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## Rediscovery and redescription of the rare, critically endangered snake *Emmochliophis miops* (Serpentes: Colubridae), with comments on its natural history, distribution, and phylogenetic relationships

### Luis Enrique Vera-Pérez,<sup>1</sup> Patrick D. Campbell,<sup>2</sup> and Giovanna Gondim Montingelli<sup>3</sup>

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### Abstract

Rediscovery and redescription of the rare, critically endangered snake *Emmochliophis miops* (Serpentes: Colubridae), with comments on its natural history, distribution, and phylogenetic relationships. The description of *Emmochliophis miops* is based on a single specimen from the Andean slopes of northwest Ecuador collected in 1897. The species had not been collected again until October 2017, when two individuals were found on the western slopes of the Cordillera Occidental of Colombia; this rediscovery is the first record of this species in this country. Herein, the holotype and the new specimens are examined, and a new diagnosis and description of *E. miops* are provided. Important data about the natural history and conservation status of the species, and comments on the systematic relationships of *Diaphorolepis, Emmochliophis* and *Synophis* are presented.

**Keywords:** Andean Shadow Snakes, Colombia, Diaphorolepidini, Ecuador, hemipenes, Parque Nacional Natural Munchique.

### Resumen

Redescubrimiento y redescripción de la serpiente rara y en peligro crítico *Emmochliophis miops* (Serpentes: Colubridae), con comentarios sobre su historia natural, distribución y relaciones filogenéticas. La descripción de *Emmochliophis miops* está basada en un solo espécimen de las laderas andinas del noroeste de Ecuador recolectado en 1897. La especie no se volvió a recolectar hasta octubre de 2017, cuando dos individuos fueron encontrados en la vertiente occidental de la Cordillera Occidental de Colombia; este redescubrimiento es el primer registro de esta especie en

Received 17 June 2019 Accepted 05 February 2020 Distributed June 2020 este país. En este estudio, el holotipo y los nuevos especímenes son examinados y se proporciona un nuevo diagnóstico y descripción de *E. miops*. Se presentan datos importantes sobre la historia natural y el estado de conservación de la especie, y comentarios sobre las relaciones sistemáticas de *Diaphorolepis, Emmochliophis* y *Synophis*.

**Palabras claves:** Colombia, Culebras Andinas de la Sombra, Diaphorolepidini, Ecuador, hemipenes, Parque Nacional Natural Munchique.

### Resumo

Redescoberta e redescrição da rara e criticamente ameaçada serpente *Emmochliophis miops* (Serpentes: Colubridae), com comentários sobre história natural, distribuição e relações filogentéticas. A descrição de *Emmochliophis miops* baseia-se em apenas um espécime procedente das vertentes Andinas do noroeste do Equador, coletado em 1897. Representantes dessa espécie não haviam sido coletados até outubro de 2017, quando dois indivíduos foram encontrados na Cordilheira Ocidental da Colômbia; esta redescoberta consiste no primeiro registro dessa espécie no país. Neste estudo, examinamos o holótipo e os novos espécimes e propomos uma nova diagnose e descrição de *E. miops*. Apresentados dados importantes sobre a história natural e o estado de conservação da espécie, além de comentários sobre as relações sistemáticas de *Diaphorolepis, Emmochliophis* e *Synophis*.

Palavras-chave: cobras-andinas-das-sombras, Colômbia, Diaphorolepidini, Equador, hemipênis, Parque Nacional Natural Munchique.

### Introduction

The colubrid tribe Diaphorolepidini currently is composed of *Diaphorolepis* (2 spp.), *Emmochliophis* (2 spp.), and *Synophis* (9 spp.); it is diagnosed by the presence of fused prefrontals, and either an expanded intervertebral scale row or expanded zygapophyses and neural spines in adults (Pyron *et al.* 2016). Members of the tribe are distributed from Darien, Panama, to the northern Andes in Colombia, Ecuador, and Peru (Bogert 1964, Hillis 1990, Sheil 1998, Sheil and Grant 2001, Pyron *et al.* 2015, 2016, Torres-Carvajal *et al.* 2015).

Recently a number of specimens of *Diaphorolepis wagneri* Jan, 1863 and *Synophis* have been collected; however, other species in Diaphorolepidini remain rare and enigmatic (Hillis 1990, Arteaga *et al.* 2013, Pyron *et al.* 2015, 2016, Torres-Carvajal *et al.* 2015). *Synophis plectovertebralis* Sheil and Grant, 2001, *Diaphorolepis laevis* Werner, 1923, *Emmochliophis fugleri* Fritts and Smith, 1969,

and *E. miops* (Boulenger, 1898) are known only by their type specimens.

*Emmochliophis* is composed of relatively small-sized terrestrial snakes restricted to the Pacific Andean slopes of northwestern Ecuador. They are easily differentiated from the other two genera of Diaphorolepidini by the absence of a loreal scale and no reduction in the number of scale rows from neck to vent (vs. loreal present and reduced scale rows from anterior to posterior body in *Synophis* and *Diaphorolepis*) (Fritts and Smith 1969, Sheil 1998, Pyron et al. 2015, 2016). Emmochliophis miops is only known from a single specimen collected in 1897 (Boulenger 1898), and is listed on the IUCN Red List as Critically Endangered, under criterion B1ab(iii) that refers to a distribution area of  $< 100 \text{ km}^2$ , a single locality, and a continued decline in range and/or habitat quality (Cisneros-Heredia and Yánez-Muñoz 2017). According to these authors, no suitable habitat remains at the type locality owing to complete deforestation for timber extraction and then cattle ranching; in addition, nearby forests have been replaced with palm oil plantations. It is thought that *E. miops* may be sensitive to changes in humidity that would not allow it to persist in disturbed forests.

In late 2015, LEV-P observed some photographs of snakes found in La Cueva sector of the Parque Nacional Natural Munchique, Colombia, taken by a colleague. Some of the photos were of a slender, black snake with a white nuchal collar that was identified as Ninia atrata (Hallowell, 1845) and not collected. However, on close examination, photographs showed that fused prefrontals were present; thus, it is not N. atrata, and the presence of a single keeled intervertebral scale row indicates that it is not in Diaphorolepis. The snake could be a Synophis, but the presence of a loreal scale could not be confirmed. The possible absence of the loreal scale led to the possibility that the snake was a species of Emmochliophis. A more thorough search was carried out at La Cueva in November 2016, but the species was not encountered. On 17 October 2017 at La Cueva. LEV-P captured a snake that resembled the specimen photographed, and on the following night a second individual was found. Neither snake has a loreal scale, thereby confirming their identification as Emmochliophis miops; this is the first record of this species in the wild in the 120 years since the collection and description of the holotype.

We compare the new specimens with the holotype, and redescribe the species, providing information on its natural history, conservation status, and some comments on the tribe Diaphorolepini.

### **Materials and Methods**

We examined the holotype of *Emmochliophis miops* (BMNH 1946.1.12.30) housed in the Natural History Museum, London (NHMUK), along with the two new specimens and a specimen of *Diaphorolepis* cf. *wagneri* that are deposited at Museo de Historia Natural of the Universidad del Cauca, Colombia (MHNUC). Specimens were collected in the La Cueva sector of the Parque Nacional Natural Munchique in the municipality of El Tambo, department of Cauca, Colombia (02°46'18.1" N, 76°58'51.5" W, 1040 m a.s.l.) under agreements TW25 of 2016 and TW63 of 2017 "Conservación de la biodiversidad en paisajes impactados por la minería en la región del Chocó Biogeográfico", issued by World Wildlife Foundation and Fundación Ecohábitats as part of monitoring program of the PNN Munchique.

Species identification is based on the taxonomic key of Pyron et al. (2016). The following morphometric characters and measurements were recorded: SL = supralabials, IL = infralabials, PrO = preoculars, PO = postoculars, Temp = temporals, PV = preventrals, V = ventrals, SC = subcaudals, DSR = dorsal scale rows, MT = maxillary teeth, SVL = snout-vent length, TL = tail length. Sexwas determined by the presence or absence of hemipenes. Ventral scale counts follow those of Dowling (1951); subcaudal scales were counted in pairs, excluding the terminal spine. Total and tail lengths were measured with a ruler to the nearest 1.0 mm; cephalic and hemipenial measurements were taken with dial calipers to the nearest 0.1 mm under a stereo microscope. Maxillary teeth were counted on both sides through a narrow incision between the supralabials and the maxillary arch with the aid of a stereo microscope.

Hemipenes were prepared following the methods of Myers and Cadle (2003) and Zaher and Prudente (2003); both were almost maximally expanded by injections of colored petroleum jelly and stained with Alizarin Red S to elucidate the calcareous spines (Uzzell 1973). Hemipenial terminology follows that of Dowling and Savage (1960), as augmented by Zaher (1999). For comparisons, hemipenial morphology of *Synophis* were taken from Zaher (1999), Sánchez-Martínez (2011) and Torres-Carvajal *et al.* (2015).

### Results

### *Emmochliophis miops* (Boulenger, 1898) (Figures 1–6) *Synophis miops* Boulenger, 1898

*Holotype.*—An adult female (BMNH 1946.1.12.30) collected by W. F. H. Rosenberg in October 1897 from Paramba, Ecuador (= Parambas, Imbabura fide Lynch and Duellman 1997).

*New specimens.*—A juvenile female (MHNUC-HE-Se-0657) and an adult male (MHNUC-HE-Se-0658), both collected by LEV-P in the La Cueva sector of the Parque Nacional Natural Munchique, municipality of El Tambo, department of Cauca, Colombia (02°46'13.1" N, 76°58'47.7" W, 1142 m a.s.l.; 02°46'13.6" N, 76°58'44.5" W, 1188 m a.s.l.; respectively).

Diagnosis.—(1 male and 2 females): Emmochliophis miops is distinguished from E. fugleri and other species of Diaphorolepidini by the combination of following characters: (1) intervertebral scale row single keeled; (2) dorsal scales keeled, in 19|19|19 rows; (3) prefrontals fused, in contact with supralabials; (4) loreal absent; (5) preoculars 1 or 2; (6) postoculars 1 or 2; (7) temporals 1+2; (8) supralabials 8, 4<sup>th</sup> and 5<sup>th</sup> in contact with orbit; (9) infralabials 8, first four in contact with first pair of chinshields; (10) ventrals 141 in male, 137 in females; (11) subcaudals more than 62 in males, 90 and 94 in females; (12) maxillary teeth 13-15; (13) dorsum dark gray with white nuchal collar after few months of preservation, and brown with a cream nuchal collar in holotype (Figures 1-2); (14) venter grayish after preservation, and cream in holotype; (15) chin tubercles present in males and juvenile females but absent in adult females (Figure 3); (16) hemipenis bilobed, semicalyculate, and semicapitate with a lateral naked pocket at base of organ (Figure 4).



Figure 1. Dorsal (upper), ventral (middle) and lateral (lower) views of the holotype BMNH 1946.1.12.30 of *Emmochliophis miops*. Scale bar = 100 mm. Photographs @The Trustees of the Natural History Museum, London.

Comparisons.—Diaphorolepidini includes slender snakes that are dark dorsolaterally, and have keeled scales, fused prefrontals and a long head. Emmochliophis miops differs from Diaphorolepis (characters in parentheses) by having an intervertebral scale row of single keeled and not expanded (double keeled and expanded), dorsal scale rows with no reduction (reduced posteriorly), loreal absent (present), infralabials 8 (10-13), maxillary teeth 13-15 (16-25), ventral scales  $\leq 141 (157-197)$ , and nuchal collar complete in juveniles and adults (incomplete and present only in juveniles). Species of Synophis also have a loreal scale, reduction in dorsal scale rows, nuchal collar absent (except in S. plectovertebralis), more maxillary teeth (16-27), more infralabials up to a maximum of 12 (except in S. calamitus Hillis, 1990, S. plectovertebralis, and S. zaheri Pyron,



Figure 2. Dorsal (upper), ventral (middle) and lateral (lower) views of new specimens of *Emmochliophis miops*: MHNUC-HE-Se-0657 (left), MHNUC-HE-Se-0658 (right). Scale bars = 40 mm. Photographs by LEV-P.

Guayasamin, Peñafiel, Bustamante and Arteaga, 2015, which can also have 7 or 8), and more ventral scales (144–193).

The differences between *Emmochliophis miops* and *E. fugleri* include the latter having no nuchal collar, 16 maxillary teeth, and unique interlocking trunk vertebrae. Juveniles of *Geophis nigroalbus* Boulenger, 1908 and some individuals of *Ninia atrata* and *N. teresitae* Angarita-Sierra and Lynch, 2017 can be confused with *E. miops* by having keeled scales and a dorsolateral black coloration with a light whitish nuchal collar. However, these three species can be easily differentiated by the absence of fused prefrontals.

*Description.*—Based on the holotype, an adult female, and a juvenile female and adult male. Slender and relatively small-sized snakes ( $\leq 258 \text{ mm SVL}$ ) (Table 1); head length 2×



Figure 3. Head of *Emmochliophis miops* in dorsal (upper), lateral (middle) and ventral (lower) views. Holotype BMNH 1946.1.12.30 (left), MHNUC-HE-Se-0657 (center), MHNUC-HE-Se-0658 (right). Scale bars = 10 mm. Photographs courtesy of @The Trustees of the Natural History Museum, London and LEV-P.



Figure 4. Hemipenis of *Emmochliophis miops* (upper, right organ of MHNUC-HE-Se-0658) and *Diaphorolepis* cf. *wagneri* (lower, left organ of MHNUC-HE-Se-0650) in asulcate (left), sulcate (center) and lateral (right) views. Scale bars = 5 mm. Photographs by LEV-P.

width, easily differentiated from neck, flattened laterally; snout truncate in dorsal and ventral views, rounded in lateral view; eye small; dark gray dorsolaterally after 3 months in preservative; nuchal collar V-shaped, white, covering temporals, part of parietals, postoculars, last supralabials and infralabials, gulars, chin shields, preventrals and first ventrals; snout black; venter pale gray anteriorly, darker posteriorly; rostral scale slightly visible from above; internasals in contact; fused prefrontals, in contact with supralabials; loreal absent; preoculars 1 or 2, with the second represented by a small subpreocular (left side of MHNUC-HE-Se-0657); supraocular 1; postoculars 1 or 2, with the second represented by a small subpostocular (right side of MHNUC-HE-Se-0657); temporals 1+2, primary 2× secondary in length; supralabials 8, with 4<sup>th</sup> and 5<sup>th</sup> bordering the orbit; infralabials 8, 1<sup>st</sup>–4<sup>th</sup> contacting anterior chin shields; dorsal scales in 19 rows with no reduction, dorsolateral rows single keeled; apical pits absent; anal plate entire; preventrals 1 or 2; ventrals 137-141, subcaudals 90 and 94 (MHNUC-HE-Se-0658 with a broken tail); chin tubercles present on first infralabials and on anterior chin shields in juvenile females (weak) and males (strongly expressed in adults). Maxillary teeth 13–15, equal in number on each side.

morphology.-Hemipenis Hemipenial bilobed, semicalyculate, and semicapitate (Figure 4, upper); 9.8 mm total in length; capitulum and body relatively similar in length (4.4 and 5.4 mm, respectively); lobes spinulate, with spines slightly larger and numerous on asulcate surface where the capitular groove forms; few spinules on the asulcate side of the lobes: sulcus spermaticus centrolineal. bifurcating at mid-portion of body and terminating at the apex; body ornamented by calcified spines, larger basally and shorter on lateral surfaces near the sulcus spermaticus; nude pocket present at the base of the organ (right side, on asulcate side).

Color in life.—Dorsolateral color of Emmochliophis miops black, interrupted only by the

Table 1.Summary of morphological characters and measurements (mm) of *Emmochliophis miops*. See Materials and<br/>Methods for abbreviations. Solidus separates the right/left sides when counts are different. Numbers in<br/>parentheses correspond to scales bordering the orbit and contacting the first pair of chin shields. Dashes<br/>separate the anterior, mid-body and posterior DSR counts. Asterisk indicates an incomplete tail.

Characters	BMNH 1946.1.12.30	MHNUC-HE-Se-0657	MHNUC-HE-Se-0658
Sex	Female	Female	Male
SVL	255	142	258
TL	135	68	91*
SL	8(4,5)	8(4,5)	8(4,5)
IL	8(1-4)	8(1-4)	8(1-4)
PrO	1	1/2	1
РО	1	2/1	1
Temp	1+2	1+2	1+2
PV	2	1	2
V	137	137	141
SC	94	90	62*
DSR	19-19-19	19-19-19	19-19-19
MT	13	14	15

white nuchal collar, which in juveniles is narrower and creamy (Figure 5). Venter light to medium gray, interrupted only by the white nuchal collar and the black snout. Ventral surface of tail dark gray.

*Distribution.*—Western slopes of the Andes in Parambas, province of Imbabura (*fide* Lynch and Duellman 1997) in northern Ecuador (type locality), and western slopes of the Cordillera Occidental in the La Cueva sector of the Parque Nacional Natural Munchique, municipality of El Tambo, department of Cauca, Colombia (Figure 6). Elevation of type locality as provided by Boulenger (1898) 1066.8 m a.s.l., or 777 and 780 m a.s.l. as corrected by Brown (1941) and Lynch and Duellman (1997), respectively. Colombian specimens were captured at 1142 and 1188 m a.s.l. *Natural history.*—The species occurs in the transition from humid premontane to tropical humid forests. It is a nocturnal terrestrial snake that moves slowly on the forest floor and among



Figure 5. Coloration in life of *Emmochliophis miops* in ventral (upper) and dorsolateral (lower). Adult male (left, MHNUC-HE-Se-0658) and juvenile female (right, MHNUC-HE-Se-0657). Photographs by LEV-P.



Figure 6. Geographical distribution of *Emmochliophis miops*. The star corresponds to the type locality in Parambas, province of Imbabura, Ecuador and the circle corresponds to the new locality record in La Cueva sector, Parque Nacional Natural Munchique, municipality of El Tambo, department of Cauca, Colombia.

rock piles. The only information on its diet is provided by Sheil (1998), from the holotype, and consists of an unidentified lizard. No stomach contents were found in the two specimens collected in this study. No defensive behaviors, such as bites, sudden movements, expulsion of excrement or secretions of the anal glands were noted upon capture. This species is delicate and highly sensitive to changes in humidity and temperature. When recently captured animals were handled, we observed that the dorsal scales became wrinkled to form a longitudinal crest, and the skin between lateral scales expanded. Minutes later, the individuals stiffened, especially along the tail, and gradually became motionless as the head flattened.

Conservation status.—Emmochliophis miops should remain as Critically Endangered, but we consider a change on criterion from B1ab(iii) (Cisneros-Heredia and Yánez-Muñoz 2017) to A1ac (IUCN 2012) because, although the species occurs within a protected area in Colombia, it has not been found in Ecuador since the holotype was collected in 1897, suggesting that it may have been extirpated in the course of degradation and loss of habitat.

### Discussion

The genus Emmochliophis was erected by Fritts and Smith (1969) and diagnosed by its unique interlocking trunk-vertebral mechanism, a condition present only in the type species E. fugleri. When Sheil (1998) included Synophis miops in Emmochliophis, he designated this character as autapomorphic of E. fugleri, and absence of fused lateral considered the projections of prezygapophyses and postzygapophyses of trunk vertebrae as a synapomorphy for Emmochliophis, a condition has not yet been evaluated in most of the Synophis group. In their description of S. plectovertebralis, Sheil and Grant (2001) reported that the only reliable characters differentiating Emmochliophis from Synophis are the non-reduction of scale rows and the absence of the loreal scale.

We suggest that the presence of a lateral naked pocket on the base of the hemipenis of Emmochliophis miops is a significant character that should be evaluated in a detailed review of the hemipenis of the holotype of E. fugleri. We need to determine whether the hemipenial pocket is an autapomorphy of E. miops or a synapomorphy for the genus because this character seems to be absent in Synophis. A review of the morphology of trunk vertebrae in the species of Synophis is needed to evaluate Sheil's (1998) proposed synapomorphy for Emmochliophis, because fusion of the lateral projections of prezygapophyses and postzygapophyses is also present in S. plectovertebralis (Sheil and Grant 2001).

Species of Diaphorolepidini have been have a similar hemipenial thought to morphology (Bogert 1964, Fritts and Smith 1969, Pyron et al. 2015). However, the hemipenes of Synophis and Emmochliophis (Figure 4, upper) are more similar to one another than they are to those of *Diaphorolepis*; the similarities include the length of the lobe and the V-shaped anterior edge of capitulum, formed by the capitular groove. See hemipenis of S. lasallei (Nicéforo-Maria, 1950) and S. bicolor Peracca, 1896 in Zaher (1999: 161-162), Sánchez-Martínez (2011: 152); S. bogerti Torres-Carvajal, Echevarría, Venegas, Chávez and Camper, 2015, S. zamora Torres-Carvajal, Echevarría, Venegas, Chávez and Camper, 2015, and S. insulomontanus Torres-Carvajal, Echevarría, Venegas, Chávez and Camper, 2015 in Torres-Carvajal et al. (2015: 162, 169, 173). Although the hemipenes of both Diaphorolepis and E. miops have a basal pocket, the hemipenis of Diaphorolepis (Remarks) is robust, with short lobes. In addition, the capitular grooves form a round shape; the sulcus spermaticus forks near the base of organ and the calcified spines of the body are larger (Figure 4, lower). These features suggest that Synophis and Emmochliophis are sister taxa, as inferred by Hillis (1990). Further molecular sampling of E. miops is key to our understanding of the systematic relationships among the Diaphorolepidini.

*Remarks.*—The hemipenial description of *Diaphorolepis* reported herein is based only on the examination of *D*. cf. *wagneri* (MHNUC-HE-Se-0650). The lack of available information on the hemipenis of *D*. *laevis* and the brief description for *D*. *wagneri* provided by Bogert (1964) is inadequate for an appropriate comparison of the hemipenial morphology within the tribe.

The rediscovery of *Emmochliophis miops* after more than 12 decades is also the first record of the species in Colombia; this extends the distribution of this Critically Endangered species at least 265.5 km from Parambas, Province of Imbabura, Ecuador, to the La Cueva sector in the Parque Nacional Natural Munchique, municipality of El Tambo, department of Cauca. It seems likely that the species may occur in other localities in the Pacific region of Colombia.

Numerous specimens of *Diaphorolepis* and *Synophis* have been collected recently and include five new species (Pyron *et al.* 2015, 2016, Torres-Carvajal *et al.* 2015); however, no additional records of *Emmochliophis miops* have appeared since its original description (Boulenger 1898). Habitat loss in the type locality and surrounding areas plus the apparent sensitivity of the species to changes in humidity reported here and inferred by Cisneros-Heredia and Yánez-Muñoz (2017) suggest that *E. miops* may be extirpated in Ecuador.

We agree with the recent proposal concerning Standard Common names for species of *Synophis* (Pyron *et al.* 2016) and we extend these criteria to all species of Diaphorolepidini. We support the following names for the remaining species (Spanish names in parentheses): *Diaphorolepis laevis* = Colombian Shadow Snake (Culebra colombiana de la Sombra); *D. wagneri* = Wagner's Shadow Snake (Culebra de la Sombra de Wagner); *Emmochliophis fugleri* = Fugler's Shadow Snake (Culebra de la Sombra de Fugler) and *E. miops* = Myopic Shadow Snake (Culebra de la Sombra miope).

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#### Appendix I. Specimens examined.

*Diaphorolepis* cf. *wagneri:* **COLOMBIA.** CAUCA: municipality of El Tambo, Parque Nacional Natural Munchique, sector La Cueva, 02°46'14.6" N, 76°58'52.4" W, 1090 m a.s.l. (MHNUC-HE-Se-0650).

*Emmochliophis miops:* **COLOMBIA.** CAUCA: municipality of El Tambo, Parque Nacional Natural Munchique, sector La Cueva: 02°46'13.1" N, 76°58'47.7" W, 1142 m a.s.l. (MHNUC-HE-Se-0657); 02°46'13.6" N, 76°58'44.5" W, 1188 m a.s.l. (MHNUC-HE-Se-0658). **ECUADOR.** IMBABURA: Parambas, 00°48'18" N, 78°21'03" W, 780 m a.s.l. (BMNH 1946.1.12.30, holotype).

# New localities and distribution models inform the conservation status of the endangered lizard *Anolis guamuhaya* (Squamata: Dactyloidae) in central Cuba

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### Abstract

New localities and distribution models inform the conservation status of the endangered lizard Anolis guamuhaya (Squamata: Dactyloidae) from central Cuba. Anolis guamuhaya is known from seven localities restricted to the Guamuhaya Massif in central Cuba and is always associated with mountane ecosystems above 300 m a.s.l. Previous evaluations of the conservation status of the species based on the estimated number of mature individuals have categorized the anole as Endangered. Eight new records of A. guamuhaya are provided here. These double the number of known localities, and two represent the first records of the species in lowland areas, apart from the Guamuhaya Massif. The new records extend the elevational range of the species from 15 m to above 1000 m. We used ecological niche modeling based on all of the locality records, along with what we considered the most appropriate IUCN criteria according to the available information (Criterion B) to reevaluate the conservation status of the species. These new records of A. guamuhaya increase its area of occupancy up to a total of 60 km<sup>2</sup>, and its extent of occurrence up to 648 km<sup>2</sup>. Despite this increase in geographic range, the species meets the IUCN criteria in the category of Endangered. We used ecological niche modeling to predict possible trends for the species under differing scenarios of global climate change, all of which portend a drastic reduction in area climatically suitable for A. guamuhaya.

Received 23 May 2018 Accepted 20 February 2020 Distributed June 2020 **Keywords:** Climate change, ecological niche modeling, natural history, Twig-giant Anole, West Indies.

### Resumen

Nuevas localidades y modelos de distribución informan sobre el estatus de conservación del lagarto amenazado Anolis guamuhaya (Squamata: Dactyloidae) de Cuba central. Anolis guamuhaya se conoce de siete localidades restringidas al Macizo de Guamuhaya, en Cuba central, siempre asociado a ecosistemas de montaña por encima de los 300 m s.n.m. Evaluaciones anteriores de su estatus de conservación basadas en el número estimado de individuos maduros categorizaron a la especie En Peligro. En este trabajo damos a conocer ocho nuevos registros de A. guamuhaya. Con estos se duplica el número de localidades conocidas y dos de ellas constituyen los primeros registros de esta especie en zonas llanas, fuera del Macizo de Guamuhaya. Estos nuevos registros expanden el rango altitudinal de la especie desde 15 m hasta por encima de los 1000 m. Teniendo en cuenta los registros previos y los nuevos, hacemos una reevaluación del estatus de conservación de la especie empleando la modelación de nicho ecológico y los criterios de la UICN que consideramos más apropiados de acuerdo a la información disponible (criterio B). Estos nuevos registros de A. guamuhaya aumentan su área de ocupación hasta un total de 60 km<sup>2</sup> y su extensión de presencia hasta 648 km<sup>2</sup>. A pesar de este incremento en su área de distribución, la especie se ajusta a los criterios de la UICN para la categoría de En Peligro. Usamos la modelación de nicho ecológico para predecir posibles tendencias de la especie bajo diferentes escenarios de cambio climático, donde todos los modelos auguran una reducción drástica del área climáticamente idónea para la especie.

Palabras claves: cambio climático, ecomorfo gigante de ramita, historia natural, modelación de nicho ecológico, Las Antillas.

### Resumo

Novas localidades e modelos de distribuição informam sobre o estado de conservação do lagarto Anolis guamuhaya (Squamata: Dactyloidae) de Cuba central. Anolis guamuhaya é conhecida de sete localidades restritas ao Maciço de Guamuhaya, no centro de Cuba, sempre associada a ecossistemas montanhosos acima de 300 m de altitude. Avaliações anteriores de seu estado de conservação baseadas no número estimado de indivíduos maduros categorizaram a espécie como Em Perigo. Neste trabalho, fornecemos oito novos registros de A. guamuhaya. Com esses registros, duplica-se o número de localidades conhecidas, e duas delas constituem os primeiros registros da espécie em zonas baixas fora do Maciço de Guamuhaya. Os novos registros expandem a amplitude altitudinal da espécie desde 15 m até acima dos 1000 m. Levando em conta os registros prévios e os novos, reavaliamos o estado de conservação da espécie empregando modelagem de nicho ecológico e os critérios da IUCN que consideramos mais apropriados de acordo com a informação disponível (Critério B). Os novos registros de A. guamuhaya aumentam sua área de ocupação para um total de 60 km<sup>2</sup> e sua extensão de presença para 648 km<sup>2</sup>. Apesar desse incremento em sua área de distribuição, a espécie ajusta-se aos critérios da IUCN para a categoria Em Perigo. Utilizamos modelagem de nicho ecológico para prever possíveis tendências para a espécie em diferentes cenários de mudanças climáticas globais, todas pressagiando uma redução drástica em sua área climática adequada.

**Palavras-chave:** anoles-graveto-gigante, Antilhas, história natural, modelagem de nicho ecológico, mudanças climáticas.

### Introduction

There are six species of anoles in the Chamaeleolis clade, all endemic to Cuba (Rodríguez et al. 2013, Poe et al. 2017). These large (154-177 mm snout-vent length), slowmoving, chameleon-like anoles have massive heads with prominent parietal bones that form a casque-like structure, and posterior molariform teeth that may enable the lizards to consume hard prey, such as gastropod mollusks (Garrido and Schwartz 1968, Díaz et al. 1998, Herrel and Holanova 2008). Among the morphological adaptations of these lizards for a strict arboreal lifestyle and use of narrow perches are a semiprehensile, non-autotomous, blunt tail and short limbs (Garrido and Schwartz 1968, Garrido 1982, Rodríguez 1999). Given the striking differences of these anoles from their congeners, they were placed in their own genus-Chamaeleolis (e.g., Garrido and Schwartz 1968, Garrido 1982, Rodríguez 1999). However, Chamaeleolis currently is considered a junior synonym of Anolis because it is nested within the Anolis radiation (Hass et al. 1993, Jackman et al. 1999, Poe 2013, Pyron et al. 2013, Poe et al. 2017).

Anolis guamuhaya (Garrido, Pérez-Beato, and Moreno, 1991) is a montane twig-giant anole currently thought to be restricted to ecosystems above 300 m a.s.l. in the Guamuhaya Massif (= Escambray) (e.g., Garrido et al. 1991, Rodríguez et al. 2013). The massif is located in south-central Cuba and is composed of two main topographical units-the Trinidad Range in the west (the largest unit) and the Sancti Spíritus Range in the east (the smallest unit); the Agabama River Basin separates these mountain ranges (Mateo-Rodríguez and Acevedo-González 1989; Figure 1). Garrido et al. (1991) stated that the holotype of A. guamuhaya was collected on the road between Jibacoa and Topes de Collantes, Trinidad Range, at about 900 m a.s.l. However, the highest elevations between these two localities is 869 m a.s.l. at Sierra Guaniquical (4 km SSW Jibacoa) and 851 m a.s.l. at Loma

Guaniquical (6 km SSE Jibacoa), and the road does not exceed 800 m a.s.l. at any point. Elevations of 900 m near to the type locality of A. guamuhaya occur only at Pico de Potrerillo (973 m a.s.l., 3.3 km SE Topes de Collantes) and Pico Tuerto (919 m a.s.l., 4.3 km WSW Jibacoa, western side of Hanabanilla Dam). Access to cartographic maps and GPS was limited when Garrido et al. (1991) described this species, and in interviews with Orlando H. Garrido, we could not confirm the exact collecting site of the holotype. Therefore, according to the International Code of Zoological Nomenclature (ITZN 1999; Article 76), the type locality of A. guamuhaya includes the entire strip of road between Jibacoa and Topes de Collantes (about 22 km). Additional specimens were collected at Topes de Collantes and surrounding areas, Sancti Spíritus Province (Garrido and Schwartz 1968, Garrido et al. 1991, Rodríguez et al. 2013), and in the vicinity of Aguacate, Cienfuegos Province (Garrido 1982, Garrido et al. 1991). The adult male collected by Wilson (1957) at Mina Carlota, Cienfuegos Province, was initially assigned to A. porcus (Cope, 1864) by Garrido and Schwartz (1968) and later to A. guamuhaya by Garrido et al. (1991). Most recently, Torres et al. (2015) reported the species from near Pico San Juan (at 1015 m a.s.l.), Cienfuegos Province.

