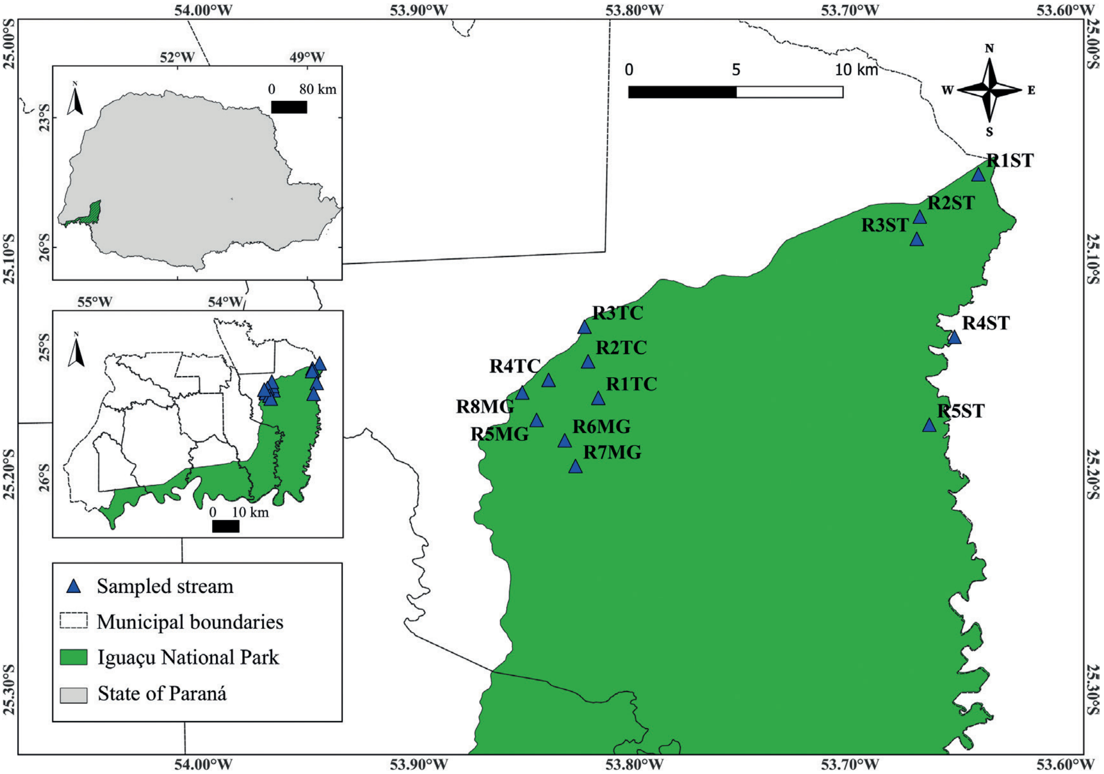

Figure 1. Map showing the location of Iguaçu National Park (green area) in the state of Paraná in southern Brazil and the distribution of sampling units at Iguaçu National Park (blue triangles). In detail, the distribution of sampling units (blue triangles) represents the location of each sampled stream. Each acronym represents a specific stream.

Habitat Data

We sampled 13 streams in primary forest of the INP, all of which were situated within Araucaria Moist Forest areas in the municipality of Céu Azul (Table 1; Figure 1). We chose to sample only small to medium-sized streams (first to third order) (Caldart *et al.* 2016) because no specimens of *Crossodactylus schmidti* were found in larger streams in previous monitoring efforts in INP (e.g., Iguaçu River) (M. V. Garey pers. obs.). We conducted our sampling during daylight and twilight periods (8:00 to 19:00 h). We established a 50-m transect in each stream, considering that this transect size has already been used in other studies with amphibians (e.g., Hazell *et al*. 2004, Wassens *et al*. 2013). The transect was permanently established upstream of the access point to the water body to minimize the impacts of researchers' presence on anurans and water. We sampled each transect three times: the first between November and December 2014, the second in January 2015, and the third in August 2015. The definition of sampling events was conducted considering climatic conditions (i.e., forecasted light rain on sampling days) and logistics. Two samplings were performed during the warmer and rainier period, while another occurred during the cooler and less rainy period. This decision was made due to uncertainty regarding whether the studied population would follow the same pattern reported for another population of the species in a more southern region (Caldart *et al.* 2016).

We collected data on the abundance of *C. schmidti* (response variable) and environmental variables describing habitat structure (predictor variables) in each stream transect. Two researchers sampled the transects for 30 min each, totaling an hour of search effort per transect per sampling campaign. During the diurnal and nocturnal searches, all encountered adult individuals were counted. Adults were defined as having SVL > 20 mm (Bastiani *et al.* 2012). The species abundance in each stream was considered equal to that of the campaign where the highest number of individuals was recorded in each stream. This procedure was adopted to mitigate the risk of overestimating abundance due to recounting individuals between different campaigns, as individuals were not marked. However, we know that the population size in each stream is larger than our observations suggest (see Greener *et al*. 2017), and that males and females may only visit the breeding site occasionally (Boyle *et al.* 2021). Considering that the visual encounter survey probably represents only a portion of the population's abundance, this limitation should be kept in mind.

We measured five environmental variables in each transect to characterize the habitat: maximum depth, width, water flow speed, predominant substrate type, and percentage of canopy cover (Table 1). We recorded environmental variables every 10 m in each transect, with six samples per stream per campaign. Water flow speed and percentage of canopy cover were measured at the middle of the stream. Maximum depth and substrate type were assessed across the entire width of the stream at each abiotic variable collection point. Maximum depth and stream width were measured in centimeters using a tape measure. We estimated water flow speed (cm/s) using the floating object method (Kauffman *et al.* 2009), where we recorded the time (s) taken for a 2-cm diameter Styrofoam ball to travel one meter. We visually classified substrate type into one of seven categories: bedrock (rock outcrop), boulder (larger rock fragments > 8 cm), coarse gravel (2–8 cm), fine gravel (1–2 cm), organic matter (i.e., leaves and stems), clay, and sand. Canopy cover was estimated by capturing photographs of the canopy with a Fujifilm FinePix XP10 camera positioned horizontally at a standardized height (approximately 140 cm above ground level). We converted the obtained images to binary black ("not sky") and white ("sky") and analyzed the proportion of white pixels in each photo, representing an estimate of visible canopy fraction or the percentage of canopy openness

Table 1. The abundance of individuals of *Crossodactylus schmidti*, structural environmental characteristics, and the location of the 13 lotic water bodies sampled in Iguaçu National Park, western Paraná state, Brazil. Values are presented as mean ± standard deviation (range). Wfs = Water flow speed (cm/s). Sw = Stream width (cm). Cc = Canopy cover (%).

Stream	Abundance	Wfs	Sw	Depth (cm)	Substrate	Cc	Latitude	Longitude
R1ST	13	22.47 ± 17.38 (12.18–46.08)	75 ± 36 (34–159)	10 ± 5 (5–26)	Clay	0.61 ± 0.08 (0.41–0.73)	-25.060555	-53.640555
R2ST	8	12.89 ± 18.80 (0.00–37.03)	100 ± 67 (30–308)	8 ± 3 (3–16)	Clay and organic matter	0.63 ± 0.07 (0.63–0.75)	-25.080277	-53.667777
R3ST	7	33.33 ± 31.17 (19.04–78.74)	178 ± 152 (62–720)	8 ± 3 (3–17)	Boulder	0.60 ± 0.12 (0.38–0.79)	-25.090555	-53.669166
R4ST	1	30.49 ± 42.76 (13.25–100)	257 ± 151 (66–486)	6 ± 2 (3–11)	Boulder	0.65 ± 0.04 (0.59–0.72)	-25.136111	-53.651666
R5ST	6	15.80 ± 25.66 (0.00–55.86)	121 ± 45 (41–179)	9 ± 7 (1–23)	Boulder	0.68 ± 0.05 (0.57–0.76)	-25.176944	-53.663333
R1TC	0	10.65 ± 35.85 (2.85–68.49)	204 ± 81 (102–370)	18 ± 10 (5–38)	Coarse gravel	0.67 ± 0.03 (0.61–0.76)	-25.164444	-53.816944
R2TC	20	22.94 ± 37.74 (12.79–82.64)	142 ± 106 (16–411)	5 ± 3 (1–13)	Clay and organic matter	0.69 ± 0.05 (0.62–0.79)	-25.147500	-53.821666
R3TC	57	10.34 ± 19.79 (5.38–49.02)	77 ± 19 (43–121)	11 ± 5 (5–19)	Clay	0.66 ± 0.05 (0.56–0.76)	-25.131388	-53.823333
R4TC	16	9.70 ± 10.15 (3.90–27.70)	120 ± 60 (16–272)	9 ± 3 (6–16)	Clay and organic matter	0.64 ± 0.06 (0.56–0.74)	-25.156111	-53.840000
R5MG	6	44.64 ± 52.96 (22.42–142.86)	439 ± 233 (170–985)	20 ± 14 (3–56)	Boulder	0.54 ± 0.09 (0.39–0.68)	-25.174722	-53.845555
R6MG	50	13.02 ± 58.70 (4.01–109.89)	256 ± 113 (84–490)	14 ± 6 (6–28)	Bedrock	0.61 ± 0.05 (0.53–0.69)	-25.184166	-53.832500
R7MG	49	11.38 ± 11.84 (4.13–32.05)	206 ± 174 (78-650)	12 ± 6 (5–25)	Coarse gravel and clay	0.67 ± 0.06 (0.55–0.75)	-25.196111	-53.827500
R8MG	8	9.31 ± 16.01 (3.71–40.00)	143 ± 85 (37—329)	9 ± 4 (2–16)	Boulder	0.68 ± 0.07 (0.52–0.78)	-25.161944	-53.852222

per point (Smith and Ramsay 2018). We analyzed the images using Image J software (Abramoff *et al.* 2004).

Microhabitat Data

We conducted two campaigns to collect data on the microhabitats used by adult *C. schmidti* in the streams of Iguaçu National Park. The first sampling was conducted in April, and the second in September 2014. For this sampling, we selected only three of the 13 streams, and we traversed transects of these three streams during both campaigns. We selected the streams for convenience, prioritizing those with easier access and shorter walking time to reach the sampling point, and ensuring they were at least 1 km apart from each other. Along the stream transect, we characterized the occurrence location (i.e., the microhabitat used) of each adult individual recorded based on seven variables: (i) position of the individual concerning the stream (i.e., inside or outside of the water), (ii) distance of the individual from the stream margin (cm), (iii) type of microhabitat (i.e., pool, riffle, or fast-flowing), (iv) substrate type (i.e., rock, coarse gravel, fine gravel, organic matter, or sand), (v) stream width (cm), (vi) maximum depth (cm), and (vii) water flow speed in the microhabitat (cm/s).

Statistical Analysis

We applied the Variance Inflation Factor (VIF) to evaluate the presence of multicollinearity among habitat environmental variables from the 13 sampled streams. A variable with a VIF value higher than three was deemed multicollinear (Zuur *et al*. 2009). The variable “substrate type” was excluded from subsequent analyses due to multicollinearity. Following the removal of this variable, the remaining variables showed no signs of multicollinearity. Subsequently, these remaining variables were standardized using Z-scores, ensuring that all variables had a mean of zero and a standard deviation of one (Legendre and Legendre 2012). This standardization procedure was implemented due to the variables being measured in different units.

We conducted Generalized Least Squares (GLS) analysis to assess the variables associated with the variation in abundance of *C. schmidti* across the streams. This method addresses both heteroscedasticity and residual autocorrelation (Pinheiro and Bates 2000), but it does not account for variation in detection probability (Mazerolle *et al.* 2007). We chose this approach because GLS models allow for explicit incorporation of spatial autocorrelation by adjusting a variance-covariance matrix to fit the data characteristics better (Dormann *et al*. 2007). Since autocorrelation is a significant source of bias, the explicit modeling of spatial autocorrelation is expected to substantially enhance the accuracy of abundance distribution predictions (Guélat and Kéry 2018). To incorporate spatial structure, we included latitude and longitude data obtained at the center of each stream transect. We tested one non-spatial model and four models with different potential spatial correlation structures: spherical, exponential, Gaussian, and rational quadratics (Zuur *et al.* 2009). The selection of the most predictive model for abundance variation was based on three criteria: (1) models with ΔAICc less than 2.0, (2) inclusion in the set of best-supported models with combined Akaike weights of 0.70 (70% confidence set), and (3) relative likelihood higher than 0.7 (Burnham and Anderson 2002, Burnham *et al.* 2011). Spatial autocorrelation in model residuals was quantified using Moran’s I test (Dormann *et al.* 2007). These analyses were conducted using R version 4.2.2 (R Core Team 2022) with the nlme (Pinheiro and Bates 2023) and qpcR (Spiess 2018) packages.

The occurrence frequencies of adults of *C. schmidti* in microhabitats with different structural environmental features were compared using the Multinomial Goodness-of-fit Test (McDonald 2014). We evaluated three microhabitat characteristics: (i) the position of the individual concerning the stream, (ii) the type of microhabitat, and (iii) the substrate type. This test involves assessing the fit of a probabilistic model to a set of observed data when sample size is small (McDonald 2014).

Results

We observed adult *Crossodactylus schmidti* in 12 of the 13 sampled streams, totaling 361 individuals. In streams occupied by *C. schmidti*, an average of 20.08 ± 19.97 individuals were found, ranging from 1 to 57 individuals per stream. These streams had a water flow speed of 18.95 ± 11.14 cm/s (range: 9.32–44.64), a maximum width of 167.46 ± 81.16 cm (range: 74.94–334.98), a maximum depth of 10.02 ± 4.07 cm (range: 4.49–20.05), and canopy coverage of 64 ± 4% (range: 54–69) (Table 1). Tadpoles of *C. schmidti* were observed in all streams where adults were found. Males engaged in vocalization activities were recorded, indicating utilization of these habitats as breeding sites.

Among the four models evaluated in the GLS analysis, both the non-spatial and Gaussian Correlation Structure models performed equally well in predicting the spatial variation in the abundance of *C. schmidti* in streams of the Atlantic Rainforest (Table 2). In the non-spatial model, only the intercept was significant, suggesting that none of the variables was individually important in explaining the abundance data without considering spatial autocorrelation. In the model with a Gaussian autocorrelation structure, none of the environmental variables were significant (Table 3), indicating that the abundance distribution of *C. schmidti* in streams is independent of both environmental and spatial factors.

Within the analyzed habitats, individuals predominantly occupied microhabitats within the stream, mainly in pool areas (54.16%), followed by riffle areas (37.5%) and fast-flowing sections (8.33%) (p = 0.0013, exact multinomial test, Appendix I). Individuals were observed to prefer microhabitats mainly composed of rock substrate (33.33% of cases) or organic matter (33.33%), with clay areas being less frequently occupied (8.33%) ($p < 0.00001$). During observations, adults were mostly found alone (75% of observations), with two adults present in the same microhabitat 25% of the time (Appendix I). Individuals were consistently found within the stream at an average distance of 23.41 ± 35.10 (0–130) cm from the margin. In these microhabitats occupied by adults, the stream had an average width of 136.75 ± 48.86 (45–228) cm, depth of 4.77 ± 2.70 (1–12) cm, and water flow speed of 19.60 ± 21.76 (0–86.96) cm/s.

Discussion

Adults of *Crossodactylus schmidti* were found in 92% of the rocky streams sampled in Iguaçu National Park. The spatial variation in the abundance of *C. schmidti* was equally explained by the model that does not consider spatial autocorrelation and by the model considering the spatial structuring of abundance. When considering or disregarding spatial structure, we found that none of the environmental variables was associated with spatial variation in the abundance of *C. schmidti*. This result suggests that individuals were randomly distributed in relation to the measured variables. The absence of a relationship observed in the model without spatial structure does not imply that there is no habitat selection by the species. Instead, selection may be predominantly influenced by environmental variables not considered in the present study, such as physicochemical characteristics of the water and biotic variables. For example, individuals of *C. gaudichaudii* Duméril and Bibron, 1841 are more likely to be found in streams with higher water temperatures and lower pH (Pereira-Ribeiro *et al.* 2023). Unfortunately, we were unable to assess physicochemical characteristics due to the lack of a multiparameter probe. Furthermore, anurans generally avoid habitats with potential predators and competitors (Buxton and Sperry 2017, Van Buskirk and Smith 2021).

Table 2. Outputs of the five models, one with a non-spatial structure and four with different correlation structures, for analyzing the relationship between the abundance of *Crossodactylus schmidti* and the environmental variables of the streams. ΔAICc = difference in Akaike's Information Criteria; Weight = weights of Akaike's Information Criteria; Relative likelihood = demonstrate the probability of one model being in favor over the other.

	df	**AIC**	**ΔAIC**	**Weight**	**Relative likelihood**
Non-spatial	6	94.2959	0.0	0.4224	1.0000
Gaussian	8	95.7291	1.4321	0.2063	0.7884
Spherical	8	96.4412	2.1453	0.1445	0.3420
Rational quadratics	8	96.5476	2.2517	0.1370	0.3244
Exponential	8	97.3947	3.0989	0.0897	0.2124

Table 3. The models' outputs were derived from the analyses of spatial variation in the abundance of *Crossodactylus schmidti* by environmental variables of streams in the Atlantic Rainforest of Iguaçu National Park. SE = standard error.

	Estimate	**SE**	***t*-value**	***p*-value**
Non-spatial				
Intercept	19.1345	5.9474	3.2173	**0.0123**
Water flow speed	-15.6946	11.1079	-1.4130	0.1954
Width	4.2976	9.4307	0.4557	0.6607
Deep	-2.5034	9.2745	-0.2699	0.7941
Canopy cover	-7.3951	10.0225	-0.7379	0.4817
Gaussian				
Intercept	19.1251	8.3516	2.2899	0.0513
Water flow speed	-11.6149	7.0416	-1.6495	0.1377
Width	2.0765	9.5453	0.2175	0.8332
Deep	-4.9967	5.2347	-0.9545	0.3678
Canopy cover	-8.4582	6.7685	-1.2496	0.2467

Habitat selection by anurans, particularly for breeding sites, tends to be strongly associated with the structural environmental features of water bodies (Welch and MacMahon 2005, Carisio *et al*. 2014, Thomas *et al.* 2019). Studies on stream-dwelling anurans have observed the importance of environmental and structural stream features such as water velocity, substrate type, depth, and width of the stream in species occurrence and abundance (e.g., Eterovick 2003, Oliveira and Eterovick 2010). In the present study, it was not possible to demonstrate the existence of preferential habitat selection by *C. schmidti* because the variation in the abundance of this species was independent and random with respect to environmental features. Despite anurans being considered organisms with limited dispersal capability (Smith and Green 2005, Wells 2007), the inclusion of spatial autocorrelation in the abundance models did not alter the result of statistical analysis. Caution must be exercised in the conclusions because GLSs do not take into account some factors that may affect abundance data collection. For example, the visual encounter survey often records lower abundances than are actually present in the stream when compared to other methods (e.g., removal sampling) (Greener *et al.* 2017), individuals may only visit the breeding site occasionally (Boyle *et al.* 2021), differences in individual detection among collectors, climatic and environmental variations that may occur between data samples, and the recounting of individuals (Mazerolle *et al.* 2007). In our study, all samples were taken by the same researchers, and individual counting was done jointly; thus, this factor did not result in bias in our study. However, environmental and structural variations may have caused bias. We observed in the field that structural variations in streams between data samples were small. Climate variation within the same campaign was also small (e.g., temperature and humidity), likely having little influence on the counting of individuals of *C. schmidti,* but variation between campaigns was more significant, which may have influenced the recorded abundance (Caldart *et al.* 2016). The recounting of individuals in the same stream or between streams in the same campaign probably did not occur due to the distance between streams and the search always being conducted against the water flow. Between campaigns, the recounting of an individual may have occurred, but with a low probability due to the limitation in the dispersal capacity of anurans (Smith and Green 2005).

At the microhabitat scale, we observed that adults of *C. schmidti* preferentially occur in stream interiors, particularly in shallow areas with rocky substrates or organic matter accumulation located in pools. The utilization of these microhabitat types by the population in Iguaçu National Park coincides with observations from other populations of *C. schmidti* (Caldart *et al.* 2010, Bastiani *et al*. 2012) and in other populations of the genus *Crossodactylus* (Almeida-Gomes *et al.* 2007, 2012). *C. schmidti* occupies these microhabitats for calling and visual communication (Almeida-Gomes *et al.* 2007, Caldart *et al.* 2010), where individuals remain partially submerged or at water level (Caldart *et al.* 2010; present study). Rocky substrates used as calling sites are strategically important in predation risk situations, including for tadpoles, because they allow easy access to water and small underwater chambers (Caldart *et al.* 2010) or even burrows located on stream banks (Pimenta *et al*. 2008). This species uses underwater chambers as spawning sites (Haddad *et al*. 2013); thus, the presence of adults in these locations may be influenced by the availability of suitable oviposition sites. Another advantage of using rocky microhabitats may be increased visibility for visual communication behaviors, which is common in many species of this genus (Caldart *et al.* 2010). The secondary use of pool microhabitats by individuals of *C. schmidti* coincides with reports for individuals of *C. trachystomus* (Reinhardt and Lütken, 1862) (Pimenta *et al.* 2008). According to Pimenta *et al.* (2008), individuals of the genus *Crossodactylus* tend to avoid occupying fast-flowing areas, especially during periods of heavy rainfall, prioritizing marginal areas with lower water volume. The preference for pool areas may be related to the energetic cost of remaining in swift-flowing areas, reducing the risk of being washed away during rainy periods. Another potential explanation is related to acoustic communication, as ambient noise can mask and interfere with acoustic communication (Goutte *et al.* 2013), which tends to be less pronounced in pool areas.

