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First record of biogenic silica in the stomach contents of South American freshwater turtles

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Abstract. Sponges of the phylum Porifera, especially those in the class Demospongiae, produce siliceous spicules, while plants form phytoliths, both of which are sources of biogenic silica in aquatic environments. Marine and freshwater turtles have diets that vary throughout their life stages and may consume these organisms. However, there are still few studies investigating the ingestion of biogenic silica by freshwater turtles. This study aimed to analyze the diversity of biogenic silica bodies in the stomachs of *Phrynops geoffroanus*. Specimens were captured in Iguaçu National Park (PNI), Paraná, a vital remnant of the Atlantic Forest. Individuals underwent biometric measurements, photographic documentation, and euthanasia using Thiopental (93 mg/kg), following strict ethical protocols. Biological material was sent to the State University of Maringá, where stomachs were extracted. Stomach contents were processed at the Laboratory of Paleoenvironmental Studies (LEPAFE) at the State University of Paraná (UNESPAR), treated with HNO_3 on a heating plate, and the resulting material was mounted on slides for analysis. Three specimens of *Phrynops geoffroanus* at different ontogenetic stages were examined. Stomach analysis revealed the presence of biogenic silica, including phytoliths, diatom frustules, and sponge spicules, with the highest concentration found in young individuals and the lowest in juveniles. The predominant phytolith types suggest interactions with grasses and Podostemaceae. Gemmuloscleres of *Oncosclera navicella* were identified in young and adult individuals, confirming predation on freshwater sponges. This study highlights the interaction between *Phrynops geoffroanus* and organisms that produce biogenic silica.

Keywords. Biomineralization, phytoliths, sponge spicules, freshwater sponge, turtles.

INTRODUCTION

Marine and freshwater turtles have a diverse diet that varies according to ontogenetic stage. Juveniles tend to be predominantly carnivorous, young individuals display benthic feeding behavior, and adults are primarily herbivorous. However, most species are considered omnivorous, feeding on sponges, fish, crustaceans, medusae, and gastropods (Márquez, 1990). In marine environments, these animals have been documented preying on biogenic silica-producing organisms such as sponges (León Bjorndal, 2002), while in freshwater habitats, they consume plants (Esteves et al., 2021). Nevertheless, research on the presence of biogenic silica in the stomachs of freshwater animals has not yet been conducted.

Sponges belong to the phylum Porifera and produce body structures composed of silica and calcium carbonate, known as spicules (Volkmer-Ribeiro and Parolin, 2010). Species in the class Demospongiae occur in both marine and freshwater environments, producing exclu-

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sively siliceous spicules, which are considered biogenic silica. These structures are bound by organic collagen filaments and are categorized into three types: megascleres (the most prominent spicules forming the skeletal structure), microscleres (smaller spicules found in the outer surface or pinacoderm of the sponge), and gemmuloscleres (the smallest spicules derived from gemmules, with taxonomic value allowing identification at the family, genus, or species level) (Kalinovsk et al., 2016). Sponges exhibit high biodiversity and can be found in both lotic and lentic environments, attached to rocks, tree roots, and other substrates (Volkmer-Ribeiro and Machado, 2017).

Biogenic silica can also be produced by certain plant species in the form of phytoliths. Phytoliths are microscopic bodies formed by plants from silica absorbed from the soil and deposited in plant tissues such as the lumen, intercellular spaces, and cell walls (Piperno, 2006). This deposition process creates isometric molds of the original plant cells, which can sometimes be taxonomically identifiable (ICPT et al., 2019; de Oliveira et al. 2023, 2024, 2025). Aerial structures such as leaves, bracts, and fruits have the greatest affinity for phytolith production (Piperno, 1991).

Phrynops geoffroanus (SCHWEIGGER, 1812) exhibits the widest geographic distribution among Chelidae turtles, occurring throughout most of Brazil, except in the states of Roraima, Amapá, and Sergipe (Costa et al., 2022). Studies in recent decades indicate a wide distribution of P. geoffroanus in South America, encompassing diverse environments and biomes (Schneider et al., 2011; de Carvalho et al., 2017; Friol, 2019). Species of this genus of freshwater turtles have a shovel-shaped jaw, a feature that allows them to feed on items deposited on the bottoms of aquatic bodies (Rhodin and Mittermeier 1983**).** In contrast, *P. geoffroanus* exhibits a more specialized feeding pattern, consuming exclusively plant fruits during the rainy season (Fachín-Terán et al., 1995; Souza, 2004).

Some aquatic species such as fish and freshwater turtles rely on sponges and plants as sources of energy, being classified as spongivorous and herbivorous (León and Bjorndal, 2002; Esteves et al., 2021). These food resources contain silica incorporated into their body structures (Piperno, 1988; Kalinovsk et al., 2016). The quantification of biogenic silica in the digestive tract of freshwater turtles will advance the understanding of their ecological role in aquatic ecosystems. Furthermore, the characterization and specific identification of freshwater sponges through their spicules, also present in the digestive tract of these turtles, allows for an indirect assessment of the distribution of freshwater sponges. In this context, this study aimed to analyze the presence of biogenic silica (sponge spicules, phytoliths, and diatom frustules) in the stomachs of *Phrynops geoffroanus* (Schweigger, 1812), a turtle species widely distributed in Brazil. This is the first study to investigate biogenic silica in the stomach contents of freshwater turtles in South America and provides evidence of predation on freshwater plants and sponges by the native species *P. geoffroanus*.