The detectability of species is sometimes a critical factor in estimating population density and other demographic parameters (e.g., Mancina and Cruz 2017). The coloration and behavior of twig-giant anoles are highly effective in camouflaging the lizards (Garrido and Schwartz 1968, Leal and Losos 2000). Because it is so difficult to detect them by day, we assume that the lizards are more abundant than they seem to be. Nevertheless, the conservation status of some species in the Chamaeleolis clade, including A. guamuhaya, has been evaluated based only on the IUCN Criterion D, which refers to the estimated number of mature individuals (IUCN 2001, 2012, IUCN Standards and Petitions Subcommittee 2014). Rodríguez (1999, 2012) listed A. guamuhaya as Endangered; however,



**Figure 1.** Distribution of *Anolis guamuhaya* in south-central Cuba with locality records from the literature (red dots) and this study (green triangles): (1) Santa Martina, (2) Boca Ambuila, (3) Mina Carlota, (4) Carso de Buenos Aires, (5) Pico San Juan, (6) ca. 6 km SW of Aguacate, (7) Río Negro Touristic Center, (8) Loma Cariblanca, (9) Trinitario Stream waterfall, (10) La Chispa, (11) Topes de Collantes and vicinity, (12) Vegas Grandes, (13) Caburní waterfall, (14) midway on the road between Jibacoa and Topes de Collantes, and (15) resurgence of the Jibacoa River. Note the two main topographical units that form the Guamuhaya Massif (the Trinidad Range in the west and the Sancti Spíritus Range in the east), divided by the Agabama River Basin.

this author commented that the population size of the species was unknown and that all records were restricted to a region of about 14 km<sup>2</sup> in the Sancti Spíritus Province, with a calculated area of occupancy of 8 km<sup>2</sup>. Also, this author overlooked two localities from Cienfuegos Province—Mina Carlota, and ca. 6 km SW of Aguacate (Wilson 1957, Garrido and Schwartz 1968, Garrido 1982, Garrido *et al.* 1991). However, the reclusive habits and camouflage of twig-giant anoles (e.g., Garrido and Schwartz 1968, Leal and Losos 2000) make it extremely difficult to estimate population size accurately by counting in searches by day, in contrast to gathering data for other more conspicuous anoles (e.g., trunk-ground anoles). Thus, we consider that the population size of *A. guamuhaya* may be substantially greater than estimates based on Criterion D suggest.

The Guamuhaya Massif has been intensively and extensively deforested, particularly during the last two centuries (e.g., Domínguez-González and Acosta-Rodríguez 2012, Mancina *et al.* 2017). Given the highly arboreal habits of twiggiant anoles, the loss of forest coverage probably has had negative effects on the population of *A. guamuhaya.* Herein we report more than twice the number of localities previously known for the species and reevaluate its conservation status applying what we consider the most appropriate IUCN criteria according to the available information and taking into account the behavioral particularities of twig-giant anoles. We used ecological niche modeling to test the possible response of *A. guamuhaya* to the effects of different future scenarios of global climate change. Last, we discussed implications for the conservation of the species.

### **Materials and Methods**

### Data Collection

The specimens of A. guamuhaya reported here were collected between 1993 and 2014; additional surveys were conducted until late-2018. Two methods were effective in detecting these lizards. In the first, small groups composed of two to five people separated by about 2 m from one another walk in line across the forest by day. When a lizard tries to hide from the first person by moving to the opposite side of the trunk or branch, it is more likely to be seen by the next person. The second method, with which we obtained the best results, is searching at night when the anoles are sleeping; by night, the twig-giant anoles are whitish in color, and therefore, easily detectable in darkness in the white light of a headlamp. It is necessary to check all vegetation strata, because these anoles may be found sleeping as low as 50 cm above the ground on shrubby vegetation and vines to more than 5 m high in the canopy (Leal and Losos 2000, authors pers. obs.).

We included seven literature records (Rodríguez *et al.* 2013, Torres *et al.* 2015) and eight new localities of *A. guamuhaya* in our analyses (Figure 1; Appendix I). To include the holotype record in this study, we assigned its locality to a point midway on the road between Jibacoa and Topes de Collantes (Figure 1). We collected geographic coordinates directly in the field with a GPS Garmin eTrex Vista HC or obtained them from MapInfo Professional ver. 10.5. *Datum* for all coordinates is WGS 84. We

also accessed existing GIS data available for voucher specimens deposited in several collections. Specimens were collected under the project "Colecciones zoológicas, su conservación y manejo II," hosted by the Instituto de Ecología y Sistemática and deposited in the zoological collection of the latter institution and the Museo de Historia Natural "Felipe Poey" (Appendix I). Acronyms and abbreviations are as follow: SVL snout-vent length, TL = tail length; = CZACC: Instituto de Ecología y Sistemática, La Habana; MFP: Museo de Historia Natural "Felipe Poey", Facultad de Biología, Universidad de La Habana, La Habana; MNHNCu: Museo Nacional de Historia Natural de Cuba. La Habana; MCZ: Museum of Comparative Zoology, Harvard University, Cambridge, Massachusetts, USA: USNM: National Museum of Natural History, Smithsonian Institution, Washington D.C., USA; NMP: National Museum of Prague, Czech Republic.

For the purposes of this work and following the concept of "location" of the IUCN Standards and Petitions Subcommittee (2014; section 4.11), we considered all locality records separated by less than 2 km to be the same location (e.g., Topes de Collantes and vicinity; Garrido and Schwartz 1968, Garrido 1982, Rodríguez et al. 2013; Appendix I) and provided a central reference coordinate and elevation. After applying this criterion, the 32 records of A. guamuhaya represent 15 different locations, eight of which are new ones reported here (Figure 1; Appendix I). For the reevaluation of the conservation status we used the IUCN Criterion B (IUCN 2001, 2012, IUCN Standards and Petitions Subcommittee 2014).

### Ecological Niche Modeling

Occurrence and study region.—We constructed Ecological Niche Models (ENMs) of A. guamuhaya with the maximum entropy algorithm Maxent (v.3.3.3; Phillips *et al.* 2006). Maxent examines the relationship between known occurrence records of the species and the environmental characteristics of that area, and then extrapolates from the areas where similar conditions occur in the study region (Phillips and Dudik 2008). We defined the study region for model calibration using a convex hull that enclosed the occurrence records, and then buffered this at a distance of 50 km (Figure 2). We considered this extent appropriate as background selection because it does not include large regions that the species does not inhabit



Figure 2. Geographical extent (white dots), convex hull (blue polygon) and background sampling (red area) considered in this study.

owing to dispersal limitations and/or biotic interactions, such as competition with similar species (Barve *et al.* 2011, Anderson and Raza 2010). We extracted climate information using ArcGIS v. 10.3 (ESRI, Redlands) and ran the model by randomly sampling 5000 background points within this extent.

Environmental variables and climatic scenarios.--As current and future predictor variables, we used 19 bioclimatic variables with a resolution of ca. 1 km<sup>2</sup> (0.74 km<sup>2</sup> for Cuba) downloaded from the WorldClim ver. 1.4 database (http://www.worldclim.com) (Hijmans et al. 2005). We first ran a model in MaxEnt ver. 3.3.3k (Phillips et al. 2006), including all variables, to identify those with the highest significance to the model and selected the bioclimatic predictors from the jackknife test of variable importance. We selected six variables with low collinearity (Pearson's r < 0.8) that could be ecologically important to reptiles, as follow: mean diurnal temperature range (Bio 2); temperature seasonality (Bio 4); maximum temperature of warmest month (Bio 5); precipitation of wettest month (Bio 13); seasonality of precipitation (Bio 15); and precipitation of driest quarter (Bio 17). The use of more predictors could generate overfitting and could reduce temporal transfer (Peterson et al. 2011, Anderson 2013, Radosavlievic and Anderson 2014).

Our prediction is based on bioclimatic envelope modeling, which changes with coupled 10 general circulation models (GCMs): BCC-CSM1-1, CCSM4, CNRM-CM5, HadGEM2-ES, MIROC5, MPI-ESM-LR, MRI-CGCM3, GISS-E2-R, NorESM1-M, IPSL-CM5A. Different GCMs and greenhouse gas scenarios will lead to various changes in distributions of species in the future. The Intergovernmental Panel on Climate Change (IPCC 2014) in its Fifth Assessment Report (AR5) proposes four Representative Concentration Pathways (RCPs). RCPs may be better than the emission scenarios developed in the Special Report on Emissions Scenarios (SRES); hence, RCPs have replaced SRES standards (Moss *et al.* 2010). The two pathways (RCP 2.6 and RCP 8.5) represent two possible radiative forcing values (+ 2.6 and + 8.5 W/m<sup>2</sup>, respectively) (Moss *et al.* 2010). We used data from 1950–2000 as baseline climate data. Ten GCMs were used for the years 2050s and 2070s. For each GCM, we used the two RCPs to evaluate different greenhouse gas scenarios. Hence, the total number of climate scenarios considered was 40 (10 GCMs, two scenarios and two-time steps).

Ecological niche modeling.-We made a correlative niche model relating environmental conditions to 15 presence records using MaxEnt software (Phillips et al. 2006). We optimized model complexity and predictive power using the R package ENMeval (Muscarella et al. 2014) to select the optimal combination of the regularization multiplier and the combination of feature classes. We tested regularization multiplier values from 0.5-6.0 with intervals of 0.5, and evaluated five settings of feature classes, as follow: linear (L); linear and quadratic (Q); hinge (H); LQH; and LQH plus product (P). The performance of model was evaluated using Akaike Information Criterion corrected for small sample sizes (AICc) (Warren and Seifert 2011). The final model was run in Maxent using 50 bootstrapping replicates and the combination of regularization multiplier and feature classes with lowest AICc using all occurrence records. Additionally, we examined the omission rate and tested AUC to assure that the model selected as optimal performed well.

We used the "minimum training presence" threshold value to discriminate suitable from nonsuitable habitat (Liu *et al.* 2005, Radosavljevic and Anderson 2014). We used the logistic output of MaxEnt (continuous model), and applied thresholds in ArcGIS to convert it into a presenceabsence model. Then we clipped the binary models by the shape files of natural forests (Estrada *et al.* 2011) and protected areas (CNAP 2013) to examine the extent of the ecological niche covered by both categories. The ecological niche model generated for *A. guamuhaya* was projected onto two future climate change scenarios and they were added for each RCPs using the median value for all projections—i.e., we used the consensus of only those areas where five or more GCM predict the occurrence of environmentally suitable areas in the future. We subtracted the future and current niche models from each other, and areas of contraction, expansion and stability were calculated.

### Reassessment of Conservation Status

Based on the information available for A. guamuhava, we consider that the most accurate evaluation of its conservation status must follow the IUCN Criterion B-viz., area of occupancy (AOO) and extent of occurrence (EOO) (IUCN 2001, 2012, IUCN Standards and Petitions Subcommittee 2014). We calculated the AOO and the EOO using ArcGIS v. 10.3 (ESRI, Redlands). For the AOO we used the standardized reference scale grid of 4 km<sup>2</sup> cells proposed by the IUCN (IUCN 2001, 2012, IUCN Standards and Petitions Subcommittee 2014). The EOO was estimated with two approaches: (1) the convex hull (e.g., IUCN Standards and Petitions Subcommittee 2014) and (2) the environmentally suitable area resulting from ecological niche modeling clipped by the layer of natural forests. Both approaches are biased toward overestimation. Thus, the convex hull can include several areas of unsuitable habitat (IUCN 2001, 2012, IUCN Standards and Petitions Subcommittee 2014) and ecological niche modeling is unable to detect environmentally suitable areas never occupied by the species (historical biogeography, limited dispersal ability) and the exact areas occupied by ecologically similar species (interference competition). However, ecological niche modeling seems a more realistic measure of habitat suitability; thus, it was used here as the final estimation of EOO. There is also a bias toward underestimation because of the highly irregular topography of the montane region that contains a larger surface area than that of a flat landscape.

Twig-giant anoles are arboreal; therefore, it is reasonable to expect the species to have a strong dependence on forested areas, where canopy connectivity may allow them to disperse. There are no estimates of the average dispersal distance in A. guamuhaya or any other member of the Chamaeleolis clade; nevertheless, using the factors mentioned above, we could qualify the species (and probably most species within this group) as having poor dispersal ability (reviewed in IUCN Standards and Petitions Subcommittee 2014). Therefore, we considered natural forest coverage to be an important variable in estimating habitat decline and EOO. Cuba has a long history of forest depletion resulting from the extensive development of the sugar industry, agriculture, and stockbreeding, which resulted in a reduction of forest coverage of more than 80% between the 16th century and the mid-20th century (Del Risco 1995, Gutiérrez-Domech and Rivero-Glean 1997). And there are few signs of recovery now (ONEI 2015). We would expect that such an extreme reduction in forest coverage has had long-lasting negative effects on populations of native, forest-dwelling species such as A. guamuhaya. The Guamuhaya Massif is a mountain range where native ecosystems have persisted. However, the primary vegetation has suffered serious impacts from forest fires and intensive felling for coffee plantations and stockbreeding (e.g., Domínguez-González and Acosta-Rodríguez 2012, Mancina et al. 2017). Consequently, we consider that the IUCN Criterion Bb(iii), which refers to continuous decline in the effective area extent and/or quality of habitat, is fully applicable to A. guamuhaya.

### Results

### Geographic Distribution and Natural History

With the eight new localities reported here, the current distribution of *A. guamuhaya* includes nearly the entire Trinidad Range and encompasses

the three central provinces—Cienfuegos, Villa Clara, and Sancti Spíritus (Figure 1; Appendix I). The three new records from Villa Clara are the first for this province. Two of the new localities are the first lowland records for *A. guamuhaya*, which was found in isolated patches of subcoastal semideciduous forest, about 20 km west of the nearest previous mountain record at Pico San Juan. The altitudinal distribution is 15–1015 m a.s.l.

The lowland and montane *A. guamuhaya* have the same scale pattern described for the holotype. In life, the dorsum of the montane lizards is greenish, whereas that of individuals from the lowlands grayish. The dewlap of montane lizards has more black areas than that of the lowland lizards (Figure 3); however, dewlap coloration can vary. These differences

may indicate local adaptations (dry subcoastal vegetation *vs.* mountain rainforest and evergreen forest) and/or genetic drift. The isolated lowland records apparently represent outlying occurrences of the species; these might reflect a reduction in suitable habitat (primary forest) in the transition zone between subcoastal and mountain areas (Ecological Niche Modeling below) and subsequent isolation of the lizards as metapopulations.

We found *Anolis guamuhaya* mostly associated with primary forest (Figure 4). However, many immature individuals were associated with ecotonal or secondary shrubby vegetation, but these areas always are in direct contact with primary forest (Table 1). The specimens deposited in the collection of the NMP have no ecological data.

Table 1. Summary of ecological data of specimens of Anolis guamuhaya examined in the field. Measurements are presented in centimeters. Juveniles (J) refer to small individuals less than 100 mm SVL and adults (A) refer to individuals larger than 100 mm SVL. Abbreviations: Veg. = vegetation type; SDF = semideciduous forest; EGF = evergreen forest; MRF = mountain rainforest; ECO = ecotonal vegetation; SEC = secondary vegetation; Ø = perch diameter; SB = shrub branch; ST = shrub trunk; TT = tree trunk; TB = tree branch; VI = vine.

<b>C</b>			Perch		h		
Specimen	Age	veg.	Туре	Ø	Height	Observations	
Cienfuegos							
MFP 12515	J	SEC	SB	1.1	305	Basking, body temperature 28°C, air temperature 27.9°C	
No voucher	J	SEC	SB	1.6	240	Pico San Juan, body temperature 28°C, air temperature 27.9°C	
CZACC 4.5871	А	MRF	ТВ	1.5	400	Sleeping at night in horizontal position.	
MFP 12.633	А	EGF	SB	1.3	50	Swallowing a snail (Zachrysia sp.)	
No voucher	А	EGF	ST	2.5	100	Carso de Buenos Aires	
CZACC 4.5846	J	ECO	ST	2.2	110	On vertical stem, by a trail by day	
MFP 12.629	А	SDF	TT	27	200	On smooth palm tree trunk, head down	
No voucher	J	ECO	VI	0.3	120	Santa Martina, on oblique twig, head up	
MFP 12.603	А	SDF	ТВ	1.8	320	Sleeping at night in vertical position, head up	
CZACC 4.5850	J	ECO	SB	0.5	50	Sleeping at night in horizontal position	
CZACC 4.5851	J	ECO	VI	0.4	200	Sleeping at night in horizontal position	
Sancti Spíritus							
No voucher	А	EGF	VI	2	200	Resurgence of the Jibacoa river, on a vertical vine near a cave entrance	



Figure 3. Selection of specimens of Anolis guamuhaya examined for this study: (A) male CZACC 4.5848 from Santa Martina; (B) female CZACC 4.5849 from Boca Ambuila; (C) female CZACC 4.5845 from Carso de Buenos Aires, notice the camouflage; and (D) male from Loma Cariblanca, kept alive at the Parque Zoológico Nacional, La Habana. Photographs by RM (A, B, D) and TMRC (C).

### Ecological Niche Modeling

The ecological niche models for *A. guamuhaya* have mean values of AUC<sub>training</sub> = 0.938  $\pm$  0.006, AUC<sub>test</sub> = 0.940  $\pm$  0.005, mean AUC<sub>diff</sub> = 0.025  $\pm$  0.0006 SD and mean of minimum training presence omission rate = 0.227  $\pm$  0.103 SD (Appendix II). These results indicate good model performance and low overfitting. The lowest values of AICc ( $\Delta$ AICc = 0) resulted from regularization multiplier of 1 with linear and quadratic feature classes. The three most important bioclimatic variables are (1) the maximum temperature of the warmest month;

(2) mean diurnal temperature range; and (3) temperature seasonality (Appendix III). The current climatically suitable area encompasses more than 1398 km<sup>2</sup> (Figure 5A), but only 46% (648 km<sup>2</sup>) is covered by natural forests (Figure 5B).

Under all climate change scenarios, the models predict a reduction of the suitable area; however, the amount of reduction is variable (Figure 6). The models suggest a reduction of suitable areas between 43–67% in 2050 (Figure 6A, B) and between 44–94% in 2070 (Figure 6C, D). The largest estimated percent loss in suitable areas occurs under RCP 8.5 scenario for 2070 (Figure



Figure 4. Different habitats of Anolis guamuhaya: (A) subcoastal semideciduous forest on limestone at Boca Ambuila;
(B) trail crossing subcoastal semideciduous forest at Santa Martina; (C) evergreen forest on limestone at Carso de Buenos Aires; and (D) mountain rainforest at Pico San Juan. Photographs by TMRC (A-C) and RM (D).

6D) in which only 77 km<sup>2</sup> (6% of the current suitable area) will remain with suitable climatic conditions for the occurrence of *A. guamuhaya*. However, all these models are clearly overestimated, because they do not include the natural forest coverage, which is expected to be drastically reduced as a collateral effect of climate change.

### **Conservation Status**

We assigned a grid size of  $4 \text{ km}^2$  per locality and summarized the 15 records from the literature and this study to estimate the range (AOO) of *A*. guamuhaya at 60 km<sup>2</sup>. The convex hull revealed an EOO of 622 km<sup>2</sup>, but the climatically suitable area clipped by the layer of natural forests revealed an EOO of 648 km<sup>2</sup>, considered herein as the most realistic approach to the EOO (Figure 5B). We consider that the loss of forest coverage during the last centuries is not only a measure of "continuing decline in area, extend and/or quality of habitat," but also a measure of the extreme decrease in the EOO. Moreover, only 21% (138 km<sup>2</sup>) of the climatically suitable area covered by natural forests (= EOO) and only seven of the fifteen localities (47%) of A. guamuhaya fall within protected areas (Figure 5B).



Figure 5. Ecological niche model for Anolis guamuhaya based on presence records (white dots) from the literature and this study. (A) Binary representation of the current environmentally suitable area (red shading) based on the "minimum training presence" threshold (consensus of the scenarios where five or more GCM coincide). (B) Current distribution of environmentally suitable areas clipped by natural forests (green shadings, assumed herein as the EOO) and then by protected areas (blue contours).



**Figure 6.** Ecological niche model percent reduction of environmentally suitable areas for *Anolis guamuhaya* projected under two future climate change scenarios (RCP: 2.6 W/m<sup>2</sup> and 8.5 W/m<sup>2</sup>) for the years 2050 and 2070. The blue shading represents the sum of those areas where five or more GCMs predict environmentally suitable conditions. The red shading represents the current environmentally suitable areas, illustrating retraction under future scenarios; no expansion was observed under any scenario.

### Discussion

Most authors have reported *A. guamuhaya* to occur above 300 m a.s.l. (Garrido *et al.* 1991, Rodríguez 2012, Rodríguez *et al.* 2010, 2013); Henderson and Powell (2009) reported an elevational distribution for this species from sea level to 900 m, although no voucher specimens from lowland localities are mentioned. The specimens reported here from the southern coast of Cienfuegos Province are the first vouchered records of this species in lowland ecosystems.

The greater number of voucher specimens from a cluster of localities in the vicinity of Topes de Collantes does not necessarily imply higher population density. It is more likely that it reflects a greater sampling effort because this area attracts many tourists, as well as researchers.

As observed here and reported in the literature, *A. guamuhaya* is both a mountain- and lowland-dwelling species, ranging from close to the sea level to above 1000 m. However, environmentally suitable areas and primary forest coverage in south-central Cuba are scarce and highly fragmented apart from the Guamuhaya

Massif (Figures 5 and 7). Montane ecosystems are the last natural refuge for this species under the worst-case scenarios of climate change in the future (Figure 6).

Climate change and the modification and fragmentation of natural forests seem to challenge the survival of *A. guamuhaya*. Changes in range size usually are assessed by considering the climatic characteristics of current distributions and the projected distribution of these climatic conditions in the future. However, the vulnerability of this lizard may be exacerbated by other factors. These anoles seem to have a limited dispersal ability; further, they are affected by the loss and fragmentation of forest patches within climatically suitable areas, because forest coverage/canopy connectivity may affect the conditions (microclimate) and resources (habitat structure) required by the species.

The model identified environmentally suitable areas for A. guamuhaya only in the Guamuhaya Massif and its nearby surroundings, including in the Sancti Spíritus Range, about 30 km east of the nearest records of the species (Figure 5). However, despite extensive surveys through the years, the only twig-giant anole that has been found so far in the latter area is A. chamaeleonides Duméril and Bibron, 1837, which also occurs in the southeastern portion of the Trinidad Range, close to A. guamuhaya (Figure 7; Garrido et al. 1991, Estrada 1994, Rodríguez et al. 2013, authors pers. obs.). All species in the Chamaeleolis clade apparently have similar ecological requirements, and sympatry rarely has been reported (e.g., Rodríguez



**Figure 7.** Records of *Anolis guamuhaya* (red dots) and *A. chamaeleonides* (orange squares) in south-central Cuba (sensu Rodríguez *et al.* 2013 and this study), superimposed to the layer of current natural forests (green shading) and protected areas (striped zones). Question mark represents the juvenile male CZACC 4.5846 from Santa Martina, tentatively assigned to *A. chamaeleonides*.

*et al.* 2013), which might be indicative of competitive exclusion (Morin 2011). Higher resource requirements in giant anoles may indicate a stronger interference competition among ecologically similar species. For example, *A. guamuhaya* and *A. chamaeleonides* apparently occur sympatrically at Topes de Collantes (Rodríguez *et al.* 2013) but the overlap in distribution seems to be minimal (Figure 7). Because no ecological studies exist on interspecific interactions between these species, this scenario provides a unique opportunity to study resource partitioning between twig-giant anoles.

We found one juvenile specimen at Santa Martina (CZACC 4.5846) that we tentatively assigned to A. chamaeleonides (because it had a scale row between the supralabials and infraoculars; Garrido et al. 1991), but its small size made it difficult to identify unambiguously (Figure 8; Appendix I). Thus, a second probable point of contact between A. guamuhaya and A. chamaeleonides might be Santa Martina. This seems plausible because the latter species is known to occur in similar ecological conditions about 20 km northwestward, near Juraguá, on the western side of Cienfuegos Bay (Figure 8; Garrido 1980, Rodríguez et al. 2013). Anolis *chamaeleonides* is widespread in Cuba (Rodríguez et al. 2013), from sea level to above 1000 m (Rodríguez et al. 2010), which suggests that it is more generalist than A. guamuhaya. Therefore, its absence throughout most of the range of A. guamuhaya in the Trinidad Range supports the hypothesis of strong competition between both species.

This is an example of the importance of ground-truthing to assess the incidence of biotic interactions (e.g., competition, predators, food availability) and abiotic factors (e.g., geographic and ecological barriers) in species distributions when using ecological niche modeling (Soberón and Nakamura 2009). The climatically suitable areas generated by the models do not necessarily represent the full distribution of the species. Given the apparently low dispersal ability of *A. guamuhaya* and strong barrier imposed by the



**Figure 8.** Juvenile male CZACC 4.5846 (52.2 mm SVL) from Santa Martina, tentatively assigned to *Anolis chamaeleonides*. The inset above shows details of the scale row between supralabials and infraoculars (arrow, also the scale row in was outlined to increase legibility), which is a diagnostic character for this species. The inset below shows details of the head of a juvenile male *A. guamuhaya* of comparable size (57 mm SVL) from the southwestern slope of Pico San Juan, notice the supralabials in contact with the infraoculars. Photographs by TMRC.

Agabama Basin, it is possible that this species never dispersed into the Sancti Spíritus Range, despite the occurrence of climatically suitable habitats in these mountains. Another possibility is that *A. chamaeleonides* has been progressively displacing *A. guamuhaya* from east to west. The severe deforestation of the Sancti Spíritus Range in the past (e.g., Domínguez-González and Acosta-Rodríguez 2012, Mancina *et al.* 2017) translates to strong habitat modifications that might have produced the retreat of *A.* guamuhaya and favored the advance of *A.* chamaeleonides. This hypothesis could be tested by using molecular markers to assess if the populations of *A.* chamaelonides found in the Sancti Spiritus range came from nearby eastern populations.

Another factor that may affect *A. guamuhaya* and potentially limit its distribution is competition with ecologically similar species such as *A. chamaeleonides*. The latter species seems more adaptable; thus, under future scenarios of climate change and overall loss/modification of suitable habitats it could displace *A. guamuhaya*. Although both forest coverage and interference competition have the potential to affect the distribution of *A. guamuhaya* significantly, they cannot be modelled predictably with current tools.

If we assume that the climatically suitable areas generated by the ecological niche model are a measure of the species range, then the EOO, the number of locations, and its AOO decrease under all future scenarios of climate change. Given these changes, the status of A. guamuhaya would fulfill the IUCN criteria in the category of Endangered (B1ab[i,ii,iii,iv], c[i]; B2ab[i,ii,iii,iv], c[i]). Nonetheless, this estimated EOO of A. guamuhaya is just a first approach to the reality because of the difficulty in determining the exact area occupied by a species on a highly irregular topography such as that of the Guamuhaya Massif. Moreover, part of this EOO is occupied by a closely related and ecologically similar species (A. chamaeleonides) that apparently can replace A. guamuhaya and further reduce its effective EOO (Figure 7; see below). However, the exact area occupied by A. chamaeleonides and the degree to which it reduces the effective EOO of A. guamuhaya is difficult to estimate.

Anolis guamuhaya remains in the Endangered category, as previously evaluated by other authors following the IUCN Criterion D (Rodríguez 1999, 2012). However, we consider

that IUCN Criterion B is more plausible for twig-giant anoles because it allows a more realistic assessment. The ecological niche models that we obtained for A. guamuhaya predict that under future scenarios of climate change, the environmentally suitable area for this species reaches a critical value under scenario RCP 8.5 W/m<sup>2</sup> for 2070, in which only 77 km<sup>2</sup> of the Guamuhaya Mountains would remain compatible with the species requirements. Moreover, it is reasonable to associate this reduction in climatically suitable habitat with a severe decline in natural forest coverage. For this reason, we consider that A. guamuhaya may fulfill the IUCN criteria for the Critically Endangered category in 2070. This situation would be exacerbated if, in fact, the species currently does not occupy all its potential distribution because of other factors apart from climatic suitability, such as forest coverage reduction and interference competition.

The fact that we found several specimens of A. guamuhaya in secondary vegetation suggests that the species can survive some degree of habitat deterioration. However, all the places where we found the species in secondary vegetation were ecotonal areas at the edge of larger patches of primary vegetation (semideciduous forest or mountain rainforest) having different degrees of canopy connectivity. These individuals inhabiting ecotonal areas may be incipient dispersers, with their presence not necessarily evidencing ecological success in deteriorated habitats. Indeed, all individuals we found in ecotonal secondary vegetation were juveniles; these lizards may be density-dependent emigrants that are dispersing in response to inbreeding and/or kin competition (Begon et al. 2006). In fact, we never found adult A. guamuhaya in isolated patches of secondary vegetation; this would seem to suggest that the species is unable to survive after a relatively low level of habitat deterioration.

More extensive, long-term studies on the demographic parameters and ecological requirements of *A. guamuhaya* are needed to assess its conservation status accurately. This is

particularly important because more than a half (53%) of all records of the species are outside the network of protected areas in Cuba. This consideration should be a matter of utmost concern when environmental authorities formulate action plans and conservation strategies.

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Appendix I. Material examined and update of localities with coordinates and elevations reported for Anolis guamuhaya and A. cf. chamaeleonides used in this study, from the literature and this paper

(N = 32 records, 15 locations). An asterisk (\*) represents voucher declared in this paper.

Acronyms and abbreviations declared in Materials and Methods.

### Anolis guamuhaya

**Type locality:** the road between Jibacoa [22.01699, -79.98912; 330 m a.s.l.], Villa Clara province, and Topes de Collantes [21.9186, -80.02060; 700 m a.s.l.], Sancti Spíritus province, including elevations of 800 m [female MNHNCu–500, holotype] (Garrido *et al.* 1991).

CIENFUEGOS province: CUMANAYAGUA municipality: -- Mina Carlota (22.07171, -80.17762; 300-600 m a.s.l.) (male MCZ R-53598, dry skeleton) (Wilson 1957, Garrido and Schwartz 1968, Garrido et al. 1991); --- southwestern slope of Pico San Juan (21.98990, -80.14939; 1,015 m a.s.l.) [juvenile male MFP 12515 (Torres et al. 2015); juvenile male, no voucher (57 mm SVL), 8.x.2013; female CZACC 4.5871\* (162 mm SVL, 121 mm TL), 25.viii.2014], collected/examined by T. M. Rodríguez-Cabrera, R. Marrero, J. Torres, P. Gutiérrez-Macías, and A. M. Rodríguez; - ca. 6 km SW of Aguacate (21.88775, -80.12651; 350 m a.s.l.) [female CZACC 4.1549; formerly IZ-5397 (Garrido 1982) and CZACC:7499 (Garrido et al. 1991)]; - Carso de Buenos Aires (21.98872, -80.18981; 760 m a.s.l.) [female MFP 12.633\* (146 mm SVL, 123 mm TL, 39 g), 19.vii.2014; adult, no voucher, 21.ii.2015], collected/examined by T. M. Rodríguez-Cabrera and J. León; - near the Trinitario stream waterfall (22.00705, -80.04250; 550 m a.s.l.) (adult, no voucher), examined by E. Morell; — Santa Martina (22.01833, -80.38369; 40 m a.s.l.) [male MFP 12.629\* (165 mm SVL, 203 mm TL, 87.2 g), 27.viii.2014; juvenile male, no voucher (49 mm SVL, 48 mm TL)], collected/examined by T. M. Rodríguez-Cabrera, J. Torres, P. Gutiérrez-Macías, A. M. Rodríguez, and L. Fernández; - Boca Ambuila, eastward of the Gavilanes river mouth (21.96478, -80.33822; 15 m a.s.l.) [female MFP 12.603\* (175 mm SVL, 167 mm TL, 80 g); juvenile female CZACC 4.5850\* (64 mm SVL, 57 mm TL, 4.5 g); juvenile female CZACC 4.5851\* (59 mm SVL, 41 mm TL, 4 g)], 24.ix.2014, collected by T. M. Rodríguez-Cabrera, R. López-Silvero, and R. Domínguez. VILLA CLARA province: MANICARAGUA municipality: — near the Río Negro Touristic Center (22.04567, -80.05894; 400 m a.s.l.) (adult, no voucher), examined by E. Morell; - southeastern slope of Loma Cariblanca (22.06025, -80.01181; 400 m a.s.l.) [male (140 mm SVL, 130 mm TL, 47.5 g), currently kept alive at the Parque Zoológico Nacional, La Habana, Cuba], collected by E. Morell. SANCTI SPÍRITUS province: TRINIDAD municipality: — Topes de Collantes (21.91194, -80.02000; 700 m a.s.l.) and vicinity, within a radius of 1 km (650-800 m a.s.l.) (USNM-156786, male NMP6V 34517, female NMP6V 71870/1, male NMP6V 71870/2, male NMP6V 71870/3, female NMP6V 71870/4, male NMP6V 71870/5, male NMP6V 71870/6, male NMP6V 71870/7, female CZACC 4.5506) (Garrido and Schwartz 1968, Garrido 1982, Rodríguez et al. 2013, Holáňová et al. 2017) — La Chispa (21.92246, -80.03304; 800 m a.s.l.) (no voucher) (Garrido et al. 1991); — Caburní waterfall (21.92076, -80.00363; 400 m a.s.l.) (no voucher) (Rodríguez et al. 2013); - ca. 2 km southeast of Topes de Collantes, near Vegas Grandes village (21.90250, -80.00500; 650 m a.s.l.) (male NMP6V 71870/8\*, male NMP6V 71871\*); — near the resurgence of the Jibacoa river (21.97158, -79.93372; 200 m a.s.l.) (adult, no voucher), examined by T. M. Rodríguez-Cabrera.