We found that adults of *C. schmidti* occur in forested streams within Iguaçu National Park, exhibiting a complex spatial structure. The species can be considered specialized in forest environments (Caldart *et al.* 2010, Bastiani *et al.* 2012), but generalist in terms of the streams it inhabits, as it occupies a wide range of streams. Furthermore, they can be considered specialists in the type of microhabitat they occupy, as they consistently inhabit similar microhabitats across different streams. These patterns may be related to the behavioral and reproductive adaptations of the species, such as specialized vocalization adapted to the typical noises of this environment (Caldart *et al.* 2011b), visual communication strategies (Caldart *et al.* 2010), and efficient escape behaviors against predation. Future studies could benefit from the inclusion of additional populations, evaluation of oviposition sites, microhabitat choice by tadpoles, and other variables that might play a significant role in habitat selection for this species, such as physicochemical variables of the water. It is crucial for future studies to consider broader spatial and temporal scales by expanding the transects to incorporate greater within-stream variability and the gradient of stream features that this species can inhabit. Simultaneously addressing spatial autocorrelation and imperfect detection by sampling unmarked individuals across multiple sites or observing individual behaviors—such as using photographic documentation of pattern variations to estimate population size, track migration, and assess the likelihood of individuals utilizing different microhabitats—could provide a better understanding of potential threats faced by this species.

Acknowledgments

We would like to thank the Iguaçu National Park staff and Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) for providing collection permits (SISBIO/40772-1 and 40772-2) and logistical support. MVG thanks the UNILA (editais PRPPG nº205/2021, and PRPPG nº 77/2022) and CNPq (PPBio - Process #441157/2023-8 and PELD – Chamada CNPq/CONFAP-FAPs/PELD nº 23/2024) for financial support. We also thank everyone who helped us in the field. We thank Alexandre Vogliotti and Cleto Kaveski Peres for critical reading and suggestions on the initial version of the manuscript. We thank Carlos Eduardo Villar Santos for creating the map shown in Figure 1. This manuscript is part of the Project: Projeto Floriano: desvendando a biodiversidade "intangível".

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Editor: J. Roger Downie

Appendix I. *Microhabitats used by individuals of* Crossodactylus schmidti*, along with their respective environmental features and the positioning of individuals in relation to streams. The data were obtained from 24 microhabitats within the streams of Iguaçu National Park, southern Brazil.*

Micro-habitat	Abundance	Position	Water flow speed (cm/s)	Width (cm)	Depth (cm)	Substrate	Type	Distance from shore (cm)
1	1	Outside	28.82	95	8	Coarse and fine gravel	Riffle	0
2	1	Outside	28.49	93	12	Rock	Riffle	10
3	1	Outside	4.17	200	7	Organic matter and coarse gravel	Pool	103
4	2	Inside	0.00	190	7	Sand	Riffle	98
5	2	Inside	0.00	63	8	Sand	Pool	8
6	1	Inside	0.00	86	7	Organic matter	Pool	10
7	1	Inside	28.09	104	6	Organic matter	Pool	7
8	1	Inside	2.73	154	1	Organic matter	Pool	2
9	1	Outside	55.25	45	3	Rock	Riffle	4
10	1	Outside	4.04	155	6	Rock	Pool	8
11	1	Outside	15.65	160	3	Rock	Pool	5
12	2	Inside	14.04	170	3	Organic matter	Pool	15
13	1	Outside	23.53	228	5	Organic matter	Pool	35
14	1	Inside	40.65	70	5	Sand and fine gravel	Riffle	30
15	1	Inside	25.91	210	2	Rock and sand	Riffle	130
16	1	Inside	0.00	153	3	Organic matter	Pool	17
17	1	Outside	86.98	145	4	Rock	Riffle	30
18	2	Inside	0.00	150	3	Organic matter	Pool	3
19	1	Outside	39.37	76	3	Fine gravel	Riffle	8
20	1	Inside	24.21	144	2	Rock	Fast-flowing	7
21	2	Inside	0.00	181	3	Rock	Pool	8
22	1	Outside	10.24	150	8	Rock	Fast-flowing	5
23	2	Inside	38.31	127	2	Rock and organic matter	Riffle	5
24	1	Inside	0.00	133	3	Organic matter	Pool	14

Appendix II. *Six of the 13 streams sampled in Iguaçu National Park for the study of the population of* Crossodactylus schmidti *(***A–F***), along with photographs of individuals in various microhabitats within the streams (***G–J***).*

Phyllomedusa 23(2):153–163, 2024
© 2024 Universidade de São Paulo - ESALQ
ISSN 1519-1397 (print) / ISSN 2316-9079 (online)
doi: http://dx.doi.org/10.11606/issn.2316-9079.v23i2p153-163

Altitudinal variation in limb size across seven populations of *Rana kukunoris* (Anura: Ranidae) fails to obey Allen's rule

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Abstract

Altitudinal variation in limb size across seven populations of *Rana kukunoris* (Anura: Ranidae) fails to obey Allen's rule. In terms of body size patterns, most research has focused on studying intra- and interspecific variation following the "Bergmannian" patterns. Allen's rule, closely connected to these patterns, has historically been given comparatively less attention. In this study, our aim was to assess the validity of Allen's rule, which suggests that the relative size of limb length should decrease as altitude increases. Here, we investigated the geographical variation in limb length among populations of the Plateau Brown Frog, *Rana kukunoris,* across seven distinct altitudes on the eastern Tibetan plateau. Our findings demonstrated a significant positive correlation between forelimb length of males relative to body size and altitude, contradicting the pattern predicted by Allen's rule. In our study system, we observed a negative correlation between elevation and temperature seasonality as well as water deficit, but a positive correlation with annual total precipitation, speculating that alternative selective forces may play a role in driving the increase in extremity length at high elevations. Long forelimbs only appear in high-altitude environments, not in aggregations with a high operational sex ratio (OSR), suggesting that individuals in stressful environments (e.g., high altitude or latitude) would drive the differentiation in relative extremity length.

Keywords: Body proportion, Environmental factor, Geographical variation, Operational sex ratio, Plateau Brown Frog.

Resumo

Variação altitudinal no tamanho dos membros em sete populações de *Rana kukunoris* (Anura: Ranidae) não obedece à regra de Allen. Em termos de padrões de tamanho do corpo, a maior parte da investigação tem-se centrado no estudo da variação intra e interespecífica segundo os padrões "Bergmannianos". A regra de Allen, intimamente ligada a esses padrões, tem merecido comparativamente menos atenção. Neste estudo, nosso objetivo foi avaliar a validade da regra de Allen, que sugere que o tamanho relativo do comprimento dos membros deve diminuir à medida que a altitude aumenta. Aqui, investigamos a variação geográfica do comprimento dos membros entre

Received 05 March 2024
Accepted 26 August 2024
Distributed December 2024

populações da rã-castanha-do-planalto, *Rana kukunoris*, em sete altitudes distintas no planalto tibetano oriental. Nossos resultados demonstram uma correlação positiva significativa entre o comprimento dos membros anteriores dos machos relativamente ao tamanho do corpo e à altitude, contradizendo o padrão previsto pela regra de Allen. Em nosso sistema de estudo, observamos uma correlação negativa entre a altitude e a sazonalidade da temperatura, bem como o déficit hídrico, mas uma correlação positiva com a precipitação anual total, sugerindo que forças seletivas alternativas podem desempenhar um papel na condução do aumento do comprimento das extremidades em altitudes elevadas. Os membros anteriores longos só aparecem em ambientes de grande altitude e não em agregações com uma elevada razão sexual operacional, sugerindo que ambientes estressantes (por exemplo, grande altitude ou latitude) levariam à diferenciação no comprimento relativo das extremidades.

Palavras-chave: Fator ambiental, Proporção corporal, Rã-castanha-do-planalto, Razão sexual operacional, Variação geográfica.

Introduction

Body size and body size proportions (e.g., extremity length) often exhibit ecogeographical patterns by adaptation to climate (Ballinger and Nachman 2022), such as those described by Bergmann's and Allen's rules (Bergmann 1847, Allen 1877). Bergmann's rule (1847) postulates that organisms inhabiting colder climates generally exhibit a larger body size compared to those in warmer climates. Allen's rule (1877) states that endotherms living in colder environments tend to have shorter appendages (e.g., ears, snout, tail, limbs) than those living in warmer environments (Tabh and Nord 2023). Both rules are supported by empirical studies (Ashton *et al.* 2000, Alhajeri *et al.* 2020), which show that endothermic animals with equal volume can possess varying surface areas, which can either facilitate or hinder their temperature regulation (Jin and Liao 2015). The gradient in body proportions arises from the allometric growth of prominent body parts across different geographic regions (Bidau and Marti 2008, Jin and Liao 2015, Hinckley *et al.* 2022). This phenomenon typically gives rise to latitudinal and/or altitudinal trends in body proportions, leading to the relative shortening of protruding body parts at higher latitudes and altitudes (Hinckley *et al.* 2022).

Ectotherms follow Bergmann's and Allen's rules because they are even more dependent on external temperature than endotherms (Volynchik 2014). Subsequent studies have shown that ectotherms, either vertebrate or invertebrate, may show geographic body size patterns that sometimes align with these rules' predictions (Ray 1960, Blanckenhorn and Demont 2004, Bidau and Marti 2007a,b). Especially regarding Bergmann's rule, which has primarily focused on the analysis of intra- and interspecific patterns conforming to the Bergmannian trend, it is noteworthy that Allen's rule has traditionally received relatively less attention (Bidau and Marti 2008).

Allen's rule has been proposed to be valid in ectotherms (Ray 1960). For example, Allen's rule has been tested in a limited number of ectothermic taxa, including arthropods (Bidau and Marti 2008), amphibians (Alho *et al.* 2011), and lizards (Langkilde 2009, Jin and Liao 2015). In ectotherms, the thermoregulatory adaptation hypothesis is less clear than in endotherms. Ectotherms primarily elevate their body temperature through exposure to external sources of heat and minimize heat absorption with a relatively smaller body surface area (Alho *et al.* 2011). This characteristic can be advantageous in thermally variable environments, allowing ectotherms to avoid overheating in hot microhabitats while conserving thermal energy in colder ones (Angilletta 2009).

Male-male competition is common in some amphibian species (Black and Brunson 1971, Davies and Halliday 1979, Lamb 1984, Reading and Clarke 1983, Hoglund 1989, Luo *et al.* 2016, Deng *et al.* 2020, de Sa *et al.* 2020). Male competition for females is expected when the operational sex ratio (OSR, the ratio of sexually competing males to fertilizable females) is male-biased (Emlen 1976, Kvarnemo and Ahnesjo 1996). As the OSR becomes increasingly biased in breeding aggregations, competitors are expected to exhibit escalating levels of aggressiveness in their efforts to defend mates (Weir *et al.* 2011). In a highly male-biased OSR, amplectant males with larger limbs form more secure pairings and are less likely to be dislodged. As a result, the size of the forelimb and hind limb of male toads may affect pairing competition (Lee 2001, Lee and Corrales 2002). Larger limbs might be expected in a highly male-biased OSR, indicative of strong competition. Relatively longer limbs in amphibians can be related to the locomotor capacity, and this, in turn, may influence the ability to avoid predators (Tejedo *et al.* 2000), help maintain communication between populations (Trochet *et al.* 2019), enhance spermatophore transfer during courtship (Morrison and Hero 2003), or aid in dispersal ability at high elevations (Phillips *et al.* 2006).

In this study, we investigated variation in limb characteristics of both female and male Plateau Brown Frogs, *Rana kukunoris* Nikolskii, 1918, along a broad elevational gradient (2000–4400 m a.s.l.) (Fei *et al.* 2012). These frogs are consequently exposed to a wide range of environmental factors. The main objectives of this work were to: (1) investigate whether the species adheres to the prediction put forth by Allen's rule, which suggests that populations at higher elevations possess relatively shorter limbs; and (2) verify whether a correlation exists between limb length and the population-specific OSR.

Materials and Methods

Study Species

Rana kukunoris is a frog species endemic to the eastern Tibetan Plateau and exhibits a broad distribution at elevated elevations (ranging from 2000 to 4400 m a.s.l.) (Fei *et al.* 2012). Similar to many other amphibian species, *R. kukunoris* exhibits female-biased size dimorphism (Yu *et al.* 2022a). Previous analysis of several populations of *R. kukunoris* found a negative relationship between body size and altitude (Yu *et al.* 2022b), which follows Bergmann's rule. This species is a typical explosive breeder (Wells 2007) and experiences a brief breeding period lasting from 9 to 21 days (Yu *et al.* 2018). Due to the colder temperatures at higher altitudes, the breeding time of high-altitude brown frogs is delayed, providing us with additional time to investigate the correlation between altitude and the development of limbs in sexually mature individuals.

Study Sites and Sample Collection

We collected a total of 417 *R. kukunoris* (157 females and 260 males) from seven populations (ranging from 2297 to 3441 m in altitude; breeding populations below 2500 m and above 3500 m are uncommon) between 2011 and 2013 during reproductive periods in the eastern Tibetan Plateau of southwest China, which includes areas such as eastern Qinghai and southern Gansu (101°34'–102°69' E, 34°29' –36°68' N; Figure 1, Table 1). Annual mean temperature (AMT), annual total precipitation (ATP), temperature seasonality (TS), and precipitation seasonality (PS) were extracted from WorldClim at a resolution of 0.167° × 0.167° grid cells (Hijmans *et al.* 2005). AET and PET were extracted at a grid cell resolution of 0.5° × 0.5°, following the methodology of Willmott and Matsuura (2001). In these locations, we observed a positive correlation between altitude and ATP by calculating Pearson's

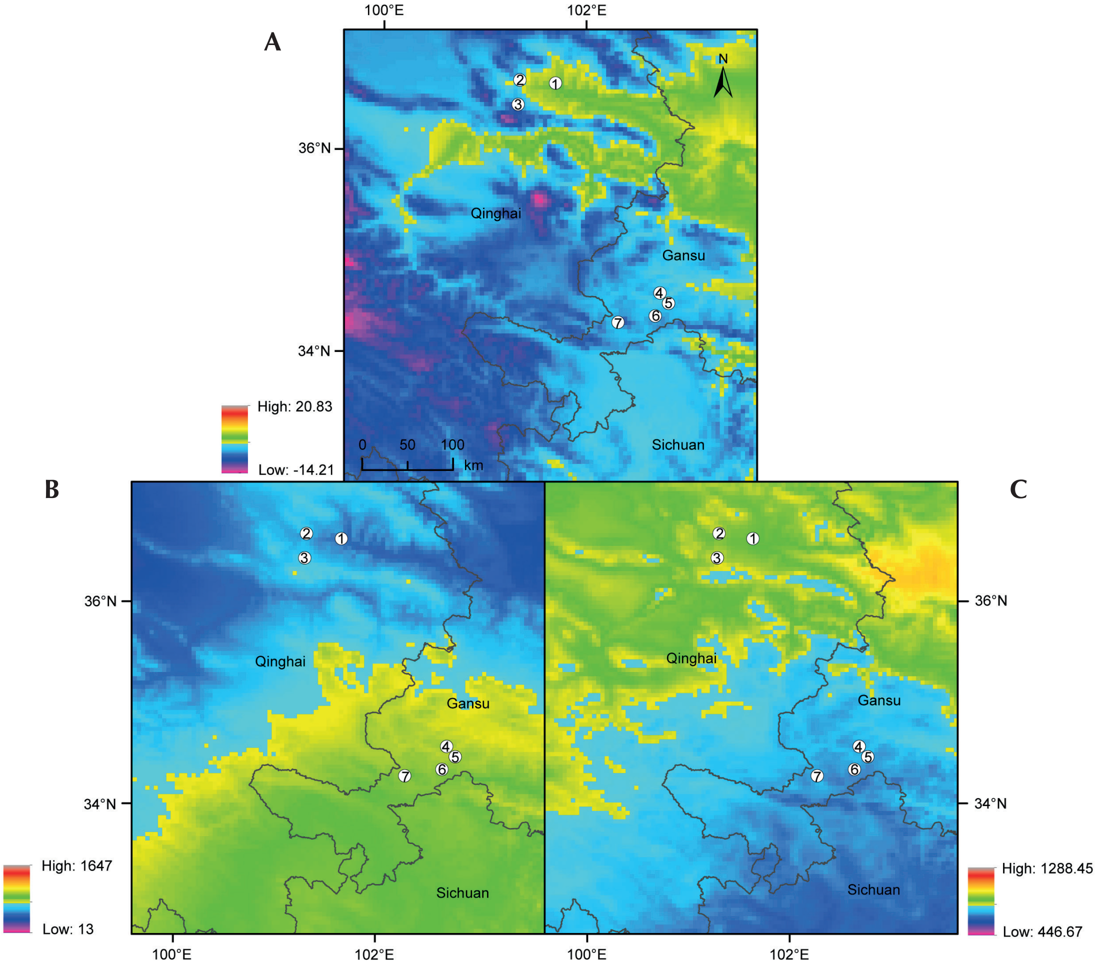

Figure 1. Topographic map showing the location of the seven study populations of *Rana kukunoris* on the eastern Tibetan plateau. Climatic layers are (**A**) annual mean temperature, (**B**) annual total precipitation, and (**C**) temperature seasonality.

correlation coefficients, while there was a negative correlation between TS and water deficit (WD, PET minus AET) (Table 2). For all sites, frogs were captured randomly and by hand with gloves every other day during their breeding seasons, particularly when they were in amplexus or actively seeking mates. Usually, sampling at each site was carried out over a period of one to three days. We verified the maturity of all individuals by directly observing the secondary sexual characteristics, such as the presence of nuptial pads on the first finger in males or an enlarged abdomen for females. Furthermore, owing to the reduced water temperature experienced during the breeding season, most sexually mature individuals congregate at the breeding ponds.

Snout–vent length (SVL), forelimb length (FL) measured from the axilla to the tip of the longest toe on the left forelimb (Yu and Lu

Table 1. Study site details, including altitude, latitude, environmental predictors, morphological traits, and sample sizes for seven high-altitude populations of *Rana kukunoris*in in the Tibet Plateau. SVL: Snout–vent length, AMT: Annual mean temperature, TS: Temperature seasonality, ATP: Annual total precipitation, PS: Precipitation seasonality, PET: Potential evapotranspiration, AET: Actual evapotranspiration, WD: Water deficit.

Sites	Altitude (m)	Longitude (degrees)	Latitude (degrees)	OSR	Sex	SVL (mm)	Forelimb length (mm)	Hind leg length (mm)	AMT	TS	ATP	PS	PET	AET	WD
1. Dabaozi'cun	2297	101.65	36.65	1.55	F	61.75 ± 6.84(8)	30.81 ± 4.46(8)	77.05 ± 7.07(8)	5.3	8347	399	92	45.07	30.93	14.138
2. Shiya'zhuang	2594	101.34	36.68	1.93	F	62.56 ± 7.23(20)	28.10 ± 3.16(20)	73.08 ± 6.40(20)	3.5	8374	419	95	34.45	30.37	4.079
					M	59.23 ± 4.72(20)	27.79 ± 2.81(20)	71.27 ± 4.35(20)							
3. Damoshi'cun	2789	101.44	36.49	2.00	F	61.19 ± 6.48(70)	28.41 ± 4.42(70)	73.29 ± 7.45(70)	0.2	7965	440	94	31.83	28.27	3.559
					M	53.50 ± 6.49(65)	24.83 ± 4.72(65)	68.68 ± 7.60(65)							
4. Zecha'zhan	3049	102.69	34.49	1.97	F	66.05 ± 4.49(20)	32.55 ± 3.07(20)	78.40 ± 5.78(20)	1.5	7198	614	84	29.71	29.7	0.003
					M	58.90 ± 2.94(25)	29.48 ± 2.89(25)	75.82 ± 5.43(25)							
5. Shibadao'wan	3060	102.69	34.47	1.67	F	61.59 ± 6.27(11)	30.45 ± 3.78(11)	75.55 ± 6.71(11)	1.4	7178	620	84	29.71	29.70	0.003
					M	53.28 ± 3.55(26)	27.00 ± 3.46(6)	84.62 ± 7.38(26)							
6. Shilin' zhan	3188	102.68	34.37	2.20	F	58.66 ± 5.40(28)	29.36 ± 4.73(28)	73.43 ± 4.35(28)	1.0	7129	631	85	29.71	29.7	0.003
					M	51.82 ± 4.94(114)	28.24 ± 4.64(30)	80.70 ± 8.27(114)							
7. Guoguo' ri	3441	102.31	34.29	1.88	M	53.33 ± 2.93(10)	28.85 ± 2.73(10)	67.90 ± 4.91(10)	0.8	7125	642	87	31.95	31.94	0.005

Table 2. Correlation coefficients between each environmental variable. AMT: Annual mean temperature, TS: Temperature seasonality, ATP: Annual total precipitation, PS: Precipitation seasonality, PET: Potential evapotranspiration, AET: Actual evapotranspiration, WD: Water deficit. **$p < 0.001$, *$p < 0.05$.

Parameters	Altitude	AMT	TS	ATP	PS	PET	AET	WD
Altitude		**-0.806***	**-0.919****	**0.923****	-0.713	**-0.796***	0.142	**-0.878****
AMT	**-0.806***		0.704	-0.634	0.417	0.879**	0.407	0.845*
TS	**-0.919****	0.704		-0.988**	0.923**	0.742	-0.048	0.801*
ATP	**0.923****	-0.634	-0.988**		-0.925**	-0.722	0.159	-0.803*
PS	-0.713	0.417	0.923**	-0.925**		0.549	-0.094	0.606
PET	**-0.796***	0.879**	0.742	-0.722	0.549		0.382	0.979**
AET	0.142	0.407	-0.048	0.159	-0.094	0.382		0.184
WD	**-0.878****	0.845*	0.801*	-0.803*	0.606	0.979**	0.184	

2010), and hindlimb length (HL) measured from the posterior midline trunk to the tip of the fourth toe on the left hindlimb (Xia *et al.* 2011) were recorded for each captured frog. The measurements were carried out using a plastic ruler, with an accuracy of up to 1 millimeter. During the process, efforts were made to fully extend the frog's limbs. To minimize any potential errors, all measurements were conducted by the same person (TLY). Body mass of each frog was measured using an electronic balance to the nearest 0.01 g. Measured single or paired frogs were temporarily placed in a designated holding container within the field laboratory, such as a 15-Liter capacity barrel, to prevent duplicate sampling and the transmission of diseases. During the three-years study period, five of the six populations were surveyed only once, thereby substantially diminishing the likelihood of duplicate sampling. Upon completion of all necessary field measurements, individuals were promptly and safely returned to their original spawning grounds, ensuring their return to their natural habitat with minimal interference.