MATERIALS AND METHODS

Study area

The sampling area is located within the boundaries of Iguaçu National Park (PNI), in the southwestern region of the state of Paraná, Brazil (25°05' to 25°41'S, 53°40' to 54°38'W). The park covers a total area of 185,262.5 hectares and represents one of the largest and most ecologically significant remnants of the Atlantic Forest biome in southern Brazil. The region is characterized by a predominantly subtropical climate and comprises two main forest types: Seasonal Semi-deciduous Forest and Ombrophilous Forest. These forest types are differentiated based on the phytogeographic characteristics of the region, including floristic composition, structure, and species distribution (Vogliotti, 2008; Alvares et al., 2013; Brocardo et al., 2019).

Several specimens of the freshwater turtle *Phrynops geoffroanus* were captured, marked, and subsequently released as part of a parallel study aimed at monitoring the species' population in the lower Iguaçu River. For specific analyses in this study, individuals Pg1, Pg3, and Pg12 were selected for euthanasia, in accordance with current ethical protocols. The captures were conducted in areas designated for public use and ecological restoration, as established in the Management Plan of Iguaçu National Park (IBDF, 1981) (Fig. 1).

Field procedures

Morphological data (biometrics) and photographic records of the captured *P. geoffroanus* individuals were collected (Fig. 2). The animals were then euthanized. Euthanasia was conducted in the field using injectable agents (barbiturates), following the guidelines recommended by CONCEA (2013). The drug used was Thiopental (diluted according to the Euthanasia Guide for Animals Used in Teaching and Research), administered intravenously at a lethal dose of 93 mg/kg. Biological specimens were sent to the State University of Maringá



Fig. 1. The study area and collection points in Parque Nacional do Iguaçu, Brazil.

(UEM) for processing and final preparation for deposition in the herpetological collection of the Capão da Imbuia Natural History Museum (MHNCI).

Animal analysis

Three specimens of *Phrynops geoffroanus* (SCHWEIGGER, 1812) were analyzed at different ontogenetic stages (Table 1). Sex determination was based on observations of shell body plan (carapace height and plastron shape), tail length, and the position of the cloacal opening relative to the distal portion of the tail (Molina, 1998; Almonacid et al., 2007). The gonadal assessment could not be performed due to limited technical expertise (Fig. 3).



Fig. 2. Specimen of *Phrynops geoffroanus* captured in the Iguaçu National Park, Brazil.

Laboratory procedures

Only the stomachs and intestines of the turtles were selected, which were processed as follows: 1) treatment with HNO_3 (65%) + H_2O_2 (v. 130) on a heating plate for 60 minutes at 100°C; 2) washing of the resulting material by centrifugation with distilled water (1000 rpm/3 min); 3) the resulting material, consisting of inorganic mineral residues and biomineralization, was collected using a 50 µL mechanical pipette, deposited on microscope slides, the slides were placed on a heated plate (~50°C) until dry and then covered with Entellan® and coverslipped. The prepared slides were stored at the Laboratory of Paleoenvironmental Studies (LEPAFE) of the State University of Paraná, UNESPAR – Campo Mourão Campus.

Slide analysis

Slides were analyzed under a biologic microscope, and images were captured using a 50-megapixel camera.

Table 1. Physical characteristics of *Phrynops geoffroanus* specimens captured in Iguaçu National Park, Brazil. Abbreviations: ID – animal identification, OE – ontogenic stage, WBM – weight (body mass) in grams, SC – size in centimeters, CL – carapace length in millimeter, CW – carapace width in millimeter, PL – plastron length in millimeter, PW – plastron width in millimeter, PH – carapace height in millimeter.

Species	ID	Sex	OE	WBM	SC	CL	CW	PL	PW	PH
Phrynops geoffroanus	Pg1	Undermined	Juvenile	13	4.9	49.4	43	42.5	32.5	17
Phrynops geoffroanus	Pg3	Female	Young	455	16.9	162.7	128.7	147.7	105.2	58.7
Phrynops geoffroanus	Pg12	Female	Adult	1,298.3	33.4	334.5	147.5	298.9	210.1	98.3

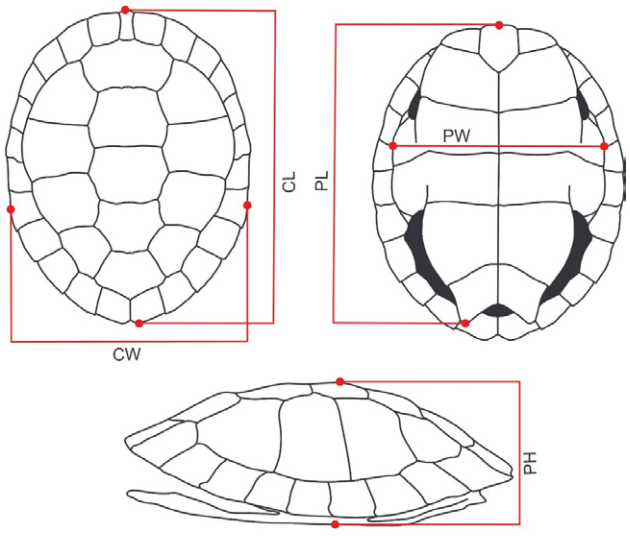


Fig. 3. Description of the morphometric measurements performed on the individuals: CL: length between the nuchal scute and the 12th marginal scute; CW: width between the 8th and 17th marginal scutes; PL: length between the intergular scute and the anal scute; PW: total width of the pectoral scutes; PH: distance from the 3rd vertebral scute to the base of the carapace (lateral view).