### Anolis cf. chamaeleonides.

CIENFUEGOS province: CUMANAYAGUA municipality: Santa Martina (22.01833, -80.38369; 40 m a.s.l.) [juvenile male CZACC 4.5846\* (52.2 mm SVL, 50 mm TL), 22.ii.2014], collected/examined by T. M. Rodríguez-Cabrera, J. Torres, P. Gutiérrez-Macías, A. M. Rodríguez, and L. Fernández.

the AICc score of best model; models with $\Delta AIC < 2$ could be considered to have substantial support.							
FC	RM	AUC	$AUC_{test} \pm SD$	AUC	OR MTP	AICc	ΔΑΙϹ
LQ	1	0.938	$0.940 \pm 0.005$	0.025	$0.227 \pm 0.103$	242.49	0
LQ	1.5	0.938	$0.934 \pm 0.009$	0.030	$0.227 \pm 0.103$	242.92	0.42
L	1	0.937	$0.940 \pm 0.005$	0.024	$0.273 \pm 0.149$	243.50	1.00
LQ	2	0.938	$0.924 \pm 0.001$	0.038	$0.227 \pm 0.103$	243.51	1.01
LQ	2.5	0.938	$0.919 \pm 0.002$	0.042	$0.227 \pm 0.103$	244.25	1.75
L	1.5	0.937	$0.935 \pm 0.0007$	0.028	$0.227 \pm 0.103$	244.30	1.80
LQHP	4.5	0.931	$0.939 \pm 0.02$	0.021	$0.227 \pm 0.103$	244.43	1.94

**Appendix II.** Settings for the best ecological niche models for Anolis guamuhaya based on lowest Akaike's information criterion (AICc).  $\Delta$ AIC represents the difference between the AICc score for each model and the AICc score of best model; models with  $\Delta$ AIC < 2 could be considered to have substantial support.

**Appendix III.** Estimates of relative contributions and permutation importance of the six bioclimatic variables selected to build the ecological niche model for Anolis guamuhaya.

Variables	Percent contribution	Permutation importance
Max. temperature of warmest month (bio 5)	55	0
Mean diurnal range (bio 2)	18.7	39.1
Temperature seasonality (bio 4)	17.4	18.2
Precipitation seasonality (bio 15)	7.3	41.9
Precipitation of driest quarter (bio 17)	1.5	0
Precipitation of the wettest month (bio 13)	0.1	0.9
# A comparative acoustic analysis of species of *Vitreorana* (Anura: Centrolenidae) from the Brazilian Atlantic Forest and Cerrado, with a description of the call of *V. baliomma* and insights into the taxonomic status of Cerrado populations

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# Abstract

A comparative acoustic analysis of species of *Vitreorana* (Anura: Centrolenidae) from the Brazilian Atlantic Forest and Cerrado, with a description of the call of *V. baliomma* and insights into the taxonomic status of Cerrado populations. Advertisement calls are the primary signaling strategy of mating in most anurans. Examining major patterns within and across frog taxa may help to delimit species and understand acoustic communication systems. Herein, the vocalization of *Vitreorana baliomma* is described and new acoustic descriptions are provided for *V. franciscana* and *V. eurygnatha*. Calls of syntopic/sympatric or allopatric populations were compared. The vocalization of *V. baliomma* is composed of two call types—A and B, with Call A being the advertisement call. The vocalization of *V. franciscana* is composed of only one call type (Call A), emitted isolated or in series. The vocalization of *V. eurygnatha* has two types of calls; Call A is similar among Cerrado populations, whereas an Atlantic Forest population has a distinct Call A pattern. The calls of each of these species of the present study is pulsed; however,

Received 03 September 2019 Accepted 20 February 2020 Distributed June 2020 the calls differ in call duration, pulse organization, or pulse duration, and these differences seem to reflect the allopatric or sympatric/syntopic occurrences of populations. The unique call pattern of the Atlantic Forest population of *V. eurygnatha* may represent the advertisement call of the nominal species, and the distinct call of the Cerrado population may indicate cryptic diversity in the species.

**Keywords:** Advertisement call, character displacement, sympatry, syntopy, vocal repertoire, *Vitreorana baliomma, Vitreorana eurygnatha, Vitreorana franciscana.* 

#### Resumo

Uma análise acústica comparativa de espécies de Vitreorana (Anura: Centrolenidae) da Mata Atlântica e do Cerrado brasileiros, com a descrição do canto de V. baliomma e considerações sobre o status taxonômico de populações do Cerrado. Cantos de anúncio representam a forma primária de sinalização para reprodução na maioria dos anuros. Avaliar os principais padrões dentro e entre táxons de anuros pode auxiliar na delimitação de espécies e no entendimento de sistemas de comunicação acústica. Nesse trabalho, a vocalização de Vitreorana baliomma é descrita e novas descrições acústicas são fornecidas para V. franciscana e V. eurygnatha. Cantos entre populações em sintopia/simpatria ou alopatria foram comparados. A vocalização de V. baliomma é composta de dois tipos de cantos—A e B, e o Canto A considerado como o canto de anúncio. A vocalização de V. franciscana é composta por um único tipo de canto (Canto A), que é emitido isoladamente ou em séries. A vocalização de V. eurygnatha possui dois tipos de cantos; o Canto A é similar entre populações do Cerrado, enquanto que uma população da Mata Atlântica possui o Canto A com um padrão distinto. Os cantos de cada espécies do presente trabalho são pulsados; no entanto, os cantos diferem em duração do canto, organização dos pulsos, ou duração dos mesmos, e essas diferenças parecem refletir a condição de alopatria ou simpatria/sintopia entre populações. O padrão único observado para a população da Mata Atlântica de V. eurygnatha provavelmente representa o canto de anúncio da espécie nominal, e o canto distinto da população do Cerrado pode indicar diversidade críptica na espécie.

**Palavras-chave:** canto de anúncio, deslocamento de caráter, repertório vocal, simpatria, sintopia, *Vitreorana baliomma, Vitreorana eurygnatha, Vitreorana franciscana.* 

#### Introduction

Advertisement calls are the primary form of communication in most anuran species. These calls are often conspicuous, stereotyped signals produced by reproductively active male frogs (Wells 2007). Female frogs react to specific call traits of conspecific males. Thus, advertisement calls can act as reproductive barriers between species (Blair 1958, 1964), and can have immediate effect on acoustic signal divergence (Boul *et al.* 2007). Vocalization traits can be used for species delimitations (Köhler *et al.* 2017), particularly when interspecific call differences emerge among frogs sharing

overlapping vocalization sites (i.e., syntopic or sympatric occurrence; Höbel and Gerhardt 2003, Hoskin *et al.* 2005, Jansen *et al.* 2016), or among morphologically cryptic species that have different acoustic traits (Angulo and Reichle 2008, Carvalho *et al.* 2019). Therefore, speciation patterns can be informed by acoustic descriptions that may reveal processes underlying the evolution of mating signals.

Although patterns of advertisement call evolution in centrolenid frogs have been evaluated at a macroevolutionary scale (Escalona *et al.* 2019), calls of some species of *Vitreorana* (Guayasamin *et al.* 2009) have not been described—viz., *V. baliomma* Pontes, Caramaschi, and Pombal, 2014 and *V. parvula* (Boulenger, 1895). The calls of about 80% of centrolenids are either unknown or lack detailed descriptions (Dautel *et al.* 2011). The lack of acoustic data impedes our understanding of the systematics of Glass Frogs such as *Vitreorana* (Castroviejo-Fisher *et al.* 2011), and hinders assessments of intraspecfic variation.

We predict that Brazilian species of Vitreorana have different advertisement calls because vocalizations usually are species specific in anurans, especially in taxa that co-occur (syntopic species) in the Atlantic Forest and Cerrado. Further, we expect that different populations of a same species will share a basic acoustic pattern, apart from variations related to environmental factors (e.g., temperature) and morphological constraints (e.g., body size). Herein, we describe the advertisement call of V. baliomma, and provide new call descriptions for V. eurygnatha (Lutz, 1925) and V. franciscana Santana, Barros, Pontes, and Feio, 2015 from other regions/biomes. Our analysis enabled us to compare major acoustic patterns combined with morphological traits between species which show allopatric and sympatric / syntopic occurrences.

# **Materials and Methods**

# Sampling

The following species of Vitreorana were sampled from Brazilian localities, as follow. Vitreorana baliomma: Estação Ecológica de Murici (09°12'32" S, 35°51'37" W, 463 m a.s.l.; datum WGS84 in all cases) in Murici, state of Alagoas (AL). Vitreorana eurygnatha and V. franciscana, which occur syntopically at the following localities: Parque Nacional da Serra da Canastra (20°18'05" S, 46°31'19" W, 850 m a.s.l.) in Vargem Bonita, state of Minas Gerais (MG); and Córrego Borrachudo (19°19'40" S, 46°00'1" W, 1123 m a.s.l.) in São Gotardo (MG). eurygnatha: Reserva Biológica Vitreorana Augusto Ruschi (19°53'49" S, 40°32'30" W, 838

Recordings and specimens of Vitreorana franciscana from Vargem Bonita were the same analyzed by Santana et al. (2015). Acoustic data for V. uranoscopa (Müller, 1924) were obtained from Haga et al. (2014) and Zaracho (2014). Appendix I contains detailed information on sound recordings, including the voucher specimens. Calls were recorded in the field with digital recorders (M-audio Microtrack® II and Marantz® PMD 660) and unidirectional microphones (Sennheiser® K6/ME67 and K6/ ME66). The recorders were adjusted to sampling rates of 44.1 kHz or 48.0 kHz and bit depth of 16 points. Files were stored as uncompressed wave files. Sound files and specimens were deposited in the following Brazilian collections: Collection of Amphibians of the Universidade Federal de Uberlândia (AAG-UFU), Uberlândia, Minas Gerais state; Museu de Zoologia João Moojen (MZUFV) at Universidade Federal de Viçosa, Viçosa, Minas Gerais state; and Herpetological Collection of the Museu de História Natural (MUFAL) at Universidade Federal de Alagoas. Maceió, Alagoas state.

# Species Identification

Specimens from Murici (N = 8 males) were identified as Vitreorana baliomma based on features from the original description (Pontes et al. 2014): snout-vent length (SVL) = 17.8-21.4 mm (mean = 19.4, SD = 1.1); vomerine teeth absent; snout truncate in dorsal view and rounded in lateral view; tympanum non-pigmented with outline barely visible and supratympanic fold poorly developed around the upper portion of the tympanum; dorsal skin texture smooth; poorly defined nuptial pad; Finger I longer than Finger II; in life, green dorsum with minute white punctuations; in preservative, dorsum cream with small and star-shaped melanophores distributed uniformly; and hands and feet yellowish with melanophores, when present, restricted to the dorsal portion of Finger IV and Toes IV and V.

Specimens of *Vitreorana eurygnatha* from Vargem Bonita (N = 1 male), São Gotardo (N = 2) and Santa Teresa (N = 2) were identified based on the following traits provided by Santana *et al.* (2015): SVL = 16.9–20.5 mm (mean = 18.5, SD = 1.4); snout truncate in dorsal and lateral views; dorsal skin texture smooth; rounded melanophores distributed uniformly on the dorsum; tympanum non-pigmented; and tubercles in the cloacal region rounded, with similar size, and poorly pigmented.

Specimens of *Vitreorana franciscana* from São Gotardo (N = 2 males) were identified on the basis of Santana *et al.* (2015): SVL = 21.3–21.5 mm (mean = 21.4, SD = 0.1); snout sub-ovoid in dorsal view; sloping in profile; dorsal skin texture shagreen; melanophores clustered on the dorsum; tympanum white; tubercles enameled, forming two prominences on each side of the cloaca; upper lip ridge of tubercles absent; ridges on the outer surfaces of forearms, shanks, and tarsi discrete; iris tan with black encircling reticulations.

# Acoustic Analysis

The following settings in Raven Pro 1.5 (Center for Conservation Bioacoustics 2014) were used for the acoustic analysis: window type = Hann, window size = 256 samples, 3dBfilter bandwidth = 270 Hz or 248 Hz, window overlap (locked) = 90%, hop size = 0.54 or 0.59ms, DFT size = 1024 samples, grid spacing = 46.9 or 43.1 Hz. Sound figures were produced using seewave 1.7.6 (Sueur et al. 2008) and tune R 1.3.2 (Ligges et al. 2018), packages implemented in R 3.6.1 (R Development Core Team 2015). using following the parameters: window type = Hann, FFT overlap = 90%, FFT size = 256 points. Spectrograms were produced with a relative amplitude color scale of 36 dB (red = maximum amplitude).

The following acoustic traits were analyzed: call duration (time from onset to offset of vocal emission), number of pulses per call (amplitude modulations within call, both complete and incomplete), pulse rate (quotient of number of pulses by each call duration), pulse duration (time comprised from onset to offset of amplitude modulations within calls), interval between pulses (duration of silent gaps between pulses), interval between calls (measured only when calls were emitted in sequence or when a different type of call was emitted shortly after main call type), and the call dominant frequency (point of greatest energy in advertisement call).

Two types of calls were recognized. Call A, the most frequently emitted signal, is the advertisement call. Call B differs from Call A in temporal structure, such as the envelope, or noticeable differences in duration, pulse number and their temporal organization. We did not use the Call B of *V. franciscana* described by Santana *et al.* (2015) because we noted that it seems to correspond to Call A of this study, although it was emitted as a calling bout with relatively lower amplitude (Results).

# Results

Calls (both A and B) of all three *Vitreorana* species analyzed are pulsed. However, they differ in temporal patterns (e.g., call duration, pulse number, and repetition rates; Table 1). Calls are emitted in different patterns as follow: in most cases, (1) a single Call A (i.e., advertisement); (2) a single Call A followed by a sequence of the same call with shorter intervals; (3) a Call B (single or in sequence) shortly after a Call A. Quantitative descriptions of each species calls are presented below.

# Vocalization of Vitreorana baliomma

The vocalization is composed of two different call types (Table 1; N = 1 male recorded from Murici, AL): Call A (N = 13 calls; Figure 1) usually followed by a Call B (N = 10 cases; N = 10 calls; Figure 1); however, Call A may also be emitted alone (N = 3 cases). Call A has complete amplitude modulations (always with 7 pulses) and increases slightly in amplitude along the

Table 1.	Acoustic features of Vitreorana baliomma, V. eurygnatha, and V. franciscana (this study), and V. uranoscopa from Haga et al. (2014) and Zaracht
	(2014). Data are presented as means, standard deviations and ranges. $N =$ number of calls analyzed. * = given as the average of all pulses in Hag.
	et al. (2014) and Zaracho (2014). Abbreviations for Brazilian states: AL = Alagoas, ES = Espírito Santo, MG = Minas Gerais, SC = Santa Catarina.

Species/Call traits	Call type	Emission pat- tern	Call dura- tion (ms)	Number of pulses	Pulse rate/s	1 <sup>st</sup> pulse duration (ms)	Last pulse duration (ms)	Interval be- tween pulses (ms)	Dominant fre- quency (Hz)	Interval be- tween calls A-B (ms)
V. baliomma	Call A $(N = 13)$	Single call	$192 \pm 4$ (185-198)	7	$36.5 \pm 1$ (35.3-37.8)	$10 \pm 2$ (8-15)	12 ± 2 (9–14)	$19 \pm 2$ (15-23)	$4,979 \pm 102$ (4,694-5,081)	235 ± 10
(Murici, AL)	Call B $(N = 10)$	Single call	$107 \pm 5$ (98-116)	$4 \pm 0.3$ (4-5)	$38.4 \pm 2.3$ (35.4-43.1)	$12 \pm 2$ (9-14)	$13 \pm 4$ (9-21)	$18 \pm 3$ (6-21)	$4,948 \pm 96$ (4,694-5,038)	(217–249)
V/ output	Call A $(N = 34)$	Single call	$168 \pm 20$ (135-226)	$3 \pm 0.4$ (3-4)	$19.7 \pm 1.9$ (15.9–24.4)	$14 \pm 3$ (7-21)	$15 \pm 4$ (8-23)	$37 \pm 8$ (7-61)	$4,796 \pm 172$ (4,453–5,016)	117 + 100
v. eurygnaura (São Gotardo, MG)	Call B $(N = 7)$	Single call or sequence of 2–3 call	$188 \pm 16$ (154-199)	$6 \pm 0.8$ (5-7)	$30.5 \pm 3.4$ (25.8–36.5)	$13 \pm 3$ (9-15)	12 ± 4 (8–20)	$25 \pm 12$ (8-42)	$4,566 \pm 97$ (4,500-4,781)	(332–560)
V. eurygnatha (Vargem Bonita, MG)	Call A $(N = 10)$	Single call	$131 \pm 5$ (123-143)	$3 \pm 0.3$ (3-4)	$23.5 \pm 1.7$ (22.4–28.0)	$10 \pm 3$ (5-15)	$12 \pm 2$ (9-15)	43 ± 7 (15-49)	$4,523 \pm 39$ (4,500-4,593)	
V. eurygnatha (Santa Teresa, ES)	Call A $(N = 6)$	Single call with 2–3 pulse clusters	$216 \pm 25$ (189–249)	18 ± 2.5 (16−22)	84.0 ± 4.5 (78.8–89.7)	$4 \pm 1$ (4-5)	10 ± 5 (4-16)		$4,830 \pm 96$ (4,694-4,995)	
V. franciscana (São Gotardo, MG)	Call A $(N = 59)$	Single call or in sequence of 3–10 calls	52 ± 10 (33–73)	$5 \pm 9.0$ (4–7)	$100.9 \pm 8.0$ (84.5-121.2)	$5 \pm 2$ (3-14)	9 ± 3 (4–15)	$4 \pm 1$ (1-7)	$4,605 \pm 91$ (4,359-4,734)	
V. franciscana (Vargem Bonita, MG)	Call A $(N = 15)$	Single call	$56 \pm 6$ (48-69)	$6 \pm 0.6$ (5-7)	$102.5 \pm 9.0$ (90.9-125.0)	$4 \pm 1$ (3-5)	$5 \pm 2$ (3-8)	$5 \pm 1$ (1-6)	$\begin{array}{c} 4,103 \pm 39 \\ (4,031-4,171) \end{array}$	
<i>V. uranoscopa</i> (Florianópolis, SC) (Haga <i>et al.</i> 2014)	Call A $(N = 46)$	Single call or in pairs	$37 \pm 0$ (20-84)	$3 \pm 0.1$ (2-4)	$77.8 \pm 4.4$ (35.7–129.0)	10 (6-	± 0* -23)		$4,893 \pm 57$ (4,651-4,996)	·
V. uranoscopa (Itapeva, MG) (Haga <i>et al.</i> 2014)	Call A ( <i>N</i> = 294)	Single call or sequence of 2–7 calls	$36 \pm 1$ (13–57)	$4 \pm 0.6$ (1-5)	$97.6 \pm 9.1$ (41.6–230.7)	10 (2-	± 2* -29)	,	$4,592 \pm 346$ (3,962-5,063)	ı
<i>V. uranoscopa</i> (San Antonio, Misiones Province, Argentina) (Zaracho 2014)	Call A ( <i>N</i> = 172)	Single call or sequence of 2–9 calls	38 ± 13 (13-85)	$3 \pm 0.4$ (1-5)	128.5 ± 11.0 (91.0–166.7)	7 ∃ (3-	± 2* -15)	·	$4,642 \pm 109$ (4,313-4,875)	ı

#### Bang et al.



Figure 1. Spectrogram and corresponding oscillogram of the vocalization of *Vitreorana baliomma* from Murici: Call A followed by a Call B. Sound file = Vitreorana\_baliomma\_Murici-Alagoas-BSLISBOA-29.IV.13\_MZ000032. See further information on recordings in Appendix I.

call. Call A duration varies from 185–198 ms. Pulses are emitted at a rate of 35.3–37.8 pulses/s. The dominant frequency ranges from 4694–5081 Hz. When Call A is followed by a Call B, the interval between them varies from 217–249 ms. Call B also is pulsed, though composed of fewer modulations. Call B duration varies from 98–116 ms and is formed by 4 or 5 pulses, which are emitted at a similar rate compared to Call A. The dominant frequency ranges from 4694–5038 Hz.

#### Vocalization of Vitreorana eurygnatha

The vocalization of the population from São Gotardo is composed of two call types (Table 1; Figures 2A; N = 3 males). Call A (N = 34 calls) pulsed and has complete amplitude is modulations. It has a slight increase in amplitude along the call. Call A duration varies from 135-226 ms and has 3 or 4 pulses. Pulses are emitted at a rate of 15.8–24.9 pulses/s. In some instances, the first pulse has remarkably lower amplitude. The dominant frequency ranges from 4453–5016 Hz. Call B (N = 3 calls; Figure 2A) was emitted by only one male as a single call (N = 2 cases) or in sequences (N = 2 cases) of two or three calls. Call B always is emitted shortly after a Call A and has the same overall pulse structure. However, Call B has different and irregular patterns of amplitude modulation, more pulses (5–7) and lower intensity (Figure 2A). The interval between Calls A and B varies from 332–560 ms. Each Call B has a duration of 154–199 ms. Pulses are emitted at a rate of 25.8–36.5 pulses/s. The dominant frequency ranges from 4500–4781 Hz. When Calls B are emitted in sequences, the interval between them varies from 389–649 ms (mean = 518, SD = 130).

Only Call A (N = 1 male) was recorded in the population of *Vitreorana eurygnatha* from Vargem Bonita, possibly due to the small sample size. The call (Figure 2B) is mostly consistent with the acoustic description for recordings from São Gotardo; however, slight differences were found in quantitative traits such as call duration, pulse rate, and dominant frequency (Table 1).

*Vitreorana eurygnatha* from Santa Teresa has a different call from those of the Cerrado populations described above. The vocalization is composed of a single call type, Call A (Figure 2C; Table 1; N = 2 males) for comparative purposes. Calls are pulsed with complete or incomplete amplitude modulations and formed by two distinct portions. The first has a progressive increase in amplitude until it reaches the second part, in which two or three pulse clusters are formed and separated by brief gaps of silence. The duration of Call A is similar to that of the Cerrado populations, ranging from 189–249 ms, but has a remarkably greater pulse number (16–22). Pulses are emitted at a higher rate (78.8–89.7 pulses/s) compared to Cerrado populations. The dominant frequency is quite similar, ranging from 4694–4995 Hz.

# Vocalization of Vitreorana franciscana

The vocalization of the population of Vitreorana franciscana from São Gotardo is composed of Call A (Figures 3A; Table 1; N = 2males; 59 calls), which is pulsed and has complete amplitude modulations. Calls are broadcast in two different ways; the first is a single call, and the second is a sequence of calls with lower amplitude that follow shortly after a first high-amplitude call (Figure 3A). Call A lasts 33-73 ms and has 4-7 pulses (Figure 3A). Pulses are emitted at a rate of 84.5-121.2 pulses/s. In some cases, last pulses can have longer duration than the others, with or without internal and incomplete amplitude modulation. The dominant frequency ranges from 4359–4734 Hz.

Only one specimen of *Vitreorana franciscana* was recorded from Vargem Bonita. The calls (Figure 3B; N = 1 male) resemble the overall structure described for calls from São Gotardo; they have similar values for temporal traits (Table 1), but have a lower dominant frequency (4031–4171 Hz).

# Discussion

The acoustic data for *Vitreorana baliomma* is informative as additional evidence to the diagnosis of this species from northeastern Brazil; however, owing to the limited sample size, we cannot assess intraspecific variation and patterns of call emission. Additional sampling should be conducted at the type locality of the species in the southern part of Bahia and from intermediate populations between the type locality and Murici, from which we obtained acoustic data (Figure 4), to assess the taxonomic identity of Vitreorana populations across coastal in Atlantic forests northeastern Brazil. Nevertheless, the description of calls of V. baliomma enables comprehensive acoustic comparisons with the calls of other Glass Frogs (Escalona et al. 2019).

Our study reveals that species of Vitreorana with calls already described from the Brazilian Atlantic Forest and Cerrado (V. eurygnatha, V. franciscana, and V. uranoscopa) diverge quantitatively, especially in cases of syntopy/ sympatry. In comparing the calls of these species, we noted two trends. First, in the case of syntopic species (e.g., V. eurygnatha and V. franciscana; Figures 2A, B, 3A, B, and 4), there are pronounced differences in the calls. Second, in the case of allopatric species (e.g., V. franciscana and V. uranoscopa; Figures 3 and 4), calls are similar. We assume that the presence or absence of selective pressures influence characteristics of acoustic mating signals in sympatric and allopatric populations of these frogs, respectively, and that these pressures are reinforced by reproductive isolation in response to sexual selection (Blair 1964, Hoskin et al. 2005). Therefore, in cases of sympatric occurrence, acoustic niche partitioning (Duellman and Pyles 1983, Höbel and Gerhardt 2003) between species of Vitreorana may have triggered selective pressures to avoid signal overlapping.

Calls of Atlantic Forest and Cerrado populations assigned to *Vitreorana eurygnatha* differ categorically (call envelope) and quantitatively in some temporal traits. The type locality of *V. eurygnatha* is located in a region of Atlantic Forest (Figure 4), which might be an indication that the calls described in our study from the Atlantic forest of Santa Teresa may belong to the call pattern of the nominal species. This is supported by a previous call description Bang et al.



Figure 2. Vocalizations of different populations of *Vitreorana eurgynatha*. (A): a cut (São Gotardo population) containing a Call A followed by a sequence of three Calls B. Lower panel detailing the section outlined: spectrogram and corresponding oscillogram of a Call A and the first Call B of the sequence (sound file = Vitreor\_eurygnathaSGotardoMG3dTRC\_AAGmt); (B) a Call A from Vargem Bonita (sound file = Vitreor\_eurygnathaCanastraMG1aTRC\_AAGmt); (C) a Call A from Santa Teresa (sound file = Vitreorana\_eurygnata\_SantaTeresa-ES\_2\_BFVT\_AAG\_m). See further information on recordings in Appendix I.



Figure 3. (A) Two Calls A of Vitreorana franciscana (São Gotardo); the second one (highlighted) is followed by a sequence of Call A. Highlighted panel corresponds to the spectrogram and oscillogram of the Call A in detail (sound file = Vitreor\_franciscanaSGotardMG1aTRC\_AAGmt); (B) a Call A of V. franciscana from Vargem Bonita (sound file = Vitreor\_franciscanaCanastraMG1cLM\_AAGmt); (C) a comparative Call A of V. uranoscopa from Itapeva, Minas Gerais, southeastern Brazil (sound file = Vitreor\_uranoscltapevaMG3aAAGm671). See further information on recordings in Appendix I.



**Figure 4.** A map of *Vitreorana* species from Cerrado or Atlantic Forest of Brazil depicting the advertisement call of each species or population from localities sampled in the present study: *Vitreorana baliomma* in black triangle = Itamaraju, type locality; white triangles = previous northernmost record, Areia Branca (SE), and northernmost record in Murici (AL) of *V. baliomma*; further occurrence records in Lisboa *et al.* (2019). *Vitreorana eurygnatha* in black circle = Serra da Bocaina, type locality; white circles = in the REBIO Augusto Ruschi, municipality of Santa Teresa (ES); and Luziânia (GO) (Cintra *et al.* 2013). *Vitreorana franciscana* in black square = PARNA da Serra da Canatra, Vargem Bonita, MG, type locality; white square = Presidente Olegário (MG). Rhomboid symbol represents São Gotardo (MG), a common occurrence point for both *V. eurygnatha* and *V. franciscana*. Inverted triangle = one point of occurrence for *V. uranoscopa* from Haga *et al.* (2014). Abbreviations for Brazilian states: AL = Alagoas; BA = Bahia; ES = Espírito Santo; GO = Goiás; MG = Minas Gerais; RJ = Rio de Janeiro; SE = Sergipe; SP = São Paulo. Scale bars for oscillograms = 0.05 ms.

of *V. eurygnatha* from Boracéia (Heyer *et al.* 1990) that agrees with the calls described by us. The striking differences between calls from the Atlantic Forest and the Cerrado emphasize the need of a taxonomic review of populations assigned to *V. eurygnatha* from Cerrado. Moreover, additional Atlantic Forest populations should have their calls evaluated to determine the extent to which acoustic variation across the range of the species coincides with genetic structure within *V. eurygnatha* (see Paz *et al.* 2018).

Although members of *Vitreorana* distributed in the Brazilian Atlantic Forest and Cerrado have a similar overall call structure, the calls differ in quantitative traits; thus, they are species specific in some cases, especially between sympatric species. This study highlights the relevance of acoustic data to inform the taxonomic status of members of the genus. This is particularly relevant to the acoustically divergent populations of *V. eurygnatha*, which are assigned to the same species based on their assumed lack of morphological differentiation. Our findings suggest that future research should investigate possible mechanisms related to acoustic niche partitioning in sympatric species of Vitreorana. To that end, acoustic divergences between sympatric populations of Vitreorana should be evaluated to determine whether the differences in calls is explained by short-term adjustments in their vocalization (behavioral plasticity) in response to acoustic niche overlap (Höbel and Gerhardt 2003) or, alternately, by fixed geographical variation in call patterns that may match variations in genetic structure (Velásquez et al. 2013) resulting from historical factors such as genetic drift (Irwin et al. 2008), natural selection (Velásquez et al. 2018), or landscape configuration (Paz et al. 2018) in sympatric populations of *Vitreorana*.

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Air (°C) Appendix I. Sound archives analyzed for each species/population of Vitteorana, with their respective recording labels; specimens voucher numbers; date, time and 19.019.024.7 20.8 22.8 22.8 20.8 20.0 19.019.019.0Date (time at recording) 30 Nov 2010 (~22:00h) 29 Apr 2013 (23:29h) 28 Jan 2012 (~00:00h) 28 Jan 2012 (~01:00h) 28 Jan 2012 (~02:00h) 30 Nov 2010 (22:05h) 04 Dec 2017 (20:22h) 04 Dec 2017 (20:27h) 28 Jan 2012 (00:34h) 28 Jan 2012 (00:43h) 01 Jan 2012 (19:47h) emperature at recording. Abbreviations for Brazilian states: AL = Alagoas, ES = Esptirto Santo, MG = Minas Gerais. Vargem Bonita, MG Vargem Bonita, MG São Gotardo, MG Santa Teresa, ES Santa Teresa, ES Itapeva, MG Murici, AL Locality AAG-UFU 6081 (19.8) AAG-UFU 1022 (16.9) AAG-UFU 1023 (17.8) AAG-UFU 6180 (20.5) AAG-UFU 1024 (21.3) AAG-UFU 1025 (21.5) MUFAL 11067 (17.8) AAG-UFU 980 (21.5) Voucher (SVL mm) **MZUFV 10735 MZUFV 10736** V. franciscana V. franciscana V. franciscana V. uranoscopa V. eurygnatha V. eurygnatha V. eurygnatha V. eurygnatha V. eurygnatha V. eurygnatha V. baliomma Species Vitreor\_eurygnathaSGotardoMG2a,bTRC\_AAGmt Vitreorana\_eurygnata\_Santa Teresa-ES\_1\_BFVT\_ Vitreorana\_eurygnata\_Santa Teresa-ES\_2\_BFVT\_ Vitreor\_eurygnathaSGotardoMG1bTRC\_AAGmt Vitreor\_franciscanaCanastraMG1b,cLM\_AAGmt Vitreor\_eurygnathaCanastraMG1aTRC\_AAGmt Vitreor franciscanaSGotardMG1aTRC AAGmt Vitreor\_franciscanaSGotardMG2aTRC\_AAGmt Vitreor\_eurygnathaSGotardoMG3a,d,cTRC\_ Vitreor\_uranoscItapevaMG3aAAGm67 Vitreorana baliomma Murici-Alagoas-BSLISBOA-29.IV.13\_MZ000032 Sound file AAG m AAG\_m AAGmt

# Reassessment of the advertisement call of topotypic Scinax squalirostris (Anura: Hylidae), with an acoustic evaluation of its occurrence in the Serra da Mantiqueira, southeastern Brazil

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#### Abstract

Reassessment of the advertisement call of topotypic Scinax squalirostris (Anura: Hylidae), with an acoustic evaluation of its occurrence in the Serra da Mantiqueira, southeastern Brazil. Scinax squalirostris (Lutz, 1925) is thought to occur along a broad range in South America. The values reported for calls of topotypes differ substantially among studies. Because vocalizations often play a key role in uncovering cryptic diversity, the call of S. squalirostris is herein redescribed based on a new sample of topotypes. The call of a population from Pocos de Caldas, Minas Gerais state, is also described. Topotypic advertisement calls have a dominant frequency between 3970 and 4125 Hz: 13–15 notes emitted at a rate of 24–27/s: call rate of 67/min and duration of 0.52–0.61 s; mid-call notes having 6 or 7 well-defined pulses, and an intra-note pulse rate of 223-266/s. Calls of the Poços de Caldas population have dominant frequency between 4083 and 4358 Hz; 15-18 notes emitted at a rate of 32-34/s; call rate of 64/min and duration of 0.46–0.56 s; mid-call notes having 6 or 8 well-defined pulses, and an intra-note pulse rate of 252–312/s. The advertisement calls of these populations have some differences with each other, and are promptly distinguished from calls of morphologically similar species. Our data to topotypes are inconsistent with some previously reported. A more detailed study of the population from Poços de Caldas is required, and more marked differences may be found in populations more distant from type locality of S. squalirostris.