We calculated operational sex ratio (OSR) within each population. OSR was determined by dividing the number of males by the number of females capable of reproduction (i.e., those capable of carrying eggs) present at a breeding site over a specific period, usually spanning several days (Emlen and Oring 1977).

Statistical Analyses

We used log10 transformation of the SVL, FL, and HL of each individual to achieve normality. The One-Sample Kolmogorov-Smirnov Test was used to test whether data met normal distribution. We used one-way analyses of variance (ANOVAs) and Welch's t-tests to analyze variation of SVL, FL, and HL between populations or between sexes. Linear mixed models (LMMs) were used to quantify the relationship between FL/HL and SVL. To test for altitudinal variation of FL/HL we used LMMs where FL/HL was treated as the dependent variable, SVL, and altitude as covariates, and population as a random effect.

We used LMMs where FL/HL was treated as the dependent variable, SVL, altitude, and OSR as covariates, and population as a random effect to test whether FL/HL covaried with OSR, as an indicator of competition levels. In the subsequent analyses of reproductive traits against competition levels, altitude was not included in the simplified models because it was a non-significant fixed effect. The software IBM SPSS Statistics 20.0 (IBM Corp., Armonk, NY, USA) was used for all analyses.

Results

Mean body size, FL, and HL differed significantly among the populations for each sex (male, SVL, $F_{5, 254} = 12.381$, $p < 0.001$; FL, $F_{5,150} = 7.171$, $p < 0.001$; HLL, $F_{5,254} = 30.310$, $p < 0.001$; female, SVL, $F_{5, 151} = 13.251$, $p = 0.008$; FL, $F_{5, 151} = 3.532$, $p = 0.005$; HLL, $F_{5, 151} = 2.386$, $p = 0.041$). Females were significantly larger in SVL, FL, and HL than males in 4, 2, and 3 of 5 populations (all $p < 0.05$). LMMs showed that mean body size of both sexes was not significantly negatively correlated with altitude (male: $t = -1.265$, $df = 4.609$, $p = 0.266$; female: $t = -0.296$, $df = 5.253$, $p = 0.779$) when controlling for population (random effect: $Z = 1.280$, $p = 0.200$; $Z = 1.051$, $p = 0.293$).

Further, LMMs showed that FL (male: $t = 7.728$, $df = 153.844$, $p < 0.001$; female: $t = 5.052$, $df = 154.640$, $p < 0.001$) and HL (male: $t = 11.546$, $df = 255.107$, $p < 0.001$; female: $t = 9.079$, $df = 150.588$, $p < 0.001$) of both sexes was significantly positively correlated with SVL when controlling for population (MFL, random effect: $Z = 1.369$, $p = 0.171$; MHLL, $Z = 1.541$, $p = 0.123$; FFL, random effect: $Z = 0.983$, $p = 0.325$; FHLL, $Z = 0.646$, $p = 0.518$). After removing the effect of SVL (male, fixed effect: $t = 8.299$, $df = 142.177$, $p < 0.001$; female, $t = 5.075$, $df = 152.704$, $p < 0.001$)) and population (male, random effect: $Z = 0.957$, $p = 0.339$; female, $Z = 0.795$ $p = 0.427$), the LMMs revealed that FL of males was significantly positively correlated with altitude ($t = 2.954$, $df = 5.439$, $p = 0.029$; Figure 2), but FL of females was not correlated with altitude ($t = 0.796$, $df = 4.831$, $p = 0.464$). When controlling for the effect of SVL (male, fixed effect: $t = 11.568$, $df = 254.334$, $p < 0.001$; female, $t = 9.097$, $df = 143.322$, $p < 0.001$) and population (male, random effect: $Z = 1.371$, $p = 0.170$; female, $Z = 0.434$, $p = 0.664$), the LMMs revealed that HL was not correlated with altitude (male, $t = 0.729$, $df = 4.044$, $p = 0.506$; female, $t = 0.688$, $df = 3.953$, $p = 0.530$; Figure 2).

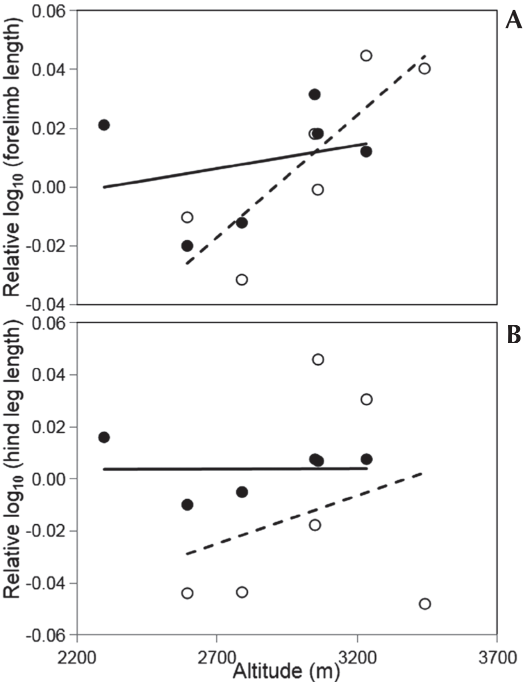

Figure 2. Relationship between altitude and (**A**) forelimb length and (**B**) hind limb length in seven populations of *Rana kukunoris*. Solid lines (filled circles): females; dashed line (open circles): males. Data points are female and male population means. Relative log10 was generated from regression of log10 (forelimb length) or log10 (hind limb length) on log10 (male body size).

The LMMs showed that FL (male: $t = 0.834$, $df = 6.218$, $p = 0.435$; female: $t = -1.640$, $df = 3.492$, $p = 0.187$) or HL (male: $t = -0.206$, $df = 2.934$, $p = 0.850$; female: $t = -1.479$,

$df = 3.695$, $p = 0.219$) was not correlated with the OSR when controlling for population (MFL, random effect: $Z = 0.885$, $p = 0.376$; FFL, $Z = 0.385$, $p = 0.700$; MHLL, random effect: $Z = 1.196$, $p = 0.232$; FHLL, $Z = 0.134$, $p = 0.894$) and the fixed effect of SVL (MFL, $t = 8.317$, $df = 146.979$, $p < 0.001$; FFL, $t = 5.111$, $df = 142.289$, $p < 0.001$; MHLL, $t = 11.574$, $df = 254.008$, $p < 0.001$; FHLL, $t = 9.032$, $df = 131.632$, $p < 0.001$), and altitude (MFL, $t = 2.757$, $df = 4.623$, $p = 0.043$; FFL, $t = 1.922$, $df = 2.828$, $p = 0.156$; MHLL, $t = 0.395$, $df = 3.012$, $p = 0.719$; FHLL, $t = 1.627$ $df = .679$, $p = 0.213$). In a reduced model controlling only for population (MFL, random effect: $Z = 1.231$, $p = 0.218$; FFL, $Z = 0.969$, $p = 0.333$; MHLL, random effect: $Z = 1.387$, $p = 0.166$; FHLL, $Z = 0.969$, $p = 0.333$), and SVL (MFL, $t = 7.953$, $df = 152.997$, $p < 0.001$; FFL, $t = 4.982$, $df = 153.886$, $p < 0.001$; MHLL, $t = 11.555$, $df = 254.680$, $p < 0.001$; FHLL, $t = 4.982$, $df = 153.886$, $p < 0.001$), FL or HL did not increase with the OSR (MFL, $t = 0.677$, $df = 5.330$, $p = 0.527$; FFL, $t = -0.515$, $df = 5.987$, $p = 0.625$; MHLL, $t = -0.191$, $df = 3.953$, $p = 0.858$; FHLL, $t = -0.634$, $df = 6.870$, $p = 0.546$).

Discussion

Based on Allen's rule, we would anticipate that natural selection would favor relatively shorter extremities in colder environments, leading to a negative correlation between altitude and relative limb length. However, our research outcomes obtained from the study on the wild population of Plateau Brown frogs contradicted this simple prediction, as we observed a clear linear correlation between altitude and forelimb length of males after adjusting for snout-vent length. This correlation indicates the presence of an underlying environmental pattern that produces a pattern that is the opposite of Allen's rule.

Ectotherms with varying surface area to volume ratios exhibit varying rates of heat exchange, potentially leading to disparate selection pressures in distinct thermal environments (Jin and Liao 2015). The relative lengths of extremities, particularly limb segments, in four lacertid species—*Phoenicolacerta laevis* (Gray, 1878), *Ophisops elegans* (Ménétries, 1832), *Acanthodactylus boskianus* (Daudin, 1802), and *Mesalina guttulate* (Lichtenstein, 1823)—have been observed to generally increase in hotter environments in accordance with Allen's rule (Volynchik 2014). Amphibians, as thermoconformers, are typically dependent on moist conditions due to their permeable skin, which poses a risk of desiccation, and the fact that their larval life history is usually aquatic (Olalla-Tarraga and Rodriguez 2007, Ficetola *et al.* 2010). In this study system, we observed that only the relative forelimb length of males increased with elevation, deviating from the expected pattern according to Allen's rule. Our finding suggests that in amphibians, the primary purpose of limbs is evidently not thermoregulation (Alho *et al.* 2011).

The environmental effects encountered during the larval stage can also extend into the adult phase (Blouin and Brown 2000, Gomez-Mestre *et al.* 2010). Different climatic or biotic factors, such as increased rainfall and water abundance, reduced nutrient availability, decreased predation pressure, and alterations in food quality and abundance (Hinckley *et al.* 2022), may potentially encourage a shift toward more terrestrial foraging at higher elevations. This pattern is similar to the observed trend along altitudinal gradients in the Plateau Brown frog. We found a negative correlation between elevation and annual mean temperature, temperature seasonality, and potential evapotranspiration as well as water deficit, but a positive correlation with annual total precipitation, thus indicating that alternative selective forces may be responsible for driving the increase in extremity length at high elevations (Kitayama 1992).

The forelimb length of Plateau Brown frogs exhibited variation along the elevational gradient after accounting for snout–vent length. As anticipated under the converse of Allen's rule, we observed a positive correlation between relative forelimb length and elevation. This result was consistent with previous studies (Jin and Liao 2015, Jaffe *et al.* 2016). Conversely, at higher elevations, having relatively longer forelimbs also could potentially provide several advantages. First, longer forelimbs could assist in effectively lifting the body, allowing for better maneuverability in steep terrains. Additionally, longer forelimbs could enlarge the field of view, enabling individuals to see further distances and detect potential threats such as predators or competitors. Moreover, elongated forelimbs might confer advantages during courtship by preventing frequent attempts by single males to displace amplexing males, ultimately increasing their breeding success. Long forelimbs only appear in high-altitude environments, not in aggregations with a high OSR, suggesting that harsh environments (high altitude or latitude) could drive the differentiation in relative extremity length.

In conclusion, our findings demonstrate a significant correlation between altitude and the relative lengths of forelimbs, which is inconsistent with Allen's rule. The varying environmental and biotic factors, such as increased rainfall and water abundance, reduced nutrient availability, decreased predation pressure, and alterations in food quality and abundance at high elevations, are likely interacting and influencing the observed increase in extremity length at high elevations.

Acknowledgments

We express our sincere gratitude to the dedicated staff of the Gahai-Zecha National Nature Reserve for their invaluable field assistance. The Gahai-Zecha National Nature Reserve Management Bureau approved this project (approval number GHZCRMB/03-212014) and gave permission for fieldwork. The study was funded by National Natural Science Foundation of China (Grant No. 31741019). All field measurements were conducted in strict adherence to the ethical guidelines set forth by the Animal Ethics Committee at Xinyang Normal University, ensuring the welfare and ethical treatment of the animals involved. We affirm that all animals utilized in this study were handled with utmost care and in accordance with ethical standards, following the guidelines established by the relevant institutional animal care regulations in China.

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Editor: Thiago R. Carvalho

Phyllomedusa 23(2):165–178, 2024
© 2024 Universidade de São Paulo - ESALQ
ISSN 1519-1397 (print) / ISSN 2316-9079 (online)
doi: http://dx.doi.org/10.11606/issn.2316-9079.v23i2p165-178

The advertisement call of *Espadarana prosoblepon* (Anura: Centrolenidae) from a population in the Central Andes of Colombia

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Abstract

The advertisement call of *Espadarana prosoblepon* (Anura: Centrolenidae) from a population in the Central Andes of Colombia. The advertisement call of the glass frog *Espadarana prosoblepon* has been described for populations from Costa Rica in Central America and Ecuador in South America, but not for populations in Colombia. Because having a detailed description of the advertisement call of several populations of the same species is important for taxonomy, phylogenetics, ecology, behavior, and evolution, we describe the advertisement call of a population of *E. prosoblepon* in Colombia, South America. We analyzed 58 temporal and spectral features of 220 advertisement calls emitted by 31 males recorded between October–December 2023 at the "Cedro Rosado" Botanical Garden in Armenia, Department of Quindío, Central Andes of Colombia. In general, the advertisement call of *E. prosoblepon* in our study population consisted of 2–4 pulsed notes (beeps) with modulated frequency. The mean duration of the call was 230 ms (range 190–430 ms) and of the notes was 40.1 ms (31.0–48.7 ms), separated by intervals of 143.5 ms (71.5–181 ms). The dominant frequency of the call was 5.2 kHz (4.9–5.6 kHz). Our results suggest slight differences in note duration between the studied population in Colombia and populations of *E. prosoblepon* in Costa Rica and Ecuador. Further studies are necessary to test for potential effects of temperature and body size on call features both at the intra- and interpopulation levels. Given the wide latitudinal and altitudinal geographic distribution of *E. prosoblepon*, intraspecific geographic variation in its call is possible.

Keywords: Acoustic communication, Advertisement call, Amphibians, bioacoustics, intraspecific geographical variation.

Resumo

Canto de anúncio de *Espadarana prosoblepon* (Anura: Centrolenidae) de uma população dos Andes Centrais da Colômbia. O canto de anúncio da rã-de-vidro *Espadarana prosoblepon* foi descrito para populações da Costa Rica, na América Central, e do Equador, na América do Sul, mas não para populações da Colômbia. Como ter uma descrição detalhada do canto de anúncio de várias

Received 21 June 2024
Accepted 14 November 2024
Distributed December 2024

populações da mesma espécie é importante para a taxonomia, filogenia, ecologia, comportamento e evolução, descrevemos o canto de anúncio de uma população de *E. prosoblepon* da Colômbia. Analisamos 58 caraterísticas temporais e espectrais de 220 cantos de anúncio emitidas por 31 machos gravados entre outubro e dezembro de 2023 no Jardim Botânico “Cedro Rosado”, em Armenia, Departamento de Quindío, Andes Centrais da Colômbia. Em geral, o canto de anúncio de *E. prosoblepon* da população estudada consistiu de 2–4 notas pulsadas (bipes) com frequência modulada. A duração média do canto foi de 230 ms (intervalo 190−430 ms) e das notas foi de 40,1 ms (31,0−48,7 ms), separados por intervalos de 143,5 ms (71,5−181 ms). A frequência dominante do canto foi de 5,2 kHz (4,9−5,6 kHz). Nossos resultados sugerem pequenas diferenças na duração da nota entre a população estudada na Colômbia e as populações de *E. prosoblepon* da Costa Rica e do Equador. São necessários mais estudos para testar os potenciais efeitos da temperatura e do tamanho do corpo nas caraterísticas do canto, tanto intra como interpopulacional. Dada a ampla distribuição geográfica latitudinal e altitudinal de *E. prosoblepon*, é possível que haja variação geográfica intraespecífica em seu canto.

Palavra-chave: Anfíbios, Bioacústica, Canto nupcial, Comunicação acústica, Variação geográfica intraespecífica.

Introduction

Centrolenidae is a Neotropical clade of 166 species of arboreal frogs that have nocturnal habits and breed along streams (Frost 2024). Among centrolenids, one of the most studied species is *Espadarana prosoblepon* (Boettger, 1892) (Figure 1). This species is distributed from sea level to approximately 1800 m elevation from the eastern region of Honduras in Central America to Colombia and Ecuador in South America (Basto-Riascos *et al.* 2017a, Frost 2024, MR pers. obs.). Adult females of this species have a body size (snout–vent length, SVL) between 25−31 mm, while in adult males it varies between 23−28 mm (Basto-Riascos *et al.* 2017a). Although variation in morphological characters includes a dorsal color pattern that is uniformly green or has blue and yellow spots (Guayasamin *et al*. 2020), the most significant diagnostic character of *E. prosoblepon* is the presence of a well-developed blade-shaped *crista dorsalis* of the humerus in adult males (Savage 2002, Kubicki 2007, Guayasamin *et al*. 2020; Figure 1). Studies on *Espadarana prosoblepon* cover aspects of phylogenetic relationships (Guayasamin *et al.* 2009, 2020), population dynamics (Robertson *et al.* 2008, Angeli *et al.* 2015), male-male agonistic behavior (Krohn and Voyles 2014, Hedman and Hughey 2015, Rios-Soto *et al.* 2017), sexual selection (Jacobson 1985, Basto-Riascos *et al.* 2017b, Goyes-Vallejos *et al.* 2021), parental care (Goyes-Vallejos *et al.* 2022, 2024), and oviposition site preference and external morphological attributes of embryos through ontogeny (Salazar-Nicholls and Del Pino 2015, Ortiz-Ross *et al.* 2020). This species has been included in macroevolutionary studies about diversification and ecology (Hutter *et al.* 2013, Castroviejo-Fisher *et al.* 2014, Delia *et al.* 2017, Escalona *et al.* 2019, Mendoza-Henao *et al.* 2023, Valencia-Aguilar *et al.* 2024, Vargas-Salinas *et al.* 2024).

The advertisement call of *E. prosoblepon* has been described based on populations from Costa Rica in Central America (Jacobson 1985, Savage 2002, Kubicki 2007) and Ecuador in South America (Freile *et al.* 2020, Guayasamin *et al.* 2020), but those studies only provide a brief description of a few of call features and its variation of relatively few individuals. Despite being one of the most widely distributed glass frog species, no quantitative and detailed call description based on Colombian populations exists (Rivera-Correa *et al.* 2021, Duarte-Marín *et al.* 2022). Call descriptions based on

Figure 1. An adult male *Espadarana prosoblepon* in the "Cedro Rosado" Botanical Garden in Armenia, Department of Quindío, Central Andes of Colombia. Note the pattern of black and yellow dots on the dorsum, which is distinctive among individuals, and the ventrolateral projection of the *crista ventralis* of the humerus (humeral spine) present only in reproductive males. Specimen not collected.

populations of Costa Rica and Ecuador have not examined the potential relationship of call features with body temperature and body size. Anurans are ectothermic vertebrates and the functioning of the muscles involved in air pumping for call production is influenced by temperature; in addition, body size determines spectral call features such as dominant frequency (Gerhardt and Huber 2002). Interspecific differences and variation in advertisement call features within and between populations of the same species may be a consequence of differences in environmental temperature and body size (Ryan and Wilczynski 1991, Gerhardt and Huber 2002, Lingnau and Bastos 2007).

Having a detailed description of the advertisement call from several populations of a frog species allows inferences to be made in taxonomy (Padial *et al.* 2008, Köhler *et al.* 2017), phylogenetics (Duellman 2007, Schneider and Sinsch 2007), ecology, behavior, and evolution (Cocroft and Ryan 1995, Bosch *et al.* 2003, Robillard *et al.* 2006, Both and Grant 2012). Furthermore, a detailed characterization of the advertisement call and its interpopulation variability allows optimization of the mathematical algorithms used for monitoring populations based on automated recordings (López-Baucells *et al.* 2019). Herein, we describe the advertisement call of *E. prosoblepon* from an urban forest fragment in the Central Andes of Colombia. We also test for the influence of temperature and body size in call features of the advertisement call.

Materials and Methods

Study Site

The study was conducted from 10 October to 19 December 2023, in an urban forest fragment known as the "Cedro Rosado" Botanical Garden (04°32'40" N, 75°46'13" W; 1490−1530 m a.s.l.), located at the University of Quindío, Armenia, Colombia. This forest has an area of 15 ha, with an average annual temperature of 19°C, relative humidity between 65–75%, and a bimodal rainfall distribution with an average annual precipitation of 2436 mm (Rodríguez 1999).

Recordings were made with a Sennheiser ME66/K6 unidirectional microphone located at 50−150 cm in front of calling males and connected to a Tascam DR-40X digital recorder. We recorded body size (snout–vent length, SVL) and temperature of the calling frog with a Mitutoyo digital caliper (precision ± 0.01) and an Extech Series 42510 infrared thermometer (precision 0.1°C), respectively. All recordings were performed between 19:00 − 00:00 h. We identified males individually based on the pattern of yellow and black dorsal dots to avoid pseudoreplication (Figure 1).

Calls were recorded in .wav format, digitized at 16 bits resolution and 44.1 kHz sampling rate; measurements of call features were made using the software RAVEN Pro 1.6 (Yang 2024). Spectrograms were created with a Fast Fourier Transformation window of 256 points and Blackman algorithm. The following call features were quantified (*sensu* Köhler *et al.* 2017): call duration (ms), number of notes, note duration (ms), interval between notes (ms), note emission rate (number of notes/call duration), dominant frequency of the note (kHz), and low and high call frequency of the note (kHz). Low and high frequencies of the note were measured at 20 dB (re 20 mPA) below the peak intensity of the dominant frequency, which is the value at which the signal energy could still be clearly distinguished from the background noise. We recorded dominant, low, and high frequency at the initial, middle, and final part of each note. Harmonic bands were evident in some recordings; we recorded the frequency of them when applicable. We recorded mean ± standard deviation and range obtained from all calls per male. None of the recorded individuals was collected. Copies of recordings obtained in this study were deposited in the Environmental Sound Collection of the Instituto de Investigación de Recursos Biológicos Alexander von Humboldt (IAvH-CSA), Villa de Leyva, Boyacá, Colombia, and the codes attached to the recordings range from IAvH-CSA-39409 to IAvH-CSA-39439 (http://colecciones.humboldt.org.co/sonidos/).