The analysis involved counting and identifying biogenic silica bodies along four randomly selected transects within the diameter of the optical field, evaluating from the base to the top of the slide. Phytoliths were classified based on morphological description, following the International Code for Phytolith Nomenclature (ICPN 2.0) (ICPT et al., 2019) (Table 2), while freshwater sponge spicules were described according to the existing literature.

RESULTS

Biogenic silica

Stomach content analysis of *Phrynops geoffroanus* turtles revealed the presence of biogenic silica bodies. These microremains were identified in all three individuals at different ontogenetic stages (juvenile, young, and adult). In addition, diatom frustules were observed in all analyzed specimens.

Phytolith analysis

The analysis of the stomach contents of the three specimens revealed 94 phytoliths distributed across 12 distinct morphotypes. The juvenile individual presented three morphotypes and 3 phytoliths (3.1% of the total), the young individual presented 6 morphotypes and 60 phytoliths (63.8%), and the adult presented 12 morphotypes and 31 phytoliths (31.9%) (Table 3).

The juvenile contained only three phytolith morphotypes: ELONGATE ENTIRE (*ELO_ENT*) (33.3%, n=1), BLOCKY PSILATE (*BLO_PSI*) (33.3%, n=1) and ACUTE BULBOUS (*ACU_BUL*) (33.3%, n=1).

In the young individual, seven distinct morphotypes were identified. Phytoliths from the family Podostemaceae accounted for 48 out of 60 phytoliths (80%). The following morphotypes were also observed: *ELO_ENT*, BULLIFORM FLABELLATE (*BUL_FLA*), BLOCKY PSILATE (*BLO_PSI*), *ACU_BUL* and RECTANGULAR SINUATE (*REC_SIN*).

The adult individual exhibited the highest diversity of phytoliths, with 12 distinct morphotypes found in the stomach content: *BLO*, RECTANGULAR ROUGH (*REC_ROU*), SADDLE (*SAD*), RECTANGULAR PSILATE (*REC_PSI*), *ELO_ENT*, SPHEROID ORNATE (*SPH_ORN*), *BUL_FLA* and POLYHEDRAL (*POL*).

Sponge spicules

Spicules of the megasclere and gemmulosclere types were identified in the stomach contents of the young and adult specimens but were absent in the juvenile. Three oxeas-type megascleres were found in the young individual and five in the adult, along with two gemmuloscleres attributed to the freshwater sponge species *Oncosclera navicella* (Carter, 1881), present in both individuals.

Table 2. Morphological description of the main phytolith morphotypes identified in the stomach contents of *Phrynops geoffroanus* captured in Iguaçu National Park, Brazil.

Morphotype	Code	Morphology
Acute bulbosus	*ACU_BUL*	Elongated and pointed
Bulliform flabellate	*BUL_FLA*	Elongated in a fan shape.
Elongate entire	*ELO_ENT*	Elongated with smooth margins and surface
Elongate dentate	*ELO_DEN*	Elongated with pointed margins
Elongate irregular	*ELO_IRR*	Irregularly elongated
Blocky	*BLO*	Form parallelepipeds similar to those typically found in Poaceae
Blocky psilate	*BLO_PSI*	Smooth-surfaced blocky
Rectangular rough	*REC_ROU*	Rectangular with wrinkled surface
Rectangular sinuate	*REC_SIN*	Rectangular with sinuous margins
Saddle	*SAD*	Saddle-shaped
Spheroid ornate	*SPH_ORN*	Spheroidal with a rough and warty surface
Polyehdral	*POL*	Polyhedral form

DISCUSSION

The stomach content analysis of three *Phrynops geoffroanus* individuals revealed the presence of preserved sponge spicules in both juvenile and adult specimens, as well as phytoliths and diatom frustules in all individuals, at different ontogenetic stages. This study constitutes the first record of *Oncosclera navicella* in the Iguaçu River which had only previously been recorded in lotic environments of the Piquiri River, Paraná, Brazil, as well as in lentic habitats in Venezuela (Ribeiro and Pauls, 2000; Volkmer-Ribeiro and Parolin, 2005).

The stomach contents of the analyzed individuals showed varying amounts of phytoliths across the different ontogenetic stages. The highest concentration was recorded in the young individual, followed by the adult, while the juvenile exhibited the lowest amount. This pattern indicates age-related differences in diet, feeding behavior, or resource selectivity, suggesting that young individuals may consume food items with a higher proportion of plant material. This diet shift according to the ontogenetic stage also occurs in the marine turtle *Caretta caretta*, while the juveniles feed on zooplankton, adults consume predominantly pelagic tunicates, bivalves, gastropods, and fish (Cardona et al., 2024). Thus, more studies are needed to verify whether this pattern can be explained by specimen size and ability to forage.

The stomach material of the juvenile, young and adult individuals contained the *ELO_ENT* morphotype, which is considered problematic in phytolith analysis. This phytolith type has low taxonomic value, as it can be produced by numerous monocotyledonous and eudicotyledonous species with different growth habits (Albert et al., 2018; de Oliveira et al., 2024, 2025).