Keywords: Amphibia, Atlantic Forest, bioacoustics, taxonomy, vocalization.

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#### Resumo

Reavaliação do canto de anúncio de Scinax squalirostris (Anura: Hylidae) topotípica, com uma avaliação acústica de sua ocorrência na Serra da Mantiqueira, sudeste do Brasil. Scinax squalirostris (Lutz, 1925) é tida como de ampla ocorrência na América do Sul. Os valores relatados para cantos de topótipos diferem substancialmente entre estudos. Como as vocalizações geralmente desempenham um papel fundamental na descoberta da diversidade críptica, o canto de topótipos de S. squalirostris é aqui redescrito com base em uma nova amostra. O canto de uma população de Poços de Caldas, estado de Minas Gerais, também é descrito. Os cantos topotípicos têm frequência dominante entre 3970 e 4125 Hz; 13-15 notas emitidas a uma taxa de 24-27/s; taxa de canto de 67/ min e duração de 0,52-0,61 s; notas do meio do canto possuindo 6 ou 7 pulsos bem definidos, e uma taxa de pulsos intra-nota de 223 a 266/s. Os cantos da população de Poços de Caldas têm frequência dominante entre 4083 e 4358 Hz; 15 a 18 notas emitidas a uma taxa de 32 a 34/s; taxa de canto de 64/min e duração de 0,46–0,56 s; notas do meio do canto possuindo 6 ou 8 pulsos bem definidos, e uma taxa de pulsos intra-nota de 252-312/s. Os cantos de anúncio dessas populações tem algumas diferenças entre si, e são prontamente distinguidos dos cantos de espécies morfologicamente semelhantes. Nossos dados para topótipos são inconsistentes com alguns relatados anteriormente. É necessário um estudo mais detalhado da população de Poços de Caldas, e diferenças mais acentuadas devem ser encontradas em populações mais distantes da localidade-tipo de S. squalirostris.

Palavras-chave: Amphibia, bioacústica, Mata Atlântica, taxonomia, vocalização.

## Introduction

*Scinax squalirostris* (Lutz, 1925) was described from the Serra da Bocaina highlands (Bokermann 1966, Lutz 1973), near the boundary between the Brazilian states of São Paulo and Rio de Janeiro. The species is thought to be distributed in the Atlantic Forest (Lutz 1973, Kwet 2001, Trevine *et al.* 2014), Cerrado (Eterovick and Sazima 2004, Pombal *et al.* 2011, São-Pedro and Feio 2011), and Pantanal (Uetanabaro *et al.* 2008) biomes in Brazil, to Uruguay, Paraguay, Argentina (Straneck *et al.* 1993), and Bolivia (De la Riva *et al.* 2000).

Barrio (1963), Haddad *et al.* (1988), Pombal *et al.* (1995, 2011), Faria *et al.* (2013), and Garey *et al.* (2018) presented data on calls of different populations of *Scinax squalirostris*, with the two latter studies also reporting on the call of the topotypic population. However, reported values for call variables of topotypes differ substantially among these studies. Given these inconsistencies and the need to elucidate the acoustic parameters of *S. squalirostris*, we redescribe its advertisement call based on a new sample of topotypes. It is

likely that S. squalirostris may comprise a species complex across its presumptive range (Pombal et al. 2011, Faria et al. 2013); thus, it is important to redescribe and authenticate its advertisement call, because vocalizations can be a key to the identification of cryptic diversity among frogs (e.g., Nunes et al. 2012, Caminer and Ron 2014, Ron et al. 2018, Carvalho et al. 2019). To determine if there is inter-populational variation in advertisement calls, we report call data of a population from the Poços de Caldas Plateau in the Mantiqueira Range in the state of Minas Gerais (MG). The call data from these two populations are compared to those reported in literature for other populations of S. squalirostris, as well to those reported for morphologically similar congeners.

# **Materials and Methods**

On 11 January 2012, calls of *Scinax* squalirostris (N = 3 males) were recorded at a site (22°43'35.16" S, 44°37'18.80" W; ca. 1500 m a.s.l.) 7 km from the type locality of the species (Fazenda do Bonito, RPPN Caburé,

around 22°44'22.99" S, 44°33'8.87" W), São José do Barreiro, São Paulo state (SP), Brazil. On 30 January 2009, calls (N = 3 males) were recorded in Poços de Caldas (MG), Brazil (21°56'57.97" S, 46°33'29.35" W; ca. 1300 m a.s.l.). The frogs were identified as *S. squalirostris* based on the descriptions of Lutz (1925) and Lutz (1973), as follow: (1) relatively small body size (males ca. 25 mm SVL); (2) elongated snout; (3) dorsolateral silvery stripe enclosed by two dark longitudinal stripes; (4) dark canthal line bordered by a white line; and (5) no transverse stripes on limbs.

Only one type of vocalization was heard and recorded. We assume this was the advertisement call because it was emitted repeatedly by males (Toledo et al. 2015, Köhler et al. 2017); it also corresponds in general to the structure of the advertisement call reported by Pombal et al. (1995) for another population. Recordings were made with a Sennheiser ME67/K6 directional microphone, connected to a Marantz PMD 671 or a M-Audio MicroTrack II digital recorder set to a sampling rate of 44.1 kHz and 16-bits resolution. Ten calls of each frog were analyzed. Call features were measured in Raven Pro 1.5 software (Bioacoustics Research Program 2014) with the following settings: 50% brightness and contrast; Hann window function at FFT width of 256 samples; 3 dB filter bandwidth of 270 Hz; overlap (locked) at 85%; and DFT size at 1024 samples. followed note-centered We the approach of Köhler et al. (2017) to define call units, therefore considering the main sound unit as a call, which is composed of subunits we treated as notes, which are in turn composed of pulses. Calls were measured from parts of the recording in which the vocalizations had higher and stable amplitudes. The acoustic terminology and definitions adopted are summarized in Table 1. Dominant frequency was obtained using the "Peak Frequency" function of Raven Pro 1.5. Sound figures were made using the Seewave package v. 1.5.9 (Sueur et al. 2008) in the R platform v. 2.12.1 (R Development Core Team 2014), using Hann window function, 85% overlap, and 256 points resolution (FFT). Call vouchers (AAG-UFU 0834–5; 1129–35) and recordings are housed in the Coleção de Anuros do Museu de Biodiversidade do Cerrado (AAG-UFU), Universidade Federal de Uberlândia, Uberlândia, Minas Gerais state, Brazil. The recordings analyzed are listed in Appendix I; a recording analyzed in Garey *et al.* (2018) was directly compared with our samples.

We sought for acoustic discrimination between topotypes and specimens from Poços de Caldas by applying the "DAPC" (Discriminant Analysis on Principal Components) function (adegenet v. 2.0.1 R package; Jombart 2008, Jombart *et al.* 2010). We searched for statistical differences between both populations based on call features that showed higher DAPC loadings (e.g., dominant frequency) through the "Exact Wilcoxon Mann Whitney Rank Sum Test" in the coin R package (Hothorn *et al.* 2008).

The morphologically similar species we considered for acoustic comparisons, are those small-sized *Scinax* with an immaculate color pattern on the hidden surfaces of thigh and groin, and with longitudinal stripes on the dorsum.

# Results

At both sites, males (Figure 1) started calling after sunset and call recordings were made between 19:22 and 20:30 h. We found no evidence of rain the day before recordings. Individuals called hidden, perched on dense, short (< 50 cm height) grassy bushes in open area marshes. Topotypes are 24.9 mm SVL (SD = 1.2; Range 22.1–26.8 mm; N = 10), and frogs from Poços de Caldas are 23.3 mm SVL (SD = 0.4; Range 23.0–23.6).

The advertisement call of topotypes (Table 2, Figure 2) is a stereotyped short, sharp, and pulsed buzz; the call increases in energy to its mid-portion and then decreases. Calls are emitted at a rate of 67/min, last 0.52–0.61 s and are spaced by inter-call intervals of 0.56–0.66 s.

 Table 1.
 Acoustic terminology adopted in the present study. Definition of most acoustic traits follows that of Köhler et al. (2017) and Raven manual (Charif et al. 2010).

Acoustic traits	Definition
Call duration (s)	Time from beginning to end of one call
Call interval (s)	Time from end of one call to beginning of the consecutive call
Call rate (calls/min)	Number of calls/time from beginning of first call to beginning of last call; the result is then multiplied by 60 to be given in calls/min
Notes/call	Number of notes present in one call
Note rate (notes/s)	Number of notes in one call/call duration
First-note duration (s)	Time from beginning to end of a note in the initial portion of the call
Mid-notes duration (s)	Time from beginning to end of a note in the middle portion of the call
Last-note duration (s)	Time from beginning to end of a note in the final portion of the call
First-note interval (s)	Time from end of a note to beginning of the consecutive note (in the initial portion of the call)
Mid-notes interval (s)	Time from end of a note to beginning of the consecutive note (in the middle portion of the call)
Last-note interval (s)	Time from end of a note to beginning of the consecutive note (in the final portion of the call)
First-notes pulse number	Number of pulses in notes from the initial portion of the call
Mid-notes pulse number	Number of pulses in notes from the middle portion of the call
Last-notes pulse number	Number of pulses in notes from the final portion of the call
Intra-note pulse rate (pulses/s)	Number of pulses in a mid-call note/note duration
Amplitude peak time (%)	Time from the beginning of the call to its point of maximum amplitude; the value is given as a percentage in relation to the total call duration
Dominant frequency (Hz)	Frequency containing the greatest sound energy of the call
5% frequency (Hz)	Frequency that divides the call into one part above and another below itself, which contain respectively 95% and 5% of the total sound energy of the call
95% frequency (Hz)	Frequency that divides the call into one part above and another below itself, which contain respectively 5% and 95% of the total sound energy of the call

Calls contain 13–15 notes emitted at a rate of 24–27/s. Notes from the mid portion of the call are slightly shorter (0.027–0.028 s) than those at the end (0.020–0.038 s). Mid-call notes have 6 or 7 well-defined pulses with an intra-note pulse rate of 223–266/s. Notes at the end of calls have pulses less clearly defined (values in Table 2 are estimates). The dominant frequency is between 3970 and 4125 Hz, with an average of 370 Hz upward frequency modulation from beginning until the end of the call; the 90% bandwidth is 1149 Hz.

Calls of frogs from Pocos de Caldas (Table 2, Figure 2) have the same structural pattern as those of the topotypes. Calls are emitted at a rate of 64/min, have durations of 0.46-0.56 s and are spaced by inter-call intervals of 0.50-0.67 s. Calls contain 15-18 notes emitted at a rate of 32-34/s. Mid-call notes are slightly longer (0.020-0.025 s) than those at the end (0.019-0.025 s)0.022 s). Notes from mid-call have 6 or 7 welldefined pulses with an intra-note pulse rate of 252-312/s. Notes at the end of call have 6-8well-defined pulses. The dominant frequency is between 4083 and 4358 Hz; two males had an average of 370 Hz upward frequency modulation from beginning to the end of call, whereas one had inconsistent variation from -500 to 500 Hz in relation to the beginning; the 90% bandwidth is 1476 Hz.

The DAPC completely discriminated (100% probability of correct membership) calls of one population from another. The number of notes per call, note rate, and mid-note duration distinguish them. Differences in dominant frequency (wilcox\_test Z = -1.53, p = 0.2), call duration (Z = 1.09, p = 0.4) and intra-note pulse rate (Z = -1.53, p = 0.2) are not significant (Figure 3).

A comparison of the advertisement calls of both populations with those available in literature for other populations of *Scinax squalirostris* is provided in Table 2. A brief comparison between the advertisement call of our topotypes with those of morphologically similar *Scinax* species is in Table 3.



Figure 1. Adult male from each population studied. Above: a topotype of *Scinax squalirostris* (Serra da Bocaina); AAG-UFU 835. Below: Poços de Caldas, Minas Gerais state; AAG-UFU 4378. Both are call vouchers.

## Discussion

Our call values of topotypic Scinax squalirostris differ from those reported by Faria et al. (2013) (Table 2). Their mean values are consistently lower than their standard deviations, suggesting that the numbers represent minimum and maximum values instead. Considering these inconsistencies, their Table 1 (p. 331; reproduced in Table 7 of Köhler et al. 2017, p. 54) seems to be erroneous. Further, their values for "calls/min" are unrealistic and contrast with those that we found; they are more similar to our values for note rate when expressed in minutes (Table 2 herein). Garey et al. (2018) also provided call data for topotypic S. squalirostris and their values for spectral traits differed somewhat from ours (Table 2); they reported a substantial high value for the

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**Figure 2.** Audiospectrograms and corresponding oscillograms of the advertisement calls of a topotypic *Scinax squalirostris* (above) and of a male from Poços de Caldas, Minas Gerais state (below). Notice the higher number of notes and shorter note duration of the Poços de Caldas population. Figured recordings: Scinax\_squalirostrisBocainaSP1cAAGm671; Scinax\_spPocosCaldasMG1aAAGmt.



Figure 3. Comparative quantitative call features of the two populations studied. Bocaina represents the type locality of *Scinax squalirostris*. Notice that the three features above are diagnostic for each. The differences in median values for dominant frequency, call duration, and intra-note pulse rate are not significant (p > 0.05). See Table 2 for descriptive statistics.

SD of dominant frequency, but did not discuss it. Based on the re-analysis of the recording of Garey *et al.* (2018), we noticed that this high SD value results from the alternation of the dominant frequency between emphasized bands along call emissions. Thus, calls in the initial portion of a call series (weak/low amplitude) had their dominant frequencies concentrated in the lower band, whereas calls of middle and last portions of the series (higher and stable amplitude) had more energy in the upper frequency band. Similar between-bands shifts in dominant frequency is known for calls of other species of *Scinax* (e.g., Magrini *et al.* 2011, Carvalho *et al.* 2015, Novaes and Zina 2016, Bang *et al.* 2017, Lopes et al. 2019). Also, the mean value of notes per call reported by Garey *et al.* (2018) should be read 10, not 1 (M. V. Garey, pers. com.).

In southeastern Brazil, other populations of frogs identified as *Scinax squalirostris* are as follow: Alto and Médio Paranapanema (Maffei *et al.* 2011, Oliveira *et al.* 2013, Maffei and Ubaid 2014), Paranapiacaba (Trevine *et al.* 2014), Ribeirão Branco (Pombal *et al.* 1995), Serra da Canastra (Pombal *et al.* 1995), Serra do Cipó (Eterovick and Sazima 2004), Serra do Ouro Branco (São-Pedro and Feio 2011), and Triângulo Mineiro (Neves *et al.* 2019).

Table 2.	Advertisement call trait values of different populations attributed to Scinax squalirostris. Values are given as Mean ± SD (SD/Mean, in %); if Mean
	was not available, Range is given. <sup>a</sup> Pulses in Haddad et al. (1988) and in Pombal et al. (2011) herein correspond to notes, and notes in Haddad et
	al. (1988) herein correspond to call. <sup>b</sup> See Discussion. <sup>c</sup> Call of this population is a single multipulsed note, and therefore this value refers to the total
	number of pulses. * Unspecified from which portion of the call measurements were taken. ** In minutes, values to our topotypes are 1530 ± 99 (see
	Discussion). Legend to Brazilian states: SP, São Paulo; MG, Minas Gerais; GO, Goiás.

	<b>Topotypes</b> (present study)	Topotypes (Faria <i>et al.</i> 2013)	<b>Topotypes</b> (Garey <i>et al.</i>	<b>Ribeirão Branco, SP</b> (Pombal <i>et al.</i> 1995)	Poços de Caldas, MG (present study)	Serra da Canastra, MG (Haddad <i>et</i>	<b>Cristalina, GO</b> (Pombal <i>et al.</i>	<b>La Paz, Bolivia</b> (Pombal <i>et al.</i>
-			2018)			<i>al.</i> 1988) <sup>a</sup>	, ,	2011)ª
Individuals recorded	m	2	9		m	1?	-	1
Call duration (s)	$0.556 \pm 0.048$ (9)	$0.420 \pm 0.550 \ (131)$	$0.5 \pm 0.1 \ (20)$	$0.6 \pm 0.04$ (7)	$0.494 \pm 0.049$ (10)	0.4	$0.664 \pm 0.020$ (3)	$0.391 \pm 0.008$ (2)
Call interval (s)	$0.599 \pm 0.052$ (9)				$0.631 \pm 0.113$ (18)	0.4		
Call rate (/min)	$67 \pm 1.9$ (3)	1584 ± 1640 (104)			$64 \pm 6.4 \ (10)$	80	62	
Notes/call	14.1 ± 0.90 (6)	11.6 ± 14.5 (125)	$10 \pm 0 \ (0)^{b}$	12.3 ± 0.67 (5)	16.1 ± 1.41 (9)	16–17	1	10
Note rate (/s)	$25.5 \pm 1.65 \ (6)^{**}$				32.5 ± 0.98 (3)			
First-note duration (s)	$0.023 \pm 0.003$ (13)				$0.010 \pm 0.006 \ (60)$			
Mid-note duration (s)	$0.027 \pm 0.001$ (4)	$0.030 \pm 0.032 \ (107)^{*}$		$0.024 \pm 0.003 \ (13)^{*}$	$0.022 \pm 0.002$ (9)			
Last-note duration (s)	$0.029 \pm 0.009$ (31)				0.020 ± 0.002 (10)		ı	·
First-note interval (s)	$0.017 \pm 0.004$ (24)				$0.019 \pm 0.007 (37)$			
Mid-note interval (s)	$0.014 \pm 0.001$ (7)			$0.026 \pm 0.001 \ (4)^{*}$	$0.011 \pm 0.003 (27)$			
Last-note interval (s)	$0.014 \pm 0.001$ (7)				0.012 ± 0.002 (17)		ı	
First-note pulse number	$4.7 \pm 0.95$ (20)			·	2.1 ± 0.91 (43)	·	ı	
Mid-note pulse number	$6.6 \pm 0.44$ (7)	7.97 ± 9.0 (113)*	$12.9 \pm 1.6 \ (12)$	$6.2 \pm 0.97 (15.64)^{*}$	$6.2 \pm 0.45$ (7)		46 ± 1 (2) °	
Last-note pulse number	8.1 ± 3.52 (43)				$6.6 \pm 0.87 (13)$			
Intra-note pulse rate (/s)	244 ± 22 (9)				283 ± 30 (11)			

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	Topotypes (present study)	<b>Topotypes</b> (Faria <i>et al.</i> 2013)	Topotypes (Garey <i>et al.</i> 2018)	<b>Ribeirão Branco, SP</b> (Pombal <i>et al.</i> 1995)	Poços de Caldas, MG (present study)	Serra da Canastra, MG (Haddad <i>et</i> <i>al.</i> 1988) <sup>a</sup>	<b>Cristalina, GO</b> (Pombal <i>et al.</i> 2011)	La Paz, Bolivia (Pombal <i>et al.</i> 2011)ª
Amplitude peak time (%)	54 ± 9.7 (18)				53 ± 4.0 (8)			
Dominant frequency (Hz)	4041 ± 78 (2)	3709 ± 4257 (115)	$3540 \pm 738.3$ (21)	3900-4600	4197 ± 143 (3)	·	4273 ± 74 (2)	4366 ± 42 (1)
5% frequency (Hz)	3187 ± 179 (6)		2375.6 ± 148 (6)	30005	3208 ± 112 (4)	3500?	2932 ± 19 (1)	3040 ± 42 (1)
95% frequency (Hz)	4336 ± 54 (1)		$4982.5 \pm 114$ (2)	20009	$4684 \pm 123$ (3)	5500?	5085 ± 19 (0.5)	4995 ± 57 (1)
SVL (mm)	22.1–26.8				23.0-23.6			
Temperature (°C)	air 15–16			air 14	air 18	air 20, water 19	air 18	air 18
Recording time	19:22–20:15 h			21:00h	20:00–20:15 h	21:00 h		

Some of these sites have elevations above 1000 m and seem to be disjunct from the topotypic population. We think that the substantial interpopulational call differences reported here (Table 2), indicate that a more detailed assessment of the population from Pocos de Caldas is needed to clarify whether these differences are within the bounds of natural variation or are diagnostic features of an undescribed taxon. One might expect even more marked differences in populations that are farther from type locality, such as those from Brazilian Cerrado (Haddad et al. 1988, Pombal et al. 2011, Santoro and Brandão 2014), Bolivia, southern Brazil, and Uruguay (Faria et al. 2013). The greater number of notes per call of the populations from Pocos de Caldas and Serra da Canastra is noteworthy, as is the longer internote interval of the population from Ribeirão Branco (Table 2). In addition, the call of the frogs from the Cristalina population, Goiás state (GO), differs from those of other populations, since it is composed of a single multi-pulsed note. Examination of different classes of data (e.g., morphometric, acoustic, and molecular) may inform species delimitation within the nominal species S. squalirostris and other populations currently assigned this name, such as the one from Pocos de Caldas. If a population of frogs at the southernmost part of the range of S. squalirostris is found to be distinct, there are available names for them from southern Uruguay, as well as northeastern and northern Argentina (Klappenbach and Langone 1992, Pinheiro et al. 2014).

Morphologically, *Scinax squalirostris* is distinguished from its congeners by the combination of its elongated snout and its dorsolateral silvery stripes (Pinheiro *et al.* 2014). In addition, it differs from morphologically similar congeners by its multi-note advertisement call in contrast to the single-note calls of these species (Table 3).

The Poços de Caldas Plateau is rich in endemic frog species (Giaretta and Sazima 1993, Vasconcelos and Giaretta 2003, Caramaschi and

	Scinax squalirostris	Scinax fuscomarginatus	Scinax madeirae	Scinax exiguus	Scinax wandae	Scinax staufferi	Scinax altae
Reference	Present study	Toledo and Haddad 2005	Jansen <i>et al.</i> 2016	Carvalho <i>et al.</i> 2017	Pyburn and Fouquette 1971	León 1969	León 1969
Call duration (s)	$0.556 \pm 0.048$ $(0.520-0.610)$	$0.540 \pm 0.062$ (0.334-0.628)	$0.825 \pm 0.126$ (0.558-1.277)	$1.148 \pm 0.334$ (0.632-1.638)	0.673 (0.653–0.696)	0.180	0.150
Call interval (s)	$0.599 \pm 0.052$ (0.560-0.660)	$3.11 \pm 2.85$ (0.580-19.59)	I	·	0.5		
Notes/call	13–15	-	1	-	-	-	1
Dominant frequency (Hz)	$4041 \pm 78$ (3970-4125)	3820 ± 220 (3070–4200)	$4085 \pm 552$ (3100-5672)	4522 ± 290 (4078–5016)	4893 (4800–5050)	1743 (1582–1872); 3056 (1962–3744) <sup>a</sup>	2008 (1853–2106); 3775 (3379–4056)ª
Individuals recorded	m	23	61	m	e	18?	23
SVL (mm)	22.1–26.8		19.3–26.8	18.0–20.8	23.4–26.9	20.7–29.0	21.7–26.0
Temperature (°C)	air 15–16, water 18–19	air 19–22	air 19–29	air 16	air 22		
Recording time	19:22–20:15 h		19:00–24:00 h				

**Table 3.** Advertisement call traits of topotypic *Scinax squalirostris* (our sample) and morphologically similar congeners. Values are given as Mean  $\pm$  SD (Minimum-Maximum). "Emphasized frequency bands. Recording localities: S. squalirostris (type locality); S. fuscomarginatus (Itirapina, SP, Brazil); Cruz 2004, Martins *et al.* 2016), suggesting that the area may have been isolated long enough to result in speciation. Finer taxonomic resolution will improve our understanding of the biogeographic patterns of frog diversification along the vegetation formations of the Atlantic Forest, as exemplified by Vasconcelos *et al.* (2014), who also verified the presence of Scinax squalirostris in the Mantiqueira Range of Minas Gerais.

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**Appendix I.** *List of analyzed sound files (\*.wav format); all housed at the collection of frogs of the Universidade Federal de Uberlândia. m671 = marantz/Sennheiser Me67; mt = microtrack/Sennheiser Me67.* 

Scinax\_squalirostrisBocainaSP1aAAGm671; Scinax\_squalirostrisBocainaSP1bAAGm671; Scinax\_squalirostrisBocainaSP1cAAGm671; Scinax\_squalirostrisBocainaSP2aAAGm671; Scinax\_squalirostrisBocainaSP2bAAGm671; Scinax\_squalirostrisBocainaSP2cAAGm671; Scinax\_squalirostrisBocainaSP3aAAGm671; Scinax\_squalirostrisPocosCaldasMG1aAAGmt; Scinax\_squalirostrisPocosCaldasMG1bAAGmt; Scinax\_squalirostrisPocosCaldasMG2aAAGmt; Scinax\_squalirostrisPocosCaldasMG2bAAGmt; Scinax\_squalirostrisPocosCaldasMG3aAAGmt; Scinax\_squalirostrisPocosCaldasMG2bAAGmt; Scinax\_squalirostrisPocosCaldasMG3aAAGmt; Scinax\_squalirostrisPocosCaldasMG2bAAGmt; Scinax\_squalirostrisPocosCaldasMG3aAAGmt; Scinax\_squalirostrisPocosCaldasMG2bAAGmt; Scinax\_squalirostrisPocosCaldasMG3aAAGmt; Scinax\_squalirostrisBocainaSP1aMVG\_CFBH.

# Acoustics suggests hidden diversity in *Scinax garbei* (Anura: Hylidae)

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## Abstract

Acoustics suggests hidden diversity in *Scinax garbei* (Anura: Hylidae). *Scinax garbei* is a treefrog species thought to be widely distributed across forest habitats of the Amazon Basin, occurring in Venezuela, Colombia, Ecuador, Peru, Bolivia, and Brazil. However, the morphological, acoustic and molecular characters of this species vary across its distribution. In view of this variation, the present study re-analyzes published advertisement calls and analyses new call data of nine populations of *S. garbei* from five countries, aiming to assess acoustic divergence. In addition, the territorial call of the species is described for the first time. Based on multivariate analyses of advertisement call data, there are three groups of populations with distinct calls, referred to herein as *S. garbei* Brazil, Northwestern, and Southwestern. *Scinax garbei* Northwestern is distinguished from *S. garbei* southwestern by temporal call traits, whereas *S. garbei* Brazil differs from the other two groups based on both temporal and spectral traits. These results indicate that *S. garbei* may represent a complex of up to three species, thereby highlighting the need for a thorough taxonomic revision of this species.

**Keywords:** Amazonia, Amphibia, bioacoustics, *Scinax rostratus* Group, species complex, taxonomy.

#### Resumo

Acústica sugere diversidade oculta em *Scinax garbei* (Anura: Hylidae). *Scinax garbei* é uma espécie de anuro arborícola tida como amplamente distribuída pelos habitats florestais da Bacia Amazônica, ocorrendo na Venezuela, Colômbia, Equador, Peru, Bolívia e Brasil. No entanto, os caracteres morfológicos, acústicos e moleculares dessa espécie variam ao longo da sua distribuição. Diante dessa variação, o presente estudo reanalisa cantos de anúncio publicados e novos dados de cantos de nove populações de *S. garbei* de cinco países, para avaliar a divergência acústica. Além

Received 02 August 2019 Accepted 25 May 2020 Distributed June 2020 disso, o canto territorial da espécie é descrito pela primeira vez. Com base em análises multivariadas de dados de cantos de anúncio, existem três grupos de populações com cantos distintos, referidas aqui como *S. garbei* Brasil, Noroeste e Sudoeste. *Scinax garbei* Noroeste se distingue de *S. garbei* Sudoeste com base em traços temporais do canto, enquanto que *S. garbei* Brasil se distingue dos outros dois grupos com base em ambos traços temporais e espectrais do canto. Esses resultados indicam que *S. garbei* pode representar um complexo de até três espécies, destacando, portanto, a necessidade de uma minuciosa revisão taxonômica dessa espécie.

Palavras-chave: Amazônia, Amphibia, bioacústica, complexo de espécies, grupo de *Scinax rostratus*, taxonomia.

# Introduction

The Neotropics harbor the greatest frog diversity in the world, especially in South America (Duellman 1999, Villalobos et al. 2013). However, this species richness currently is underestimated given the existence of many complexes of morphologically cryptic species, particularly in the Amazonian region (e.g., Fouquet et al. 2007, 2016, Funk et al. 2011, Ferrão et al. 2016. Caminer et al. 2017. Vacher et al. 2017, 2020, Rivadeneira et al. 2018, Chasiluisa et al. 2020, Mota et al. 2020). In this context, integrative approaches that incorporate distinct lines of evidence (e.g., acoustics, molecular, cytogenetics, etc.) have effectively solved taxonomic problems (e.g., Padial and De la Riva 2009, Caminer and Ron 2014, 2020, Baldo et al. 2019).

Frogs emit different types of acoustic signals that are categorized based on the social context in which the vocalizations are made (Toledo *et al.* 2015, Köhler *et al.* 2017). The most common type is the advertisement call; this is emitted by males to attract conspecific females and maintain between-males spacing in a chorus (Duellman and Trueb 1994, Wells 2007, Toledo *et al.* 2015, Köhler *et al.* 2017). As this call transmits species-specific information related to prezygotic isolation (Köhler *et al.* 2017), it is a useful diagnostic tool to reveal morphologically cryptic species (e.g., Nunes *et al.* 2012, Ron *et al.* 2018, Carvalho *et al.* 2019). Another common acoustic signal is the territorial call, which is emitted in aggressive contexts that involve the defense of a territory (e.g., calling site) (Toledo *et al.* 2015, Köhler *et al.* 2017).

Scinax Wagler, 1830 currently comprises 72 species of treefrogs distributed throughout the Neotropics (Frost 2020). Scinax garbei (Miranda-Ribeiro, 1926) is a large-sized species of the S. rostratus Species Group (Faivovich 2002, Faivovich et al. 2005, Wiens et al. 2010), described from the Rio Juruá, Eirunepé, state of Amazonas (AM), Brazil. This species is thought to be widely distributed across forest habitats of the Amazon Basin, with occurrences reported from Venezuela, Colombia, Ecuador, Peru, Bolivia (La Marca 1992, Duellman and Wiens 1993, De la Riva et al. 1994, Barrio-Amorós et al. 2019), and from the Brazilian states of Acre (Bernarde et al. 2011, 2013), Amapá (Silva e Silva and Costa-Campos 2014), Amazonas (Miranda-Ribeiro 1926, Lima et al. 2006, Pantoja and Fraga 2012), Mato Grosso (São-Pedro et al. 2009, Noronha et al. 2015, present study), Pará (Ávila-Pires et al. 2010, Pinheiro et al. 2012), and Rondônia (Bernarde 2007, Piatti et al. 2012).