Call features of advertisement calls of anurans are generally inter-correlated; therefore, after verifying such inter-correlation in our data, the number of variables (spectral and call features) was reduced by conducting a principal component analysis (PCA) with Varimax-rotation (Johnson and Wichern 2014). Since we detected and quantified the frequency of harmonics for some males but not others, these call features were not included in the PCA. The number of resulting components was determined considering an explained variance greater than 80% and eigenvalues > 1 (Cliff 1988, Peres-Neto 2005). The principal components (PCs) obtained from this analysis were used as dependent variables in four multiple linear regressions (Zar 1984). Each of these regressions was performed to test the relationship between call features in the PCs with temperature and body size as independent variables. Both the PCA and the multiple regression analysis were performed using the software SPSS v.21.0.0.0 (SPSS Inc 1999).

Results

The following description is based on 220 calls recorded from 31 males of *Espadarana prosoblepon*. The advertisement call of this species consists of 2.1 ± 0.2 "beeps" in average (range 2–4 beeps) pulsatile notes with amplitude and frequency modulation (Figure 2). Notes last 40.1 ± 4.8 ms (range 31.0–48.7 ms; $N = 31$ males), and consecutive notes are separated by silent intervals of 143.5 ± 18.7 ms (71.5–181 ms). Mean call duration was 230 ± 40 ms (190–430 ms), and call duration increased with the number of notes per call (Pearson correlation: $r = 0.81, p < 0.001$). Note emission rate was 9.2 ± 0.9 calls/s (7.4–11 calls/s). The dominant frequency of the first note was 5.3 ± 0.2 kHz (4.9–5.6 kHz), of the second note was 5.2 ± 0.2 kHz (4.9–5.6 kHz), and of the third note was 5.1 ± 0.1 kHz (4.9–5.2 kHz; $N = 3$). Only one male emitted a call with four notes; the mean dominant frequency of this fourth note was 5.1 kHz. The low frequency of the first note was 4.8 ± 0.2 kHz (4.5–5.2 kHz; $N = 31$), and the high frequency was 5.8 ± 0.2 kHz (5.6–6.1 kHz); for the second note the low frequency was 4.8 ± 0.2 kHz (4.4–5.1 kHz), and the high frequency was 5.8 ± 0.2 kHz (5.5–6.1 kHz); and for the third note the low frequency was 4.8 ± 0.2 kHz (4.6–5.0 kHz; $N = 3$), and the high frequency was 5.6 ± 0.1 kHz (5.5–5.8 kHz; $N = 3$). The low and high frequency for the fourth note was 4.8 kHz and 5.9 kHz, respectively ($N = 1$ male). After pooling the data of dominant, low, and high frequency for all the notes and males, we determined that the mean dominant call frequency of *E. prosoblepon* in the studied population was 5.2 ± 0.2 kHz (4.9–5.6 kHz; $N = 31$), the low frequency was 4.8 ± 0.2 kHz (4.5–5.2 kHz), and the high frequency was 5.8 ± 0.2 kHz (5.6–6.1 kHz). Detailed data of the dominant, low, and high frequency at the initial, middle, and final part of each note is summarized in Table 1. We recorded up to three harmonics in the call of some males; the first harmonic was at 10.3 ± 0.4 kHz (9.7–

11.1 kHz; *N* = 30), the second harmonic was at 15.6 ± 0.6 kHz (14.3–16.7 kHz; *N* = 26), and the third harmonic was at 14.9 kHz in only one male.

The variability in the 26 advertisement call features included in the PCA was summarized into four PCs (Table 2). PC1 and PC2 grouped all the spectral features of the call, while PC3 and PC4 grouped temporal features of the call. When we tested the relationship of these PCs with temperature and body size (Figure 3), we found that temperature does not relate to PC1 (β = 0.001, t = 0.006, *p* = 0.995), PC2 (β = 0.018, t = 0.097, *p* = 0.923), and PC3 (β = -0.017, t = -0.1, *p* = 0.921), but relates in a negative way with PC4 (β = -0.58, t = -3.83, *p* = 0.001), while body size does not relate to PC1 (β = -0.073, t = -0.388, *p* = 0.701), PC2 (β = -0.043, t = -0.229, *p* = 0.820) and PC4 (β = 0.166, t = 1.094, *p* = 0.283), but relates in a negative way with PC3 (β = -0.455, t = -2.703, *p* = 0.012).

Discussion

The genus *Espadarana* is composed of five species (Frost 2024). The advertisement call has been described for *E. prosoblepon* (Jacobson 1985, Savage 2002, Kubicki 2007, Freile *et al.* 2020, Guayasamín *et al.* 2020), *E. andina*

Table 1. Summary of the dominant, low, and high frequency (kHz) values per note of the advertisement call of the glass frog *Espadarana prosoblepon* in the "Cedro Rosado" Botanical Garden in Armenia, Department of Quindío, Central Andes of Colombia. Data are presented as mean ± standard deviation and range. Sample size (number of males recorded, *N*) varies because not all males emitted advertisement calls with the same number of notes. See text for other temporal and spectral features of the calls.

	Note 1 *N* = 31	**Note 2** *N* = 31	**Note 3** *N* = 5	**Note 4** *N* = 1
Initial part of the note				
Dominant frequency	5.23 ± 0.17 (4.91 – 5.51)	5.22 ± 0.17 (4.99 – 5.49)	5.12 ± 0.14 (4.99 – 5.34)	5.17 ± 5.17
Low frequency	4.81 ± 0.19 (4.48 – 5.20)	4.82 ± 0.18 (4.40 – 5.24)	4.73 ± 0.15 (4.59 – 4.99)	4.68
High frequency	5.67 ± 0.18 (5.37 – 6.05)	5.64 ± 0.19 (5.35 – 6.03)	5.54 ± 0.09 (5.43 – 5.68)	5.55
Middle part of the note				
Dominant frequency	5.45 ± 0.16 (5.17 – 5.81)	5.43 ± 0.17 (5.17 – 5.76)	5.20 ± 0.08 (5.17 – 5.34)	5.68
Low frequency	5.06 ± 0.15 (4.82 – 5.42)	5.01 ± 0.16 (4.65 – 5.37)	4.81 ± 0.15 (4.59 – 4.98)	4.78
High frequency	5.87 ± 0.17 (5.56 – 6.21)	5.85 ± 0.19 (5.53 – 6.15)	5.66 ± 0.11 (5.60 – 5.85)	5.99
Final part of the note				
Dominant frequency	5.53 ± 0.18 (5.26 – 5.97)	5.45 ± 0.22 (5.10 – 5.98)	5.23 ± 0.16 (5.11 – 5.51)	5.51
Low frequency	5.06 ± 0.20 (4.79 – 5.47)	4.96 ± 0.18 (4.62 – 5.36)	4.76 ± 0.16 (4.64 – 5.05)	4.63
High frequency	5.96 ± 0.22 (5.65 – 6.42)	5.94 ± 0.24 (5.60 – 6.53)	5.66 ± 0.11 (5.55 – 5.83)	6.00

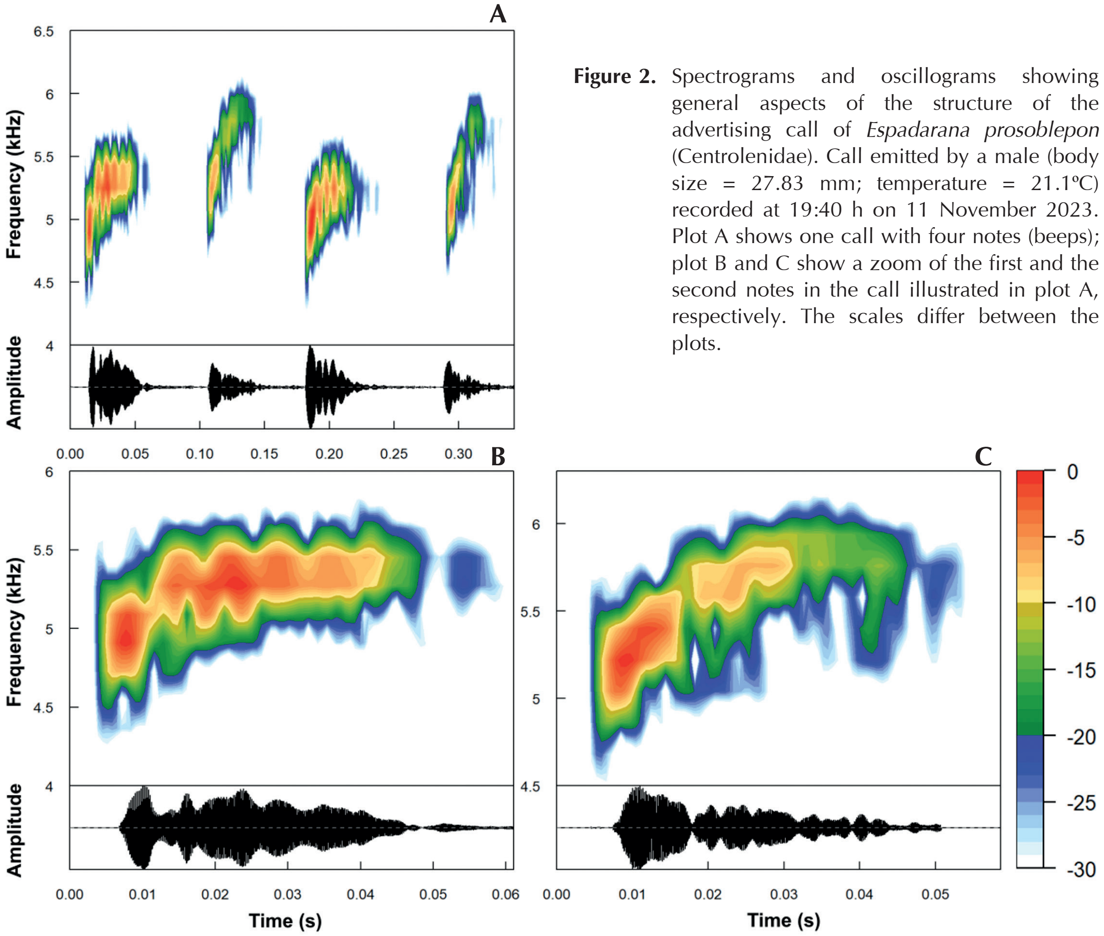

Figure 2. Spectrograms and oscillograms showing general aspects of the structure of the advertising call of *Espadarana prosoblepon* (Centrolenidae). Call emitted by a male (body size = 27.83 mm; temperature = 21.1ºC) recorded at 19:40 h on 11 November 2023. Plot A shows one call with four notes (beeps); plot B and C show a zoom of the first and the second notes in the call illustrated in plot A, respectively. The scales differ between the plots.

(Rivero, 1968) (Señaris and Ayarzaguena 2005, Cabanzo-Olarte and Ortega-Chinchilla 2017), *E. audax* (Lynch and Duellman, 1973) and *E. callistomma* (Guayasamin and Trueb, 2007) (Guayasamin *et al.* 2020), but not for *E. durrellorum* (Cisneros-Heredia, 2007) (Cisneros-Heredia 2007, Guayasamin *et al.* 2020). The advertisement call of *E. prosoblepon* follows a type trii call structure, as do other species in the genus *Espadarana* (Duarte-Marín *et al.* 2022). Some specific differences occur in features such as call duration, note duration, number of notes per call, and dominant frequency (Table 2). For instance, the number of notes per call in *E. prosoblepon* (2–5 notes) is similar between sister species, *i.e., E. callistomma* (3–4 notes), but higher than in *E. andina* and *E. audax* (1 note). A similar pattern is detected for call duration (Table 3). This tendency could reflect a low-to-absent phylogenetic signal in these temporal call features (Escalona *et al.* 2019, Vargas-Salinas *et al.* 2024). Comparisons beyond those summarized in Table 2 are not possible at present because different call features have been recorded for the taxa. Call descriptions of *E. andina, E. audax,* and *E. callistomma* (Table 2) do not include data and analysis of potential effects of temperature and body size.

Our call description includes other features in addition to those described previously for

Table 2. Results of the principal component analysis summarizing variation in 26 advertisement call features for males of the glass frog *Espadarana prosoblepon* in a population located in central Andes of Colombia. Variables (call features) were assigned to a principal component if their loading was > |0.6| (highlighted in bold font). Note that a negative load value indicates that the variable (i.e., call feature) relates in a negative way with the corresponding PC. When applicable, temporal call features were measured in ms and spectral call features in kHz.

Call features	**Principal Components**			
	PC1	**PC2**	**PC3**	**PC4**
Initial high frequency Note 2	**0.96**	0.13	-0.05	-0.03
Initial high frequency Note 1	**0.94**	0.15	0.10	-0.13
Initial dominant frequency Note 2	**0.93**	0.24	-0.00	-0.09
Initial dominant frequency Note 1	**0.91**	0.22	0.17	-0.15
Low frequency of the whole call	**0.93**	0.31	0.07	-0.08
Middle high frequency Note 2	**0.90**	0.35	0.23	-0.07
Middle dominant frequency Note 2	**0.90**	0.40	0.06	-0.04
Initial low frequency Note 2	**0.89**	0.20	-0.01	-0.13
Dominant frequency of the whole call	**0.87**	0.10	0.12	0.04
Middle dominant frequency Note 1	**0.86**	0.40	0.14	0.07
Initial low frequency Note 1	**0.85**	0.24	0.31	-0.06
High frequency of the whole call	**0.85**	0.46	0.13	0.00
Middle high frequency Note 1	**0.85**	0.40	0.11	0.07
Middle low frequency Note 1	**0.80**	0.44	0.13	0.06
Middle low frequency Note 2	**0.78**	0.47	0.08	0.03
Final high frequency Note 2	**0.71**	0.47	0.08	0.00
Final low frequency Note 2	**0.69**	0.55	0.08	0.07
Final dominant frequency Note 2	**0.67**	0.63	0.06	-0.09
Final high frequency Note 1	0.52	**0.79**	0.13	-0.00
Final dominant frequency Note 1	0.52	**0.78**	0.14	-0.02
Final low frequency Note 1	0.44	**0.71**	0.33	0.06
Number of notes	-0.12	-0.19	**-0.94**	-0.15
Call duration	-0.10	-0.11	**-0.90**	0.41
Note emission rate	0.04	-0.03	0.41	**-0.88**
Interval between notes	-0.09	0.24	0.07	**0.85**
Duration of notes	-0.01	-0.40	0.09	**0.63**
Eigenvalue	**17.323**	**2.473**	**1.908**	**1.152**
Percentage of variance explained	**66.627**	**9.511**	**7.339**	**4.429**

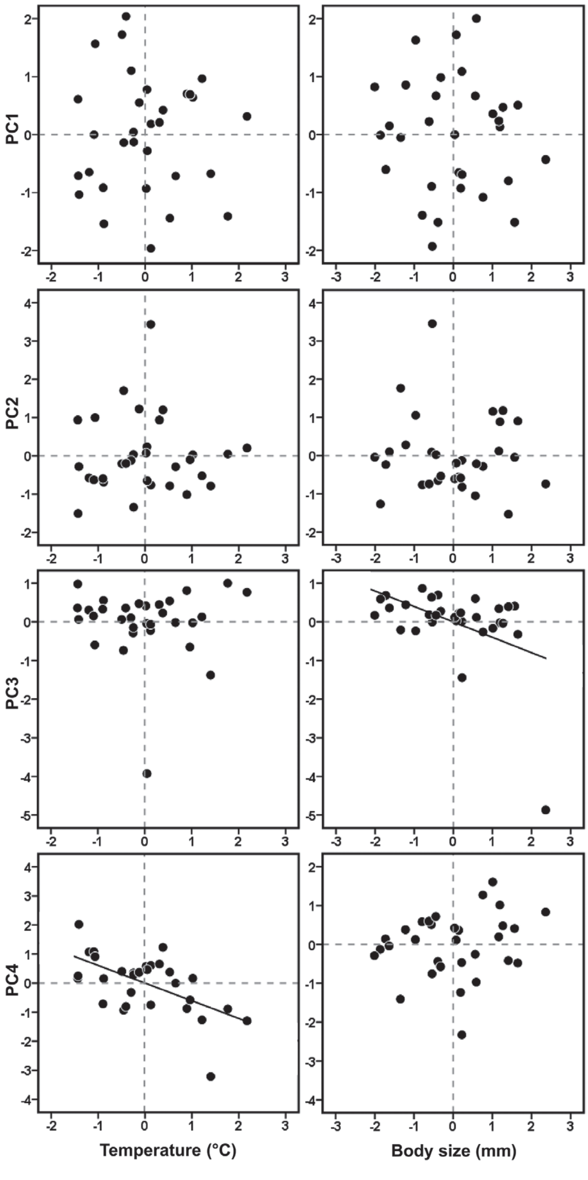

Figure 3. Partial regression plots showing the relationship between call features (summarized in four principal components, PCs) with temperature (first column) and body size (second column) of males of the glass frog *Espadarana prosoblepon*. Values in y-axis (left) and x-axis (bottom) are residuals of the multiple regression analysis. The regression line is shown only in those plots for which there was a significant relationship between variables at an alpha = 0.05. PC1 and PC2 summarize spectral call features while PC3 and PC4 summarize temporal call features. Details of call features included in each PC are indicated in Table 2. Note that the inverse relationship between body size and PC3 is highly influenced by an outlier corresponding to the largest recorded male (body size = 29.92 mm) with the higher average value of number of notes (3 notes) and call duration (427 ms). When the multiple regression analysis was run without this male, the relationship of PC3 with temperature disappeared (β = -0.021, t = -0.115, p = 0.910) and with body size remained as non-significant (β = -0.271, t = -1.466, p = 0.154).

Table 3. Summary of some advertisement call features (number of notes per call, note duration, call duration, dominant frequency) in glass frogs of the genus *Espadarana*. Data are presented as mean ± standard deviation and range. Sample size refers to number of males recorded/number of calls analyzed. * Values of this range correspond to the mean dominant frequency at the beginning of the note and the end of the note.

Species	Number of notes per call	Call duration (ms)	Note duration (ms)	Dominant frequency of the call (kHz)	Sample size	Source
E. andina	1	31.8 ± 5.5 (25.3–48.0)	31.8 ± 5.5 (25.3–48.0)	5.7 ± 0.1 (5.5–5.9)	4/24	Señaris and Ayarzaguena 2005
E. audax	1	38.0 ± 8.2 (26.0–53.0)	38.0 ± 8.2 (26.0–53.0)	6.1 ± 0.4 (5.4–6.7)	2/4	Guayasamin *et al.* 2020
E. callistomma	3–4	380.0 ± 66.0 (280–440)	30.0 ± 7.0 (14.0–40.0)	(5.3–5.8)	1/10	Guayasamin *et al.* 2020
E. prosoblepon	2–5	–	–	–	–	Freile *et al.* 2020
E. prosoblepon	2	212 ± 7 (207–223)	40 ± 8 (32–54)	(5.8–6.3) *	1/4	Guayasamin *et al.* 2020
E. prosoblepon	2.1±0.2 (2–4)	230.0 ± 40.0 (190–430)	41.1 ± 4.8 (31.0–48.7)	5.2 ± 0.2 (4.9–5.6)	31/220	This study

populations of *Espadarana prosoblepon* in Costa Rica (Jacobson 1985, Savage 2002, Kubicki 2007) and Ecuador (Freile *et al.* 2020, Guayasamin *et al.* 2020). Since the description by Jacobson (1985) is the most detailed and often cited, we compare our calls with results published by this author. General tendencies mentioned below agree with data shown in the other descriptions. The average dominant frequency of the call is slightly higher in the Costa Rica population (5.8 kHz, range 5.3–6.0 kHz; Jacobson 1985) than ours (5.2 kHz, range 4.9–5.6 kHz). This tendency is not attributed to interpopulation differences in body size because in any of these populations, a relationship existed between spectral features of the call and body size. The absence of an inverse relationship between male body size and call frequency in our data of *E. prosoblepon* was unexpected because in most anurans such traits relate in an inverse way (Vargas-Salinas and Amézquita 2014, Tonini *et al*. 2020). Some exceptions to this tendency occur, typically in species associated with noisy habitats (Tonini *et al*. 2020, Maria *et al*. 2023) such as those in which glass frogs call and breed (i.e., streams). Regarding glass frogs, a weak negative relationship has been found between body size of males and dominant call frequency in studies of multiple species (Escalona *et al*. 2019, Mendoza-Henao *et al*. 2023, Vargas-Salinas *et al*. 2024). It is possible that at the intraspecific level, such an inverse relationship could be even weaker or absent in some species, in part because of a low variability in body size of males within a given population.

Note duration might show a contrary tendency than dominant frequency; that is, it was slightly shorter in the Costa Rica population (40 ms, range 25–50 ms) than in our population (41.9 ms, range 32.7–57.7 ms). This comparison should be taken as preliminary since PC3 (that includes the feature "note duration") was inversely correlated to temperature in our study (but see Figure 3), and Jacobson (1985) did not test this potential effect. The number of notes per call apparently do not differ between populations; Jacobson (1985) recorded up to five notes per call while we found up to four notes per call, but in the field, we were able to hear calls with five notes. A more detailed comparison of the advertisement call between populations of *E. prosoblepon* is not feasible to date, because 1) no data is available for Costa Rica populations regarding call features such as duration of interval between notes, dominant frequency per note, and minimum and maximum frequency per note (Jacobson 1985, Savage 2002, Kubicki 2007), and 2) the best description for an Ecuadorian population is based on only one male (Guayasamin *et al.* 2020).