The stomach contents of the young individual revealed phytolith morphotypes characteristic of the Podostemaceae family (da Costa et al., 2011; da Costa et al., 2021), indicating that the young turtle consumed vegetation from this group. Podostemaceae species are riparian plants adapted to anchor onto rocks and other solid substrates in fast-flowing freshwater environments, such as rapids and waterfalls (Ruhfel et al., 2024). The presence of these phytoliths in the stomach material therefore suggests that the young individual foraged in areas associated with these habitats. The *ACU_BUL* type identified displayed two distinct morphologies; the elongated and pointed variant resembled forms found in several eudicot families (Cannabaceae, Boraginaceae, Ulmaceae, Moraceae), commonly referred to as "Hair" (Wu et al., 2017; de Oliveira et al., 2024), and the short, pointed variant resembled the "Prickle" morphotype commonly found in monocot grasses (Fig. 4 a2) (Tripathi et al., 2014; Wroth et al., 2019).

The stomach contents of the adult individual showed the greatest diversity of phytoliths (ICPT, 2019; Qader et al., 2024). The *BUL_FLA* morphotype is a significant taxonomic marker, often associated with Poaceae species under water stress (Kondo et al., 1994). The *SAD* morphotype is widely distributed in the Chloridoideae, Bambusoideae, and Arundinoideae subfamilies (Lu and Liu, 2003). Woody species morphotypes were also identified in the adult turtle's stomach content. The *SPH_ORN*

Table 3. Quantidade e diversidade de fitólitos encontrados no material estomacal de *Phrynops geoffroanus* captured in Iguaçu National Park, Brazil.

Animal	Total phytoliths	Diversity and quantity of phytoliths
Juvenile	3	*ELO_ENT* (33.3%, n=1), *BLO_PSI* (33.3%, n=1), *ACU_BUL* (33.3%, n=1).
Young	60	*POD* (80%, n= 60), *ACU_BUL* (33.3, n=2), *BLO_PSI* (6.6%, n=4), *SAD* (5%, n=3), *BUL_FLA* (1.6%, n=1), *ELO_ENT* (1.6%, n=1) and *REC_SIN* (1.6%, n=1).
Adult	31	*BLO* (38.7%, n=12), *REC_ROU* (12.9%, n=4), *SAD* (12.9%, n=4), *REC_PSI* (9.6%, n=3), *ELO_ENT* (9.6%, n=3), *SPH_ORN* (6.4%, n=), *BUL_FLA* (6.4%, n=2), *SPH_ORN* (6.4%, n=2) and *POL* (3.2%, n=1).

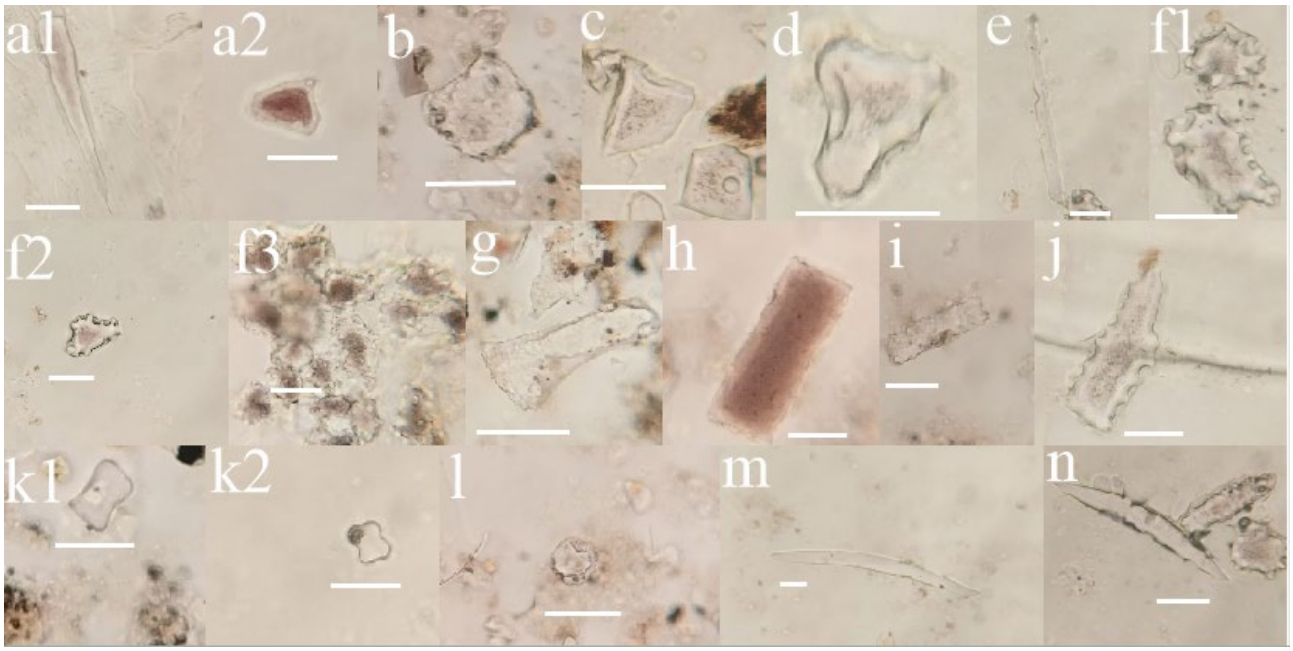


Fig. 4. The main morphotypes of phytoliths and sponge spicules found in turtles' stomach material from the Lower Iguaçu River. Scale (10 µm). Sigle a1 and a2 – *ACU_BUL*, b – *BLO*, c – *BLO_PSI*, d – *BUL_FLA*, e – *ELO_ENT*, f1, f2 e f3 –Podostemaceae phytoliths, g – *POL*, h – *REC_PSI*, i – *REC_ROU*, j – *REC_VEL*, k1 e k2 – *SAD*, o – *SPH_ORN*, m – megascleres, n gemoscleres.