Some of the advertisement calls of *Scinax* garbei from populations in Ecuador, Peru, and Bolivia differ markedly from one another (Duellman 1970, 1972, 1978, 2005, Duellman and Pyles 1983, Duellman and Wiens 1993, De la Riva *et al.* 1994). Although Zimmerman (1983) reported the dominant frequency and pulse rate for a specimen from Manaus (AM), the advertisement call of *S. garbei* never has

been formally described from Brazil. Moreover, the species varies considerably in body size, thigh color pattern, and degree of development of the proboscis and of the heel tubercle (Duellman 1970, 1972, Heyer 1977, Duellman and Wiens 1993). In addition, recent molecular studies have shown that the populations from Ecuador and southeastern Peru do not form a monophyletic group (Jansen *et al.* 2011; Ron *et al.* 2018). These inconsistencies suggest that *S. garbei* represents a species complex, thereby illustrating the need of a better assessment of the taxonomic status of the different populations currently under this name.

Call divergences between some populations of *Scinax garbei* may elucidate their respective identities. However, many of the apparent discrepancies may reflect the different technologies and methodologies used in the analyses of calls; thus, reliable comparisons cannot be made. Herein, we re-analyze published data and also analyze original recordings of *S. garbei* from a total of nine populations from five countries. Acoustic variation among some populations is discussed, as well as the taxonomic implications of the observed variation. Lastly, we provide the first description of the territorial call of the species.

# **Materials and Methods**

# Data Collection

Vocalizations were recorded on two occasions: (1) on 24 January 2011 on the Campus of the Universidade Federal do Amazonas, Manaus, state of Amazonas (AM), northern Brazil (03°06'0.86" S, 59°58'35.59" W, 79 m a.s.l., datum WGS84; ca. 1,170 km east-northeast of the type locality of *Scinax garbei*); and (2) on 11 January 2019 in the municipality of Alta Floresta, state of Mato Grosso (MT), Brazil (09°38'34.22" S, 56°16'17.07" W, 273 m a.s.l., datum WGS84; ca. 1,540 km east-southeast of the type locality).

Specimens collected in Alta Floresta are housed in the Collection of Frogs of the Museu de Biodiversidade do Cerrado, Universidade Federal de Uberlândia (AAG-UFU 6498–6502, with specimens 6498 and 6499 being call vouchers). Recordings (sampling rates of 44.1 or 48.0 kHz; 16 bits resolution) are also deposited in the same collection. See Appendix I for further details about recordings.

# Additional Recordings

Fourteen recordings from the following localities were provided by the Fonoteca Zoologica (FonoZoo) from the Museo Nacional de Ciencias Naturales de Madri: Puerto Almacén (Bolivia), Abel Iturralde Province (Bolivia), Leticia (Colombia), Pucaurquillo (Peru), Pilcopata (Peru), and Santa Cecilia (Ecuador). Vocalizations of Scinax garbei from some of these localities had been analyzed previouslye.g., Pilcopata (Duellman 1972, Duellman and Wiens 1993), Santa Cecilia (Duellman 1970, 1972, 1978, Duellman and Wiens 1993), and Puerto Almacén (De la Riva et al. 1994). One voucher specimen from Pilcopata and two from Santa Cecilia are housed in the KU Herpetology Collection of the Biodiversity Institute at the University of Kansas (respectively KU 139242, 125603, 125604). These recordings were made with analogue recorders and digitized at a sampling rate of at least 44.1 kHz and 16 bits resolution. A recording from Tambopata-Candamo National Park (Peru) published on a CD by Cocroft et al. (2001) also was analyzed. Appendix I provides recording details.

# Specimen Identification

Voucher specimens from Santa Cecilia and Pilcopata were identified as *Scinax garbei* by Duellman (1970, 1972) and Duellman and Wiens (1993). We confirmed the identity of other recordings attributed to *S. garbei* based on the similarity of the advertisement call to those of either of these two populations. Based on the resemblance of the advertisement calls, we determined that the recording from Manaus was of the same species as that from Alta Floresta. Characters of the frogs from Alta Floresta match the diagnosis provided for S. garbei by Duellman and Wiens (1993), as follow: (1) snout-vent length (SVL) 41.4–44.0 mm [mean: 42.9; standard deviation (SD): 1.1]; (2) snout acuminate in dorsal and lateral profiles, with a proboscis that present different degrees of development; (3) conical ulnar and tarsal tubercles present (more or less distinct); (4) enlarged heel tubercle present; (5) tubercles present on lower jaw; (6) skin on dorsum smooth to tuberculate, with tubercles often conical; (7) dorsum brown (brown or dull green in life), with dark triangular mark with apex directed posteriorly and corners on eyelids; (8) large dark marks posterodorsal to axillae; (9) dashes or transverse marks in sacral region; (10) transverse bars on dorsal surface of limbs; (11) flanks cream with brown flecks; (12) broad cream (orange in life) and dark vertical bars on posterior surfaces of thighs; and (13) iris pale creamy bronze, with a median horizontal reddish-brown streak.

## Acoustic Analyses

A high-pass filter up to 500 Hz and a lowpass filter up to 5000 Hz were applied to recordings to reduce background noise. After filtering, recordings with low amplitude levels were normalized (peak -1.0 dB) with Audacity v. 2.2.2 software (Audacity Team 2020). Advertisement calls (N = 237) from nine populations and territorial calls (N = 97) from seven populations were analyzed in Raven Pro v. 1.5 software (Center for Conservation Bioacoustics 2014) with the following settings: window size = 256 samples; 3 dB filter bandwidth = 248, 270or 539 Hz; window type = Hann; overlap = 89.8% (locked); hop size = 0.271, 0.542 or 0.590 ms; DFT size = 1024 samples; grid spacing = 43.1, 46.9 or 93.8 Hz (depending on the sampling rate). Temporal traits were obtained from

oscillograms. Dominant, maximum, and minimum frequency values were obtained through "Peak Frequency," "Frequency 95%," and "Frequency 5%" functions of Raven, respectively. We used the acoustic terminology and definitions of Bang Giaretta (2016) with the following and exceptions. The call rate is that of Carvalho et al. (2017). Pulse rate was calculated from a section of 100 ms in the middle of the call, as follows: (number of pulses - 1) / time between the onset of first and last pulses. The callcentered approach of Köhler et al. (2017) was adopted. Mean and standard deviation (SD) values were obtained from mean values of each individual, whereas ranges (i.e., minimummaximum) include all values from the raw dataset. Sound figures were generated with the seewave package v. 1.7.6 (Sueur et al. 2008) in the R platform v. 3.5.2 (R Core Team 2018) with the following settings: window = Hanning, overlap = 85%, and FFT = 256.

# Classification of Call Types

Two types of call were recognized. Advertisement calls are the most common, emitted repeatedly in a stereotyped way, and sometimes antiphonally by neighboring males (Köhler *et al.* 2017). Territorial calls (*sensu* Toledo *et al.* 2015) are those often emitted by males in close-range interactions in Alta Floresta. Advertisement and territorial calls from other recordings were categorized as such based on the context of the recording and by comparison with recordings from Alta Floresta.

# Statistical Analyses

Multivariate analysis included all traits measured for call descriptions and were based on mean values of each individual. Among-population/groups acoustic discrimination was sought through the randomForest (RF) (randomForest v. 4.6-14 package, Liaw and Wiener 2002) and DAPC (Discriminant Analysis on Principal Components) (adegenet v. 2.1.1 package; Jombart 2008, Jombart et al. 2010) functions in R platform. The RF algorithm constructs several (by default 500) classification trees using bootstrap samples from the original dataset, with each split using the best predictors among those randomly chosen at each node; then it generates classifiers and aggregates results by voting to classes (Breiman 2001, Liaw and Wiener 2002). Unsupervised (i.e., no pre-established groups) RF analyses were performed a priori to detect putative clustering among samples; subsequent multivariate analyses were carried over the recognized clusters. RF analyses also result in between-objects distance estimates, which are subject to a Multidimensional Scaling Analysis (MDS) and displayed graphically through the proximityPlot function (rfPermute package; Archer 2014). The DAPC runs on the Principal Component Scores (Jombart 2008, Jombart et al. 2010). A Discriminant Analysis (DA) was applied on a few axes (retaining ca. 95% of the variance) of the Principal Component Analysis (PCA), improving the imbalance between objects and traits (Jombart et al. 2010). The DAPC analysis was carried out to test for reciprocal congruence between it and RF.

Traits indicated as important for amonggroups discrimination by RF and DAPC, were assessed for statistical significance with the "Exact Wilcoxon Mann Whitney Rank Sum Test", by applying the function wilcox\_test (coin package; Hothorn *et al.* 2008) in R. As this test is performed between pairs, the significance levels (p) were adjusted by the Holm method applying the p.adjust function. Statistical significance was assumed when p-value < 0.05.

# Results

Unsupervised RF analysis resulted in three clusters, hereinafter designated by their geographic location (Figure 1): (1) *Scinax garbei* Brazil.—Alta Floresta (N = 4 males; 81 calls) and Manaus (N = 1; 12); (2) *Scinax garbei* Southwestern.—Abel Iturralde (N = 2; 7), Puerto Almacén (N = 1; 20), Tambopata-Candamo

National Park (N = 1; 16), and Pilcopata (N = 3; 7); and (3) *Scinax garbei* Northwestern.— Pucaurquillo (N = 1; 6), Leticia (N = 1; 11), and Santa Cecilia (N = 6; 77).

# Descriptions of Vocalizations

Advertisement calls.—Calls of all groups (Figures 2, 3) consist of a single multi-pulsed note composed of two emphasized frequency bands [hereinafter called lower (LFB) and higher (HFB) bands]. Calls of Scinax garbei Brazil and Southwestern have similar envelopes, with a fast-ascending amplitude modulation at the onset and quickly reaching a plateau that is sustained for the duration. Calls of S. garbei Northwestern have an irregular, elliptical envelope, with variation in amplitude modulation in the middle and final portions of the call; calls of frogs from Leticia lack this downward modulation. Calls of S. garbei Brazil and Southwestern have variable durations, whereas call duration of S. garbei Northwestern is more regular. First and final pulses have more variable durations and intervals; pulses of S. garbei Northwestern occasionally have deeper internal amplitude modulations (Figure 3). In both S. garbei Brazil and Southwestern, the HFB has a fast-upward modulation at the call onset and quickly reaches a stable frequency that is sustained for the duration of the call. In contrast, the HFB of S. garbei Northwestern has a long, gradual upward modulation from the call onset to some point at the middle or final portion, at which it reaches a stable frequency that is sustained for the call duration. Unlike HFB, the LFB of all groups maintains a stable frequency throughout the call. In all groups, the dominant frequency can alternate between the LFB and HFB along call emissions; it alternated between bands in three individuals of S. garbei Brazil, in two of S. garbei Southwestern, and in six of S. garbei Northwestern. Conversely, dominant frequency corresponds only to HFB in two S. garbei Brazil, in two S. garbei Southwestern, and in one S. garbei Northwestern. Also, in one S. garbei



Figure 1. Distribution map of the three groups of *Scinax garbei* recognized here (ellipses: pink = *S. garbei* Brazil; green = *S. garbei* Northwestern; blue = *S. garbei* Southwestern). Star = type locality (Juruá River, Eirunepé); 1 = Alta Floresta; 2 = Manaus; 3 = Leticia; 4 = Pucaurquillo; 5 = Santa Cecilia; 6 = Pilcopata; 7 = Tambopata; 8 = Abel Iturralde; 9 = Puerto Almacén. Abbreviations for Brazilian states: AC = Acre, AM = Amazonas, MT = Mato Grosso, RO = Rondônia, RR = Roraima.

Northwestern, the dominant frequency corresponded only to LFB. Descriptive statistics of call traits of each group are in Table 1 and of each population in Appendix II.

*Territorial calls.*—These calls were emitted by individuals from all populations, except for those from Puerto Almacén and Tambopata. Calls of all groups (Figure 4) consist of a single multi-pulsed note with irregular amplitude modulations; usually there is an ascending amplitude modulation in the first part of the call and a downward modulation in the final portion. Calls of *S. garbei* Northwestern and Southwestern vary in duration, whereas call duration of *S. garbei* Brazil is more regular. Pulses in the middle portion of the call usually are better defined. Territorial calls often are emitted shortly after advertisement calls. However, these calls also are emitted without having been preceded by an advertisement call, and not all advertisement calls are followed by territorial ones (52% of advertisement calls of *S. garbei* Brazil, 26% of *S. garbei* Southwestern, and 13% of *S. garbei* Northwestern were followed by territorial calls.) Territorial calls also have two main frequency bands in which dominant frequency alternates along call emissions. Descriptive statistics are in Table 2.

Call characteristics	<b>Brazil</b> ( <i>N</i> = 5/93)	Northwestern $(N = 8/94)$	Southwestern $(N = 7/50)$
Duration (s)	$1.9 \pm 0.5 (0.3 - 3.0)$	$0.5 \pm 0.1 (0.3-0.7)$	$0.9 \pm 0.2 \ (0.3-1.9)$
Rate (calls/min)	15.5 ± 10.9 (2.1–27.1)	$46.4 \pm 19.5 \ (9.9-69.1)$	$22.1 \pm 9.9 (9.4 - 38.7)$
Interval (s)	$4.0 \pm 6.4 \; (0.469.7)$	$1.5 \pm 2.0 \ (0.2 - 30.0)$	$2.3 \pm 2.0 \ (0.5-7.8)$
1 <sup>st</sup> pulse duration (ms)	$5.3 \pm 1.0 \ (2.0 - 11.0)$	$6.3 \pm 1.7 (3.0 - 17.0)$	$7.4 \pm 1.3 (3.0 - 12.0)$
Mid-portion pulses duration (ms)	$7.8 \pm 0.8 (5.0 - 11.0)$	$7.4 \pm 1.6 \ (4.0-12.0)$	$7.5 \pm 1.1 \ (4.0 - 11.0)$
Final pulse duration (ms)	$10.1 \pm 0.7 (6.0 - 15.0)$	$9.6 \pm 1.8 (5.0 - 20.0)$	$10.6 \pm 2.7 (5.0 - 14.0)$
1 <sup>st</sup> pulse interval (ms)	4.1 ± 1.3 (1.0–19.0)	5.7 ± 1.2 (2.0–17.0)	$7.8 \pm 2.2 (3.0-22.0)$
Mid-portion pulse interval (ms)	$6.4 \pm 0.5 (5.0 - 9.0)$	$4.3 \pm 0.9 (1.0 - 8.0)$	$8.1 \pm 0.9 (5.0 - 11.0)$
Final pulse interval (ms)	$7.0 \pm 0.6 \ (4.0-10.0)$	$4.8 \pm 1.2 \ (2.0 - 9.0)$	$8.6 \pm 1.3 (5.0-14.0)$
Pulse rate (pulses/s)	$72.7 \pm 2.1 \ (69.0-76.9)$	88.5 ± 11.7 (74.5–108.7)	$66.0 \pm 5.4 (54.9 - 72.2)$
Minimum frequency (Hz)	843 ± 59 (750–938)	1304 ± 48 (1219–1406)	1423 ± 131 (1292–1723)
Maximum frequency (Hz)	3289 ± 228 (2813–3703)	3700 ± 245 (3230–4313)	4165 ± 393 (3618–4996)
Dominant frequency of LFB (Hz)	993 ± 22 (938–1219)	1590 ± 85 (1464–1781)	1644 ± 99 (1421–1781)
Dominant frequency of HFB (Hz)	2903 ± 125 (2627–3188)	3355 ± 284 (2756–4031)	3475 ± 165 (3058–3876)

 Table 1.
 Advertisement call traits of the three groups of *Scinax garbei* recognized here. N = number of individuals/ number of calls analyzed. Values are given as mean ± SD (minimum–maximum).

# Statistical Comparisons

Supervised analysis of RF (Figure 5) of the main groups resulted in 0% error rate-i.e., recordings from all populations were recovered in their own groups. Scinax garbei Northwestern is completely discriminated from S. garbei Brazil along both axes; it is distinguished from S. garbei Southwestern along the first axis and slightly separate along the second axis. Scinax garbei Brazil and S. garbei Southwestern are completely separated along the second axis and overlap along the first axis. Likewise, DAPC analysis resulted in all individuals reassigned to their original clusters. In this discriminant analysis, S. garbei Northwestern and Brazil overlap along the second axis but are completely separated along the first axis; S. garbei Southwestern is separated from the other groups along both axes.

Six acoustic traits indicated by the multivariate analyses as important for discrimination have statistically significant differences (Figure 6; Table 3). Scinax garbei Brazil is diagnosed from S. garbei Southwestern by its longer call and lower dominant frequencies to both LFB and HFB. Scinax garbei Brazil is diagnosed from S. garbei Northwestern by its longer call, longer pulse intervals in the middle and final parts of the call, and by its lower pulse rate and dominant frequency of LFB. Scinax garbei Southwestern is diagnosed from S. garbei Northwestern by its longer pulse intervals in the middle and final parts of the call, and lower pulse rate.

# Discussion

Published descriptions of the advertisement call of *Scinax garbei* are somewhat discordant.

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Figure 2. Audiospectrograms and respective oscillograms of the advertisement calls of *Scinax garbei* Brazil (A), Northwestern (B) and Southwestern (C). Sound files: *Scinax\_garbei*AltaFlorestaMT1bPM\_AAGm671 (A); 7807 (FonoZoo) (B); Track 40 (CD Frogs of Tambopata) (C). Further recordings details are in Appendix I.


Figure 3. Audiospectrograms and respective oscillograms of 100-ms sections extracted from the midpoint of each call depicted in Figure 2, detailing pulses shape: *Scinax garbei* Brazil (A), Northwestern (B) and Southwestern (C).

Call characteristics	<b>Brazil</b> ( <i>N</i> = 5/56)	Northwestern $(N = 6/17)$	Southwestern $(N = 4/14)$
Duration (s)	$0.045 \pm 0.008$	0.038 ± 0.026	0.057 ± 0.020
	(0.033-0.059)	(0.013–0.102)	(0.020–0.105)
Interval after advertisement call (s)	$0.031 \pm 0.004$	$0.031 \pm 0.010$	$0.017 \pm 0.010$
	(0.022-0.048)	(0.003-0.076)	(0.003-0.032)
Minimum frequency (Hz)	903 ± 154	1326 ± 76	1484 ± 118
	(750–1359)	(1219–1500)	(1335–1688)
Maximum frequency (Hz)	3385 ± 299	3879 ± 350	3967 ± 532
	(3000–4091)	(3469–4500)	(3445–5063)
Dominant frequency of LFB (Hz)	1050 ± 190	1631 ± 79	1693 ± 195
	(818–1723)	(1378–1938)	(1464–2063)
Dominant frequency of HFB (Hz)	2846 ± 109	3529 ± 351	3345 ± 147
	(2578–3058)	(3058–4125)	(2842–3656)

Table 2.	Territorial call traits of the three groups of <i>Scinax garbei</i> recognized here. <i>N</i> = number of individuals/numbe
	of calls analyzed. Values are given as mean ± SD (minimum–maximum).



Figure 4. Audiospectrograms and respective oscillograms of the territorial call of *Scinax garbei* Brazil (A), Southwestern (B) and Northwestern (C). Territorial call preceded by an advertisement call (D), and three territorial calls emitted alone (E). Aggressive interaction between two individuals, with the foreground male emitting territorial calls in response to the background one (F). Red arrows indicate territorial calls. Sound files: *Scinax\_garbei*AltaFlorestaMT1bPM\_AAGm671 (A, D, E); 7679 (FonoZoo) (B); 7806 (FonoZoo) (C); 7808 (FonoZoo) (F). Further recordings details are in Appendix I.

**Table 3.** Pairwise comparisons between the three groups of *Scinax garbei* recognized here, regarding statistical significance (Wilcoxon Mann Whitney Rank Sum Test) of call traits indicated as having higher loadings (RF and DAPC) for discrimination among groups. Values are significant when p < 0.05.

Trait / Pair comparison	Brazil vs.	Brazil vs.	Southwestern vs.
	Southwestern	Northwestern	Northwestern
Call duration	Z = 2.8723	Z = 2.9646	Z = -3.1202
	p < 0.007	<i>p</i> < 0.007	p < 0.006
Mid-portion pulse interval	Z = -2.6029	Z = 2.9277	Z = -3.2404
	p < 0.01	p < 0.007	p < 0.004
Pulse rate	Z = 2.3548	Z = -2.9277	Z = 3.2404
	p < 0.02	p < 0.007	p < 0.004
Final portion pulse interval	Z = -2.1962	Z = 2.9358	Z = -3.2433
	p < 0.03	p < 0.007	p < 0.004
Dominant frequency of the LFB	Z = -2.842	Z = -2.9358	Z = -1.1593
	p < 0.01	p < 0.01	p > 0.2
Dominant frequency of the HFB	Z = -2.842	Z = -2.7813	Z = -1.273
	p < 0.02	p < 0.02	p > 0.2

#### Brazil • Northwestern • Southwestern



Figure 5. Plots of the two first axes of a Multidimensional Scaling (MDS) on the Random Forest result for advertisement call data of the three groups of *Scinax garbei* recognized here.



Figure 6. Boxplots of the most important acoustic traits to discrimination among the three groups of *Scinax garbei* recognized here. See Table 3 for statistical significance values of each trait.

Duellman (1970) first described the advertisement call of the species (as Hyla garbei) from Santa Cecilia (Ecuador); these call values appeared again in Duellman (1972, 1978) and Duellman and Wiens (1993). However, in Duellman and Pyles' (1983) description of the call from Santa Cecilia, there are substantial differences in the values for call rate, pulse rate, and fundamental and dominant frequencies relative to previous studies. Duellman (1972) also reported on the call of a population from Pilcopata (Peru) (as Hyla epacrorhina), and Duellman and Wiens (1993) reported on the call of a population from Tambopata (Peru). The calls of these Peruvian populations resemble one another (except for differences in pulse rate), and differ in call duration, pulse rate, and dominant frequency from the calls of the Ecuadorian populations. In addition, Zimmerman (1983) reported values for pulse rate and dominant frequency for a frog from Manaus (Brazil) that differ from previous works. De la Riva et al. (1994) reported on the advertisement call from Puerto Almacén (Bolivia), and pointed out that their data are substantially different from those of Ecuadorian populations regarding call duration, call rate, pulse rate and dominant frequency. The call data for frogs from Puerto Almacén do not match those for frogs from Brazil, but are quite similar to those reported for Peruvian populations (except for some differences in pulse rate). Duellman (2005) provided some call values for a frog population at the Reserva Cusco Amazónico (Peru); these are mostly consistent with data reported for the Bolivian and other Peruvian populations.

The call values we obtained in our reanalyses of recordings from Pilcopata, Puerto Almacén, and Santa Cecilia, for the most part match the values provided by previous studies, except for the strikingly higher pulse rate (195– 240 pulses/s *vs.* 76–103 pulses/s in our study) and the strikingly lower fundamental frequency (391–482 Hz *vs.* 1500–1781 Hz of LFB in our study) respectively found by Duellman (1970) and Duellman and Pyles (1983), for the population from Santa Cecilia. Our call values for the population from Manaus differ from those reported by Zimmerman (1983) in dominant frequency (2350 Hz *vs.* 1007 Hz of LFB and 2894 Hz of HFB in our study) and pulse rate (100 pulses/s *vs.* 75 pulses/s in our study). Also, our call values for the population from Tambopata differ from those reported by Duellman and Wiens (1993) in pulse rate (110 pulses/s *vs.* 63 pulses/s in our study). Most of these discrepancies may reflect the technology employed in call recordings and analyses.

Our multivariate analyses separate the populations of S. garbei into three main groups with distinct advertisement calls. Scinax garbei Southwestern and S. garbei Northwestern are diagnosed from each other only by temporal call traits, whereas S. garbei Brazil is diagnosed from the other two groups by both temporal and spectral call traits. Besides quantitative call differences, the call of S. garbei Northwestern has temporal and spectral structures quite distinct from those of the other groups (Figure 2). Although there are statistically significant differences in both minimum and maximum frequencies of S. garbei Brazil in relation to the other groups, we do not consider these traits as diagnostic because the calls may have been affected by the normalization (Materials and Methods).

Although Duellman (1972) reported that the advertisement call of Scinax garbei from Pilcopata was followed by one or two secondary notes, we concluded that these notes are territorial calls. The population from Alta Floresta had the highest emission rate of territorial calls; this can be explained by the high density of males in close-range interactions (Allan 1973, Toledo et al. 2015). Despite being alone, the individual from Manaus also emitted territorial calls, although at a lower rate (as occurs in other species of Scinax; pers. obs. of the authors). Territorial calls of the different groups share a somewhat similar envelope shape, but vary in duration, amplitude modulation levels, and values of spectral traits.

Although variation in advertisement calls might occur intra-specifically (e.g., Castellano et al. 2002, Heyer and Reid 2003, Smith et al. 2003, Velásquez et al. 2013, Forti et al. 2017, Zaracho et al. 2018), the call divergences we report exceed those usually reported for intraspecific variation. These call differences, along with previous studies reporting on morphological (e.g., Duellman 1972, Heyer 1977, Duellman and Wiens 1993) and molecular (Jansen et al. 2011, Ron et al. 2018) divergences in this species, provide strong evidence that S. garbei may represent a complex of up to three species. Thus, this may be another case of what was assumed to be a widely distributed species representing a species complex, as has been recently demonstrated for other Amazonian frog and salamander species (e.g., Jungfer et al. 2010, Simões et al. 2010, Caminer and Ron 2014, Gehara et al. 2014. Caminer et al. 2017. Rivadeneira et al. 2018, Jaramillo et al. 2020).

If more than one species exists within Scinax garbei, there are two names available for two of the populations sampled herein. One is Hyla lutzi, described by Melin (1941) based on two specimens from Manaus and "Rio Uaupés" (São Gabriel da Cachoeira), state of Amazonas, Brazil. After examining the holotype of S. garbei and syntypes of *H. lutzi*, together with a series of specimens from the upper Amazon Basin, Duellman (1970) concluded that despite a minor difference in size between Brazilian and Ecuadorian specimens all of them corresponded to a single taxon, and therefore synonymized H. lutzi with S. garbei. The other synonym of S. garbei is S. epacrorhina, described by Duellman (1972) from Pilcopata, Cusco Department, Peru. After examining the type series of S. epacrorhina and several specimens from Ecuador and Peru, Duellman and Wiens (1993) concluded that morphological and call differences between these species reflected geographic variation, and therefore synonymized it with S. garbei. Given our new data and the historical background, a taxonomic revision including specimens and DNA sequences from localities within the

regions sampled here, in addition to data from topotypes, is necessary to elucidate the taxonomic status of the different populations currently assigned to *S. garbei*.

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Appendix I. Sound files (.wav) analyzed in the ME67); mt (Microtrack II / Semtheis	present study and associated metadata. Equipmen er K6/ME67); WM-D6C (Sony WM-D6C / Semhei U4000 (Uher 4000 / Uher).	it (recorder / ser ME80);	microphone): n 41120 (Sanyo M	1671 (Marantz 671 / Sen) 1120 / Sennheiser ME80	ıheiser K6/ );
Recording label/code	Locality	Time (h)	Date	Air temperature (°C)	Equipment
Scinax_ garbeiAltaFlorestaMT2cDLB_ AAGm671	Alta Floresta, Mato Grosso, Brazil	19:13	11 Jan 2019	24.5	m671
Scinax_ garbeiAltaFlorestaMT3aDLB_ AAGm671	Alta Floresta, Mato Grosso, Brazil	19:20	11 Jan 2019	24.5	m671
Scinax_ garbeiAltaFlorestaMT3bDLB_ AAGm671	Alta Floresta, Mato Grosso, Brazil	19:21	11 Jan 2019	24.5	m671
Scinax_ garbeiAltaFlorestaMT1aPM_ AAGm671	Alta Floresta, Mato Grosso, Brazil	19:37	11 Jan 2019	24.5	m671
Scinax_ garbeiAltaFlorestaMT1bPM_ AAGm671	Alta Floresta, Mato Grosso, Brazil	19:44	11 Jan 2019	24.5	m671
Scinax_ garbeiAltaFlorestaMT2aPM_ AAGm671	Alta Floresta, Mato Grosso, Brazil	19:44	11 Jan 2019	24.5	m671
Scinax_ garbeiAltaFlorestaMT2cPM_ AAGm671	Alta Floresta, Mato Grosso, Brazil	19:46	11 Jan 2019	24.5	m671
Scinax_ garbeiManausAM2aTRCmt	Manaus, Amazonas, Brazil	18:07	24 Jan 2011	25	mt
Scinax_ garbeiManausAM2bTRCmt	Manaus, Amazonas, Brazil	18:11	24 Jan 2011	25	mt
Scinax_ garbeiManausAM2cTRCmt	Manaus, Amazonas, Brazil	18:14	24 Jan 2011	25	mt

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	Appendix I. Continued.				
Recording label/code	Locality	Time (h)	Date	Air temperature (°C)	Equipment
10516 (FonoZoo)	Puerto Almacén, Santa Cruz Department, Bolivia	ı	ı	·	WM-D6C or M1120
8391 (FonoZoo)	Heath River WildLife Center, Bolivian shore of the Heath River, Abel Iturralde Province, La Paz Department, Bolivia	21:30	12 Mar 2009	26	WM-D6C
8393 (FonoZoo)	Heath River WildLife Center, Bolivian shore of the Heath River, Abel Iturralde Province, La Paz Department, Bolivia	22:00	12 Mar 2009	24	WM-D6C
8367 (FonoZoo)	Leticia, Amazonas Department, Colombia	21:15	28 Dec 2009	24.8	WM-D6C
8308 (FonoZoo)	Pucaurquillo, Loreto Department, Peru	20:10	03 Dec 2009	25	WM-D6C
7679 (FonoZoo)	Pilcopata, Cusco Department, Peru	22:45	15 Jan 1971	23	U4000
7680 (FonoZoo)	Pilcopata, Cusco Department, Peru	22:55	15 Jan 1971	23	U4000
7681 (FonoZoo)	Pilcopata, Cusco Department, Peru	23:20	15 Jan 1971	23	U4000
7803 (FonoZoo)	Santa Cecilia, Sucumbíos Province, Ecuador	21:00	03 May 1969	26	U4000
7804 (FonoZoo)	Santa Cecilia, Sucumbíos Province, Ecuador	22:30	03 May 1969	25	U4000
7805 (FonoZoo)	Santa Cecilia, Sucumbíos Province, Ecuador	22:23	05 May 1969	23	U4000
7806 (FonoZoo)	Santa Cecilia, Sucumbíos Province, Ecuador	23:00	14 May 1969	23	U4000
7807 (FonoZoo)	Santa Cecilia, Sucumbíos Province, Ecuador	23:05	14 May 1969	23	U4000
7808 (FonoZoo)	Santa Cecilia, Sucumbíos Province, Ecuador	23:25	14 May 1969	23	U4000
Track 40 (CD Frogs of Tambopata)	Tambopata-Candamo National Park, Madre de Dios Department, Peru	ı		24	