The available information for Costa Rica populations (Jacobson 1985, Savage 2002, Kubicky 2007) and other populations from Costa Rica, Panamá (Appendix I) and Ecuador (Table 2) suggests little differentiation in advertisement call features between populations of *Espadarana prosoblepon* examined here. More recordings and data are necessary for a robust conclusion. In these studies, it will be necessary to test potential effects of temperature and body size on call features.

Acknowledgments

We thank Fonozoo and Richard Lehtinen for allowing the use of their recordings of males in Panamá and Costa Rica. Members of the Evolution, Ecology and Conservation Research Group (EECO) at the Universidad del Quindío kindly supported our fieldwork and analysis of data. Finally, thanks to the Biology Program at the Universidad del Quindío, Colombia, for supporting this research. A previous version of this manuscript was greatly improved by comments from Juan Manuel Guayasamin, Moises Escalona, and Ignacio de la Riva. We complied with all ethical norms for care and handling of specimens dictated by international and regional institutions.

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Appendix I. *Summary advertisement call features for two males belonging to populations of* Espadarana prosoblepon *in Bocas del Toro, Panamá (code Fonozoo.com = ID: 19092, FZ-SOUND-CODE: 7616) and Las Cruces biological station, Costa Rica (code Fonozoo.com = ID: 22957, FZ-SOUND-CODE: 12939). Body size of the recorded male was not given in either case. When applicable, temporal call features are expressed in ms and spectral call features in kHz.*

Attributes	**Bocas del Toro, Panama**	**Las Cruces, Costa Rica**
Temperature (°C)	16.6	–
Number of calls	1	1
Call duration	270.7	395.6
Number of notes	2	3
Duration Note 1	31.6	34.2
Duration Note 2	35.9	29.5
Duration Note 3	–	33.5
Interval between Note 1 and 2	203.1	130.1
Interval between Note 2 and 3	–	167
Dominant frequency Note 1	6.4	6.6
Dominant frequency Note 2	6.2	6.6
Dominant frequency Note 3	–	6.5
Low frequency Note 1	5.5	6
High frequency Note 1	7.2	7.5
Low frequency Note2	5.4	6
High frequency Note 2	7.0	7.3
Low frequency Note 3	–	6
High frequency Note 3	–	7.1
Initial dominant frequency Note 1	6.4	6.7
Initial dominant frequency Note 2	6.0	6.7
Initial dominant frequency Note 3	–	6.6
Initial low frequency Note 1	5.7	6.0
Initial high frequency Note 1	7.2	7.4
Initial low frequency Note 2	5.4	6.0
Initial high frequency Note 2	7.0	7.3
Initial low frequency Note 3	–	5.4
Initial high frequency Note 3	–	7.1
Middle dominant frequency Note 1	6.4	6.7
Middle dominant frequency Note 2	6.4	6.7
Middle dominant frequency Note 3	–	6.6
Middle low frequency Note 1	5.5	6
Middle high frequency Note 1	7.2	7.6
Middle low frequency Note 2	5.4	6
Middle high frequency Note 2	7.1	7.4

Appendix I. *Continued.*

Attributes	Bocas del Toro, Panama	Las Cruces, Costa Rica
Middle low frequency Note 3	–	5.9
Middle high frequency Note 3	–	7.3
Final dominant frequency Note 1	6.4	6.9
Final dominant frequency Note 2	6.4	6.6
Final dominant frequency Note 3	–	6.6
Final low frequency Note 1	5.2	6.1
Final high frequency Note 1	7.2	7.6
Final low frequency Note 2	5.4	6.2
Final high frequency Note 2	7.1	7.2
Final low frequency Note 3	–	6.1
Final high frequency Note 3	–	7

Phyllomedusa 23(2):179–190, 2024

ISSN 1519-1397 (print) / ISSN 2316-9079 (online)
doi: http://dx.doi.org/10.11606/issn.2316-9079.v23i2p179-190

Differential antipredator behavioral responses in tadpoles of *Duttaphrynus melanostictus* (Anura: Bufonidae): an experimental demonstration

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Abstract

Differential antipredator behavioral responses in tadpoles of *Duttaphrynus melanostictus* (Anura: Bufonidae): an experimental demonstration. In aquatic systems, many prey animals including larval anurans predominantly use chemical cues to assess predation risk. In such systems, a variety of predators (e.g., insects or their larvae, sympatric carnivorous/omnivorous tadpoles) can affect the behavioral responses and life history of prey tadpoles. Many anuran tadpoles are able to discriminate chemical cues of different predators and exhibit differential antipredator behavioral responses according to the perceived risk. The behavioral responses of tadpoles of *Duttaphrynus melanostictus* to different predators (predaceous insects, *Lethocerus* sp., omnivorous tadpoles of *Euphlyctis cyanophlyctis,* and carnivorous tadpoles of *Hoplobatrachus tigerinus*) were studied in the laboratory. The predator's diet-derived metabolites released in the excreta of the predators after consumption of conspecific (*D. melanostictus*) prey tadpoles were used to simulate predation threat. The *D. melanostictus* tadpoles showed antipredator behavioral responses i.e., reduced swimming movements and overall time spent swimming, and had a higher burst speed in response to water-borne cues released from the excreta of all predators that were fed with conspecific prey. Further, *D. melanostictus* tadpoles showed the strongest antipredator behavioral responses to cues released by carnivorous, active predatory tadpoles, *H. tigerinus,* moderate responses to the cues of the sit-and-wait carnivorous insect, *Lethocerus* sp. and low responses to those of omnivorous *E. cyanophlyctis* tadpoles. The hierarchy of antipredator behavioral responses in *D. melanostictus* tadpoles to different predators is *H. tigerinus* > *Lethocerus* sp. > *E. cyanophlyctis* > chemical blank solution. The findings of the present study thus show that tadpoles of *D. melanostictus* appear to modulate the intensity of their defense behavior in accordance with the level of threat posed by each predator.

Keywords: Anuran larvae, Chemical and visual cues, Dietary metabolites, Predator, Prey, Reduced activity, Swimming speed.

Received 22 May 2024
Accepted 12 November 2024
Distributed December 2024

Resumo

Respostas comportamentais antipredador diferenciais em girinos de *Duttaphrynus melanostictus* (Anura: Bufonidae): uma demonstração experimental. Nos sistemas aquáticos, muitas presas, incluindo larvas de anuros, utilizam predominantemente sinais químicos para avaliar o risco de predação. Nesses sistemas, uma variedade de predadores (por exemplo, insetos ou suas larvas, girinos carnívoros/onívoros simpátricos) pode afetar as respostas comportamentais e a história de vida dos girinos. Muitos girinos são capazes de discriminar sinais químicos de diferentes predadores e apresentam respostas comportamentais antipredadores diferenciadas de acordo com o risco percebido. As respostas comportamentais dos girinos de *Duttaphrynus melanostictus* a diferentes predadores (insectos predadores, *Lethocerus* sp., girinos onívoros de *Euphlyctis cyanophlyctis* e girinos carnívoros de *Hoplobatrachus tigerinus*) foram estudadas em laboratório. Os metabólitos derivados da dieta do predador liberados nas excreções após o consumo de girinos de presas conspecíficas (*D. melanostictus*) foram utilizados para simular a ameaça de predação. Os girinos de *D. melanostictus* apresentaram respostas comportamentais antipredadores, isto é, reduziram os movimentos e o tempo total gasto na natação e tiveram uma maior velocidade de explosão em resposta a sinais de água liberados pelos excrementos de todos os predadores que foram alimentados com presas específicas. Além disso, os girinos de *D. melanostictus* apresentaram as respostas comportamentais anti-predador mais fortes aos sinais liberados pelos girinos predadores carnívoros e ativos, *H. tigerinus*, respostas moderadas aos sinais do inseto carnívoro *Lethocerus* sp. e respostas baixas às dos girinos onívoros de *E. cyanophlyctis*. A hierarquia das respostas comportamentais antipredador nos girinos de *D. melanostictus* a diferentes predadores é *H. tigerinus* > *Lethocerus* sp. > *E. cyanophlyctis* > solução tampão. Os resultados do presente estudo mostram que os girinos de *D. melanostictus* parecem modular a intensidade do seu comportamento de defesa de acordo com o nível de ameaça representado por cada predador.

Palavras-chave: Atividade reduzida, Larvas de anuros, Metabólitos da dieta, Pistas químicas e visuais, Predador, Presa, Velocidade de natação.

Introduction

Anuran amphibians often breed opportunistically in ephemeral water bodies during the rainy season, and the larval anurans live in such waters until metamorphosis (Saidapur 2001, Cogălniceanu *et al.* 2012, Goldberg *et al.* 2012, Mogali *et al.*2020). Hence, tadpoles of different anuran species that co-occur in such waters face threats from desiccation, crowding, competition for food and space, and most importantly predation (Skelly 1997, Loman 1999, Lardner 2000, Benard 2004, Mogali *et al.* 2011, 2016, 2020). Ephemeral ponds can house diverse types of predators, the most common of which are aquatic insects and their larvae, and omnivorous and carnivorous predatory tadpoles (Heyer *et al.*1975, Skelly 1997, Relyea 2001a, Saidapur 2001, Mogali *et al.* 2020). Furthermore, in the ephemeral water bodies where different species of tadpoles reside, the water is often turbid or filled with aquatic vegetation or leaf litter, causing poor visibility (Hoff *et al.* 1999, Mogali 2018, Mogali *et al.* 2023a,b). Moreover, most species of anuran tadpoles are near sighted (Kiesecker *et al.* 1996, Hoff *et al.* 1999, Mogali 2018). In such conditions, chemical cues are more useful than visual cues to detect food or predators (Kiesecker *et al.* 1996, Nystrom and Abjornsson 2000, Hickman *et al.* 2004, Fraker 2008, Saidapur *et al.* 2009). Earlier studies have shown that the source of chemical cues emanating from predators and detected by prey tadpoles may differ (Takahara *et al.* 2008, Smith and Awan 2009, Ferrari *et al.* 2010). Chemical cues may arise from the starved predators, kairomones (Petranka *et al.* 1987, Schoeppner and Relyea 2005, Mogali 2018), dietary metabolites derived following consumption of conspecific prey and released through feces (Wilson and Lefcort 1993, Laurila *et al.* 1997, Chivers and Mirza 2001, Kiesecker *et al.* 2002, Mogali *et al.* 2012, Scherer and Smee 2016), alarm pheromones released by injured or damaged prey (Hews and Blaustein 1985, Hews 1988, Summey and Mathis 1998, Schoeppner and Relyea 2005, Carlson *et al.* 2015), or a combination of these factors (Schoeppner and Relyea 2009a,b). Earlier studies also revealed that anuran tadpoles showed a variety of antipredator behaviors when exposed to chemical cues of predators including increased activity levels or high swimming speed to escape predators (Hews 1988, Van Buskirk and McCollum 2000, Dayton *et al.* 2005, Mogali *et al.* 2021), reduction in activity levels (Lawler 1989, Saidapur *et al.* 2009, Mogali *et al.* 2012), formation of dense aggregations or schools (Spieler and Linsenmair 1999), or increased use of refuge sites (Stauffer and Semlitsch 1993, Relyea 2003, Hossie and Murray 2010, Mogali *et al.* 2022), depending on the species.

The Asian common toad, *Duttaphrynus melanostictus* (Schneider, 1799) (Anura: Bufonidae), is widely distributed in India. In South India, during early monsoon season, it generally breeds in ephemeral water bodies along with other sympatric anuran species (Mogali *et al.* 2011, 2017, 2023a). The temporary water bodies where herbivorous tadpoles of *D. melanostictus* reside are also home to a variety of invertebrate predators such as dragonfly and damselfly larvae, giant water bugs, crabs, and beetles as well as vertebrate predators including an omnivorous [*Euphlyctis cyanophlyctis* (Schneider, 1799)], and carnivorous tadpoles [*Hoplobatrachus tigerinus* (Daudin, 1802)]. During our regular field visits, we noticed that herbivorous tadpoles of *D. melanostictus* are preyed upon by all these predators. Earlier studies suggest that different predators present different levels of predation risk to prey tadpoles (Relyea 2001a,b). Hence, in the present work, we studied the behavioral responses of *D. melanostictus* tadpoles to chemical cues (of a dietary origin) of three different types of predators. They are giant water bugs, *Lethocerus* sp. (Hemiptera: Belostomatidae) which are sit-and-wait insect predators exclusively carnivorous in nature, tadpoles of *E. cyanophlyctis* (Anura: Dicroglossidae), which are omnivorous in nature and basically feed on detritus or algae and also on other sympatric anuran tadpoles including *D. melanostictus*. On the other hand, tadpoles of *H. tigerinus* (Anura: Dicroglossidae) are primarily predators; they are carnivorous, active in nature, feeding on other sympatric anuran tadpoles.

In the present study we hypothesized that *D. melanostictus* tadpoles which coexist with all these predators in the natural water bodies should show antipredatory behavioral responses to predators' chemical cues. Additionally, we hypothesized that the antipredatory responses of *D. melanostictus* tadpoles should depend on the level of threat presented by each type of predator. We expected that *D. melanostictus* tadpoles would show strong antipredator behavioral responses to *H. tigerinus* tadpoles because these tadpoles are basically carnivorous in nature, active hunters and also detect their prey by means of both visual and chemical senses (Saidapur *et al.* 2009). We expected moderate antipredator behavioral responses in *D. melanostictus* tadpoles to *Lethocerus* sp. because these are carnivorous insects but sit-and-wait predators. We expected weak antipredator responses to *E. cyanophlyctis* because these are omnivorous tadpoles mainly feeding on detritus or decayed matter or algae, and also on other sympatric tadpoles. Evaluating these hypotheses will provide novel information in the field of behavioral ecology of anuran tadpoles.

Materials and Methods

Three egg clutches of *Duttaphrynus melanostictus* were collected from an ephemeral pond in the Karnatak University Campus, Dharwad (latitude 15.440407° N, longitude 74.985246° E), Karnataka state, India in the early monsoon period and were immediately transported to the laboratory. They were placed separately in plastic tubs (32 cm diameter and 14 cm depth) containing 5 L of aged (dechlorinated) tap water. The eggs from all clutches hatched synchronously at stage 19 (Gosner 1960) the next day. The tadpoles were mixed and reared for stocking in two separate glass aquaria (75 × 45 × 15 cm) containing 20 L of aged tap water. Approximately two hundred tadpoles were stocked in each aquarium. Tadpoles of *D. melanostictus* from stage 25 (Gosner 1960) onwards were fed with sufficient amount of boiled spinach. The carnivorous predatory insect, *Lethocerus* sp. (*N* = 20; 42.50 ± 0.60 mm total length, mean ± SE), omnivorous tadpoles of *Euphlyctis cyanophlyctis* (Gosner stages 33–34; *N* = 20; 42.02 ± 0.65 mm total length) and carnivorous tadpoles of *Hoplobatrachus tigerinus* (Gosner stages 33–34; *N* = 20; 41.15 ± 0.40 mm total length) were collected with the help of net from the same temporary pond where the eggs of *D. melanostictus* were obtained. All three species of predators were reared individually to avoid cannibalism in separate plastic tubs (19 cm diameter and 7 cm depth) containing 0.5 L of aged tap water. Tadpoles of *E. cyanophlyctis* are omnivorous in nature so they were provided with boiled spinach and tadpoles of *D. melanostictus.* The tadpoles of *H. tigerinus* and the insect, *Lethocerus* sp. are carnivorous and were therefore fed exclusively with tadpoles of *D. melanostictus*. The behavioral responses of the prey (*D. melanostictus*) were studied by exposing them to stimulus solutions of dietary metabolites of predators (either *E. cyanophlyctis* or *Lethocerus* sp. or *H. tigerinus*) exclusively fed with conspecific tadpoles (*D. melanostictus*). The preparation of stimulus solution was as follows.

Preparation of Dietary Cues of Conspecific Origin

Dietary cues of E. cyanophlyctis *fed conspecific tadpoles.*—A single *E. cyanophlyctis* tadpole was placed in a plastic tub (*N* = 10 tubs; 19 cm diameter and 7 cm depth) containing 200 mL of aged tap water along with four tadpoles of *D. melanostictus* at Gosner stages 29–30 (at about 08:30 h). The tadpoles of *E. cyanophlyctis* consumed all the tadpoles provided to them by the evening (18:30 h). On the following day between 09:30 h and 11:30 h, predators were removed and the water from the tubs was filtered using fine cheesecloth. The filtrate served as the stimulus solution containing the diet-derived excretory metabolites or substances of predators (*E. cyanophlyctis*) fed conspecific prey 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, Chivers *et al.* 2013). Thus, 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.

Dietary cues of Lethocerus *sp. fed conspecific tadpoles.*—A single *Lethocerus* sp. was placed in a plastic tub (*N* = 10 tubs; 19 cm diameter and 7 cm depth) containing 200 mL of aged tap water along with four tadpoles of *D. melanostictus* at Gosner stages 29–30 (at about 08:30 h). *Lethocerus* sp. consumed all the tadpoles provided to them by the evening (18:30 h). On the following day filtrate was obtained and served as a stimulus solution.

Dietary cues of H. tigerinus *fed conspecific tadpoles.*—A single *H. tigerinus* tadpole was placed in a plastic tub (*N* = 10 tubs; 19 cm diameter and 7 cm depth) containing 200 mL of aged tap water along with four tadpoles of *D. melanostictus* at Gosner stages 29–30 (at about 08:30 h). The tadpoles of *H. tigerinus* consumed all the tadpoles provided to them by the evening (18:30 h). On the following day filtrate was obtained and served as a stimulus solution.

Behavioral Responses of D. melanostictus *Tadpoles to Dietary Cues of Different Predators*

Behavioral responses of D. melanostictus *tadpoles to dietary cues of omnivorous tadpole* *predator,* E. cyanophlyctis *fed with* D. melanostictus.—The behavioral responses of *D. melanostictus* tadpoles to dietary cues (water conditioned with predators fed with conspecific tadpoles) were recorded by placing a single tadpole (*D. melanostictus*; Gosner stage 29–30; mean total length 24.15 ± 0.35 mm) 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 such that it recorded the entire area. The video 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 stimulus solution (dietary cues) to the test tank. The Ethovision system was used to record swimming activities such as maximum swimming speed (V_{max}), distance traversed by the tadpole, number of swimming spurts and time spent swimming during an entire trial. For each trial, a new tadpole of *D. melanostictus* 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 then added at the rate of ~1 mL/s to simulate the disturbance the later chemical cue would make. The burette was then removed gently. Movement of the tadpole was then 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 dietary cues of the predator (*E. cyanophlyctis*) fed with *D. melanostictus* tadpoles was added as described above. Movement of the tadpole was recorded for another 5 min to determine the activity pattern after exposure to dietary cues.

Similarly, the behavioral responses of *D. melanostictus* tadpoles to dietary cues were recorded for the other predators, the carnivorous insect, *Lethocerus* sp. and carnivorous tadpoles, *H. tigerinus* fed with *D. melanostictus.* The testing procedure was exactly the same as that of the earlier one. A new test tadpole was used for each trial. Twenty-five trials were conducted for each group (25 trials × 3 types of predators = 75 trials altogether).

Statistical Analysis

After checking for normality, initially data were analyzed using one-way MANOVAs for various swimming activities (response variables are maximum swimming speed, frequency of swimming spurts, time spent swimming and total distance moved). After getting significant MANOVAs, Further the data on the behavioral responses of *D. melanostictus* tadpoles to chemical blank solution vs. different predator type (*Euphlyctis cyanophlyctis, Lethocerus* sp., *Hoplobatrachus tigerinus*) and similarly stimulus solution vs. predator type were analyzed separately by one-way ANOVA followed by Tukey's HSD *post-hoc* test. Finally, the data on the behavioral responses of *D. melanostictus* tadpoles, before and after addition of the stimulus solution (dietary cues) of each predator were compared separately by using the paired-samples t test. All the statistical tests were performed using SPSS ver. 16.0.

Results

Intensity of Behaviors in D. melanostictus *Tadpoles to Different Predators*

The results of the one-way MANOVAs clearly showed that each swimming activities (V_{max}, no. of swimming spurts, time spent swimming and total distance moved) of *D. melanostictus* tadpoles are statistically significant difference by predator type (*Euphlyctis cyanophlyctis, Lethocerus* sp., *Hoplobatrachus tigerinus*) and treatment type (chemical blank solution, stimulus solution) (Table 1). Further, results of one-way ANOVA with Tukey's HSD *post-hoc* test revealed that there was no significant difference in the various swimming activities (V_{max}, no. of swimming spurts, time spent swimming and total distance moved) of *D. melanostictus* tadpoles between chemical blank solution vs. different predator type (*Euphlyctis cyanophlyctis, Lethocerus* sp., *Hoplobatrachus tigerinus*) (Table 2). However, there was a significant difference in the V_{max}, swimming spurts, time spent swimming and total distance travelled by *D. melanostictus* tadpoles between stimulus solution vs. different predator type (Table 2). The test tadpoles exposed to the stimulus solution of *H. tigerinus* exhibited significantly higher V_{max} ($p < 0.01$) but overall significantly reduced their activity i.e., spent less time swimming ($p < 0.01$) with reduced number of swimming spurts ($p < 0.01$) and moved a shorter distance ($p < 0.01$) compared to any

Table 1. Results of one-way MANOVAs for various swimming activities of *Duttaphrynus melanostictus* tadpoles (the response variables are maximum swimming speed, frequency of swimming spurts, time spent in swimming and total distance moved) to chemical cues of predator type (*Euphlyctis cyanophlyctis, Lethocerus* sp., *Hoplobatrachus tigerinus*) and treatment type (chemical blank solution, stimulus solution). Asterisks indicate significant difference.

Source	Wilks' Lambda	*F*	*p*
Swimming speed ($V_{max,}$ cm/s)	0.087	770.60	< 0.01*
Number of swimming spurts	0.079	855.60	< 0.01*
Time spent swimming (s)	0.081	836.92	< 0.01*
Distance moved (cm)	0.119	544.30	< 0.01*

Table 2. Results of one-way ANOVAs with Tukey's HSD *post hoc* tests for various swimming activities of *Duttaphrynus melanostictus* tadpoles (the response variables are maximum swimming speed, frequency of swimming spurts, time spent in swimming and total distance moved) to chemical blank vs. predator type (*Euphlyctis cyanophlyctis, Lethocerus* sp., *Hoplobatrachus tigerinus*) and stimulus solution vs. predator type. Dissimilar letters along the same column indicate significant difference between different treatment groups.