morphotype is commonly found in leaf tissues of eudicots (de Oliveira et al., 2024, 2025). When present in stems or trunks, this morphotype can serve as a diagnostic marker for tree species (Bremond et al., 2008). The *POL* morphotype observed in this specimen's stomach content resembles those found in the leaves of woody species from Norway (Lisztes-Szabó et al., 2019). The large number of phytolith morphotypes derived from grasses and riparian plants confirms the selective use of these plant resources by freshwater turtles. This phytolithic interpretation is consistent with studies conducted in Australia, where similar plants were also recorded in the stomach and fecal contents of freshwater turtles (Kennett et al., 1996; Armstrong et al., 2005).

Another type of biogenic silica identified in the stomach content of *P. geoffroanus* consisted of freshwater sponge spicules. These structures were observed as megascleres, which lack taxonomic value, and gemmuloscleres, which originate from gemmules and have taxonomic significance, allowing species-level identification (Kalinovsk et al., 2016). The gemmuloscleres found in the young and adult individuals were confirmed to be from *O. navicella*.

Predation of freshwater sponges by freshwater turtles has previously been documented in different regions of the world, including the Burnett River basin in Australia (Armstrong et al., 2005), seasonally ephemeral freshwater pools on coastal floodplains of the wet–dry tropics of northern Australia (Kennett et al., 1996), and the Kawai Nui wetlands in Hawaii, USA (Works et al., 2018). Furthermore, the detection of gemmuloscleres in the stomach contents of young and adult freshwater turtles, and their absence in juveniles has not previously been observed, with other studies even describing an exclusively spongivorous diet for juveniles (León and Bjorndal, 2002). This highlights a similarity in predation patterns between freshwater and marine turtle species. The marine turtle *Eretmochelys imbricata*, for example, feeds almost exclusively on sponges, including toxic ones, and its diet is geographically uniform (Meylan, 1988). Thus, further studies with *Phrynops geoffroanus* could investigate whether its diet is geographically uniform and whether juveniles could be less resistant to toxic components than young and adults, thus elucidating the absence of sponges in juveniles. However, the diet of *Caretta caretta* juveniles differ from adults (Cardona et al., 2024), hence, the reasons for this shift should be studied for *Phrynops geoffroanus*.

CONCLUSION

The results of this study confirmed the ingestion of phytoliths by *Phrynops geoffroanus*, with differences in

abundance and diversity across ontogenetic stages. The young individual presented the highest concentration of phytoliths, while the juvenile had the lowest. The predominance of morphotypes characteristic of grasses and Podostemaceae suggests a strong feeding interaction with aquatic and riparian environments. Furthermore, the identification of gemmuloscleres of *O. navicella* in young and adult specimens indicates that this turtle species consumes freshwater sponges. Thus, the record of *O. navicella* presented in this study for the Iguaçu River reinforces previously documented evidence of the species' occurrence in both lotic and lentic environments in South America.

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AUTHORIZATIONS

The study was approved by the Ethics Committee on Animal Use (CEUA), protocol 9251160223, and authorized by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) under license 86706-4.

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The Data Deficient *Leptobrachella nokrekensis* is a junior synonym of the supposedly range-restricted and Critically Endangered *Leptobrachella khasiorum* (Amphibia: Anura: Megophryidae)

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Abstract. Based on a molecular phylogeny using 16S ribosomal RNA gene fragment and an examination of the external morphology of topotypic specimens we demonstrate that *Leptobrachella nokrekensis* syn. nov. from Garo hills is a junior synonym of *Leptobrachella khasiorum*. These data indicate that *Leptobrachella khasiorum* which was previously considered restricted to the Khasi Hills and Critically Endangered is more widespread than previously known. Genetic evidences also suggests that *L. tamdil* from Lushai hills may also be conspecific with *L. khasiorum*, warranting further investigations. A reassessment of its threat status is required based on this taxonomic revision and the consequent extension of *L. khasiorum*'s distribution and elevational range.

Keywords. 16S rRNA, data deficient, endemism, amphibians, IUCN Red List, Eastern Himalayas, taxonomic revision, threat status reassessment, Darwinian shortfall.