Call characteristics	Bra	ızıl		Southw	estern			Northwestern	
	Alta Floresta, Brazil	Manaus, Brazil	Abel Iturralde, Bolivia	Puerto Almacén, Bolivia	Pilcopata, Peru	Tambopata, Peru	Santa Cecilia, Ecuador	Pucaurquillo, Peru	Leticia, Colombia
	(N = 4/81)	(N = 1/12)	(N = 2/7)	(N = 1/20)	(N = 3/7)	(N = 1/16)	(N = 6/77)	(N = 1/6)	(N = 1/11)
Duration (s)	$2.0 \pm 0.5$	$1.4 \pm 1.0$	$0.8 \pm 0.1$	$0.9 \pm 0.3$	$0.8 \pm 0.2$	$1.3 \pm 0.2$	$0.5 \pm 0.1$	$0.5 \pm 0.1$	$0.5 \pm 0.1$
	(0.4-3.0)	(0.3-2.6)	(0.6-1.1)	(0.3-1.5)	(0.6-1.9)	(0.7-1.7)	(0.4-0.7)	(0.3-0.6)	(0.3-0.7)
Rate (calls/min)	$18.9 \pm 9.2$ (6.3-27.1)	2.1	$30.0 \pm 12.2$ (21.4-38.7)	25.7	$10.5 \pm 1.6$ (9.4-11.6)	27.5	$48.9 \pm 27.0$ (9.9-69.1)	30.1	39.0
Interval (s)	$1.2 \pm 0.3$	$15.5 \pm 21.8$	$1.4 \pm 0.9$	$1.4 \pm 1.1$	$3.6 \pm 2.7$	$0.9 \pm 0.4$	$1.8 \pm 2.9$	$1.2 \pm 1.2$	$0.5 \pm 0.2$
	(0.4-2.0)	(4.2-69.6)	(0.7-3.4)	(0.6-5.0)	(0.5-7.8)	(0.5-1.9)	(0.2-30.1)	(0.4-3.0)	(0.3-0.8)
1st pulse duration (ms)	$5.3 \pm 1.1$	$5.2 \pm 2.6$	$7.6 \pm 2.3$	$8.4 \pm 1.7$	$6.7 \pm 0.9$	$8.2 \pm 1.8$	$6.3 \pm 2.0$	$5.8 \pm 1.2$	$6.4 \pm 1.9$
	(2.0-11.0)	(2.0-10.0)	(5.0-10.0)	(4.0-11.0)	(3.0-9.0)	(5.0-12.0)	(3.0-17.0)	(4.0-7.0)	(3.0-10.0)
Mid-portion pulse	$7.7 \pm 0.8$	$8.2 \pm 1.1$	$6.6 \pm 1.5$	$8.9 \pm 1.3$	$7.2 \pm 0.2$	$8.6 \pm 0.7$	$7.7 \pm 1.6$	$5.0 \pm 0.9$	$8.0 \pm 1.5$
duration (ms)	(5.0-10.0)	(6.0-11.0)	(4.0-8.0)	(7.0-11.0)	(6.0-10.0)	(7.0-9.0)	(5.0-12.0)	(4.0-6.0)	(5.0-10.0)
Final pulse duration (ms)	$9.9 \pm 0.5$	$11.2 \pm 1.9$	$8.4 \pm 1.3$	$9.1 \pm 2.2$	$13.0 \pm 2.4$	$9.4 \pm 1.7$	$9.8 \pm 1.9$	$7.8 \pm 2.6$	$10.5 \pm 2.7$
	(6.0-15.0)	(7.0-14.0)	(5.0-12.0)	(6.0-13.0)	(10.0-15.0)	(6.0-11.0)	(5.0-20.0)	(5.0-11.0)	(7.0-17.0)
1st pulse interval (ms)	$4.2 \pm 1.4$	$3.7 \pm 2.4$	$5.8 \pm 0.8$	$8.9 \pm 1.7$	$9.2 \pm 2.5$	$6.6 \pm 1.1$	$5.6 \pm 1.2$	$6.8 \pm 5.1$	$4.7 \pm 1.8$
	(1.0-19.0)	(1.0-9.0)	(3.0-9.0)	(5.0-13.0)	(6.0-22.0)	(5.0-9.0)	(2.0-16.0)	(4.0-17.0)	(2.0-8.0)
Mid-portion pulse	$6.4 \pm 0.6$	$6.0 \pm 0.6$	$7.6 \pm 1.3$	8.9 ± 1.2	$8.3 \pm 0.7$	$7.4 \pm 0.7$	$4.1 \pm 0.9$	$4.8 \pm 0.8$	$5.2 \pm 1.4$
interval (ms)	(5.0-9.0)	(5.0-7.0)	(6.0-11.0)	(7.0–11.0)	(5.0-9.0)	(7.0-9.0)	(1.0-7.0)	(4.0-6.0)	(3.0-8.0)
Final pulse interval	$7.2 \pm 0.5$	$6.2 \pm 1.2$	$8.2 \pm 2.6$	$9.8 \pm 2.5$	$8.8 \pm 0.8$	$7.9 \pm 0.9$	$4.4 \pm 1.1$	$5.7 \pm 1.6$	$6.1 \pm 1.8$
(ms)	(4.0-10.0)	(5.0-8.0)	(5.0-11.0)	(6.0-14.0)	(7.0-12.0)	(6.0-10.0)	(2.0-9.0)	(3.0-8.0)	(4.0-9.0)
Pulse rate (pulses/s)	$72.1 \pm 1.8$	$75.0 \pm 0.6$	$71.3 \pm 0.7$	$55.8 \pm 0.4$	$66.8 \pm 1.0$	$62.99 \pm 0.38$	$87.8 \pm 10.5$	$104.7 \pm 5.4$	$76.5 \pm 1.0$
	(69.0–76.9)	(74.5-76.1)	(69.0–72.2)	(54.9-56.2)	(65.2-68.2)	( $62.50-63.82$ )	(76.1–103.1)	(96.8-108.7)	(74.5-78.7)
Minimum frequency	825 ± 50	$914 \pm 24$	$1319 \pm 38$	$1652 \pm 51$	$1375 \pm 54$	$1545 \pm 15$	$1294 \pm 51$	$1328 \pm 50$	$1343 \pm 17$
(Hz)	(750–938)	(891–938)	(1292–1378)	(1550-1723)	(1313-1406)	( $1507 - 1550$ )	(1219-1406)	(1249–1378)	(1335–1378)
Maximum frequency	$3268 \pm 257$	$3371 \pm 120$	$3705 \pm 79$	4462 ± 247	$4281 \pm 384$	4436 ± 88	$3727 \pm 262$	$3445 \pm 177$	$3790 \pm 82$
(Hz)	(2813-3703)	(3188-3609)	(3618-3790)	(4221–4996)	(3938-4781)	(4264-4522)	(3375-4313)	(3230–3618)	(3618-3876)
Dominant frequency of	$989 \pm 23$	$1007 \pm 24$	$1513 \pm 69$	$1677 \pm 49$	$1719 \pm 31$	$1650 \pm 21$	$1616 \pm 84$	$1515 \pm 32$	$1515 \pm 26$
LFB (Hz)	(938-1219)	(984–1031)	(1421–1594)	(1594-1723)	(1688-1781)	( $1637 - 1680$ )	(1500-1781)	(1464-1550)	(1464-1550)
Dominant frequency of HFB (Hz)	$2905 \pm 145$	$2894 \pm 77$	$3286 \pm 63$	$3697 \pm 159$	$3521 \pm 126$	$3491 \pm 43$	$3367 \pm 311$	$3122 \pm 214$	$3512 \pm 91$
	(2627-3188)	(2718-2953)	(3058-3359)	(3488-3876)	(3281–3656)	(3445-3575)	(2906-4031)	(2756-3316)	( $3273-3618$ )

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# Causes of embryonic mortality in *Espadarana* prosoblepon (Anura: Centrolenidae) from Costa Rica

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### Abstract

Causes of embryonic mortality in Espadarana prosoblepon (Anura: Centrolenidae) from Costa Rica. Members of the family Centrolenidae-commonly known as "glass frogs"-exhibit arboreal egg-laying behavior, depositing their clutches on riparian vegetation. Few studies have investigated specific causes of mortality during embryonic stages, perhaps the most vulnerable stage during the anuran life cycle. The Emerald Glass Frog, Espadarana prosoblepon, was used as a case study to investigate the causes of embryonic mortality in a species with short-term (i.e., less than 1 day) parental care. The specific sources of mortality of eggs of *E. prosoblepon* were quantified and overall rates of survival (hatching success) were estimated. Nineteen egg clutches were transferred from permanent outside enclosures to the wild. Clutch development was monitored daily until hatching; five mortality causes were quantified: desiccation, failure to develop, fungal infection, predation, and "rain-stripped." The main causes of mortality were predation (often by katydids and wasps) and embryos stripped from the leaf during heavy rains. The results were compared to those of previous studies of centrolenids exhibiting parental care, and discussed in the context of the importance of the natural history data for these frogs with regard to understanding the evolutionary history of parental care in glass frogs.

Keywords: Egg clutch, Emerald Glass Frog, hatching success, predation.

#### Resumo

Causas de mortalidade embrionária em *Espadarana prosoblepon* (Anura: Centrolenidae) da Costa Rica. Membros da família Centrolenidae—comumente conhecidos como "pererecas-devidro"—exibem o comportamento arborícola de postura de ovos, depositando suas desovas na vegetação ripária. Poucos estudos investigaram causas específicas de mortalidade durante estágios embrionários, talvez o estágio mais vulnerável durante o ciclo de vida dos anuros. A perereca-devidro-esmeralda, *Espadarana prosoblepon*, foi usada em um estudo de caso para investigar as causas

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da mortalidade embrionária em uma espécie com curto período de cuidado parental (menos de 1 dia). As causas específicas de mortalidade de ovos de *E. prosoblepon* foram quantificadas e as taxas gerais de sobrevivência (sucesso de incubação) foram estimadas. Dezenove desovas foram transferidas de recintos externos permanentes para a natureza. O desenvolvimento da desova foi monitorado diariamente até a eclosão; foram quantificadas cinco causas de mortalidade: dessecamento, falha no desenvolvimento, infecção fúngica, predação e retirada pela chuva. As principais causas de mortalidade foram a predação (geralmente por gafanhotos e vespas) e embriões arrancados da folha durante fortes chuvas. Os resultados foram comparados com os de estudos anteriores de centrolenídeos que exibem cuidados parentais e discutidos no contexto da importância dos dados da história natural para essas pererecas no que diz respeito à compreensão da história evolutiva do cuidado parental em pererecas-de-vidro.

Palavras-chave: desova, perereca-de-vidro-esmeralda, predação, sucesso de eclosão.

# Introduction

Some amphibian species have evolved nonaquatic oviposition (terrestrial, arboreal; reviewed in Wells 2007). It is thought that laying eggs in terrestrial environments evolved as a result of the selective pressure of aquatic predators (Magnusson and Hero 1991). Clutches laid in terrestrial or arboreal environments benefit from increased oxygen exchange and the "release" from aquatic predators (Duellman and Trueb 1986); however, other risks, such as desiccation, predation, and fungal infection, arise during this vulnerable life stage (Vonesh 2000, Warkentin 2000, Warkentin et al. 2001, Touchon and Warkentin 2009).

Members of the glass frog family (Anura: Centrolenidae) deposit clutches out of the water, on surfaces of leaves or other vegetation, overhanging streams. Centrolenids are particularly interesting, because in some species, parental care involves egg attendance (McDiarmid 1978, Guayasamin et al. 2009). However, the breeding and parental care behavior of only a handful of the 157 species (12 genera; Frost 2019) has been studied in detail. Much remains to be learned about other aspects of centrolenid reproductive ecology and the natural history of most species of glass frogs (Cisneros-Heredia and McDiarmid 2007, Delia et al. 2010). Thus, comparative studies to explore

the evolution of these behaviors are challenging (but see Delia *et al.* 2017) and additional studies are needed to provide a broader understanding of extrinsic factors that may have favored behavioral strategies to maximize embryonic and larval survival.

Embryonic stages are vulnerable and those that develop out of the water, face specific risks (Warkentin 2011). Several studies of the reproductive ecology and parental care behavior in glass frogs have assessed specific causes of embryonic mortality (Vockenhuber et al. 2009, Delia et al. 2013, 2019, Lehtinen et al. 2014, Bravo-Valencia and Delia 2016, Hughey et al. 2017, Salgado and Guayasamin 2018). But few have focused on the causes of embryonic mortality in species with short-term (i.e., less than 1 day) parental care, and embryonic mortality sources have been identified in only two taxa—Teratohyla pulverata (Peters, 1873) and Cochranella granulosa (Taylor, 1949)-(Hawley 2006, Delia et al. 2017). We investigated the causes of embryonic mortality in the Emerald Glass Frog, Espadarana prosoblepon (Boettger, 1892), a species in which the female remains with the clutch fewer than 3 hr after oviposition (Jacobson 1985).

*Espadarana prosoblepon* occurs in Central and South America from Honduras to Ecuador (Savage 2002). Males have humeral "spines" on their forearms, and use these spines in territorial combats (Figure 1). Females lack humeral spines and are slightly larger than males (Hedman and Hughey 2014). Males call from the upper surfaces of leaves and other vegetation near small streams to attract females. After amplexus, females lay their eggs on the upper surfaces of leaves, moss-covered rocks, tree trunks, or branches (Jacobson 1985).

Despite its wide geographic range, relatively little is known about the reproductive biology of Espadarana prosoblepon—especially its parental care behavior and other aspects of its embryonic development. Jacobson (1985) studied а population from Monteverde (northwestern Costa Rica) and described key aspects of its mating behavior and reproductive biology, including clutch size, male reproductive success, and amplexus behavior. Jacobson reported that about half of the observed clutches hatched successfully, with "success" defined as more than 70% of the embryos hatching; however, the sources of embryonic mortality were not described in detail. Research exploring other aspects of the natural history of this species has increased recently and include experimental studies on the use of humeral spines and malemale aggressive behavior (Hedman and Hughey 2014, Krohn and Voyles 2014), estimates of survival and abundance (McCaffery and Lips 2013), and population declines after the arrival of the amphibian killing fungus Batrachochytrium dendrobatidis Longcore, Pessier, and D.K. Nichols, 1999 (Angeli et al. 2015). Basto-Riascos et al. (2017a) reported several aspects of the reproductive biology of E. prosoblepon in a population from the Andes of Colombia, including oviposition-site use and mating patterns. Additionally, a few natural history observations on fungal infections (Guayara-Barragán et al. 2010) and predation of embryos by spiders (Basto-Riascos et al. 2017b) have been published. However, there are no studies investigating embryonic survival or specific causes of embryonic mortality other than these opportunistic accounts.



Figure 1. Sexual dimorphism in *Espadarana prosoblepon.*(A) Males have noticeable humeral spines on their forearms (white arrow). (B) Females lack humeral spines. Photo: JGV.

# **Materials and Methods**

# Study Area and Fieldwork

This study took place between 11 June and 12 July 2018, at Las Cruces Biological Station, San Vito, Puntarenas Province, Costa Rica (8.7858° N, 82.9589° W). Every night, we searched for pairs of *Espadarana prosoblepon* in amplexus, starting at 20:00 h (Figure 2A). The study area was about 500 m<sup>2</sup>, surrounding a 60-m segment of a small stream ("Culvert Creek") at the field station. When an amplectant pair was found, we transferred them (handling the pair carefully, to avoid dislodging the male) to a permanent enclosure that was located adjacent to the stream, thereby ensuring natural

temperature and humidity conditions. The enclosure had four partitions adjacent to each other, consisting of  $38 \times 50 \times 75$ -cm chambers, surrounded by green wire mesh (Figure 2B). Each partition was provided with leaf litter for refuge during the day, a water bowl, and a pinna (single leaflet) of a fern, Angiopteris evecta (G. Forst.) Hoffm., hung from the corner; the fern is an oviposition substrate observed in the field (JGV, pers. obs.). We controlled for one potential source of variation that might affect hatching success by providing identical oviposition sites. Pairs were left overnight and the following morning, we checked whether oviposition had occurred (Figure 2C). After successful oviposition, both males and females were measured (snout-vent length in mm), toeclipped, and photographed for individual identification, and then released where they had been captured.

# Embryonic Mortality Causes and Hatching Success

To evaluate the sources of embryonic mortality, we transferred clutches obtained in captivity to sites where natural clutches had been observed during the weeks prior to the start of this study (JGV, pers. obs.). Pairs of *Espadarana prosoblepon* deposited clutches on the surface of individual pinnules of *Angiopteris evecta* provided inside the enclosures. Each clutch was transferred along with the pinnule and secured to a natural substrate (overhanging vegetation) with a cable tie (Figure 3D). We marked the location of the clutch with flagging tape, counted the number of eggs, and recorded the height at which each clutch was placed above water; all clutches were separated by at least 1 m (Figure 2E).

We monitored all clutches three times per day, and for each observation, we counted the total number of eggs (to determine the mean percentage of clutch mortality), noted developmental stage, and identified sources of embryonic mortality. Based on previously published descriptions (McDiarmid 1978, Warkentin 2000, Hawley 2006), five categories of mortality were recognized: (1) unfertilized eggs or embryos that failed to develop, with eggs turning white and disintegrating, or embryos that cease development before or at Gosner Stage 18 (Gosner 1960); (2) fungal infection, with eggs turning cloudy and hyphae apparent on the surface; (3) desiccation, with eggs failing to develop and having almost no jelly around them; (4) drowning or "rain-stripped," with the entire clutch disappearing after periods of heavy precipitation; and (5) predation, with eggs or late-stage embryos disappearing, especially around the edges of the clutch with some embryos remaining in the center, or by "empty" gelatinous masses before hatching occurred. Clutches were monitored until all surviving embryos hatched into a plastic cup placed each clutch (Hayes 1983). beneath We determined embryonic hatching success as the number of hatched tadpoles relative to the initial number of eggs per clutch. Also, we calculated the mean percentage of clutch mortality for each of the identified sources of mortality.

# Results

We monitored 19 clutches in this study. The average clutch size was  $25 \pm 6$  SD eggs (range = 15-41). Clutches were relocated at an average height of  $0.98 \pm 0.24$  m (range = 0.55-1.45). The average embryonic hatching success per clutch was 14.5% (± 26.3 SD, range = 0–84.2%), with only two clutches having hatching success greater than 70%. We identified five causes of embryo mortality: desiccation (1 of 19 clutches); failure to develop or failure to fertilize the eggs (14 of 19 clutches); fungal infection (2 of 19 clutches); predation (9 of 19 clutches); and loss of egg clutches in heavy rain (10 of 19 clutches). The mean percentages of clutch mortality attributed to different mortality causes are shown in Figure 3. Predation caused the most clutch mortality (40.1%, Figure 4), followed by rainstripped embryos (31.2%), and failure to develop (12.4%). We observed wasps (Polibya sp.), and a katydid (Copiphora cultricornis Pictet, 1888)



Figure 2. Transfer of egg clutches of *Espadarana prosoblepon*. (A) A pair of *E. prosoblepon* in amplexus. (B) Pairs were captured and placed in an enclosure and left overnight. (C, D) After successful oviposition, the leaflets with the clutch were cut and transferred to a natural substrate. (E) Clutch location was marked with flagging tape and included clutch number, clutch size, and day of oviposition.

preying upon embryos on two and one occasions, respectively. In most of the clutches, up to 53.8% of embryos failed to develop. In contrast, predation and heavy rain caused the loss of more than 75% of the embryos in at least eight (42% of clutches) and five (26% of clutches) of the clutches, respectively.

#### Discussion

# Clutch Size and Hatching Success in Espadarana prosoblepon

The clutch sizes in *Espadarana prosoblepon* are similar to those previously reported for the Monteverde population (Jacobson 1985). However, a study conducted in the Colombian Andes reported an average clutch size of  $35.4 \pm 4.8 \text{ eggs}$  (range = 30-50, N = 29; Basto-Riascos *et al.* 2017a), which is substantially greater than those we observed.

Embryonic hatching success was low (14%) relative to previously reported hatching success rates in other centrolenid species. In Teratohyla pulverata, a species with short-term parental care, embryonic survivorship is high (85%) with low levels of predation and with most clutches suffering from developmental abnormalities (Hawley 2006). In a Mexican population of Hyalinobatrachium fleischmanni (Boettger, 1893), a species in which males brood the eggs, the average embryonic survival is 90%, with scarcely any predation occurring (Delia et al. 2013). The wet season at our study site in Puntarenas, Costa Rica, extends from May-November, with a short drought called "El veranillo de San Juan" lasting about 10 days in mid-June. Most of the monitored clutches of E. prosoblepon from Monteverde were laid after mid-August, with estimates of embryonic survival of 47% (Jacobson 1985). As the wet season progresses, it is possible that embryonic survival in E. prosoblepon increases. Also, we should point out that given changes in temperature and precipitation patterns with climate change (Blaustein et al. 2010),



**Figure 3.** Mean percentage ± SE of egg/embryo mortality in clutches of *E. prosoblepon* resulting from desiccation, failure to develop, fungal infection, predation, and rain-stripping.

centrolenid populations may experience longterm declines as a result of desiccation of egg clutches during dry periods or rain-stripping of the clutches during periods of unusually heavy rain (e.g., El Niño years) (Pounds *et al.* 1999).

# Causes of Embryonic Mortality in Espadarana prosoblepon

The main causes of embryonic mortality in Espadarana prosoblepon were predation and rain stripping of clutches. Removal experiments comparing survival and hatching success between attended and unattended clutches in centrolenids have shown that egg attendance reduces dessication and sometimes provided protection against predators. (Vockenhuber et al. 2009, Delia et al. 2013, 2017, Lehtinen et al. 2014, Bravo-Valencia and Delia 2016, Hughey et al. 2017, Salgado and Guayasamin 2018). In only one case, Hyalinobatrachium valerioi (Dunn, 1931), was predation the main cause of mortality in both attended and unattended clutches (Vockenhuber et al. 2009). These observations suggest that the risk of desiccation may be a stronger driver of the evolution of egg-brooding behavior than the risk of predation.



Figure 4. A katydid *Copiphora cultricornis* (A) and a wasp *Polybia* sp. (B) consuming embryos of *Espadarana* prosoblepon. Photos: JGV.

Future studies should investigate whether predation rates change throughout the breeding season in *E. prosoblepon.* For example, in *Hyalinobatrachium orientale* (Rivero, 1968), predation is more prevalent in unattended clutches during the wet season than during the dry season (Lehtinen *et al.* 2014). Rain-stripping does not seem to be a primary source of embryonic mortality in other centrolenid species as it is in *E. prosoblepon*, in which "clutch drowning" affected more than half of the clutches. In most species for which rain-stripping has been reported as a source of embryonic mortality, less than 10% of the embryos were lost to heavy rains (Delia *et al.* 2013, 2017, Bravo-Valencia and Delia 2016, Hughey *et al.* 2017).

Environmental conditions (i.e., humidity, temperature, and rainfall) in our mesh enclosure were the same as in the surrounding area. Height and substrate of oviposition sites are known to influence embryonic survival in *E. prosoblepon* (Jacobson 1985). By controlling for both of these factors, we attempted to reduce variation in mortality causes resulting from properties of the oviposition site itself, such as antimicrobial properties of the leaf, as suggested by Basto-Riascos *et al.* (2017a) or the amount of water held by the substrate (e.g., moss vs. leaf surface). To the best of our knowledge, no studies have

investigated the adaptive benefits of ovipositionsite choice in centrolenids. Additional studies are necessary to determine whether oviposition-site preferences counteract the effect of predation, and whether behavioral strategies (e.g., laying eggs in hidden places sheltered from heavy rains and predators) decrease mortality risks. Future studies should combine experimental approaches with natural history observations, to test whether the choice of oviposition sites has an impact on embryonic survival.

We have reported a high percentage of developmental abnormalities or unfertilized eggs in contrast to the results of other studies in which these sources of mortality accounted for less than 5% of embryo deaths (Delia et al. 2013, 2017, Lehtinen et al. 2014, Bravo-Valencia and Delia 2016, Hughey et al. 2017). Possibly, the process of moving pairs into captivity during amplexus and oviposition disturbed the frogs such that the males did not fertilize all the eggs in the clutch. As an alternative, it is possible that this is a characteristic of this species, as reports of E. prosoblepon brooding behavior in the wild indicate that in more than half of the observed oviposition events, females only remained in partial contact with their clutches and do not exhibit the "egg rotation" movements observed in other species with parental care, a behavior that may aid in egg fertilization (Jacobson 1985). Further studies are needed to confirm or reject this observation. However, it is important to note that, the actual percentage of developmental abnormalities in the wild may be difficult to determine given that other sources of mortality (i.e., fungal infections, predation) take place before failure to develop is detected (Bravo-Valencia and Delia 2016); therefore, natural observations may underestimate the ocurrence of this source of mortality in species with shortterm care such as E. prosoblepon.

Our results contribute information about the natural history of *Espadarana prosoblepon* in a population from southwestern Costa Rica, contributing to the existing literature about the reproductive biology of this species (Jacobson

1985, Basto-Riascos *et al.* 2017a). We provide information about specific sources of embryo mortality, types of predators, and differences in clutch size, contributing useful data for future comparative studies of the reproductive and behavioral ecology of this species. Establishing the primary differences in the causes of mortality among species with prolonged parental care, short-term parental care, and without parental care, will facilitate future comprehensive studies within Centrolenidae and may elucidate the ecological factors involved in the evolution of male and female parental care behavior in glass frogs.

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# Behavioral responses of tadpoles of *Duttaphrynus melanostictus* (Anura: Bufonidae) to cues of starved and fed dragonfly larvae

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# Abstract

Behavioral responses of tadpoles of *Duttaphrynus melanostictus* (Anura: Bufonidae) to cues of starved and fed dragonfly larvae. Tadpoles of *Duttaphrynus melanostictus* use chemoreception to detect kairomonal cues and excretory metabolites from predatory anuran tadpoles (*Hoplobatrachus tigerinus*) that consume them. We describe here the behavioral responses of tadpoles of *D. melanostictus* to predatory dragonfly larvae (*Pantala flavescens*). The predator's kairomones (water conditioned by the starved predator) or its diet-derived metabolites released in excreta of predator after consumption of conspecific prey tadpoles were used to simulate predation risk. The tadpoles of *D. melanostictus* had no behavioral response to predator kairomones. However, the larvae reduced swimming movements and overall time spent in swimming, and had a higher burst speed/swimming velocity in response to water borne cues released from the excreta of predators fed conspecific prey. Thus, just the presence of dragonfly larvae does not elicit defense behaviors in tadpoles of *D. melanostictus*, but when predation risk is recognized as real (i.e., when tadpoles are exposed to excretory metabolites of predators fed conspecific tadpoles), defense behaviors are activated.

**Keywords:** Defense behavior, dietary cues, kairomones, *Pantala flavescens*, predatorprey interactions, tadpoles, Wandering Glider.

#### Resumo

Respostas comportamentais de girinos de *Duttaphrynus melanostictus* (Anura: Bufonidae) a sinais de larvas de libélula famintas e alimentadas. Os girinos de *Duttaphrynus melanostictus* usam a quimiorrecepção para detectar sinais cairomonais e metabólitos excretados por girinos predadores (*Hoplobatrachus tigerinus*) que os consomem. Descrevemos aqui as respostas comportamentais de girinos de *D. melanostictus* a larvas de libélulas predadoras (*Pantala flavescens*). Os cairomônios do predador (água condicionada pelo predador faminto) ou seus metabólitos derivados da dieta liberados nos excretas do predador após o consumo de girinos coespecíficos foram utilizados para simular o risco de predação. Os girinos de *D. melanostictus* não apresentaram resposta

Received 02 August 2019 Accepted 06 March 2020 Distributed June 2020 comportamental aos cairomônios dos predadores. Contudo, as larvas reduziram os movimentos de natação e o tempo total gasto na natação e apresentaram uma maior velocidade de partida e de natação em resposta a estímulos transmitidos pela água liberados dos excretas de predadores que se alimentaram de presas coespecíficas. Assim, apenas a presença de larvas de libélula não elicia comportamentos de defesa nos girinos de *D. melanostictus*, mas os comportamentos de defesa são ativados quando o risco de predação é reconhecido como real (isto é, quando os girinos são expostos a metabólitos excretados pelos predadores alimentados com girinos coespecíficos).

**Palavras-chave:** cairomônios, comportamento de defesa, estímulos alimentares, girinos, interações predador-presa, *Pantala flavescens*, planador-errante.

# Introduction

In nature, predation is a significant selective force acting on prey that drives the evolution of strategies for assessment of predation risk and the development of antipredator defense strategies in order to optimize survival and fitness. Detection of predators before an encounter can give prey the opportunity to respond behaviorally and reduce the probability of being detected, and hence eaten (Lima and Dill 1990). However, inducing anticipatory antipredator behavior has direct energetic costs, as well as costs associated with reduced opportunity to feed, that affect growth and development of tadpoles (Lima and Dill 1990, Ferrari et al. 2010). Aquatic anuran tadpoles predation risk using chemosensory assess mechanisms before responding with defense behaviors (Saidapur et al. 2009, Ferrari et al. 2010, Mogali et al. 2012, 2015). Specifically, they perceive alarm cues released by injured prey, kairomones (chemicals originating from the body of predators), and dietary cues (excretory metabolites or substances of predators fed conspecific/heterospecific prey) to alter behavior to escape predation (Wisenden 2000, Hagman 2008, Schoeppner and Relyea 2009a, Mogali et al. 2011, Carlson et al. 2015, Scherer and Smee 2016).

In southern India, most anurans including the common toad, *Duttaphrynus melanostictus* (Schneider, 1799), breed in ephemeral ponds following south-western monsoon rains. This leads to the larvae of several species coexisting sympatrically (Saidapur 2001). Some of these

tadpoles are carnivorous and even cannibalistic. For example, tadpoles of the Indian bullfrog, Hoplobatrachus tigerinus (Daudin, 1802), are voracious predators that hunt actively and also are cannibalistic (Saidapur 2001, Rajput et al. 2011). The ponds in this region also harbor many predatory aquatic insects such as water beetles, water scorpions, and dragonfly larvae. The larvae of the dragonfly Pantala flavescens (Odonata: Libellulidae) are "sit-and-wait" predators that move slowly and wait for the prey to approach before attacking. We have shown that toad tadpoles detect kairomones of tadpoles of H. tigerinus, as well as dietary metabolites or substances from a predator that consumed toad tadpoles (Saidapur et al. 2009, Mogali et al. 2011). Herein, we describe antipredator behavioral responses of tadpoles of D. melanostictus in the presence of dragonfly larvae.

# **Materials and Methods**

Egg strings (N = 4) of *Duttaphrynus* melanostictus were collected from temporary ponds in and around the Karnatak University Campus, Dharwad, India (15.44° N, 74.98° E), in June 2010. In the laboratory, they were placed in separate plastic tubs (42 cm diameter and 16 cm depth) containing 10 L of aged tap water. All eggs hatched almost simultaneously at Gosner stage 19 (Gosner 1960) a day after their collection. Subsequently, hatchlings of the different clutches were mixed (N = 100 per clutch × 4 clutches = 400 hatchlings) and reared in a glass aquarium (75 L × 45 W × 15 H cm)

with 15 L of aged tap water. From this stock, tadpoles of similar size  $(16.28 \pm 0.80 \text{ mm total})$ length) and developmental stage (Gosner stages 27 - 28) randomly chosen for the were experiments. The final instar larvae of P. flavescens (N = 50;  $30.2 \pm 1.3$  mm total length) were obtained from the same ponds where the egg clutches were collected. Dragonfly larvae were individually reared in plastic bowls (14 cm diameter and 7 cm depth) with 200 mL of aged tap water, to avoid cannibalism. Tadpoles in the stock tanks were fed boiled spinach. Dragonfly larvae were fed toad tadpoles. The behavioral responses of the prey were studied by exposing them to "stimulus solution" of either predator kairomones or dietary metabolites of predators fed conspecific tadpoles. The preparation of stimulus solutions was, as follows.

**Preparation** of kairomones.—Individual dragonfly larvae were placed in separate plastic bowls (N = 10 bowls; 19 cm diameter and 7 cm depth) with 200 mL of aged tap water without food for 96 h to eliminate diet-derived excretory metabolites from the stimulus solution, resulting in a stimulus solution with only kairomones.

Preparation of dietary cues of conspecific origin.-Three dragonfly larvae were placed in plastic bowls (N = 5 bowls; 19 cm diameter and 7 cm depth) with 600 mL of aged tap water along with 12 toad tadpoles at Gosner stages 27-28 (at about 08:30 h). The dragonfly larvae 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 bowls was filtered using fine cheese cloth. The filtrate served as the stimulus solution containing the diet-derived excretory metabolites or substances of predators fed conspecific prey and are unlikely to have contained the alarm cues of prey. Prey alarm cues are known to be labile in nature (Ferrari et al. 2008, Wisenden et al. 2009, 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.

# Behavioral Responses of Prey to Predator's Kairomones

The behavioral responses of prey tadpoles to kairomones (water conditioned with starved predators) were recorded by placing single tadpole in a rectangular glass tank (28 L  $\times$  15 W  $\times$  15 H cm) with 600 mL of aged tap water. A handycam (Sony, DCR-SR300/E) was positioned above the tank to record activity in the entire tank. The handycam 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 to the test tank. The Ethovision system was used to record maximum swimming speed  $(V_{\rm max})$ , distance traversed by the tadpole, number of swimming spurts, and time spent swimming during an entire trial. For each trial, a tadpole put 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) was then added at the rate of  $\sim 1 \text{ mL/s}$ . 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 kairomones was added as described above. Movement of the tadpole was recorded for another 5 min to determine the activity pattern after exposure to kairomones.

# Behavioral Responses of Prey to Dietary Cues of Conspecific Origin

In this experiment, the stimulus solution contained chemical cues from excretory metabolites or substances of dragonfly larvae fed toad tadpoles instead of only kairomones of the predator. The behavioral responses of tadpoles were recorded as described above, before and after the addition of stimulus solutions.