One-way ANOVA for chemical blank vs. predator type				
Predator type	**Swimming speed (Vmax, cm/s)**	**No. swimming spurts**	**Time spent swimming (s)**	**Distance moved (cm)**
Euphlyctis cyanophlyctis	11.80 ± 0.08a	65.92 ± 1.43a	63.10 ± 1.31a	399.11 ± 8.81a
Lethocerus sp.	11.75 ± 0.07a	65.60 ± 1.46a	63.59 ± 1.45a	399.63 ± 8.52a
Hoplobatrachus tigerinus	11.82 ± 0.07a	67.00 ± 1.35a	65.43 ± 1.57a	394.48 ± 5.22a
F value	F2,72 = 0.226	F2,72 = 0.267	F2,72 = 0.795	F2,72 = 0.112
p value	p = 0.798	p = 0.766	p = 0.456	p = 0.894
One-way ANOVA for stimulus solution vs. predator type				
Predator type	**Swimming speed (Vmax, cm/s)**	**No. swimming spurts**	**Time spent swimming (s)**	**Distance moved (cm)**
Euphlyctis cyanophlyctis	18.48 ± 0.70a	31.00 ± 0.56a	29.36 ± 0.55a	256.48 ± 3.89a
Lethocerus sp.	21.15 ± 0.22b	22.36 ± 0.70b	21.05 ± 0.68b	195.97 ± 3.51b
Hoplobatrachus tigerinus	24.55 ± 0.30c	14.52 ± 0.53c	13.47 ± 0.49c	148.51 ± 3.83c
F value	F2,72 = 151.768	F2,72 = 183.101	F2,72 = 187.64	F2,72 = 197.846
p value	p < 0.01	p < 0.01	p < 0.01	p < 0.01

other groups (Table 2B). The test tadpoles exposed to the stimulus solution of *Lethocerus* sp. exhibited significantly higher V_{max} ($p < 0.01$) and overall reduced their swimming activities ($p < 0.01$) compared to the stimulus solution of *E. cyanophlyctis* and stimulus free solution (Table 2B). The test tadpoles exposed to the stimulus solution of *E. cyanophlyctis* exhibited significantly higher V_{max} ($p < 0.01$) and overall reduced their swimming activities ($p < 0.01$) compared to the chemical blank solution or stimulus-free solution (Table 2B). The degree of antipredator behavioral responses of test tadpoles (*D. melanostictus*) was greatest when exposed to dietary cues of carnivorous *H. tigerinus*, followed by carnivorous *Lethocerus* sp. (intermediate) and least to omnivorous *E. cyanophlyctis* (Table 2B).

Behavioral Responses of D. melanostictus *Tadpoles to Dietary Cues of Different Predators*

Behavioral Responses of D. melanostictus *tadpoles to dietary cues of omnivorous tadpole predator,* E. cyanophlyctis *fed with* D. melanostictus.—Upon exposure to dietary cues of omnivorous tadpole predator, *E. cyanophlyctis* fed with conspecific prey (*D. melanostictus*), test tadpoles (*D. melanostictus*) showed a significant increase in V_{max} ($t = -39.540$, $df = 24$, $p < 0.01$; Figure 1A), and a significant decrease in the number of swimming spurts ($t = 11.864$, $df = 24$, $p < 0.01$; Figure 1B), time spent swimming($t = 11.613$, $df = 24$, $p < 0.01$; Figure 1C) and total distance moved($t = 9.261$, $df = 24$, $p < 0.01$; Figure 1D) when compared to their baseline activities in stimulus-free water (Figure 1).

Behavioral responses of D. melanostictus *tadpoles to dietary cues of carnivorous insect predator,* Lethocerus *sp. fed with* D. melanostictus.—Upon exposure to dietary cues of the carnivorous insect predator, *Lethocerus* sp. fed with conspecific prey (*D. melanostictus*), test tadpoles (*D. melanostictus*) showed a significant increase in V_{max} ($t = -24.771$, $df = 24$, $p < 0.01$; Figure 2A), and a significant decrease in the number of swimming spurts ($t = 15.906$, $df = 24$, $p < 0.01$; Figure 2B), time spent swimming ($t = 14.968$, $df = 24$, $p < 0.01$; Figure 2C) and total distance moved ($t = 14.079$, $df = 24$, $p < 0.01$; Figure 2D) when compared to their baseline activities in stimulus-free water (Figure 2).

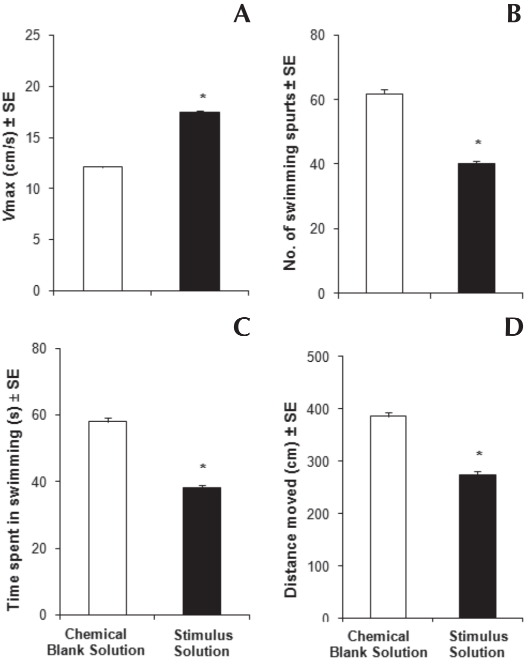

Figure 1. Maximum swimming speed (V_{max}) (**A**), Swimming spurts (**B**), Time spent in swimming (**C**), and Distance moved (**D**) by tadpoles of *Duttaphrynus melanostictus* exposed to chemical blank solution (aged tap water) or stimulus solution (dietary cues) of predator, *Euphlyctis cyanophlyctis* 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 difference between the treatment groups.

Behavioral responses of D. melanostictus *tadpoles to dietary cues of carnivorous tadpole predator,* H. tigerinus *fed with* D. melanostictus.—

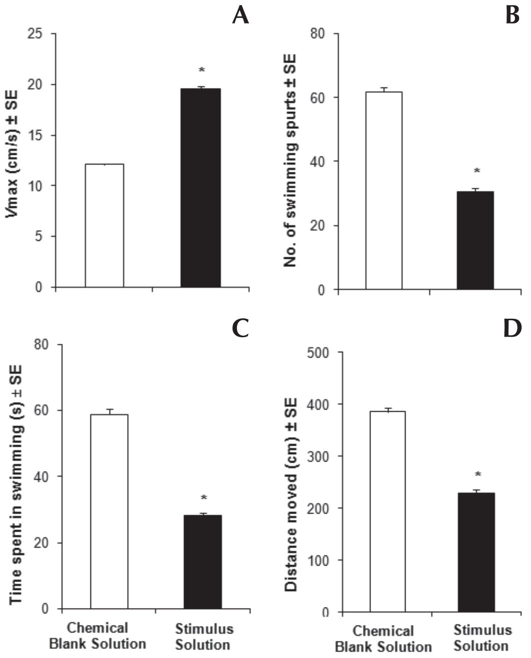

Figure 2. Maximum swimming speed (V_{max}) (**A**), Swimming spurts (**B**), Time spent in swimming (**C**), and Distance moved (**D**) by tadpoles of *Duttaphrynus melanostictus* exposed to chemical blank solution (aged tap water) or stimulus solution (dietary cues) of predator, *Lethocerus* sp. fed 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 difference between the treatment groups.

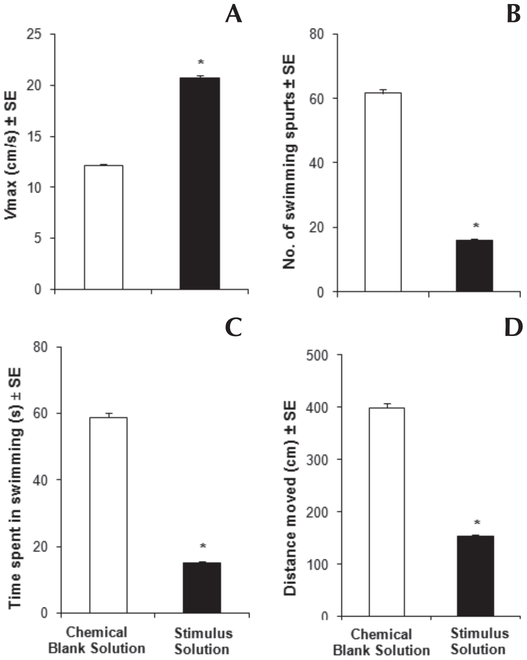

Figure 3. Maximum swimming speed (V_{max}) (**A**), Swimming spurts (**B**), Time spent in swimming (**C**), and Distance moved (**D**) by tadpoles of *Duttaphrynus melanostictus* exposed to chemical blank solution (aged tap water) or stimulus solution (dietary cues) of predator, *Hoplobatrachus tigerinus* 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 difference between the treatment groups.

Upon exposure to dietary cues of the carnivorous tadpole predator, *H. tigerinus* fed with conspecific prey (*D. melanostictus*), test tadpoles (*D. melanostictus*) showed a significant increase in V_{max} (t = -31.555, df = 24, $p < 0.01$; Figure 3A), and a significant decrease in the number of swimming spurts (t = 28.541, df = 24, $p < 0.01$; Figure 3B), time spent swimming (t = 27.237, df = 24, $p < 0.01$; Figure 3C) and total distance moved (t = 24.302, df = 24, $p < 0.01$; Figure 3D) when compared to their baseline activities in stimulus-free water (Figure 3).

Discussion

In natural aquatic environments, many prey organisms including larval anurans are at threat of predation, but the level of their threat is dependent on their defenses, which have evolved to promote their escape from predators and promote survival (Lima and Dill 1990, Kats and Dill 1998, Relyea 2001b, Schmidt and Amézquita 2001, Jara and Perotti 2010, Schalk 2016). In aquatic environments, a variety of chemical cues (e.g., kairomones of predators, alarm cues of damaged conspecifics, disturbance cues and dietary metabolites of predators fed with conspecific prey items) affect the behavioral responses of prey (Wilson and Lefcort 1993, Wisenden 2000, Van Buskirk and Arioli 2002, Schoeppner and Relyea 2005, Ferrari *et al.* 2010, Mogali *et al.* 2012, Scherer and Smee 2016, Rivera-Harnãndez *et al.* 2022). The dietary cues of predators, especially those from predators fed with conspecific prey items, elicit strong antipredator behavior in many prey animals including anuran tadpoles (Kats and Dill 1998, Ferrari *et al.* 2010, Mogali *et al.* 2011, 2012, Scherer and Smee 2016). The results of the present study showed that tadpoles of *D. melanostictus* sensed dietary cues of all predators (*Lethocerus* sp., tadpoles of *E. cyanophlyctis* and *H. tigerinus*) when predators were fed with conspecific prey tadpoles and test tadpoles quickly decreased their activity levels during the trial period (i.e., less time spent in swimming, fewer swimming spurts and less distance travelled). Furthermore, it is interesting to note that whenever the *D. melanostictus* tadpole moved in the stimulus solution, their burst speed (V_{max}) was higher than in the stimulus blank solution, indicating their efforts to escape from the perceived risk upon exposure to dietary cues of predator. Our results are in conformity with earlier studies on tadpoles of *Rana clamitans* (Latreille, 1801) (Fraker 2009), *Rana temporalis* (Mogali *et al.* 2012) and *Clinotarsus curtipes* (Jerdon, 1853) (Mogali *et al.* 2023c). Thus, *D. melanostictus* tadpoles primarily appear to perceive *Lethocerus* sp. and tadpoles of *E. cyanophlyctis* and *H. tigerinus* as potential predators. This may be because long ecological co-existence of *D. melanostictus* tadpoles with sympatric *Lethocerus* sp. and omnivorous/carnivorous tadpoles may have led to the evolution of antipredator defense strategies in response to dietary cues of these predators.

The results of the present study also clearly showed that the antipredator behavioral responses of *D. melanostictus* tadpoles to all predators are not the same. The tadpoles of *D. melanostictus* clearly discriminated among the predators, and as a consequence they exhibited differential antipredator behavioral responses to perceived predator risk. They showed the strongest antipredator behavioral responses to dietary cues of carnivorous tadpoles, *H. tigerinus,* intermediate antipredator behavioral responses to dietary cues of *Lethocerus* sp., and the weakest antipredator behavioral responses to dietary cues of omnivorous tadpoles, *E. cyanophlyctis.* It is clear that, among these three predators; *D. melanostictus* tadpoles react to *H. tigerinus* tadpoles as the most dangerous predators; this is probably because *H. tigerinus* are basically carnivorous and also active hunters. More importantly, they detect their prey items by means of both visual and chemical cues (Saidapur 2001, Saidapur *et al.* 2009). The tadpoles of *D. melanostictus* also react to *Lethocerus* sp. as dangerous predators. They are basically carnivorous insects, sit-and-wait predators but they detect their prey through their strong vision and mechanoreceptor movements. The tadpoles of *D. melanostictus* probably show weak antipredator responses to *E. cyanophlyctis* tadpoles because these potential predators are omnivorous in nature feeding primarily on detritus matter or algae and also on other sympatric herbivorous tadpoles and detect their prey or food items only through chemical senses but not by visual senses (Mogali *et al.* 2023d).

In summary, tadpoles of *D. melanostictus* show antipredator behavioral responses to all their naturally co-existing predators and the strength of these responses depends upon likely predation risk. The findings of our study reinforce the idea that the antipredator behaviors of anuran tadpoles incorporate complex tradeoffs between risk and benefit.

Acknowledgments

This study was supported by a grant from the Department of Science and Technology (SP/SO/AS-38/2009), New Delhi, awarded to BAS and SKS. SMM was supported as a Project Assistant in the project. The study was conducted as per the ethical guidelines of CPCSEA, New Delhi, India (registration no. 639/02/a/CPCSEA).

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Editor: Ross Alford

Phyllomedusa 23(2):191–195, 2024
© 2024 Universidade de São Paulo - ESALQ
ISSN 1519-1397 (print) / ISSN 2316-9079 (online)
doi: http://dx.doi.org/10.11606/issn.2316-9079.v23i2p191-195

SHORT COMMUNICATION

Sexual dimorphism of snout–vent length in *Liolaemus nigroviridis* (Squamata: Liolaemidae)

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Keywords: Black-green Tree Iguana, Body size, Highlands, Sky island lizard, South America.

Palavras-chave: América do Sul, Iguana-arborícola-verde-e-preta, Lagarto-de-ilha-do-céu, Tamanho do corpo, Terras altas.

Sexual dimorphism in body size exists in many reptile species (see e.g. Olsson *et al.* 2002, Valdecantos *et al.* 2019, Liang *et al.* 2021, Rodríguez-Rodríguez and Calderón-Espinosa 2024). *Liolaemus* Wiegmann, 1834 is the predominant reptile genus in the southern cone of South America, currently comprising at least 289 recognized species (Uetz *et al.* 2024). For several species of the group, it has been described that males are larger than females (see Cabrera *et al.* 2013, Mella 2017). However, for most species in the genus there are no quantitative studies analyzing size differences between males and females (although see Cabrera *et al.* 2013, Maia-Carneiro and Rocha 2013). Usually, snout–vent length (SVL) is one of the most commonly used characteristics for measuring body size in herpetozoans, which is the measurement from the tip of the snout to the posterior margin of the precloacal scales (Breitman *et al.* 2015).

Received 18 April 2024
Accepted 04 September 2024
Distributed December 2024

Liolaemus nigroviridis Müller and Hellmich, 1932 is an endemic Chilean lizard inhabiting the sky islands, distributed from the southern Coquimbo Region (30° S) to the northern O'Higgins Region (34° S) (Cianferoni *et al.* 2013, Mella-Romero *et al.* 2023). *Liolaemus nigroviridis* populations inhabit the Coastal and Andean mountain ranges. The highest concentration of records for the species occurs between 2,000 and 2,800 m a.s.l. (Mella-Romero *et al.* 2023). Although *L. nigroviridis* is considered by the IUCN as Least Concern, this species would be facing threats derived from climate and land-use change (Mella-Romero *et al.* 2024a, Mella-Romero *et al.* 2024b, Moya *et al.* 2024). Natural history observations suggest that males of this species are larger and more robust than females (Donoso-Barros 1966, Mella 2017). However, this has not been quantified nor evaluated.

In this context, we hypothesize that there is sexual dimorphism in size in *L. nigroviridis,* with males of the species having a larger SVL than females.

To obtain data and test our hypothesis, we conducted herpetological sampling in the locality of Farellones (33°21'31.75" S, 70°17'42.40" W), in the Andes mountains in the Metropolitan Region of Chile. On 17 December 2022, we captured 98 specimens (47 females and 51 males) of *L. nigroviridis* between 09:15 h and 19:45 h. The area covered by the study was 9 ha. The captures were performed with the permission of the pertinent national agency (SAG, R.E. N° 5116/2022) and followed the protocol authorized by the Institutional Animal Care and Use Committee (Comité Institucional de Uso y Cuidado de Animales; CICUA) of the University of Chile (Certificado N° 22605-FCS-UCH).

We considered only adult specimens (juveniles were discarded and released). Juveniles were identified by (i) head length < 13 mm (Fuentes 1976), (ii) greyish-brown tone (without greenish scales), and (iii) presence of a discontinuous vertebral line (Donoso-Barros 1966, Mella 2017). We identified adult females and males of *L. nigroviridis* by the shape of the cloaca (rounded in females, square in males), and by the presence of precloacal pores, which are present in males and absent in females (Ruiz de Gamboa 2021). For each individual, we recorded its SVL using a digital caliper (Stainless Hardened, 0.01 mm) (Breitman *et al.* 2015). Measurements were always made by one specialist (JM-R). The lizards were retained in individual cloth bags until all measurements were completed. After the measurements had been taken, *L. nigroviridis* individuals were released at the same capture site.

To determine if there are differences in body size between females and males of *L. nigroviridis* using SVL, we performed data characterization by analyzing the assumptions of normality and homoscedasticity of both data sets (Cabrera *et al.* 2013). Since the distribution of the data for female lizards differed from a normal distribution ($p < 0.05$) and complied with the homoscedasticity principle ($p > 0.05$), we applied the Mann-Whitney U test for differences between two data sets (females and males) for nonparametric data, with the subsequent power test of the analysis. We selected a non-parametric test over data transformation since the distribution and categorization of our data fit the requirements of the Mann-Whitney U test (Conover 1999, Newbold *et al.* 2013). To perform the statistical analysis and elaborate the figures shown below, we used the software RStudio version 3.4.1 (Wickman 2016).

We found significant differences in SVL between females and males ($W = 2283$; $p < 0.001$) with a statistical power of 0.69 ($d = 0.5$; $p = 0.05$). Females had a mean SVL of 6.07 ± 0.49 cm (SD) with a higher frequency of data between 6.1 and 6.4 cm (min. 4.68 cm; max. 7.01 cm). For males, the mean SVL was 7.37 ± 0.56 cm (SD) with a higher frequency of data between 7.0 and 7.7 cm (min. 5.98 cm; max. 8.42 cm) (Figures 1 and 2). Females had a median of 6.08 cm, and males had a median of 7.43 cm (Figures 1 and 2).

We corroborated our hypothesis of sexual size dimorphism in *L. nigroviridis,* with males of the species having a higher SVL than females.

For some species of the genus *Liolaemus,* it is assumed that males are larger and more robust than females (see Mella 2017). Some studies report SVL, but do not analyze whether this characteristic shows sexual dimorphism (e.g. Campos-Soto *et al.* 2023). Of the studies that analyzed this variable, some do not find differences between females and males (e.g. Mella *et al.* 2023), while others do (e.g. Cabrera *et al.* 2013, Maia-Carneiro and Rocha 2013, Villamil *et al.* 2017). When sexual size dimorphism occurs in *Liolaemus,* there are cases in which males are larger, while in other cases, females are larger (Valdecantos *et al.* 2021). For example, in Cabrera *et al.* (2013), 22 species of *Liolaemus* were analyzed for sexual dimorphism in body size. In 16 of the 22 species, males were larger, while females were the larger sex in only six species. Our results showed that in *L. nigroviridis* males are significantly larger than females.

Sexual dimorphism in body size in species of lizards would be related, in ecological terms, to intra and intersexual selection (Carothers 1984,

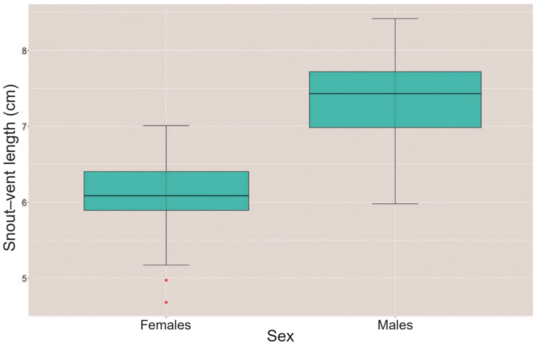

Figure 1. Snout–vent length for females and males of *Liolaemus nigroviridis.* Red dots indicate outliers. The horizontal black line in the boxplots indicates the mean.

Olsson *et al.* 2002). Thus, males would be larger because this favors them in aggressive encounters with other males or because larger males are better evaluated by females (Carothers 1984, Carothers *et al.* 1998, Cabrera *et al.* 2013). Since males of *L. nigroviridis* are territorial (see Carothers 1987), it is very likely that having a large body size is useful in intrasexual fights and territorial vigilance, which would provide an ecological explanation for the larger body size of males in the species (Valdecantos *et al.* 2021). Although intersexual selection (e.g. courtships by the males involving physical displays; Dunham and Wilczynski 2014) could be another explanation for sexual size dimorphism in *L. nigroviridis,* this has not been studied.