INTRODUCTION

The Khasi, Garo and Lushai hills of Northeast India are disconnected mountain ranges with interesting biogeographical connections to the Eastern Himalayas, the Brahmaputra Valley and the Indo-Malayan region including the Malayan Peninsula (Olson and Dinerstein, 2002; Pawar et al., 2007; Badavath and Sahoo, 2025). The region's amphibian fauna has been studied since the mid-19th century, yet many taxonomic revisions are ongoing as recent collections continue to provide new insights into species boundaries and diversity (Kamei et al., 2012; Biju et al., 2019; Saikia and Sinha 2019; Mahony et al., 2020; Patel et al., 2021; Saikia et al., 2022; Boruah et al., 2023; Naveen et al., 2023). There are a few examples of congeneric herpetofaunal diversity that are distinct between these ranges, probably due to the geographic disconnectivity (e.g., Lalronunga et al., 2021; Naveen et al., 2023; Mirza et al., 2024) while many others are known to occur across them (e.g., Siammawii et al., 2021; Muansanga et al., 2022; Naveen et al., 2025).

Four species of *Leptobrachella* Cope, 1865 are known from India, of which the three species dealt with in this study were described within a short span between January and June 2010, from the Khasi, Garo, and Lushai Hills. *Leptobrachella khasiorum* (Das, Tron, Rangad, and Hooroo, 2010), originally described as *Leptolalax khasiorum* from Mawphlang Sacred Grove in the Khasi Hills is believed to be endemic to just this region. Consequently, it is categorized as Critically Endangered due to its extremely narrow Extent Of Occurrence. *Leptolalax tamdil* (Sengupta, Sailo, Lalremsanga, Das, and Das, 2010) was described from the nearby Tamdil wetlands in Mizoram. This species was subsequently trans-

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ferred to the genus *Leptobrachella* and reported from a few other locations in central Mizoram, as well as from another more northeastern location in Manipur (Decemson et al., 2021). A third species, described from the Garo hills, *Leptobrachium nokrekensis,* later transferred to the genus *Lepobrachella,* although claimed to be described in "2009" by Mathew and Sen (2009), is now considered *Leptobrachella nokrekensis* (Mathew and Sen, 2010 "2009"), since Das and Deuti (2011) convincingly argued that the name was only made available on June 3, 2010, the official date of distribution of this printed publication.

Leptobrachella is one of the most diverse genera in the family Megophryidae Bonaparte, 1850 with 117 species distributed across southern China, northeastern India, Myanmar, Thailand, Vietnam, Malaya, Borneo, and the Natuna Islands (Frost 2025). The members of this genus exhibit a high degree of localised endemism and morphological overlap (Nguyen et al., 2021; Wu et al., 2025). But despite their cryptic morphology, new species continue to be identified, based on evidence from molecular analyses (Lin et al., 2022; Hoang et al., 2024; Luo et al., 2025; Wu et al., 2025). However, in the case of the northeastern Indian species, *Leptobrachella khasiorum*, *Leptobrachella nokrekensis*, and *Leptobrachella tamdil*, even though all of them exhibit a high degree of morphological similarity, none of their original descriptions included molecular data (Das et al., 2010; Mathew and Sen, 2010 "2009"). In the current study, the taxonomic validity of *Leptobrachella nokrekensis* is re-examined, based on fresh topotypical material collected from the Garo and Khasi hills, and further studies on *Leptobrachella tamdil* from Mizoram to assess its status is recommended.

MATERIALS AND METHODS

Sampling

Specimens were collected from two locations of the northeast Indian state of Meghalaya: Sakalgre Village, Garo hills (25.515992, 90.381045; 925 m asl): Three specimens – one adult male (PU RSN 23) and two adult females (PU RSN 24, PU RSN 34), collected by RSN from a stream near the village in October 2024.

Near Mawphlang Sacred Grove, Khasi hills (25.436459, 91.759246; 1575 m asl): Two specimens – one adult male (PU RSN 32) and one adult female (PU RSN 33), collected by RSN from a stream flowing out of the grove in October 2024.

Specimens were collected under the following permits (No. WC/Research/157/608). They were photographed in life before being euthanized using approximately 0.10 ml of a 20% benzocaine solution applied to the ventral surface. Liver tissues were extracted from freshly euthanized specimens for molecular analyses and stored in 99% molecular-grade ethanol. The specimens were then fixed in 10% buffered formalin and subsequently stored in 75% ethanol. All specimens were deposited in the collections of the Department of Ecology and Environmental Sciences, Pondicherry University (PU).

Measurements

Morphological data from fixed specimens were measured to the nearest 0.02 mm with INSIZE digital callipers. The following measurements were taken: Snout-Vent Length (SVL); Axilla-to-Groin Length (AGL); Mid-Body Width (MBW); Head Width (HW) (measured at the angle of the jaws); Head Length (HLD) (from posterior end of the mouth snout tip); Nostril-to-Snout Distance (NS); Internarial Distance (IN); Maximum Upper Eyelid Width (UEW); Eye Diameter (ED); Interorbital Distance (IO) (shortest distance between the upper eyelids); Eye-to-Nostril Distance (EN); Tympanum Diameter (TYD); Tympanum-to-Posterior Corner of Eye Distance (TE); Upper Arm Length (UAL); Forearm Length (FAL) (previously Forelimb Length) (measured from the elbow to the base of the outer palmar tubercle); Palm Length (PAL) (measured from the base of the outer palmar tubercle to the tip of the third finger); Thigh Length (THL); Shank Length (SL) (approximated by measuring the crus); and, Foot Length (FL) (measured from the base of the inner metatarsal tubercle to the tip of the fourth toe).