In both experiments, a total of 25 trials were run using a new tadpole each time. The test tank was cleaned and replenished with aged tap water between trials.

#### Statistical Analysis

The data on  $V_{\text{max}}$ , swimming spurts, time spent swimming and total distance traversed were log transformed prior to analysis to meet the assumption of normality. The behavioral responses of tadpoles before and after the addition of stimulus solutions (predator kairomones diet-derived or excretory metabolites) were compared using Paired-Samples t-test. Statistical tests were performed using SPSS ver. 16.0.

#### Results

The  $V_{\text{max}}$  (t = -0.373, df = 24, p = 0.712, Figure 1A), frequency of swimming spurts (t = 0.528, df = 24, p = 0.602, Figure 1B), time spent swimming (t = 0.126, df = 24, p = 0.901, Figure 1C) and total distance traversed (t = 0.123, df = 24, p = 0.903, Figure 1D) by tadpoles exposed to chemical blank water were similar to those exposed to kairomones.

Upon exposure to diet-derived metabolites of a predator after it consumed conspecific prey, tadpoles showed a significant increase in  $V_{max}$  (t = -5.092, df = 24, p < 0.001, Figure 2A), and significant declines in the number of swimming spurts (t = 7.154, df = 24, p < 0.001, Figure 2B), time spent swimming (t = 7.242, df = 24, p < 0.001, Figure 2C) and total distance moved (t = 6.879, df = 24, p < 0.001, Figure 2D) when compared to their baseline activities in stimulusfree water.

#### Discussion

Tadpoles of *Duttaphrynus melanostictus* do not alter their behavior in response to only kairomones of predatory larvae of *P. flavescens*, thereby suggesting that they do not perceive cues of the dragonfly larvae as a serious predation threat. Similar results have been reported for tadpoles of *Indosylvirana temporalis* (Günther, 1864) (Mogali *et al.* 2012) and *Euphlyctis cyanophlyctis* (Schneider, 1799) (Supekar and



**Figure 1.** Maximum swimming speed  $(V_{max})$  (**A**), Swimming spurts (**B**), Time spent in swimming (**C**), and Distance travelled (**D**) by tadpoles of *Duttaphrynus melanostictus* exposed to chemical blank water (aged tap water) or kairomonal stimuli of *Pantala flavescens*. Data are represented as mean ± SE; N = 25 trials; data analyzed by Paired-Samples t-test.

Gramapurohit 2018). In contrast, toad tadpoles exhibit strong behavioral responses (i.e., reduced swimming movements and high burst speed) to kairomones of predatory sympatric tadpoles of *Hoplobatrachus tigerinus* (Mogali *et al.* 2011), which locate prey visually and prey on coexisting sympatric anuran tadpoles (Saidapur 2001, Saidapur *et al.* 2009). Thus, frog species such as *H. tigerinus* pose a serious predation threat. The long ecological coexistence of toad tadpoles with sympatric carnivorous tadpoles such as *H. tigerinus* may have led to the evolution of antipredator defense strategies in response to kairomones of these predators.

In contrast, larvae of dragonfly are "sit-andwait" predators that move slowly and usually wait for prey to come near before attacking



speed  $(V_{max})$ Figure 2. Maximum swimming (**A**), Swimming spurts (B), Time spent in swimming (C), and Distance travelled (D) by tadpoles of Duttaphrynus melanostictus exposed to chemical blank water (aged tap water) or dissolved diet-derived excretory metabolites of conspecific prey fed Pantala flavescens. Data are represented as mean  $\pm$  SE; N = 25 trials; data analyzed by Paired-Samples t-test. Asterisks over the bars indicate significant difference between chemical blank water and diet-derived metabolites of predators from excreta groups.

(Miller *et al.* 2014). They seem to be perceived by the prey as less dangerous and they may pose a lower predation threat. There is intense selection pressure on "sit-and-wait" predators to suppress chemical (e.g., kairomones) evidence of their presence (Miller *et al.* 2015) because they need the prey to approach them closely. Also, it is possible that the predation pressure of dragonfly larvae on toad tadpoles is low. If so, toad tadpoles are better served by conserving their energy by not inducing antipredator defenses in response to kairomones, if any, of dragonfly larvae. In contrast, water-soluble substances in the excreta of larvae of *P. flavescens* following consumption of conspecific prey seems to indicate intense predation threat and elicit strong behavioral changes in toad tadpoles in the form of reduced swimming movements and high burst speed. Apparently, evidence of recent predation on conspecific members of the group indicates high predation risk for tadpoles in the vicinity.

Our results are consistent with those reported for tadpoles of Dryophytes versicolor (LeConte, 1825) (Schoeppner and Relyea 2009b). We rule out the influence of alarm cues released by toad tadpoles as there were neither surviving injured individuals nor dead remains of tadpoles in the bowls for 15 h prior to harvesting the stimulus solution, and alarm cues are highly labile in nature (Ferrari et al. 2008, Wisenden et al. 2009, Chivers et al. 2013). Hence, the antipredator defense behavior of toad tadpoles in the present study is specifically in response to diet-derived excretory metabolites or substances released by dragonfly larvae that have consumed conspecific prey. In conclusion, toad tadpoles can exhibit different behavioral responses to different predator-related cues.

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# Chondrocranial and hyobranchial structure in two South American suctorial tadpoles of the genus *Telmatobufo* (Anura: Calyptocephalellidae)

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#### Abstract

**Chondrocranial and hyobranchial structure in two South American suctorial tadpoles of the genus** *Telmatobufo* (Anura: Calyptocephalellidae). The chondrocranium, hyobranchium, rectus abdominis muscle, and epaxial musculature of *Telmatobufo australis* and *T. ignotus* are described. In addition, these structures were compared with those of the non-suctorial *Calyptocephalella gayi*, the sister group of *Telmatobufo*.

Keywords: Evolution, larval morphology, southern Chile, suctorial tadpoles.

#### Resumen

Estructura del condrocráneo y aparato hiobranquial de dos renacuajos suctores sudamericanos del género *Telmatobufo* (Anura: Calyptocephallidae). Se describen los condrocráneos, aparatos hiobranquiales, músculo recto abdominal, y la musculatura epaxial de *Telmabufo australis* y *T. ignotus*. En adición, los renacuajos de *Telmatobufo* se comparan con los de *Calyptocephalella gayi*, el grupo hermano de *Telmatobufo*.

Palabras claves: evolución, morfología larvaria, renacuajos suctores, sur de Chile.

#### Resumo

Estrutura do condrocrânio e do aparelho hiobranquial de dois girinos suctoriais sulamericanos do gênero *Telmatobufo* (Anura: Calyptocephalellidae). Descrevemos aqui o condrocrânio, o aparelho hiobranquial, o músculo reto-abdominal e a musculatura epiaxial de *Telmabufo australis* e *T. ignotus*. Além disso, comparamos os girinos de *Telmatobufo* aos de *Calyptocephalella gayi*, o grupo-irmão de *Telmatobufo*.

Palavras-chave: evolução, girinos suctoriais, morfologia larval, sul do Chile.

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# Introduction

Anurans of the family Calyptocephalellidae (genera Telmatobufo Schmidt, 1952 and Calvptocephalella Strand, 1928) are a small, old Gondwanan lineage (Clade Australobatrachoidea; San Mauro et al. 2005) that inhabits lentic (*Calyptocephalella*) and lotic (*Telmatobufo*) environments associated with the trans-Andean temperate Nothofagus forests of South America. Pyron and Wiens (2011) suggested that Telmatobufo and Calyptocephalella are sister groups that probably diverged during the Oligocene-Miocene in southern South America (Nuñez and Formas 2000, Frazão et al. 2015). The species of Telmatobufo-T. australis Formas, 1972 (Formas 1972) (Figure 1 A, B), T. bullocki Schmidt, 1952 (Formas 1988), T. venustus (Philippi, 1899) (Díaz et al. 1983), and T. ignotus Cuevas, 2010 (Cuevas 2010) (Figure 1 C, D)-have a characteristic larval ecomorphotype. It is a mountain stream-type with a highly modified suctorial oral disc, robust and depressed body, well-developed tail muscles, and low tail fins. The pond-type tadpoles of Calyptocephalella gayi (Duméril and Bibron, 1841) differ from those of *Telmatobufo* in having anteroventral non-suctorial mouth. normal development of tail musculature, and moderatesized caudal fins (Cei 1962) (Figure 1 E, F). Herein, we describe the chondrocranium, hyobranchial apparatus, epaxial musculature, and rectus abdominis muscle of Telmatobufo australis and T. ignotus. We compare the morphology of these species with that of the sister species Calyptocephalella gavi, and comment on the evolution of the tadpoles of Telmatobufo.

#### **Materials and Methods**

Twelve tadpoles of the following species were examined: *Telmatobufo australis* (N = 6, IZUA-3652), *T. ignotus* (N = 4, IZUA-3653) and *Calyptocephalella gayi* (N = 3, IZUA-



Figure 1. Tadpoles (Stage 30; lateral and ventral views) of *Telmatobufo australis* (A, B), *T. ignotus* (C, D) and *Calyptocephalella gayi* (E, F). The arrow indicates the mouth of *C. gayi*. Bar = 20 mm.

3656). Following the Song and Parenti (1995) technique, we stained and cleared the chondrocranium and the hyobranchial apparatus of four tadpoles of *T. australis* (Stage 30), three *T. ignotus* (Stage 31), and one *C. gayi* (Stage 30). We performed manual dissections to examine the rectus abdominis and epaxial muscles of two tadpoles of *T. australis* (Stage 30), one *T. ignotus* (Stage 31) and one *C. gayi* (Stage 30), one *T. ignotus* (Stage 31) and one *C. gayi* (Stage 33).

The tadpoles of *Telmatobufo australis* were collected in February 1972 in a small stream in secondary forest of the coastal mountain range at Chivería (39°57'79" S, 73°28'52" W), Valdivia Province in southern Chile. The larvae of *T. ignotus* were collected in December 2007 in a stream located in a remnant of Maulino Forest in the coastal mountain range at Ramadillas Ravine (35°56'57" S, 72°36'57" W), Cauquenes Province in central Chile. The tadpoles of *Calyptocephalella gayi* were collected in January 2004 in a permanent lagoon in the city of Valdivia (39°49'28" S, 73°13'26" W), Valdivia Province in southern Chile.

The tadpoles were euthanized following strict biosafety protocols, staged according Gosner (1960), and cleared and stained. The adopted the terminology of chondrocranial morphology of Larson and de Sá (1998).

The myological terminology follows that of Haas and Richars (1998), and the rectus abdominis muscle is described in the manner of Carr and Altig (1992). The larvae examined are housed in the Laboratorio de Sistemática, Instituto de Ciencias Marinas y Limnológicas, Universidad Austral de Chile.

# Results

# Chondrocranium

The chondrocranium and hyobranchial apparatus of larvae of Telmatobufo ignotus and T. australis are morphologically similar. Thus, the following description is based on *T. australis*; features that differ in T. ignotus are described parenthetically. These differences should be considered with caution and confirmed by examination of more extensive samples. At Stage 30, the chondrocranium is open dorsally and  $1.3 \times$  longer than wide. The maximum width, at the level of the anterior edge of the pars articularis quadrati, is 79% of the total length of the chondrocranium. The maximum height at the level of the processus muscularis is about 35% of the length.

# Neurocranium

The length of the trabecular horns of Telmatobufo australis is about 32% of the chondrocranial length (Figure 2A). The anterior third of each horn is expanded and diverges laterally from its counterpart; the expansion is about  $3 \times$  the width of the horn at the level of the bifurcation. According to Haas (2003), the cornu trabeculae proportion (ratio of the width of the cornua at their base divided by their length) is 0.85. Accordingly, the trabecular horns have an extensive transverse articulation with the dorsal margin of the suprarostral cartilage (Figure 2B). The short and pointed lateral process lies at the base of each horn and receives a well-developed quadratoethmoidale ligamentum from the quadratoethmoid process of the quadratocranial commisure. The olfactory foramen is well defined. The frontoparietal fenestra occupies the 57% of the length of the chondrocranium. The lateral walls of the braincase are formed by the orbital cartilages. The prootic foramen, large and elongate, is located between the anterior edge of the otic capsule and the posterior margin of the orbital cartilage. Two other large foramina are visible in the posteroventral portion of the orbital cartilage. The smallest is posterior to the oculomotor foramen and the anterior edge of the optic foramen. The pila metoptica separates the foramina. The pila antotica lies between the oculomotor foramen and the anterior edge of the prootic foramen. The oculomotor foramen is rounded and its diameter is less than the oval optic foramen. The subocular fenestra is elongate (round in T. ignotus) and approximately onesixth the length of the chondrocranium. The commisura quadratocranialis anterior is onethird the length of the chondrocranium and  $1.5 \times$ the maximum width of the otic capsule. Dorsally, the otic capsules are rhomboid with the maximum dimension being  $1.2 \times$  greater than the width as measured at a right angle to the axial axis; the greatest dimension of the capsule is about 23% of the chondrocranial length. There are three semicircular canals: the anterior and the lateral

are well defined, but the posterior is diffuse. The otic capsules are connected dorsally via the tectum synoticum; ventrally, they contact the basicranial floor. The fenestra ovalis is small, located in the ventrolateral wall of the otic capsule. The occipital arch forms the posterior part of the chondrocranium. The ventral occipital condules are oriented laterally; they are slightly pedunculate and separated by the notochordal canal. The jugular foramen is near the ventral region of the occipital arch and the otic capsule. The suprarostral cartilage supports the upper jaw. In T. australis, it consists of a medial corpus and two lateral alae (Figure 2B). The pars corporis and the pars alaris are arranged in an arched line that bears the jaw sheath. The cornu trabeculae has a wide synchondrotic articulation of a flexible cartilage with suprarostral cartilage. The partes corporis of the suprarostral cartilage are broadly fused in a large plate about 7× wider than high. The height of the suprarostral plate is uniform; however, its medial portion deviates ventrally, as indicated by the concave dorsal margin. Both the pars corporis plate and the pars alaris are broadly fused. The pars corporis is almost perpendicular to axis the body. The pars alaris is positioned laterally to the sagittal axis of the body and concentric to the pars corporis. The posterior process of the pars alaris is single, with a rounded distal tip and dorsal orientation. The adrostral cartilages are large and elongate and parasagittally oriented. They are close to the suprarostral cartilage (pars alaris), but are not in contact; a thin rod of cartilaginous tissue separates them.

# Visceral Components

*Palatoquadrate.*—The long and markedly broad palatoquadrate lies parallel to the longitudinal body axis (Figure 2A). The pars articularis quadrati is expanded anteriorly and has three well-defined processes on the anterior margin. The lateral circumoral ligament extends from the tip of the trabecular horns to the anterolateral margins of the partes articularis



Figure 2. Chondrocranium and hyobranchial apparatus of *Telmatobufo australis* (Stage 30). Dorsal view of the chondrocranium and mandibular arch (A), frontal view of the lower jaw and suprarostral (B), ventral view of the hyobranchial apparatus (C), and ventral view of the lower jaw (D). Dorsal view of the chondrocanium and mandibular arch (E, F) and ventral view of the hyobranchial apparatus of *Calyptocephalella gayi* (Stage 33). The dotted area (E) indicates the ossification of the frontoparietal, parasphenoid, and exooccipital bones. Bar = 5 mm.

quadrati. The processus muscularis quadrati is triangular and broad; it is pointed in lateral view, rounded dorsally, inclined medially, and does not exceed the height of the chondrocranium. The ligamentum tectum attaches the muscular process of the palatoquadrate to the anterior end of the processus anteorbitalis. Posteriorly, the palatoquadrate attaches to the orbital cartilage via the process ascendens. This narrow, thin cartilage contacts the pila antotica at the level of the oculomotor foramen ("intermediate" level of attachment; Sokol 1981) and it is located obliquely to the longitudinal axis of the chondrocranium oblique to the central axis of the chondrocranium.

*Meckel's cartilage and cartilage labialis inferioris.*—Meckel's cartilage is broad, robust, and sigmoid in shape; it is about 27% of the chondrocranium length (Figure 2D). The cartilage is oblique to the medial axis of the chondrocranium, forming an angle of nearly 45°. It articulates with the infrarostral cartilage rostrally and with the pars articularis quadrati caudall; the latter articulation is saddle-shaped. The anterior and posterior margins of the paired infrarostral cartilages are slightly curved and broader medially than laterally; medially they are connected by a syndesmotic commissura intermandibularis.

# Hyobranchial Apparatus

The hyobranchial apparatus of Telmatobufo australis is composed of the ceratohyals and the branchial baskets (Figure 2C). The subrectangular, wide, and flattened ceratohyals are medially connected by a cartilaginous pars reuniens. A small basihyal lies anterior to the pars reuniens. Each ceratohyal bears a rounded anterior process and a pointed and divergent posterior process. The anterolateral process is not evident. The process lateralis is elongate and its distal end is rounded (acuminate in T. ignotus). The basibranchial is rhomboidal and bears a rounded, posteriorly directed urobranchial process. The subtriangular hypobranchial plate forms a well-defined hypobranchial sinus. The branchial baskets consist of four curved ceratobranchials distally joined via commissure terminals. Ceratobranchials I-IV are fused with the hypobranchial plate. The proximal end of the Ceratobranchial I is contiguous with the hypobranchial plate and has a pointed processus anterior brachialis, similar to the branchial processes that the remainder ceratobranchials

bear. Ceratobranchials I–III are curved and bear lateral projections, except for the inner side of Ceratobranchial III. The short, broad Ceratobranchial IV is fused to the hypobranchial plate. There are three curved spicules (I–III) that are about 25% of the length the respective ceratobranchial. The distal tips of Spicules I and III are truncate, whereas that of Spicule II is bifurcate. Spicule IV is not evident.

# Musculature

The epaxial postcranial musculature is well developed and extends over the otic capsule to insert on the anterior third of the capsule. The rectus abdominis muscle reaches the anterior end of the ceratohyals. The muscle consists of two thin, well-defined bands composed of seven myotomes that diverge anteriorly. The medial aponeurosis between both bands is ample-i.e., at the level of the fifth myotome the area corresponds to a sixth the length of the muscle and a quarter at the level of the first myotome. The gut coils are visible through the aponeurosis. Myotomes 2-7 are quadrangular, and the most anterior is conical. The length of the myotomes increases from back to front; the most anterior myotome is approximately  $1.7 \times$  than the seventh. All myotomes have densely packed fibers. (In T. ignotus, the rectus abdominis is more extensive and robust than in *T. australis*).

# Discussion

Haas and Richards (1998) concluded that the morphological specializations of suctorial tadpoles result from convergent evolution of external and internal characters (e.g., oral suctorial disc, robust and depressed body, active tail muscle and low tail fins, fusion of the suprarostrals, expanded trabecular horns, robust infrarostrals, and palatoquadrate).

Phylogenetically, Pyron and Wiens (2011) suggested that the genera *Telmatobufo* and *Calyptocephalella* (Calyptocephallidae) are sister groups. However, the taxa differ osteologically (e.g., atlas and axis fused in Telmatobufo; cranium heavily ossified in Calvptocephalella; Lynch 1978), as well as in external larval morphology; Telmatobufo has suctorial tadpoles, whereas Calvptocephalella gayi has generalized, pond-type larvae (Orton 1953). The differences in the external and internal characters of the tadpoles of Telmatobufo (T. australis, T. ignotus) and Calyptocephalella gavi (Figure 2E, F) are summarized in Table 1. These foregoing distinctions suggest that there may have been divergent evolutionary trajectories acting on the adult and larval frogs. In the case of larval Telmatobufo, evolution produced novelties (i.e., synapomorphies) that distinguish the genus. During the Miocene-Oligocene, southern South America underwent significant orogenic changes, characterized mainly by the Andean uplift (Dott et al. 1977). The Andes acted as a barrier to wind circulation (Westerlies), causing a rain shadow that led to increased desertification east of the Andes (Ruzzante and Rabassa 2011).

Phylogenetically, it seems evident that the common ancestor of Calyptocephalella and Telmatobufo probablywas an anuran that was not hyperossified and that had a generalized, pondtype larvae. We postulate that members of this anuran clade (i.e., the ancestor and all of its descendants; Wiley and Lieberman 2011) diverged from one another during the Miocene-Oligocene when the Andes were being uplifted. The ancestor of Calyptocephalella may have been isolated east of the Andes, where some species disappeared (e.g., Calyptocephalella spp.; Muzzopappa and Báez 2009, Agnolin 2012) and others adapted to the xeric conditions, giving rise to species, such as the hyperossified C. gavi that retains a generalized larval type. In contrast, the ancestral lineage leading to *Telmatobufo* seems to have evolved a specialized suctorial larva as an adaptation to the lotic environments that became available along the southwestern edge of Gondwana with the uplift of the Andes in what today is southern Chile. The particular suite of evolutionary novelties

shared by the tadpoles of *Telmatobufo* is unique among australobatrachian anurans; it defines the genus taxonomically and supports its monophyly.

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Table 1. Morphological ch.	aracters of the tadpoles of Telmatobufo spp. and Calyptocepha	<i>alella gayi.</i> ¹Cei 1962, ²Muzzopappa 2013.
	Telmatobufo spp.	Calyptocephalella gayi
External morphology		
Ecomorphological type	Suctorial	Pond-type
Mouth	Broad suctorial disc	Oral disc small <sup>1</sup>
Vent tube	Dextral, covered with a triangular skin fold	Dextral <sup>1</sup>
Tail	Paddle-shaped, distal tip slightly rounded	Distal tip pointed, more homogeneus fins'
Chondrocranium and hyobranchial skeleton		
General aspect	Rectangular	Oval <sup>2</sup>
Cornua trabeculae	Wide anteriorly and fused about $arsigma$ its length	Narrow and independent along its entire length <sup>2</sup>
Cartilage labialis superior	Components robust and fused into single structure	Partes corporis and alaris fused; partes corporis not fused <sup>2</sup>
Maximum width	At jaw articulation	External edge of the palatoquadrate <sup>2</sup>
Palatoquadrate	Robust and connected to the chondrocranium by a wide commissure quadratocranialis anterior	Connected to the chondrocranium by a narrow commissure quadratocranialis anterior <sup>2</sup>
Meckel's cartilage	Oblique in relation of the longitudinal axis of the cranium	Perpendicular in relation of the longitudinal axis of the cranium <sup>2</sup>
Adrosal cartilages	Present	Absent <sup>2</sup>
Ossification	Absent (Stage 30)	Frontoparietals, parasphenoid, and exoccipital (Stage 30) <sup>2</sup>
Processus lateralis ceratohyal	Narrow, laterally directed	Broad, posteriorly curved
Musculature		
Epaxial musculature	Extends to anterior parts of otic capsules	Extends to the posterior parts of otic capsules
Hypaxial musculature	M. rectus abdominis underlies the branchial baskets up to the level the ceratohyals	M. rectus abdominis never overpasses the level of the septum transversum, that marks the limit body-head (this study)

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# New prey records for the Atlantic Central American Milksnake *Lampropeltis polyzona* (Serpentes: Colubridae)

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Keywords: Anolis sagrei, diet, ophiophagy, Sceloporus variabilis, Scincella. Palabras claves: Anolis sagrei, dieta, ofiofagismo, Sceloporus variabilis, Scincella. Palavras-chave: Anolis sagrei, dieta, ofiofagia, Sceloporus variabilis, Scincella.

The Atlantic Central American Milksnake (also known as the Mexican false coral snake), Lampropeltis polyzona Cope, 1860, is a New World taxon with a complicated taxonomic history. Formerly, it was considered a subspecies of the widespread and polytypic Lampropeltis triangulum (Lacépède, 1789), but recent phylogenetic analyses by Ruane et al. (2014) and Chambers and Hillis (2020) indicate that the taxon likely warrants species-level recognition. Although these two works differ in the geographic ranges they ascribe to L. polyzona, both indicate that the snake ranges widely along both the Pacific and Atlantic coasts of Mexico, from southern Sonora and northern Veracruz south to at least northern Guerrero and northern Oaxaca. Across this range, L. polyzona occurs in a wide array of vegetation types including spiny subtropical forest, seasonal evergreen forest, cloud forest, pine-oak forest and tropical

deciduous forest (Heimes 2016, Uetz *et al.* 2019). The recent species-level recognition of *L. polyzona* merits an update of the food items known to compose the diet of this snake. Herein I report seven new prey items in wild-caught *L. polyzona* from central Veracruz, Mexico; these data were obtained through fieldwork and dissection of museum specimens. I also provide an updated list of the diet items recorded in the literature for this species. My observations reveal the first cases of ophiophagy in *L. polyzona* and demonstrate an interesting food web linkage involving the non-native lizard *Anolis sagrei*.

During fieldwork across central Veracruz from 2014–2019, I opportunistically gathered dietary data from six *Lampropeltis polyzona*. In each case, I deposited either a specimen voucher at the Colección Herpetológica del Museo de Zoología "Alfonso L. Herrera," Facultad de Ciencias, Universidad Nacional Autónoma de México (MZFC-HE), or a digital photographic voucher at the Natural History Museum of Los Angeles County (LACM PC). Additionally, I dissected and gathered dietary data from a

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preserved specimen of *L. polyzona* in the collection of the Instituto Tecnológico Superior de Zongolica (ITSZ-R). Subsequently, I compiled a database of prey items of *L. polyzona* based on an exhaustive literature review and included only those records that mentioned at least the genus of the prey (Table 1). Each previously unpublished dietary record of *L. polyzona* is detailed below.

On 21 August 2014 at approximately 20:00 h, in the Colonia Agrícola Rincón de las Flores (18°42'48.79" N, 96°51'6.75" W; WGS 84; 1,259 m a.s.l.), municipality of Tezonapa, I salvaged a dead juvenile *Lampropeltis polyzona* (MZFC-HE 34402) that contained a partially digested adult *Scincella* sp. (Figure 1A). On 04 September 2015 at approximately 10:00 h, in a mixed coffee plantation at the same locality, I salvaged a dead adult *L. polyzona* (MZFC-HE 34403) that contained a partially digested adult *Sceloporus variabilis* Wiegmann, 1834 (Figure 1B).

On 14 April 2016 at approximately 20:00 h in the tourist center of Rancho Fermín (18°54'6.45" N, 96°48'16.42" W; WGS 84; 590 m a.s.l.), municipality of Atoyac, I observed and photographed an adult *L. polyzona* eating an adult *Coniophanes fissidens* (Günther, 1858) (Figure 1C) in a shade-grown coffee plantation.

On 17 June 2017 at approximately 02:02 h near Los Túneles Ferroviarios, 1 airline km NW of Atoyac (18°55'13.15" N, 96°46'12.79" W; WGS 84; 489 m a.s.l.), municipality of Atoyac, I captured and subsequently released an adult male *Lampropeltis polyzona* [snout–vent length (SVL) 850 mm, tail length (TL) 100 mm] in an area planted with palm (*Chamaedorea* sp.) and mango trees (*Mangifera indica* L.). Prior to release, this snake regurgitated a juvenile *Leptodeira septentrionalis* (Kennicott, 1859) (Figure 1D), which given its good condition, probably had been ingested only a few hours earlier.

On 23 May 2017 at approximately 18:00 h in a greenhouse in an industrial area on the outskirts of the town of Yanga (18°50'10.78" N, 96°48'25.27" W; WGS 84; 540 m a.s.l.), municipality of Yanga, Veracruz, I observed a juvenile *Lampropeltis polyzona* feeding on a sub-adult male

Anolis sagrei Duméril and Bibron, 1837 (Figure 1E). I did not observe the capture of the Anolis.

On 09 December 2019 at approximately 10:00 h in a small area of secondary vegetation 2 km NW of Coatepec (19°27'32.0" N, 96°56'39.0" W; WGS 84; 1,179 m a.s.l.), municipality of Coatepec, I found a dead adult *Lampropeltis polyzona* (LACM PC 2495: SVL 700 mm, TL 83 mm) that contained a *Sceloporus variabilis* tail and a reptile eggshell in its stomach (Figure 1F).

In the ITSZ-R collection, I examined four preserved specimens of *Lampropeltis polyzona;* only one (ITSZ-R-109, male, SVL 1056 mm) contained identifiable stomach contents. These consisted of hair and a lower mandible from a *Mus musculus* (Linnaeus, 1758) (Figure 1G).

In total I recorded seven novel prey items, adding to the 13 prey items for Lampropeltis *polyzona* previously recorded in the literature. The composition of the updated diet of this species is, as follows: 9 (45%) of the 20 types of prey items consumed are lizards; 4 (20%) are mammals; 3(15%) are reptile eggs; 2(10%) are snakes; and 2 (10%) are birds (Table 1). Authors of previous reports stated that the diet of L. polyzona is mostly composed of small mammals (e.g., Heimes 2016, Rorabaugh and Lemos-Espinal 2016), but this expanded dataset (although small) suggests that the snake feeds primarily on reptiles (55%), occasionally on mammals (20%) and rarely on birds and reptile eggs (25%). Although the genus Lampropeltis is known to be ophiophagous (e.g., Fitch and Fleet 1970, Ernst and Ernst 2003, Cotten et al. 2008), there were no reported cases of snake predation by L. polyzona until the present study. The lizard Anolis sagrei is considered a non-native, invasive species in Veracruz, Mexico, and has become widely established worldwide. The ecological impact of introduced A. sagrei is usually considered negative (e.g., Delaney et al. 2014, Thawley et al. 2019), but my data suggest that A. sagrei represents a potentially common and valuable food resource for L. polyzona in the vicinity of Yanga, Veracruz.



Figure 1. (A) Remains of a *Scincella* sp. from the stomach of MZFC 34402. (B) Remains of *Sceloporus variabilis* from the stomach of MZFC 34403. (C) Adult *Lampropeltis polyzona* preying on an adult *Coniophanes fissidens*. (D) Adult male *L. polyzona* with a recently regurgitated juvenile *Leptodeira septentrionalis*. (E) Juvenile *L. polyzona* feeding on a subadult male *Anolis sagrei*. (F) Tail of *Sceloporus variabilis* and reptile eggshell from the stomach of LACM PC 2495. (G) Lower jaw of *Mus musculus* from the stomach of ITSZ-R-109.

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Prey	Observation	State	Reference
IGUANIDAE			
Ctenosaura pectinata	In situ observation	Isla Isabel, Nayarit	Rodríguez and Drummond 2000
C. pectinata (eggs)	Dissection	Isla Isabel, Nayarit	Rodríguez and Drummond 2000
PHRYNOSOMATIDAE			
Sceloporus variabilis	Dissection	Veracruz	This study
Sceloporus sp.	Captive observation	Jalisco	Mitchell 1980
SCINCIDAE			
Plestiodon sp.	Captive observation	Jalisco	Mitchell 1980
Plestiodon sp.	—	Veracruz	Pérez-Higareda et al. 2007
Scincella sp.	Dissection	Veracruz	This study
DACTYLOIDAE			
Anolis sagrei	In situ observation	Veracruz	This study
TEIIDAE			
Aspidoscelis costata	In situ observation	Isla Isabel, Nayarit	Rodríguez and Drummond 2000
A. costata (eggs)	In situ observation	Isla Isabel, Nayarit	Rodríguez and Drummond 2000
Holcosus sp.	_	Veracruz	Pérez-Higareda <i>et al</i> . 2007
DIPSADIDAE			
Coniophanes fissidens	In situ observation	Veracruz	This study
Leptodeira septentrionalis	In situ observation	Veracruz	This study
Reptile egg shell	Dissection	Veracruz	This study
AVES: SULIDAE			
Sula leucogaster	In situ observation	Isla Isabel, Nayarit	Cohen 1988
Sula nebouxii	In situ observation	Isla Isabel, Nayarit	Rodríguez and Drummond 2000
MAMMALIA: SORICIDAE			
Cryptotis parva	In situ observation	Veracruz	Aguilar-López and Pineda 2013
Sorex saussurei	Dissection	Hidalgo	Mendoza-Quijano and Ruíz-Piña 1995
MAMMALIA: MURIDAE			
Reithrodontomys sp.	Dissection	Michoacán	Williams 1978
Mus musculus	Dissection	Veracruz	This study

 Table 1. List of dietary items recorded for the Atlantic Central American Milksnake Lampropeltis polyzona.

# Contributions to the knowledge of the natural history of *Claudius angustatus* (Testudines: Kinosternidae) in Veracruz, Mexico

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Keywords: Diet, ectoparasites, leech, *Trachemys venusta*. Palabras claves: dieta, ectoparasitos, sanguijuelas, *Trachemys venusta*. Palavras-chave: dieta, ectoparasitas, sanguessugas, *Trachemys venusta*.