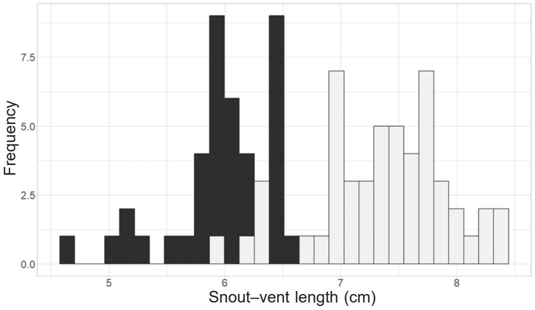

Figure 2. Frequencies of snout–vent length data for females (black bars) and males (white bars) of *Liolaemus nigroviridis.*

We should mention that in *Liolaemus* the SVL would not be the only characteristic of the males proposed as a subject of sexual selection, but also the head size (Vanhooydonck *et al.* 2010, Cabrera *et al.* 2013), both having relation with advantages in the territorial encounters between males (Valdecantos *et al.* 2021).

We consider that the relationship between male body size and intra or intersexual selection is an interesting avenue to investigate to answer ecological questions about *Liolaemus* species. Studies such as this one offer quantitative support to natural history observations and fill information gaps about these lizards, which constitute the most diverse reptile genus in the southern cone of South America.

Acknowledgments.—J.M.-R. thanks to ANID; CONICYT-PCHA, Doctorado Nacional/2019-21190472 for financing his postgraduate studies. K.A.-R. thanks to ANID; Magister Nacional 2023/ 22231399 for financing her postgraduate studies. J.M.-R. thanks to F. Moya, L. Alquinta, J. Mella, and P. Lamilla for their support in the fieldworks related to his doctoral thesis. We also thank Servicio Agrícola y Ganadero for the capture permit R.E. N° 5116/2022. We are especially grateful to S. Sparks and two reviewers, for their comments on the original version that optimized the manuscript.

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Editor: Jaime Bertoluci

Phyllomedusa 23(2):197–200, 2024
© 2024 Universidade de São Paulo - ESALQ
ISSN 1519-1397 (print) / ISSN 2316-9079 (online)
doi: http://dx.doi.org/10.11606/issn.2316-9079.v23i2p197-200

SHORT COMMUNICATION

An observation on the diving behavior of *Cyrtodactylus consobrinus* (Squamata: Gekkonidae) from Borneo

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Keywords: Escape strategy, Lizard, Peters' Bow-fingered Gecko, Riparian.

Palavras-chave: Comportamento de mergulho, Estratégia de fuga, Lagartixa-de-dedos-arqueados-de-peter, Ripário.

Cyrtodactylus consobrinus (Peters, 1871) is a forest-inhabiting gekkonid endemic to Southeast Asia. It has been known to occur in Peninsular Malaysia, extending to Sumatra, Pulau Sinkep and Borneo (Das 2010, Uetz *et al*. 2024), while a recent study restricted the species to western Borneo (Davis *et al*. 2023a, b), where it inhabits lowland forests and often observed foraging on tree trunks (Das 2010). This species was described under the name *Gymnodactylus consobrinus* by the German naturalist Wilhelm Karl Hartwich Peters (1815–1883), based on a single specimen collected from Sarawak, currently a Malaysian state on the island of Borneo (Peters 1871). Subsequently, it was listed under the genus *Cyrtodactylus* by Grandison (1972). Little is known about several aspects of the natural history of *C. consobrinus*, including its behavior. In this paper, we present an additional account on the anti-predator behavioral response of *C. consobrinus*.

During a field trip on 13 August 2024 in Kubah National Park (Kuching, Sarawak, Malaysia, which lies within the type locality of the species, the Matang Range), an adult *C. consobrinus* was observed resting on rock boulder at the riparian zone at 20:06 h. As the authors approached, the gecko instinctively dived into the stream and stayed underwater at a depth of 30–40 cm. The gecko was observed hiding its head on the small rock and stayed submerged for about three minutes (Figure 1). Short video clips of this observation were

Received 11 September 2024
Accepted 25 October 2024
Distributed December 2024

Figure 1. Adult *Cyrtodactylus consobrinus* submerged underwater to escape a perceived threat (yellow arrow) (Photo H.-S. Chen).

deposited as video vouchers to the Zoological Reference Collection, Lee Kong Chian Natural History Museum, National University of Singapore: ZRC (IMG) 2.685a and ZRC (IMG) 2.685b.

Diverse anti-predatory behaviors and strategies have been long reported across different vertebrate taxa as mechanisms to avoid detection, give warning to the group, and/or escape predators in active pursuit (Magurran 1990, Caro 2014, Saavedra and Amo 2020, Hernández-Palma *et al*. 2023, James *et al*. 2023). In squamate reptiles, active and passive defense traits include mimicry, aposematic displays, caudal autotomy, thanatosis, agony vocalization, and rapid locomotory escape (Cooper *et al*. 2004, Labra *et al*. 2013, Fuentes *et al*. 2021, Miranda *et al*. 2022, Kojima *et al*. 2024). Some lizard species have been recorded to utilize aquatic environment as temporary refugium to escape from predators. These semi-aquatic species, such as *Anolis aquaticus* Taylor, 1956 (Dactyloidae) and *Potamites erythrocularis* Chávez and Catenazzi, 2014 (Gymnophthalmidae), tend to dive underwater and can stay for a relatively long duration before surfacing (Arrivillaga and Quinkert 2019, Swierk 2019, Martin *et al*. 2024). Hare and Miller (2009) tested the usefulness of diving as a measure of performance in *Oligosoma smithi* (Gray, 1845) and suggested that submergence in water was for foraging and/or escape predators. The agamid *Gonocephalus grandis* (Gray, 1845) has been reported to dive into waterbodies. This was observed by Malkmus *et al*. (2022), where *G. grandis* leaped into waterbodies when it sensed danger. Manthey and Schuster (1996) reported juveniles and females of *G. grandis* as often are found by and in water. In addition, an adult male of *G. grandis*, initially observed on a tree branch overhanging a hill stream, was observed to dive into the water at Nanga Ulai, near Betong, Sri Aman Division, Sarawak. It remained underwater for over five minutes and was thereafter collected (ZRC 2.4941).

Diving behavior as strategy for predator escape is uncommon and rarely observed and documented in the wild on non-aquatic/ non-riparian group such as gekkonids. Our observation represents the first published documentation of diving behavior as predator escape strategy of *C. consobrinus*. Earlier account on diving behavior in gekkonids as an attempt to escape predator was observed in *Cnemaspis affinis* (Stolickza, 1870) (reported as *Gonatodes affinis*), wherein Annandale (1905) reported this phenomenon based on his field observation of *C. affinis* from Peninsular Malaysia. In his account, *C. affinis* have been observed frequently slipping and subsequently submerged on water accumulated in hollow areas of tree trunks and remained submerged for a few minutes. Similarly, this behavior was also reported for *Cyrtodactylus majulah* Grismer, Wood, and Lim, 2012 (Singapore Bent-toed Gecko) from the Central Catchment Nature Reserve in Singapore (Groenewoud 2015). This species is known to inhabit the lowland secondary forests with small streams in Singapore and the Riau Archipelago of Indonesia (Grismer *et al*. 2012). In addition, *C. patiensis* Grismer, Chan, Grismer, Wood, and Belabut, 2008 (Panti Mountain Bent-toed Gecko) have been reported by Grismer (2011) to have an escape strategy similar to *C. consobrinus* and *C. majulah,* wherein they jump into streams and run fast on the surface to evade capture.

Diving underwater as an escape strategy against predators is rare in gekkonids and merits further documentation, especially within the genus *Cyrtodactylus*. In addition, it is also interesting to investigate the duration of submergence and relative lung capacity across *Cyrtodactylus* species capable of this strategy.

Acknowledgments.—We thank Bruce Teo for company and assistance in the field; and to Kelvin K.P. Lim (National University of Singapore) for archiving our video documentation and providing us the video voucher number. ID thanks the Sarawak Forestry Corporation for permission to access an area under their jurisdiction, and the Institute of Biodiversity and Environmental Conservation, Universiti Malaysia Sarawak, and a Niche Research Grant from the Ministry of Higher Education, Government of Malaysia (NRGS/1087/2013(01)) for support.

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Editor: Jaime Bertoluci

Phyllomedusa 23(2):201–206, 2024

ISSN 1519-1397 (print) / ISSN 2316-9079 (online)
doi: http://dx.doi.org/10.11606/issn.2316-9079.v23i2p201-206

SHORT COMMUNICATION

Spawning site plasticity in *Cycloramphus boraceiensis* (Anura: Cycloramphidae)

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Keywords: Amphibians, Atlantic Forest, Brazil, Natural history, Reproductive mode, Rocky streams, Tadpoles.

Palavras-chave: Anfíbios, Brasil, Girinos, História natural, Mata Atlântica, Modo reprodutivo, Riachos rochosos.

Reproductive biology is likely the most studied subject in amphibian natural history (e.g., Womack *et al.* 2022). In part, this observation is explained by the conspicuous and easy-to-observe breeding behavior of amphibians, but also because a standardized classification system exists for amphibian reproductive modes (recently reviewed by Nunes-de-Almeida *et al.* 2021). This classification lists 74 reproductive modes (RM) for amphibians, and 71 RM for anurans (Nunes-de-Almeida *et al.* 2021). Based on the variation in the number of RM proposed by different authors (e.g., Duellman and Trueb 1986, Haddad and Prado 2005, Nunes-de-Almeida *et al.* 2021), it is clear that given the diversity of reproductive strategies, more information will be valuable.

The reproductive strategies of species in the genus *Cycloramphus* Tschudi, 1838 roughly fall into two categories: terrestrial, in which tadpoles are nidicolous (Lutz 1944, Heyer and Crombie 1979, Almeida-Silva *et al.* 2019, Verdade *et al.* 2023) or semi-terrestrial, in which tadpoles are saxicolous, exotrophic, and mostly associated with streams (Heyer 1983a,b, Haddad and Sazima 1989, Lima *et al.* 2010, Silva and Overnay 2012, Nunes-de-Almeida *et al.* 2016, Verdade *et al.* 2019). Terrestrial reproduction has evolved independently multiple times from saxicolous species according to the phylogeny of the genus (Sá *et al.* 2020). Variation observed in the reproduction of saxicolous species are classified under RM 17, in which females lay eggs on humid rock surfaces or inside rock crevices above the water in streams, and RM 19 for *Cycloramphus dubius* (Miranda-Ribeiro, 1920), in which eggs are deposited on wet rocks or between roots at the edges or within streams, according to Nunes-de-Almeida *et al.* (2021).

Received 19 June 2024
Accepted 09 August 2024
Distributed December 2024

The semi-terrestrial tadpoles of *Cycloramphus* spp. were thought to have restricted microhabitats, but recent observations in the field (e.g., *C. boraceiensis* Heyer, 1983; Pedrozo *et al.* 2024) and in captivity (e.g., *C. bandeirensis;* Verdade *et al.* 2019) have expanded our knowledge of their microhabitats and other features. Egg site deposition, development, and diet of *Cycloramphus* tadpoles are more variable than once thought. Egg masses of saxicolous species of *Cycloramphus* have been found on humid rocks, or more rarely, on plant stems near the splash zone of waterfalls within streams. We present herein natural history observations made in the field that increase our knowledge of variation of spawning sites of *C. boraceiensis*.

We conducted observations in the Projeto Dacnis private reserve, an area of 136 ha located within lowland Atlantic Forest in the municipality of Ubatuba, state of São Paulo, Southeastern Brazil (23°27'46" S, 45°07'58" W; WGS-84; 15–500 m a.s.l.). The region is characterized by paludal forests (patches of forests with waterlogged soil) in lowland areas and patches of primary and secondary dry forests on steep terrain. Climate in the region is classified as humid subtropical without distinct seasonality (Rolim *et al.* 2007). In 14 years monitoring the area, we found two saxicolous species in forest streams: *Thoropa taophora* (Miranda-Ribeiro, 1923) and *C. boraceiensis,* both with semi-terrestrial tadpoles that can be diagnosed by differences in body shape, position of eyes, and color pattern (Heyer *et al.* 1990, Moura *et al.* 2019, Colaço *et al.* 2021).

We monitored three spawning events, one in a cavity in the soil (Figure 1A–D) and two in a hole in a liana stem (Figure 2A–B). We encountered the first mass of eggs of *C. boraceiensis* on 13 October 2015 and observed the development of the embryos in three visits over two weeks. Forty eggs were laid on soil in a dry cavity in a vertical ravine 2 m from the stream. An adult male was observed near the clutch (Figure 1B). After six days, the larvae remained in the egg capsules but with visible eyes and body pigmentation (Figure 1C). After another six days, the tadpoles were well developed, moving among the gelatinous mass from the empty egg capsules (Figure 1D). Following a heavy rain on 28 October 2015, neither the eggs, tadpoles, nor the adult male were seen at the spawning site, having been washed away.

On 4 May 2023, we observed a second egg mass of *C. boraceiensis* inside a hole in a liana stem that was hanging approximately 30 cm above a dripping rocky wall. The spawning site was kept consistently humid by a small trickle of water running down the liana and by spray from a nearby small waterfall. We counted 36 eggs at a very early stage of development (Figure 2B). We visited the site another three times. On 8 May 2023, we observed eye pigmentation and the emergence of gills on the embryos inside the egg capsules (Figure 2C). After 7 days, free-living tadpoles were clustered inside the liana hole (Figure 2D), and from 19 May 2023 onward tadpoles were found on the rocky wall bellow the liana stem (Figure 2E). An adult *C. boraceiensis* remained close to the spawning site inside a small crevice on the rocky wall throughout the observation period. Seven months later, on 14 December 2023, another egg mass was located in the same liana hole, again with an adult nearby. Unfortunately, we were unable to determine if it was the same frog observed in May. Site fidelity is a possibility in stream-associated *Cycloramphus* spp., as high quality reproductive sites may be a limiting resource.

Our observations are the first to describe an egg mass of a saxicolous stream-associated species of *Cycloramphus* in a dry cavity on soil away from wet rocks or the splash zone of a waterfall. Our observations of spawning on the stems of lianas confirm those reported for *C. boraceiensis* (Pedrozo *et al.* 2024). Based on the current reproductive mode classification system of Nunes-de-Almeida *et al.* (2021), *C. boraceiensis* would be categorized in RM 17 (for saxicolous species of the genus), RM 19 (previously known only for *C. dubius*), and RM

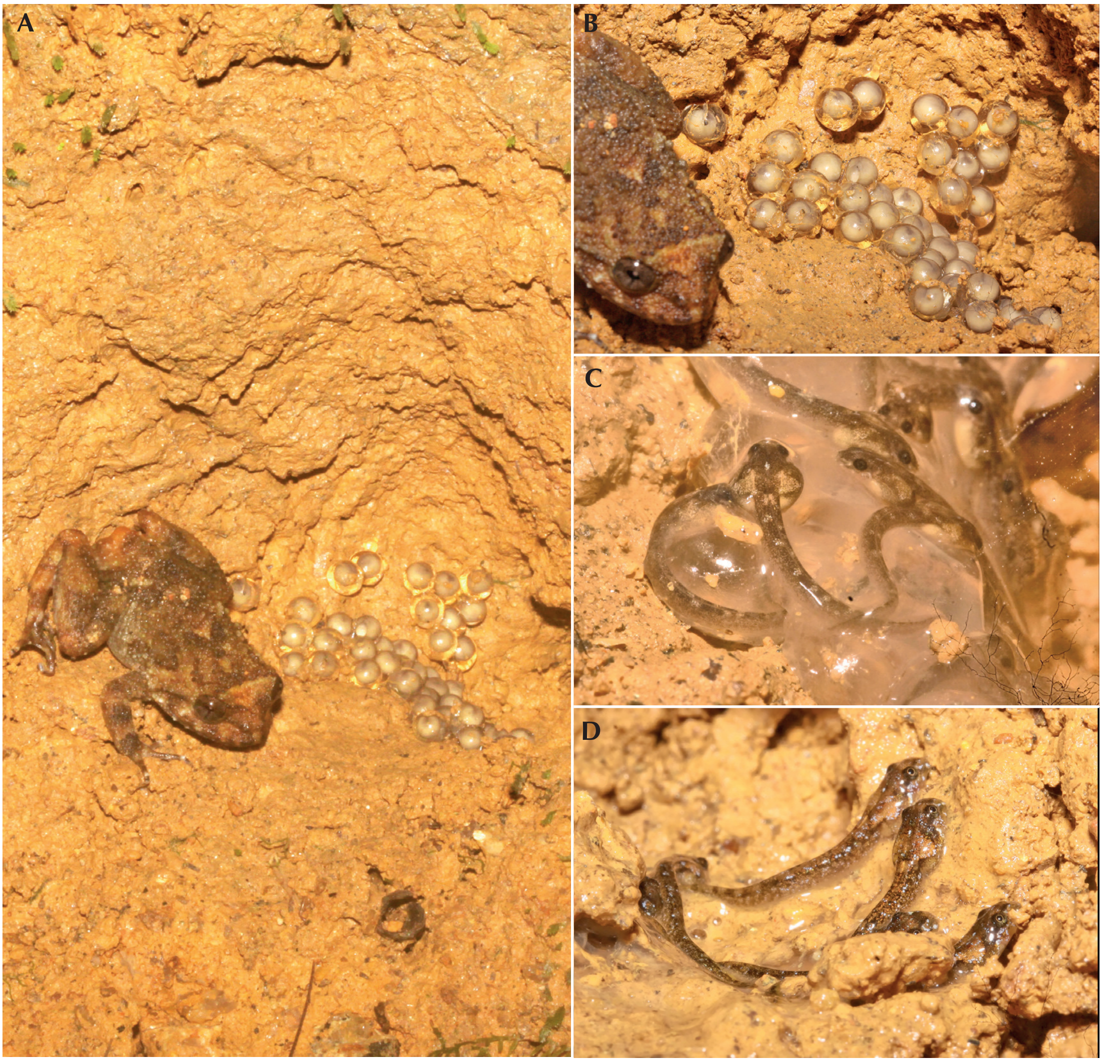

Figure 1. (**A**) Egg mass of *Cycloramphus boraceiensis* in a permanently dry ravine cavity; (**B**) a male guarding eggs in an early stage of development; (**C**) larvae with visible eyes and body pigmentation; (**D**) hatched tadpoles still in the gelatinous mucus inside the ravine cavity.

32 (reported here for the first time) by depositing eggs in dry cavities on soil.

Our observations expand those of Pedrozo *et al.* (2024) on the use of humid cavities in the soil at the steep edges of streams for reproduction in *C. boraceiensis*. The plasticity in spawning sites observed in *C. boraceiensis* indicates how little we know about the requirements associated with high quality sites for reproduction, and how the limitation of this resource could lead to differential survivorship of eggs and tadpoles. Understanding selective pressures induced by humidity levels, risk of displacement by heavy rainfall, and effective

Figure 2. (**A**) Egg mass of *Cycloramphus boraceiensis* within a consistently humid vine hole situated above a rock wall with a continuous water layer; (**B**) an egg mass with embryos at an early stage; (**C**) larvae with ocular pigmentation and gills; (**D**) hatched tadpoles still within the liana cavity; (**E**) tadpoles adhered to the rocky wall.

parental care are crucial to understanding the evolution of terrestrial reproduction in the genus. The future of populations of *Cycloramphus* spp. may include a scenario of climate change that may affect rainfall volume and periodicity.

Acknowledgments.—We would like to thank Elsie Laura Rotenberg for the final review of the text. Ariovaldo A. Giaretta, Geoff Smith, and anonymous reviewers provided comments on earlier versions of the manuscript. Grants and fellowships were provided by São Paulo Research Foundation (FAPESP #2022/11096-8) and the National Council for Scientific and Technological Development (CNPq #302834/2020-6).

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Editor: Geoffrey R. Smith

Phyllomedusa 23(2):207–211, 2024
© 2024 Universidade de São Paulo - ESALQ
ISSN 1519-1397 (print) / ISSN 2316-9079 (online)
doi: http://dx.doi.org/10.11606/issn.2316-9079.v23i2p207-211

SHORT COMMUNICATION

Defensive repertoire of *Leptodactylus rhodomystax* (Anura: Leptodactylidae) in the Brazilian Amazon

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Keywords: Antipredatory behavior, Anuran amphibians, Defensive strategies.

Palavras-chave: Anfíbios anuros, Comportamento antipredatório, Estratégias defensivas.

Anuran amphibians are common prey for a wide variety of vertebrate and invertebrate predators (Duellman and Trueb 1994, Toledo *et al*. 2007). In response to the selective pressures exerted by these predators, this group has developed a series of antipredator defense mechanisms that may include ecological, morphological, physiological, and/or behavioral aspects (Toledo *et al*. 2011, Ferreira *et al*. 2019). These strategies can be divided into three main phases: avoiding detection by predators (e.g., camouflage), preventing attack (e.g., aposematism), and reacting during attack (e.g., use of skin secretions) (Ferreira *et al*. 2019). These strategies can be employed independently in the presence of a potential predator (e.g., venom, aposematism, camouflage) or used in the presence of and/or direct interaction with the predator (e.g., fleeing, feigning death, biting) (Edmunds 1974, Toledo *et al*. 2011).