Molecular analysis

Total genomic DNA was extracted from two specimens of *Leptobrachella* from Meghalaya (PU RSN 33 and PU RSN 24) with a DNA extraction and purification kit, following the manufacturer's protocols. A fragment of the16S rRNA gene was amplified using the primers 16sAR-L (5' CGCCTGTTTATCAAAAACAT-3') and 16sBR-H (5'CCGGTCTGAACTCAGATCACGT 3') respectively (Kocher et al., 1989). Amplifications were performed in an Applied Bio Systems Veriti 96 well thermal cycler: 20 µl reactions with 4 µl of 5X Phusion HF buffer, 0.4 µl of 10mM dNTP, 0.2 µl of Phusion DNA Polymerase, 0.1 µl each of forward and reverse primers, 2.0 µl of DNA template and 13.2 µl of nuclease free water with the following procedure: initial denaturation of DNA at 95°C for 5 min, 35 cycles of: denaturation at

95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and at last, final extension at 72°C for 10 min. The amplicon was checked by running it through an agarose gel electrophoresis for a clear band of the desired region in the amplified PCR product. The amplified PCR product was purified and sequenced commercially at Barcode BioSciences Pvt.Ltd (BBS), Bangalore, India. The new sequences were then checked on the Basic Local Alignment Search Tool, BLAST (The National Centre for Biotechnology Information) (Altschul et al. 1990) to verify their approximate identity.

Phylogenetic analysis

The new topotypic sequences of *Leptobrachella khasiorum* (PZ625872) and *Leptobrachella nokrekensis* (PZ625865) were assembled along with 52 other congeners from the Indo-Burma, Indo-China and southwestern China regions, with *Leptobrachium sylheticum* and *Xenophrys major* as the outgroups (see Fig. 1). The single existing sequence from the type locality of *Leptobrachella khasiorum* and all available homologous sequences of *Leptobrachella tamdil* were also added to the dataset. The resultant dataset of 61 sequences were aligned with Muscle and optimized manually, in MEGA X (Kumar et al., 2018). This alignment was then used to determine the uncorrected pairwise genetic distances between the samples with MEGA X. For the phylogenetic analysis, the best-fit model of nucleotide substitution was selected in jModelTest2 (Darriba et al., 2012). MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) was used to perform the Bayesian analysis under the GTR+I+G model. Two independent analyses, each consisting of four Metropolis-coupled Markov chain Monte Carlo (MCMC) chains, were run for 20 million generations, with parameters sampled every 1,000 generations. Convergence was evaluated by ensuring that the average standard deviation of split frequencies fell below 0.01 and that potential scale reduction factors approached 1.0. Stationarity and effective sample sizes (ESS) were monitored within MrBayes. The first 25% of sampled trees were discarded as burn-in following Huelsenbeck et al. (2001), and clade support was assessed using Bayesian posterior probabilities (BPP), with values ≥ 0.95 considered strong (Leaché and Reeder, 2002). Maximum-likelihood (ML) analyses were carried out in IQ-TREE v1.6.12 (Nguyen et al., 2015) under the GTR+F+I+G4 model. Branch support was estimated using 10,000 ultrafast bootstrap (UFB) replicates (Hoang et al., 2018). Nodes with UFB ≥ 95 were considered strongly supported (Minh et al., 2013).

RESULTS

The Bayesian Inference and Maximum Likelihood trees obtained were similar in topology and largely congruent with existing recent large scale phylogenies of *Leptobrachella*. The relationships between the focal group, comprising *Leptobrachella khasiorum*, *L. nokrekensis* and *L. tamdil*, were well resolved and the new sequences of *L. khasiorum* and *L. nokrekensis* grouped together with the sequence of *L. khasiorum* by Mahony et al., 2017 from the type locality. The Indian taxa formed a strongly supported monophyletic clade (UFB 100; BPP 1) which showed a sister relationship to a recntly described species *L. aurantirosea*. The uncorrected pairwise genetic distance at the 16S mitochondrial gene between the new samples from this study and the available sequence of *L. khasiorum* from the GenBank was 0.2-1.3% (Table 1). These shallow divergences fall within the range typically considered conspecific among members of this genus and anurans as a whole for the 16S mtDNA gene.

Furthermore, morphological characteristics were similar among newly collected specimens. Based on comparative examination of specimens of *L. khasiorum* with the original descriptions of *L. nokrekensis* provided in Mathew and Sen, ("2009" 2010), Das and Deuti, (2011) point out, the following differences between these two species, *L. nokrekensis* (vs. *L. khasiorum*) "*reduced tympanum, being less than half orbit diameter (vs. half orbit diameter); area around pectoral region pale (vs. with dark pattern); paired red tubercles anterior to tympanum (vs. absent); loreal region concave (vs. sloping); and dorsum with longitudinal folds (vs. absent)*".

In contrast, the series of topotypic *Leptobrachella* specimens collected and examined in this study, including adult specimens from both the Garo and Khasi Hills, consistently show a tympanum size greater than half the orbit diameter, an absence of longitudinal folds on the dorsum, and a consistently concave loreal region in all specimens. Other differences in coloration and tuberculation noted by Das and Deuti, (2011), these traits are now known to be highly polymorphic in this genus and are not always reliable for species delimitation (see intraspecific variation section in Nguyen et al., 2021; Lin et al., 2022; Hoang et al., 2024; Luo et al., 2025; Wu et al., 2025) (Fig. 2, Table 2).