The Narrow-bridged Musk Turtle (or Chopontil), Claudius angustatus Cope, 1865 is a small turtle (maximum carapace length = 165mm; Legler and Vogt 2013) distributed from sea level to 300 m elevation in the Mexican states of Veracruz, Tabasco, Oaxaca, Chiapas, Campeche, and Quintana Roo, as well as Guatemala and Belize (Rhodin et al. 2017, Uetz 2019). This species is considered "Lower Risk / Near Threatened" by the International Union for Conservation of Nature (IUCN 2019) and Endangered (en Peligro de Extinción) by the Mexican federal government (SEMARNAT 2010). The primary threats to the species are the loss and transformation of its habitats with agricultural and livestock use, as well as the use of the species for its meat (Cázares 2015). Available ecological information about this species is limited, but includes aspects of its reproductive biology, habitat use, predators, distribution, parasites, and natural diet (Thatcher

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1963, Flores-Villela and Zug 1995, Vogt 1997a,b, Aguirre-Léon *et al.* 2002, Cázares 2015, Rhodin *et al.* 2017). *Claudius angustatus,* is carnivorous; its natural diet consists of crustaceans, aquatic insects and their larvae, and occasionally, vegetation (Vogt 1997b, Aguirre-Léon *et al.* 2002). Additionally, Hausmann (1968) reported that *C. angustatus* is not selective, because in captivity, the turtle will eat meat, fish, shrimps, and worms.

At the end of the rainy season and during a pre-harvest burning of a sugarcane field on 19 November 2018 at about 11:30 h, Miguel A. Sánchez captured an adult male C. angustatus (carapace length = 124 mm; Figure 1A). The locality from which the turtle was collected was Palmillas in the Municipality of Yanga, Veracruz, Mexico (18.8239° N, 96.7693° W; WGS 84; 458 m a.s.l.). The turtle was held captive for 2 days until it could be relocated. We deposited a photograph in the collections of the Natural History Museum of Los Angeles (LACM PC 2465). We photographed two leeches on the lower part of the right foot of this turtle. When we removed the leeches, one released young leeches. Based on the photos, we determined that

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the leeches belong to Glossiphoniidae (Figure 1 B), a family characterized by parental care (Siddall 2005).

Two feces pellets of *C. angustatus* were collected; subsequent fecal analysis revealed several skull fragments, a scute precentral bone, two cervical vertebrae, phalanges, shell fragments, and scutes of a turtle, as well as remains of a beetle (Figure 1C). The scutes were identified as *Trachemys venusta* (Gray, 1855)

(Emydidae) by Marco Antonio López-Luna (Research Professor, División Académica de Ciencias Biológicas de la Universidad Juárez Autónoma de Tabasco). The saddle-shaped (in lateral aspect) cervical vertebrae are diagnostic of *Chelodina* (Herrel 2008), we think that the rest of the bones are also from *T. venusta*. Based on the sizes of the shields, we think that the prey was a juvenile *T. venusta* (carapace length = 108 mm).



Figure 1. A male *Claudius angustatus* (LACM PC 2465) from Palmillas, Municipality of Yanga, Veracruz, México (A). Two freshwater leeches of the family Glossiphoniidae (B) and remains of the turtle *Trachemys venusta* recovered from fecal content (C).

The presence of leeches in freshwater turtles has been documented (e.g., Carr and Mast 1988, Richardson et al. 2017, Perera et al. 2019). Most the documented leeches of belong to Glossiphoniidae, a family of freshwater leeches that use their proboscises to feed on the blood of their vertebrate hosts (Sawyer 1986). This is the first report of leeches in C. angustatus, a species that typically, is buried in the mud during the rainy season; when the bodies of water begin to dry, the turtle remains inactive for six months (Cázares 2015).

This is the first reported occurrence of a vertebrate and T. venusta in the natural diet of C. angustatus. This event either represents a case of predation or scavenging by the turtle. Cannibalism (Polis and Myers 1985), attacks between turtle species (Moll and Legler 1971), and scavenging (Forsberg and Geluso 2017) occur in nature. Vogt and Guzman (1988) reported a small turtle in the natural diet of Staurotypus triporcatus (Wiegmann, 1828), a member of the same family of C. angustatus. In turtles, this behavior has been associated with habitat loss, Moll and Legler (1971) mention an increase in injuries and cases of cannibalism in turtles in disturbed sites. Another consequence of habitat loss is interspecific and intraspecific competition for shelter and food (Moll and Legler 1971).

The collecting locality is the first record for the municipality of Yanga, and Palmillas is 4 km from the nearest previously reported locality in the municipality of Cuitlahuac, Veracruz (TNHC 32774: Smith and Smith 1979). The maximum elevation at which C. angustatus was known to occur was 300 m a.s.l. (Legler and Vogt 2013, Cázares 2015, Rhodin et al. 2017); however, we found the turtle at an elevation of 458 m a.s.l. Additionally, there are two specimens in the herpetological collections of the University of Kansas Biodiversity Institute (KU 24450, 27069; Vertnet 2016) from Potrero Viejo in the municipality of Amatlán de los Reyes, Veracruz, at an altitude of 611 m a.s.l.; this is maximum recorded elevation for C. angustatus.

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# First report of overwintering in tadpoles of Odontophrynus occidentalis (Anura: Odontophrynidae) from Argentina

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Keywords: Amphibians, Arid Chaco, developmental stage, San Juan, winter activity.

Palabras claves: actividad invernal, anfibios, Chaco Árido, estadio de desarrollo, San Juan.

Palavras-chave: atividade invernal, anfíbios, chaco árido, estágio de desenvolvimento, San Juan.

Fellers *et al.* (2001) defined overwintering in anuran larvae as spending the winter (i.e., June– September in the Southern Hemisphere) as tadpoles. Several environmental factors influence growth and development rates in larval anurans (Saha and Grupta 2011). Among them are temperature (Kaplan 1980, Saidapur and Hoque 1995), photoperiod (Saidapur 1989), rainfall (Lynch and Wilczynski 2005), food quality (Alvarez and Nicieza 2002), and hydroperiod (Ryan and Winne 2001).

We know little about larval overwintering sites in anurans and this is one of the major gaps in our understanding of amphibian ecology. Because of this, it is not possible to make a

Received 06 May 2019 Accepted 18 May 2020 Distributed June 2020 universal statement about the physicochemical environmental requirements of overwintering amphibians. Two factors that should be important are temperature and dissolved oxygen (Glenn *et al.* 2008).

Overwintering tadpoles have been reported in at least 17 genera and 40 species of frogs in the northern and southern hemispheres. Included are: Rana (= Lithobates), Ascaphus, Alytes, Alsodes, Atelognathus, Batrachyla, Hylorina, *Calvptocephalella*, Chaltenobatrachus, and Polypedates (Martof 1956, Bradford 1983, Díaz and Valencia 1985, Thiesmeier 1992, Úbeda 1998, Úbeda et al. 1999, Hulse et al. 2001, Logares and Úbeda 2004, 2006, Cuello and Perotti 2006, Tattersall and Ultsch 2008, Navas et al. 2010, Basso et al. 2011, Hsu et al. 2012). In South America, larval overwintering has only been reported for temperate species such as Alsodes gargola Gallardo, 1970 (Logares and Úbeda 2004, 2006), *A. tumultuosus* Veloso *et al.*, 1979, and *A. montanus* (Lataste, 1902) (Díaz and Valencia 1985) and two species of *Atelognathus* [*A. nitoi* Barrio, 1973 (Úbeda *et al.* 1999) and *A. patagonicus* (Gallardo, 1963) (Cuello and Perotti 2006, Cuello *et al.* 2014)]. Basso *et al.* (2011) suggested that overwintering might occur in *Chaltenobatrachus grandisonae* (Lynch, 1975). Herein, we report the occurrence of overwintering in tadpoles of *Odontophrynus occidentalis*.

The study area corresponds to the western part of the Chaco Ecoregion (Cabrera and Willink 1980, Márquez *et al.* 2014, Morrone 2014). In the Arid Chaco, rainfall is low (300 mm at its western limit) and there is a marked summer regime because 70% of the rains occur in the warmer months, from November–February. The annual air mean temperature is 17.1°C, with a mean monthly air temperature of the warmest month (January) of 26°C, but frequently, the maximum temperatures exceed 40°C. Winters are temperate; the mean monthly air temperature of the coldest month is 12°C (Karlin *et al.* 2013).

We searched for tadpoles of the streambreeding frog *Odontophrynus occidentalis* Berg, 1896 in the Río La Majadita (30°42'47.016" S, 67°29'44.015" W; 976 m a.s.l.) in San Juan Province of the Chaco ecoregion in Argentina in July 2018. We also undertook subsequent field trips to the study area to observe the frogs. *Odontophrynus* from this region originally was identified as *O. barrioi*, but this species was placed in the synonymy of *O. occidentalis* by Martino *et al.* (2019). We searched the headwaters of the Río Majadita by day looking for tadpoles in every potential habitat (e.g., under rocks and vegetation, and in crevices). We used dipnets to catch the tadpoles and determine the developmental stages (Gosner 1960).

The six tadpoles of **Odontophrynus** occidentalis were collected on 16 July 2018 in the headwaters of Río La Majadita were in Stages 35–36 (Gosner 1960). In subsequent field trips, we found the following: 15 tadpoles in August 2012; an amplectant pair of the species in April 2014; one metamorph in September 2014; and 53 tadpoles in October 2014 in La Mesada Stream (31°1'18.768" S, 67°17'42.719" W; 867 m a.s.l.). We observed tadpoles in advanced stages and metamorphs in the spring when they are more frequently observed than adults (Juan Acosta pers. com.). The advanced stages and metamorphs found in the spring may not be young of that year and possibly overwintered as larvae. Also, the wide range of sizes and stages of individuals found in October 2014 (Figure 1) might reflect the co-occurrence



Figure 1. Odontophrynus occidentalis ranging from Gosner (1960) Stages 21–45 captured in October 2014 in La Mesada Stream. Scale bar: 2 cm.

of overwintering larvae and young of the year. Our observation of an amplectant pair of frogs in the early autumn suggests that offspring from this mating may overwinter as larvae.

Grenat *et al.* (2011) reported long developmental times for tadpoles of *Odontophrynus. cordobae* Martino and Sinsch, 2002 in nature (2–13 mo after oviposition) when the larvae overwinter. This protracted development is similar to that reported by Fernández and Fernández (1921) for *Odontophrynus* sp. from northern Córdoba (11–13 mo), and by Gallardo (1963) for *O. americanus* (Duméril and Bribon, 1841) from Buenos Aires (7 or 8 mo). Given the relatively prolonged development of other species of *Odontophrynus*, we speculate that overwintering in *O. occidentalis* may reflect a long developmental period.

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# Predation on *Hylodes phyllodes* (Anura: Hylodidae) by the harvestman *Heteromitobates discolor* (Arachnida: Opiliones)

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Keywords: Amphibians, arthropods, diet, feeding, predator-prey interaction.

Palavras-chave: alimentação, anfíbios, artrópodes, dieta, interação predador-presa, opilião.

Anurans are considered an important part of the trophic networks of several ecosystems (Wells 2007). Adult frogs are mostly generalist carnivores that consume a large number of invertebrate prey as well as small vertebrates (Duellman and Lizana 1994, Duellman and Trueb 1994). Furthermore, anurans are also part of the diet of a wide variety of animals such as birds, mammals, reptiles, fish, and some groups of invertebrates (Toledo et al. 2005, 2007). Arthropods are the most prominent invertebrate group that has been reported preying on anurans (Toledo 2005). Among them, spiders (Muscat et al. 2014), ants (Allen et al. 2004), and giant water bugs (Toledo 2003) are particularly prone to consume adult frogs and tadpoles regularly. Other groups of arthropods, such as beetles, wasps, and harvestmen also prey on anurans, but these predatory interactions seem to be less frequent (Warkentin 2000, Castanho and Pintoda-Rocha 2005, Wizen and Gasith 2011). There is only one report of a predatory interaction

between a harvestman and an anuran (Castanho and Pinto-da-Rocha 2005). Given the similar microhabitats of frogs and harvestmen, one might expect encounters between different species of both taxa; a predatory interaction is a possible because harvestmen are opportunistic feeders.

*Hylodes phyllodes* (Heyer and Crocoft, 1986) is a small, endemic frog of the Atlantic Forest of southeastern Brazil. It is closely associated with small rocky streams of coastal forests in the states of Rio de Janeiro and São Paulo (Heyer et al. 1990). This diurnal species forages and vocalizes on rocks located in streams or even on rocks near stream banks (Hartmann et al. 2006). They are locally abundant and are part of the diet of different vertebrate and invertebrate species (pers. obs.). When threatened, H. phyllodes usually take refuge in stone crevices (Hartmann et al. 2006), but they also have a series of defensive behaviors that can be used when they are attacked by a predator (Toledo et al. 2011). At night, individuals perch on the broad leaves of small trees or bushes, or even on tree trunks, usually close to the ground and never far away from their activity site (pers. obs.).

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The harvestman *Heteromitobates discolor* (Soerensen, 1884) is a medium-sized arachnid, endemic to a long system of mountain ranges and escarpments called Serra do Mar, in the Atlantic Forest Biome (Silva and Gnaspini 2010). *Heteromitobates discolor* usually is nocturnal, spending the day hidden in rocky crevices near rocky streams. At night, individuals forage on rocks and in low vegetation; they stand upright with their bodies raised and leg pair number two extended to detect any possible prey (Silva and Willemart 2015).

In the southeastern Atlantic Forest, Hylodes phyllodes and Heteromitobates discolor share the same microhabitat. Although both species are commonly found along rocky streams, this is the first report of a predatory event involving Hy. phyllodes by He. discolor. On two different nights we observed two distinct events that, together, constitute a full predatory event. First, we observed an individual Hy. phyllodes being subjugated by He. discolor; second, we observed a harvestman consuming a frog. Both events were recorded during fieldwork developed by the Projeto Dacnis (Atlantic Forest preservation and research NGO) research team in its lowland Atlantic Forest private reserve located in Ubatuba, state of São Paulo, southeastern Brazil. Both events took place in the same area (23°27'50.70" S, 45°07'48.50" W, 70 m a.s.l.) along the margins of one of the many small, rocky streams in the area. The sizes of the animals were estimated visually to avoid interfering with the predation event.

On 08 June 2017, at 20:00 h, an adult *Heteromitobates discolor* (~9-mm dorsal shield and ~100-mm leg span) was found grasping and carrying an adult *Hylodes phyllodes* (SVL ~ 30 mm) on a moist vertical rock (Figure 1A). After walking along the rocky surface, the harvestman entered one of the rock crevices with the anuran trapped between its legs. The frog was still alive (the gular region moving slightly, accompanied by muscular body spasms) but apparently, it was unable to escape. Owing to the depth of the rock crevice and its small opening, we could not see the harvestman consuming the frog.

On 20 November 2018, at 20:15 h, about 2 m away from the previous spot, an adult *Heteromitobates discolor* (~9-mm dorsal shield and ~100-mm leg span) was found ingesting a juvenile *Hylodes phyllodes* (~20 mm SVL). Feeding activity occurred 1.5 m from the stream edge, on the leaf of a fern (*Olfersia cervina*) attached to a rock. The frog was dead and part of fore- and hind limbs had already been consumed (Figure 1B).

Harvestmen are omnivores that are opportunistic, feeding on plant matter, fruit, fungi, small invertebrates, and sometimes, small, dead vertebrates (Acosta and Machado 2007). In fact, predation on vertebrates is rare event; there is only one record of the arachnid preying on live vertebrates (abandoned chicks). Thus, most of the data about harvestmen feeding on vertebrates is restricted to consumption of dead vertebrates (Benson and Chartier 2010). Castanho and Pinto-da-Rocha (2005) reported two events in which harvestmen consumed dead frogs; thus, no subjugation was observed. Our record of Heteromitobates discolor grasping and carrying a live frog to a shelter proves that these arachnids can subjugate relatively large vertebrates. Our second supports the findings of other authors (Castanho and Pinto-da-Rocha 2005) that harvestmen feed on frogs. It is important to note that both observations were made at night when Heteromitobates discolor is active and Hylodes phyllodes usually is inactive. Thus, the arachnids may employ an active hunting strategy that predisposes it to find an inactive frog.

Unlike spiders that prey most often on amphibians, harvestmen have no venom to subjugate their prey (Acosta and Machado 2007); they rely on the function and the strength of their legs to restrain prey. Anurans, on the other hand, have an incredible variety of defensive strategies to deal with potentially more dangerous predators than harvestmen, such as snakes, birds, and mammals (Toledo *et al.* 2011). The rich defensive repertoire of anurans combined with their large size relative to



Figure 1. Heteromitobates discolor grasping and carrying a live Hylodes phyllodes to a rock crevice (A) and feeding on a dead individual (B)

harvestmen can frogs such as *Hylodes phyllodes* difficult to catch and potentially dangerous prey for a non-venomous arachnid such as *Heteromitobates discolor*, and may explain the rarity of this kind of predatory event in nature.

Predatory interactions are important ecological factors driving the evolution and population metrics of both prey and predator. However, as noted here, some predatory events are rare and observed only fortuitously. Future work exploring the effect of primary predators, as well as evaluating the importance of unusual and opportunistic predators, will be a useful asset to our understanding of the natural history of different taxa.

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# Death-feigning behavior in *Microhyla berdmorei*, *M. butleri*, and *M. heymonsi* (Anura: Microhylidae) from Peninsular Malaysia

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Keywords: Amphibia, defense, forest, stream, thanatosis.

Palavras-chave: Amphibia, defesa, floresta, riacho, tanatose.

Anurans live in various types of environments and are exposed to different predators, such as snakes, fishes, birds, small mammals, and larger invertebrates such as spiders (Duellman and Trueb 1986, Wells 2007). Anurans have evolved a wide array of defensive mechanisms to survive; these include immobility, escape, death feigning (thanatosis), leg stiffening, legs interweaving, skin secretions, body contraction, and the unken reflex (Duellman and Trueb 1986, Toledo et al. 2011). Death feigning-a state of immobility that many species of anurans employ in response to external stimuli-has been considered to be a defensive mechanism (Mivatake et al. 2004). In death feigning, the animal "pretends" to be dead when it is threatened or attacked by a potential predator (McFarland 1982, Toledo et al. 2010). According to Humphreys and Ruxton (2018), death feigning or tonic immobility (TI) is the innate adoption of a motionless posture by a

prey individual that is triggered by physical contact or the close proximity of a predator. This has been observed in various animal taxa, including amphibians such as Dendrophryniscus brevipollicatus and D. leucomystax (Bertoluci et al. 2007), Physalaemus kroyeri (Gally et al. 2012), Osornophryne percrassa (Escobar-Lasso and Gonzalez-Duran 2012), Odontophrynus carvalhoi, and O. cultripes (Borges-Nojosa et al. 2016). In Peninsular Malaysia, this defensive strategy has been documented in several frog species, including Occidozyga laevis, Pulchrana picturata, P. laterimaculata, and Polypedates leucomystax (Shahriza 2016). To supplement knowledge of anuran defensive mechanisms, I report here the incidence of death feigning in three species of microhylids-Microhyla berdmorei, M. butleri, and M. heymonsi.

*Microhyla berdmorei* is a forest frog with snout-vent lengths of 25–45 mm (Berry 1975). The widespread species ranges across southern Yunnan Province, northeast India, Bangladesh, through Myanmar, Thailand, Laos, Cambodia, Vietnam, south to peninsular Malaysia, Sumatra,

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and Borneo, but does not occur in Singapore (Van Dijk et al. 2004c). In peninsular Malaysia, *M. berdmorei* lives on the forest floor of primary rainforests and along logging tracts (Berry 1975). The two smaller microhylids, M. butleri and M. heymonsi, have total lengths of 22.5-26.0 and 20.0-21.5 mm, respectively. Both occur in central and southern China, including Taiwan and Hong Kong, throughout Myanmar, Thailand, Laos, Cambodia, Vietnam, peninsular Malaysia, and Singapore (Van Dijk et al. 2004a, b). The range of M. heymonsi extends to Sumatra, Siberut, Phuket Island, and Great Nicobar Island (Van Dijk et al. 2004b). In peninsular Malaysia, both species are found in cleared and disturbed areas, such as gardens, roadsides, low bushes, among grasses and shrubs (Berry 1975).

In November 2017, two Microhyla berdmorei were collected from Sungai Sedim Recreational Forest, Kedah (SSRF) (05°25' N, 100°46' E; elevation < 150 m a.s.l.). Both specimens were caught while actively calling under leaf litter, near an intermediate-sized rock pool. The clear water rock pool had a sandy-gravel bed and the bottom was covered by leaf litter and twigs. A single M. butleri and four individuals of M. heymonsi were captured from Ulu Paip Recreational Forest, Kedah (UPRF) (05°23' N,  $100^{\circ}39'$  E; elevation < 150 m a.s.l.). The single M. butleri was collected while hiding under a rotten tree buttress, near a small ditch, which consists of shallow water. The four specimens of M. heymonsi were captured among the grasses, near an oil palm plantation, along the way to Ulu Paip. Both species were caught in December 2017, after heavy downpour. All the specimens were collected at night, between 20:00 and 22:00 h, via active searching and chance encounters. Frogs were captured by hand, aided with head lamps or flash lights. They were placed in the plastic aquaria  $(30 \times 20 \times 20 \text{ cm})$  according to the species, and transported back to the laboratory by car. The distance between the laboratory and sampling location is 54 km and took about 50 min to arrive. Wet leaf-litter and twigs were added into the aquaria as hiding places for the

frogs. In the laboratory (Universiti Sains Malaysia), snout-vent length (SVL), head width (HW) and mass (W) of each frog were measured by using digital calliper and electronic balance. The live specimens and defensive postures of the frogs were photographed using an Olympus digital camera, model SP800UZ with 30× optical zoom. The frogs were kept for approximately 24 hours before being stimulated. After being stimulated and photographed, all the frogs were euthanized using tricane, fixed with 10% formalin, stored in 70% ethanol and deposited at School Pharmaceutical the of Sciences. Universiti Sains Malaysia for future reference (17USM-SS-MBe-05; 17USM-UP-MBu-01; 17USM-UP-MH-01,02). Frogs with no sign of death feigning were also preserved (17USM-SS-MBe-06; 17USM-UP-MH-03,04). The experiments were conducted in the laboratory with temperature between 25–30°C. The sample sizes were small because the frogs are very difficult to locate and capture.

During this study, a single specimen of M. berdmorei (adult, SVL = 35 mm, HW = 10 mm, W = 8 g) and *M. butleri* (adult, SVL = 25 mm, HW = 6 mm, W = 2 g), and two specimens of M. heymonsi (SVL = 24 and 22 mm, HW = 7 and 6 mm, W = 2 and 1 g) demonstrated death feigning (Figure 1). This behavior was performed by the frogs in the laboratory, after being approached or handled. When handled, the frogs leaped in an erratic pattern, at various angles for approximately 4-5 min, before displaying a death feigning posture. While in this position, several characteristics were recorded; venter region turned upwards, dorsal region turned downwards, exposing throat and belly surfaces; body inflated, forelimbs raised upward, exposing palmar surfaces; hindlimbs held close to body, exposing plantar surfaces and exposing thigh surfaces; eyes partially closed, mouth closed and whole frog staying motionless. All the frogs retained an immobilized position between 1-2 min, before turning to normal position, and leaping away. Additionally, one of the M. heymonsi specimens displayed a crouching down posture (Figure 1G).



Figure 1. Death feigning behavior in microhylids from Kedah, Peninsular Malaysia. (A-B) Microhyla berdmorei, (C-D) M. butleri, (E-F) M. heymonsi. Crouching down behavior displayed by M. heymonsi (G).

After performing death feigning, the frog leaped and crawled under dead leaves to hide. Shortly, the leaves were removed, and the frog was found in a crouched posture. Several features were noted; the ventral region was adpressed to the substrate, the dorsal region slightly arched up, head and hindlimbs were flexed onto body, forelimbs were held close to body, the head facing down and both eyes opened. The frog remained immobilized for approximately 1 min, before leaping away.

In the present study, all the observed specimens were fleeing away in attempt to escape. As noted by Toledo et al. (2011), this behavior may be used by all anuran species, and can be either quick and erratic, or slower but directed. In our case, the frogs leaped and fled in quick and erratic patterns. Only a single individual of *M. berdmorei* and *M.* butleri, and two individuals of M. heymonsi demonstrated death feigning mechanisms. This was displayed by the frogs, after they leaped in various irregular directions for approximately 4 to 5 min. After being fatigued and failed to escape, they performed this action. Thus, I propose this as a secondary defensive mechanism. Several frog species from different families, including Pulchrana laterimaculata, P. picturata (Ranidae), Polypedates leucomystax (Rhacophoridae) and Occidozyga laevis (Dicroglossidae), show the same leaping pattern before displaying a death feigning posture (Shahriza 2016). Toledo et al. (2010) reported that this behavior was shown by anurans after a short series of jumps.

While displaying death feigning, the frogs inverted their bodies and remained motionless, like a dead animal. Since many predators do not consume dead organisms, this type of behavior might prevent the frogs from being attacked (Toledo *et al.* 2011). This also can reduce the motivation of predators, which specialize in capturing live prey (Rovee *et al.* 1976). Additionally, death feigning can enhance escape opportunities from predators, which handle prey gently or momentarily release them prior to feeding (Ratner and Thompson 1960). However, the success or failure of this depends on the foraging mode of the predator (Honma *et al.* 2006). Death feigning may be associated with other features, including aposematic coloration (Toledo *et al.* 2010) and stiff-legged posture (Bertoluci *et al.* 2007). However, these were not displayed by the observed individuals.

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# Morphological abnormalities in a population of *Pleurodeles waltl* (Caudata: Salamandridae) from southwestern Spain

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**Keywords:** Brachydactyly, brachymelia, hypodactyly, Iberian Ribbed Newt, polydactyly, skin webbing, syndactyly, tail bifurcation, urodele.

**Palavras-chave:** bifurcação da cauda, braquidactilia, braquimelia, hipodactilia, membranas cutâneas, polidactilia, sindactilia, tritão-de-costelas-salientes, urodelos.

Prevalence of morphological abnormalities usually is less than 5% in most amphibian populations (Ouellet et al. 1997, Vandenlangenberg et al. 2003, Mester et al. 2015). Morphology is closely related to whole-organism performance in amphibians (Zamora-Camacho 2018, Zamora-Camacho and Aragón 2019a), and thus, is under strong selection (Watkins 1996). Therefore, this low frequency likely reflects the negative effects of abnormalities on whole-organism performance (Zamora-Camacho and Aragón 2019b). However, amphibian populations are experiencing an alarming increase in morphological abnormality rates worldwide (Lanoo 2008, Johnson and Bowerman 2010, Laurentino et al. 2016). Amongst these, the most common are limb malformations, such as misshapen or fused limbs, and missing, or presence of extra limbs

and toes (Harris *et al.* 2008, Johnson and Bowerman 2010, Reeves *et al.* 2013).

Herein, I report six cases of morphological abnormalities recorded in a sample of 172 ribbed newts (Pleurodeles Iberian waltl Michahelles, 1830) from southwestern Spain. This species occurs across the central and southern Iberian Peninsula, as well as northwestern Africa (Salvador 2015). It is the largest salamandrid across its range; adults often exceed 30 cm in total length, roughly half of which corresponds to the tail (Salvador 2015). Normal newts have two forelimbs with four digits and two slightly larger hind limbs with five digits (Salvador 2015). The species is fairly aquatic, mainly from the autumn through the spring, when it inhabits several types of bodies of water-mainly medium-to-large temporary ponds (Salvador 2015). However, P. waltl typically aestivates during the summer, and can be found wandering on land on rainy nights.

Sampling was conducted in Pinares de Cartaya (37°19' N, 70°11' W, 60 m a.s.l.), an

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11,000-ha Pinus pinea L. grove with an undergrowth dominated by Cistus ladanifer L., Pistacia lentiscus L., and Rosmarinus officinalis L. scrub. Although it is unclear whether this formation is natural or human-introduced, there is evidence of its predominant role in the area from at least the last 4000 years; so, it is widely considered as a natural landscape (Martínez and Montero 2004). Between 2016 and 2018, the area was sampled 21 times for adult Pleurodeles waltl. Sampling was conducted on rainy autumn and winter nights, when the salamanders were intercepted during their terrestrial activity. A total of 172 individuals (93 females and 79 males) were captured. I checked morphology of each newt in search of morphological abnormalities. Newts were released after toe-clipping as a part of a long-term research project. Toeclipping is a common marking and tissuesampling technique with little effect on urodele behavior, growth, and survival (Ott and Scott 1999, Kinkead et al. 2006, Pfleeger et al. 2016). Therefore, recaptures were identified and not counted more than once.

In total, 3.49% of the sample (six individuals) had abnormalities of some type; the proportion was greater in females (4.30%, four individuals) than in males (1.53%, two individuals). Specifically, the abnormal individuals encountered were one female with polydactyly, syndactyly, and/or brachydactyly in both forelimbs and in one hindlimb (described in Zamora-Camacho 2016), along with the individuals shown in Figure 1.

In this sample, brachydactyly was the most frequent abnormality (1.74% of individuals in the sample), followed by syndactyly (1.16%), hypodactyly, polydactyly, brachymelia, interdigital skin webbing, and bifurcated tail (0.58%). These findings match previous ones that the prevalence of morphological abnormalities in amphibian populations is usually around or below 3% (Gillilland *et al.* 2001, Mester *et al.* 2015, Laurentino *et al.* 2016, Zambrano-Fernández *et al.* 2020); however, the frequency can be higher in some local populations (Worthington 1974,



Figure 1. Morphological abnormalities found in *Pleuro-deles waltl*. (A) A female with brachydactyly and syndactyly in right hindlimb, (B) a male with hypodactyly in left forelimb, (C) a female with brachymelia in left forelimb, (D) a female with brachydactyly and interdigital skin webbing in right hind limb, and (E) a male with bifurcate tail.

Hanken 1983, Zamora-Camacho and Medina-Gálvez 2019). In an isolated population of *Pleurodeles waltl* in southern Spain, three of eighteen individuals (16.7%) were polyphalangic (Torres and Hidalgo 2016).

These results are consistent with previous reports that brachydactyly is amongst the most frequent, and hypodactyly amongst the least frequent. morphological abnormalities in amphibians (Stocum 2000, Williams et al. 2008). However, the prevalence of syndactyly was higher, and that of polydactyly was lower, in this sample than in other cases reported (Stocum 2000, Williams et al. 2008). Three individuals had multiple abnormalities, which is considered to be rare (Stocum 2000, Williams et al. 2008). However, taken together, these limb abnormalities are relatively common in urodeles (Johnson et al. 2003, Martínez-Silvestre et al. 2014, Laurentino 2016). Skin webbing (not necessarily interdigital) is relatively common in anurans (Gardiner and Hoppe 1999, Meteyer et al. 2000, Johnson et al. 2001a, 2001b, Ankley et al. 2004), but not many such cases are known in urodeles (Meyer-Rochow and Asashima 1988, Johnson et al. 2006, D'Amen et al. 2008, Laurentino et al. 2016). Likewise, tail bifurcation is uncommon in wild adult urodeles (reviewed in Henle et al. 2012).

The potential causes of these abnormalities are unclear. In many cases, habitat pollution has been reported as a trigger of morphological abnormalities in amphibians (Álvarez et al. 1995, Taylor et al. 2005, D'Amen et al. 2008). However, the individuals described here were sampled in a well-preserved natural habitat; thus, pollution does not seem plausible. Excess ultraviolet radiation (Pahkala et al. 2001) and anomalous temperatures (Worthington 1974) during embryonic development also can provoke morphological abnormalities in amphibians. But again, this was a low-elevation, covered-canopy forest, which makes both phenomena unlikely. Parasite infections also can produce abnormalities (Johnson et al. 2003), sometimes synergistically with predation events (Johnson et al. 2006). Also, failed predator attacks often result in lost limbs and/or tails that, in this (Elewa *et al.* 2017) and other urodeles (reviewed in Nye *et al.* 2003), can be regenerated. An aberrant regeneration may lead to a morphological anomaly (Young 1977). For example, skin webbing may appear in newts (*Notophthalmus viridescens*) that have been forced experimentally to regenerate a limb several times (Dearlove and Dresden 1976). Finally, a genetic origin of these abnormalities cannot be dismissed (Droin and Fischberg 1980).

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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.

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

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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.

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