The genus *Leptodactylus* Fitzinger, 1826 is one of the most diverse groups of anurans, widely distributed across the Neotropical region (de Sá *et al*. 2014, Frost 2024). It is comprised of medium- to large-sized species that exhibit diverse ecology, occupying various environmental niches (Heyer 1979, de Sá *et al*. 2014). *Leptodactylus rhodomystax* Boulenger, 1884, belonging to the *L. pentadactylus* group, is characterized by its medium size (maximum SVL of 91.4 mm in females and 89.6 mm in males), the presence of a light stripe on the upper lip, and distinct light spots (usually yellow or white) on the posterior surface of the thigh against a dark background (Heyer 1979). This species has terrestrial and nocturnal habits, typically occupying forest environments (Barrio-Amorós *et al*. 2019, Gagliardi-Urrutia *et al*. 2022). It has a wide distribution, found in the Amazonian regions of the Guianas (including Suriname and Venezuela), extending through Brazil, Colombia, Ecuador, and Peru (Frost 2024). The defensive repertoire of this species is poorly known, with records limited to death-

Received 09 August 2024
Accepted 25 October 2024
Distributed December 2024

feigning (Ramalho *et al*. 2019) and stretching limbs (Pedroso-Santos *et al*. 2022). Here, we present a compilation of the defensive strategies used by *L. rhodomystax,* including documentation of new behaviors employed by the species.

Data were obtained during field expeditions conducted in 2009 and 2013 in the municipalities of Canutama (06°32'02'' S, 64°22'58'' W, WGS 84; Figure 1B) in the state of Amazonas, Brazil, and in the municipalities of Cotriguaçu (09°51'28'' S, 58°24'50'' W, WGS 84; Figure 1E) and Paranaíta (09°39'54'' S, 56°28'37'' W, WGS 84; Figure 1A, C–D, F), both located in the state of Mato Grosso, Brazil. All strategies displayed by individuals were performed in non-natural environments during *ex situ* photographic sessions of the specimens. We photographed all defensive behaviors exhibited and classified the strategies according to the terminology proposed by Ferreira *et al.* (2019).

We recorded five types of defensive strategies performed by four individuals of *L. rhodomystax:* (1) hidden aposematism, (2) rear elevation, (3) body inflation, (4) body elevation (full and partial), and (5) skin secretion. Exhibitions of body inflation and rear elevation, along with display of the internal patterns of the thighs (hidden aposematism), were performed synergistically by Individual 1 (Figure 1B); full body elevation was exhibited simultaneously with body inflation by Individual 2 (Figure 1C, D); partial body elevation was performed by Individual 3 (Figure 1E); and skin secretions were recorded in Individual 4 (Figure 1F). The observed behaviors can be utilized in phases of attack prevention (hidden aposematism, rear elevation, body inflation, and body elevation) and counter-attack (skin secretion).

The behavior of rear elevation, also known as body-raising (Toledo *et al*. 2011), involves raising the posterior part of the body by fully or partially extending the hind legs (Ferreira *et al*. 2019). During this display, *L. rhodomystax* exhibits aposematic patterns characterized by spots, usually vibrant yellow, on the posterior region of the thighs that strongly contrast with the black background (Figure 1A). These spots are also visible during displays of stretching limbs, a strategy that can be classified as a type of hidden aposematism (Ferreira *et al*. 2019, Pedroso-Santos *et al*. 2022).

The strategy of body inflation occurred synergistically with displays of rear elevation and full body elevation. In this behavior, the individual inflates its lungs with air, thereby increasing its size (Toledo *et al*. 2011, Ferreira *et al*. 2019). During full body elevation, the individual extends both its front and hind limbs completely to appear larger and more intimidating to potential predators (Figure 1D). In contrast, during partial body elevation, the individual extends only its front limbs, spreading them slightly apart and adopting a more upright posture. This position not only increases the individual's size but may also expose the spines located on the phalanges and chest region of sexually active males, potentially facilitating spine aggression (Toledo *et al*. 2005, Oliveira-Santos *et al*. 2023). Additional presumed functions of these mechanisms include making it more difficult for predators to ingest an individual and making it harder for predators to grasp an individual (Ferreira *et al*. 2019).

Many amphibians use skin secretions as anti-predatory defense mechanisms (Ferreira *et al*. 2019). These secretions can have odoriferous, adhesive, slippery, and/or toxic properties (Brizzi and Corti 2007, Toledo *et al*. 2005). When handled, *L. rhodomystax* can produce a foamy substance with a viscous and slippery appearance, concentrated mainly in the inguinal region (Figure 1F). Although biochemical tests to confirm the compounds in the secretion could not be conducted, we believe that it has slippery and/or toxic effects like species in the *L. pentadactylus* group, commonly known as Pepper Frogs, that produce substances rich in peptides with toxic effects (Toledo *et al*. 2005, Carrillo *et al*. 2024).

Although the strategies described here are the first records for *L. rhodomystax* (except for hidden aposematism; see Pedroso-Santos *et al*. 2022), there are reports of similar behaviors in other species within the genus, especially in the

Figure 1. Defensive strategies used by *Leptodactylus rhodomystax* in the Brazilian Amazon. (**A**) Posterior thigh region displaying hidden aposematic patterns; (**B**) rear elevation; (**C–D**) full body elevation with body inflation; (**E**) partial body elevation; (**F**) exudation of skin secretion. Photos: RWA (A, C, D, F), VTC (B), RAK-R (E).

L. pentadactylus group. These include displays of posture mechanisms (body elevation, body inflation, and/or rear elevation) in *L. knudseni* Heyer, 1972, *L. labyrinthicus* (Spix, 1824), *L. pentadactylus* (Laurenti, 1768), *L. rhodonotus* (Günther, 1869), *L. stenodema* Jiménez de la Espada, 1875, and *L. vastus* Lutz, 1930; skin secretion in *L. flavopictus* Lutz, 1926, *L. knudseni, L. labyrinthicus, L. pentadactylus, L. savagei* Heyer, 2005, and *L. vastus;* and hidden aposematic patterns in *L. knudseni, L. labyrinthicus, L. pentadactylus, L. rhodonotus,* and *L. vastus* (Toledo *et al*. 2005, 2011, Castro *et al*. 2017, Ferreira *et al*. 2019).

Even while remaining motionless, anurans can employ a variety of strategic mechanisms to deter predator attacks (Toledo *et al.* 2011). Postural mechanisms, such as rear elevation, are frequently accompanied by the simultaneous display of hidden aposematic coloration, skin secretion production, and body inflation (Castro *et al.* 2017, Ferreira *et al.* 2019). Our observations of *L. rhodomystax,* along with reports from other species within the *L. pentadactylus* group, suggest that species in this group may synergistically utilize this sequence of behaviors to enhance their survival against predator attacks. These findings contribute to our understanding of the defensive strategies employed by these species and the behavioral patterns adopted by phylogenetically related species.

Acknowledgments.—We thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the research grant awarded to ARLR. We thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for a research grant (307722/2021-0) awarded to RWA.

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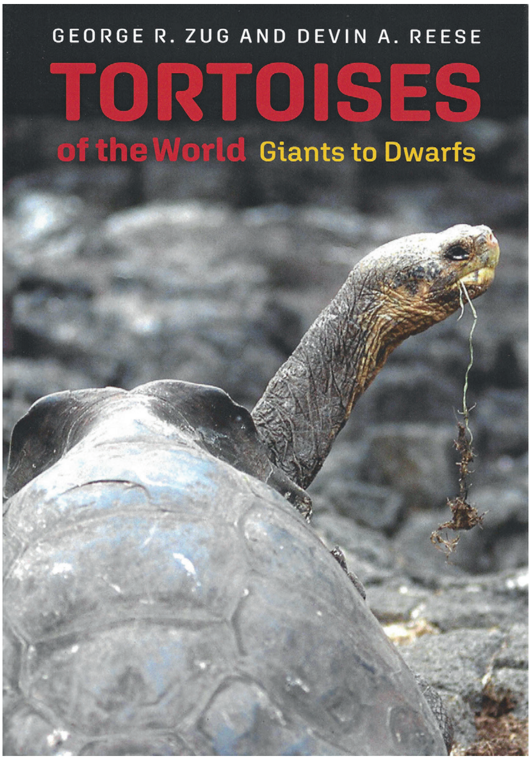
Editor: Vanessa K. Verdade

Zug, G. R. and D. A. Reese. 2024. **Tortoises of the World: Giants to Dwarfs.** Johns Hopkins University Press, Baltimore, Maryland, USA (www.press.jhu.edu). x + 228 pp.

Hardcover and e-Book. US $49.95 ISBN: 978-1-4214-4835-0 (Hardcover), 978-1-4214-4836-7 (eBook).



The tortoise wins again. Any time you put the tortoise in a race against the hare, the tortoise wins. The best case is the race home; tortoises carry their homes with them. This book explains why in many ways, tortoises come up winners but why they are faring so very badly in the modern world.

The title suggests that the book is focused on size and perhaps the mechanisms involved in the evolution of giants (*Megalochelys* was 2 m long) and dwarfs (adults < 20 cm) that are found in various land tortoise lineages, but the focus is instead quite broad, summarizing essentially all of the biology of land tortoises, the Family Testudinidae. The authors provide a "deep look" at land tortoises using a sound-bite approach. Topics are broken down into very short sections, often half a page or less, with catchy titles. The overall message of the book is the very interesting biology and diversity of tortoises and the perils that have threatened and continue to threaten them on a global scale

There is much in this book about the biology and anatomy of tortoises that explains their success as a group over tens of millions of years. Most are herbivores and their low metabolic rate allows them to tolerate conditions that mammalian herbivores (e.g., rabbits) cannot. The importance of the shell and how tortoises can move while carrying a large box are well explained. The very large bladder and large lungs in the top of the shell turn out to be very important in survival and dispersal. Dorsal lungs explain why tortoises float so well and land tortoise physiology includes multiple advantages that allow them to go for long periods without food or water. Among the very nice set of color plates, is the photo of the Aldabra tortoise that walked up a beach in Tanzania covered with goose barnacles (plate 20). It provides definitive illustration that tortoises are capable of very long distance, open ocean dispersal (740 km from Aldabra to Tanzania) and supports the books contention that over-water-dispersal has been a major feature of tortoise evolutionary history.

The authors consider the biological importance of a long life for which tortoises are famous, and then point out that this is no longer the case for many populations in the modern world. Those individuals that do survive are able to continue to reproduce for their entire lifetime which improves the likelihood that they will eventually produce offspring that survive and maintain populations.

Although it likely adds to the readability of the book, it is unfortunate that there are no citations in this volume. Instead, a .url for an online bibliography of source material is provided. The absence of citations is unfortunate on multiple fronts. First it keeps the reader from pursuing the exact source of information for statements in the text and the authors of those works don't receive the credit they are due. It also means that the timing of discoveries about tortoise biology is lost to the reader. Perhaps most unfortunate is that a large part of the work that the authors put into the book is not available to the reader; the authors knew exactly which sources they were citing but that connection has been lost.

The evolution and diversity of tortoises is treated via chapters on modern diversity and evolution. It is pointed out that tortoises as a family are the youngest major group (~50 million years) in the turtle lineage that is at least 220 million years old. There are useful descriptions of variation in size, shape and thickness of the shell that led to the remarkable differences among species. It might have been of interest to point out that all tortoise species start out with exactly the same bones in the shell and nearly the same scales. It is remarkable that such morphological diversity should arise from a single basic starting point. The book also makes it clear that our understanding of the diversity of living tortoises is still changing. For example, in recent decades the number of *Kinixys* species has gone from 3 to 8 and the western-most gopher tortoise in North America (formerly placed in *Gopherus agassizii*) actually represents three species.

For this reviewer, a cladogram of living land tortoises would have been a very useful addition to the coverage of tortoise evolution. Although there is frequent consideration of relationships among tortoises and between tortoises and other turtles, there is nothing like a branching diagram to illustrate these important concepts. It is also unfortunate that only family-level distribution maps by continent are included. More detailed range maps would have added greatly to the volume by illustrating such points as the remarkable diversity of tortoises in southern Africa and Madagascar and the very limited ranges of many of the species that make up that diversity.

Tortoise conservation is taken up in the final chapter. It covers topics like legal protection for tortoises, translocation, head-starting and captive colonies. It highlights some of the major ongoing conservation programs like those for Galapagos tortoises, Aldabra tortoises, the Bolson gopher tortoise, and the many African and Malagasy endemics that are among the most threatened species of turtle in the world. But the key to keeping land tortoises in the wild will be to maintain the natural ecosystems that they require. As human populations grow, and the effects of climate change result in changes in land use, the problem of maintaining good tortoise habitats will get even more serious than it is now.

There are some minor errors that should have been caught before publication. It is stated that all turtles have scutes, two families do not. It is stated that scutes are dermal in origin, they are epidermal. *Astrochelys yniphora* is said to be the world's rarest turtle, what about *Rafetus swinhoei?* It is reported that forearms are thickly scaled, but it is more than scales, many species have limbs that are armored with bone that is covered by scales.

These small problems do not detract from the very important message that land tortoises face a very challenging survival outlook. The family has a long history of remarkable dispersal and diversity, with size being a major component of variation among lineages. Tortoises help to shape entire ecosystems, and their disappearance reflects badly on the human race. The fact that interactions with humans over millennia have led to the extinction of many species and in some cases, entire lineages, is well documented. The absence of giant tortoises from continents is shown to be related to the rise of human populations and the diversity of island-dwelling giants has also been sharply reduced. A summary table of tortoise species shows that two-thirds of modern forms are extinct or face some serious level of threat. This book clearly makes the case that without concerted conservation efforts, land tortoises will no longer be able to win the race by running slowly.

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Author Index - Volume 23 (2024)

Subject Index - Volume 23 (2024)

INSTRUCTIONS TO AUTHORS

General Information. *Phyllomedusa* publishes articles dealing with the entire field of herpetology. The journal also maintains sections for Short Communications and Book Reviews. Manuscripts are considered on the conditions that they: (1) have not been published elsewhere; (2) are not under consideration for publication, in whole or in part, in another journal or book; and (3) are submitted by the authors in the format and style of *Phyllomedusa* and in accordance with the specifications included in the Instructions to Authors. Manuscripts should be submitted as a single Microsoft Word document via e-mail. High-quality color images are accepted. Manuscripts must be written in English with appropriate abstracts in alternate languages. If English is not your primary language, arrange to have your manuscript reviewed for English usage before you submit it. Direct any questions about manuscript submission to the primary editor. Publication in *Phyllomedusa,* including color images, is free of charge.

Scope. Manuscripts must contain significant new findings of fundamental and general herpetological interest. Surveys and taxonomic descriptions are published only if there is sufficient new biological information or taxonomic revision to render the paper of general herpetological interest. Lower priority is accorded confirmatory studies, investigations primarily of localized interest, range extensions, technique papers with narrow application, descriptions of phenomena based on insufficient data, and descriptive work that is not placed in a significant context. Manuscripts should include a clear statement of the purpose of the study or the hypothesis that was tested.

Peer Review. At least two referees, an Associate Editor, and the Editor will review each manuscript that is deemed to fall within the scope of *Phyllomedusa.* Authors will be notified of the status of their manuscript within 90 days. Revised manuscripts accepted for publication will be edited for English usage and syntax prior to final acceptance for publication.

Manuscript Style and Format. Use the active voice when possible; thus, you should write "I/we studied the frog," rather than "The frog was studied by me/us" (passive voice). Use American spelling and punctuation. Double space the entire manuscript, including references, tables, table captions, and legends for illustrations. Use Times New Roman 12-point font, and set up document with margins of at least 2.54 cm (1 in.) on each side. Do not justify the text; it should be left aligned and ragged right. Number manuscript pages consecutively and lines continuously, **followging the arrangement and format outlined below exactly.**

- **Title:** Bold-faced caps and lower-case Roman; sentence capped, left aligned; use colons to separate ranked taxonomic names.
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  Abstract
  Title of paper in bold-faced Roman. Content of abstract follows in light-faced Roman; left alignment.
- **Keywords:** Light-faced Roman; separate words with commas; capitalize only proper nouns; include descriptors not contained in the title in alphabetical order.
- **Body of Article:** The text of the article will include the following parts indicated by **primary headings in bold-faced Roman aligned to the left (except for References, which should be centered).**

Introduction
Materials and Methods
Results
Discussion
Acknowledgments
References

Secondary headings within major sections are title-capped, italics aligned left. **Tertiary headings** follow a paragraph indentation; they are sentence capped, and set in italics. Tertiary headers are followed by a point and an em-dash. Follow example:
Material and Methods [Primary header]
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- **Body of Short Communication or Book Review:** These shorter articles do not include the primary headings Introduction, Materials and Methods, Results, and Discussion. "Acknowledgments" is treated as a third-level, or tertiary header.
- **Tables:** Number tables consecutively with Arabic numbers. Refer to tables in text as Table 1, Tables 2 and 3, and Tables 2–5. Exceedingly long tables should be placed in appendices. Table captions should be placed above the table. Horizontal rules may be used in the table header and at the foot of the table. No rules (horizontal or vertical) should appear in the body of a table. Consult Vol. 20 (2) of *Phyllomedusa* for proper format of table captions and contents.
- **Appendices:** Number appendices consecutively with Roman numerals. Refer to tables in text as Appendix I, Appendices II and III, and Appendices II–V. Appendix captions should be placed above the appendix content. Most appendices should follow the format instructions for tables. Extensive lists of specimens examined should be included as an appendix. Consult Vol. 18 (2) of *Phyllomedusa* for proper format and arrangement of specimens examined.
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  - ✓ Use the **double-digit rule** for numbers other than measurements. Numbers less than 10 are spelled out—e.g., "… nine animals were sampled"; numbers of 10 and more are denoted in Arabic—e.g., "… but 10 larvae were collected."
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Vanzolini, P. E. 1993. A new species of turtle, genus *Trachemys,* from the state of Maranhão, Brazil (Testudines, Emydidae). *Revista Brasileira de Biologia 55:* 111–125.

✓ **Two authors in a journal series:**

Zamudio, K. R. and H. W. Greene. 1997. Phylogeography of the bushmaster (*Lachesis muta:* Viperidae): implications for Neotropical biogeography, systematics, and conservation. *Biological Journal of the Linnean Society 62:* 421–442.

✓ **More than two authors in a journal series:**

Hero, J.-M., W. E. Magnusson, C. F. D. Rocha, and C. P. Catterall. 2001. Antipredator defenses influence the distribution of amphibian prey species in the central Amazon rain forest. *Biotropica 33:* 131–141.

✓ **Chapter in an edited volume:**

Hedges, S. B. 1999. Distribution patterns of amphibians in the West Indies. Pp. 211–254 *in* W. E. Duellman (ed.), *Patterns of Distribution of Amphibians. A Global Perpective.* Baltimore and London. The Johns Hopkins University Press.

✓ **Unpublished thesis or dissertation:**

Verdade, V. K. 2001. Revisão das espécies de *Colostethus* Cope, 1866 da Mata Atlântica (Anura, Dendrobatidae). Unpublished M.Sc. Dissertation. Universidade de São Paulo, Brazil.

✓ **Book:**

McDiarmid R. W. and R. Altig (eds.). 1999. *Tadpoles. The Biology of Anuran Larvae.* Chicago and London. The University of Chicago Press. 633 pp.

✓ **Material from the World Wide Web:**

Frost, D. R. (ed.). 2010. Amphibian Species of the World: an Online Reference. Version 5.4 (8 April 2010). Electronic Database accessible at http: // research.amnh.org/vz/herpetology/amphibia/American Museum of Natural History, New York, USA. Captured on 22 August 2010.

✓ **Software:**

Maddison, W. P. and D. R. Madison. 2010. Mesquite. A Modular System for Evolutionary Analysis. Version 2.73. URL: http://mesquiteproject.org

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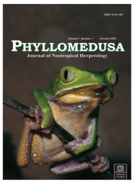
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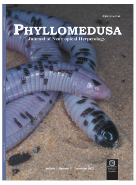
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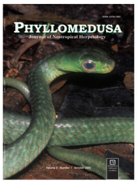
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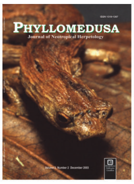
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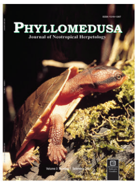
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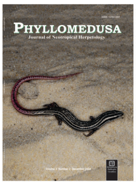
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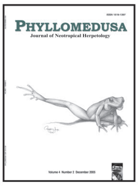
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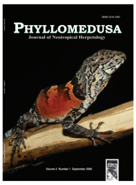
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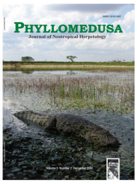
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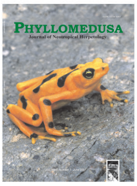
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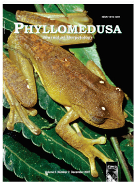
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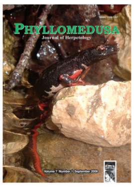
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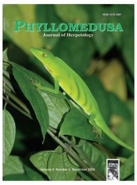
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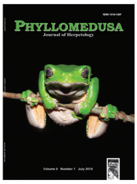
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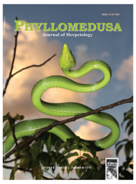
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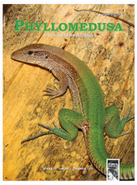
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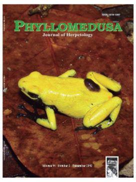
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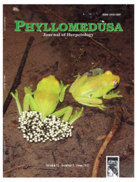
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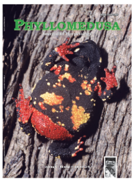
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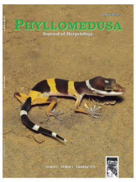
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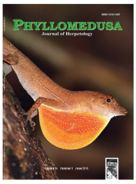
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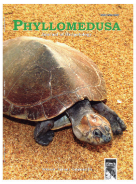
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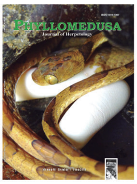
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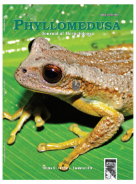
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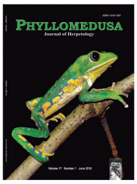
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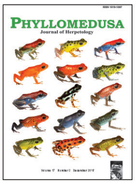
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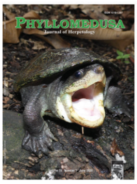
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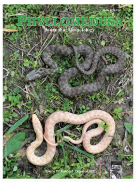
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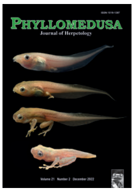
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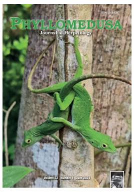
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