Based on these morphological and molecular evidences from the topotypes, it's evident that *Leptobrachella khasiorum* and *L. nokrekensis* are not two distinct species. The remaining question then is nomenclatural priority. Although *L. nokrekensis* was initially believed to have been described earlier, Das and Deuti, (2011) clarified that its official date of availability was on June 3, 2010. Thus, the nomen *L. khasiorum*, made available on

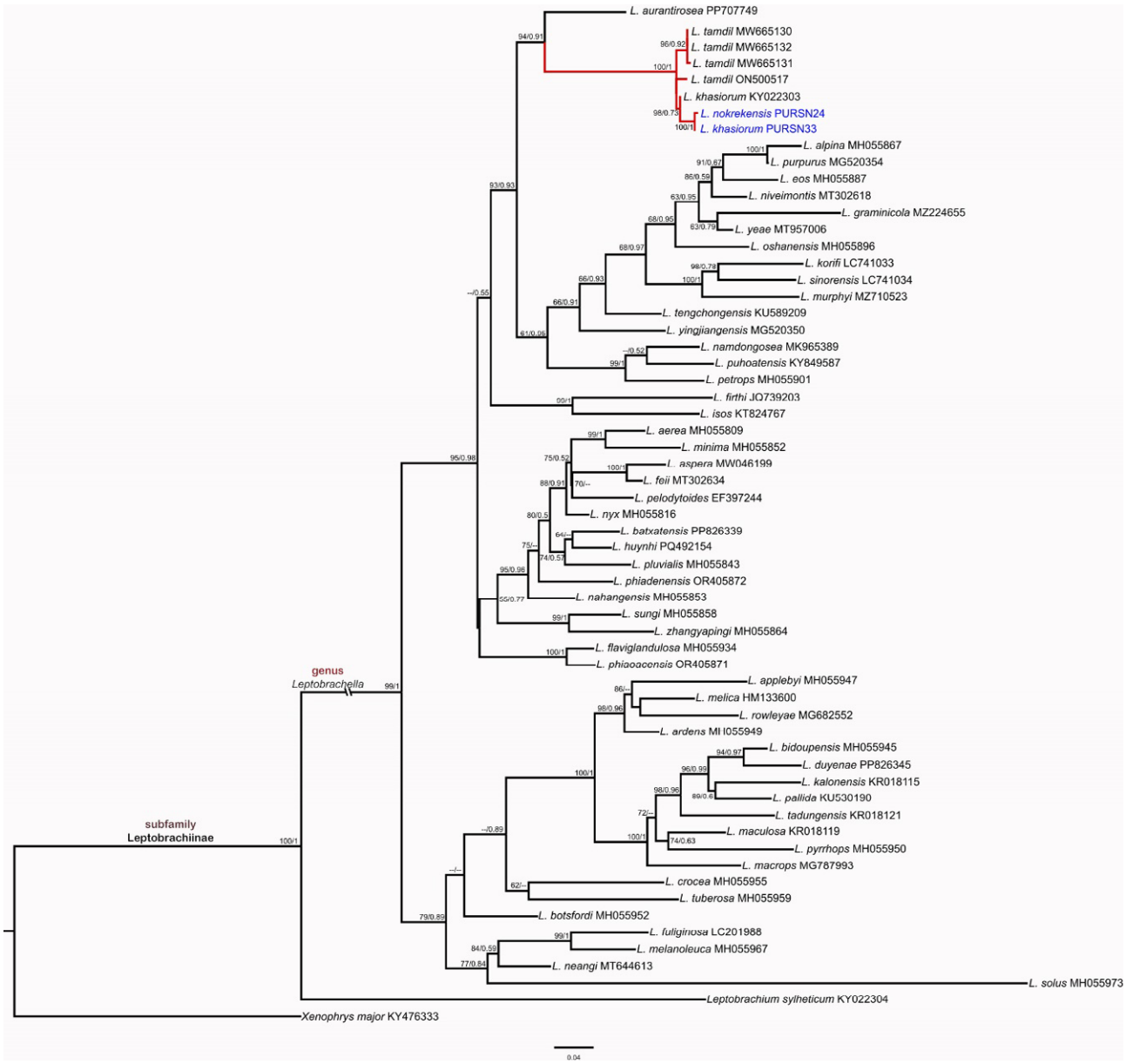


Fig. 1. Maximum Likelihood tree based on the 16S mtDNA gene for *Leptobrachella* species of India studied here and congeners from nearby regions. The clade containing the Indian taxa is highlighted in red, and newly collected topotypic samples are shown in blue. Branch support values represent Ultrafast Bootstrap Support (UFB > 50%) and Bayesian Posterior Probabilities (BPP > 0.50), respectively.

January 10, 2010, in Das et al., (2010), is the first available among the two. Therefore, *L. khasiorum* is the valid nomen for this species, and *Leptobrachella nokrekensis* syn. nov. is its junior synonym.

The preliminary molecular data presented here suggest that *L. tamdil* too is a probable junior synonym of *Leptobrachella khasiorum* (uncorrected p-distance 1.0 – 2.6%). However, this warrants further studies, as the current study was unable to examine any fresh specimens from Mizoram or the specimens associated with the sequences used from GenBank in this study and also the available GenBank sequences are not from the type locality but from other localities in Mizoram and Manipur. Therefore, this is left unresolved here for further studies to resolve.



Fig. 2a. *Leptobrachella khasiorum* (PU RSN 33) from Mawphlang, Khasi hills.

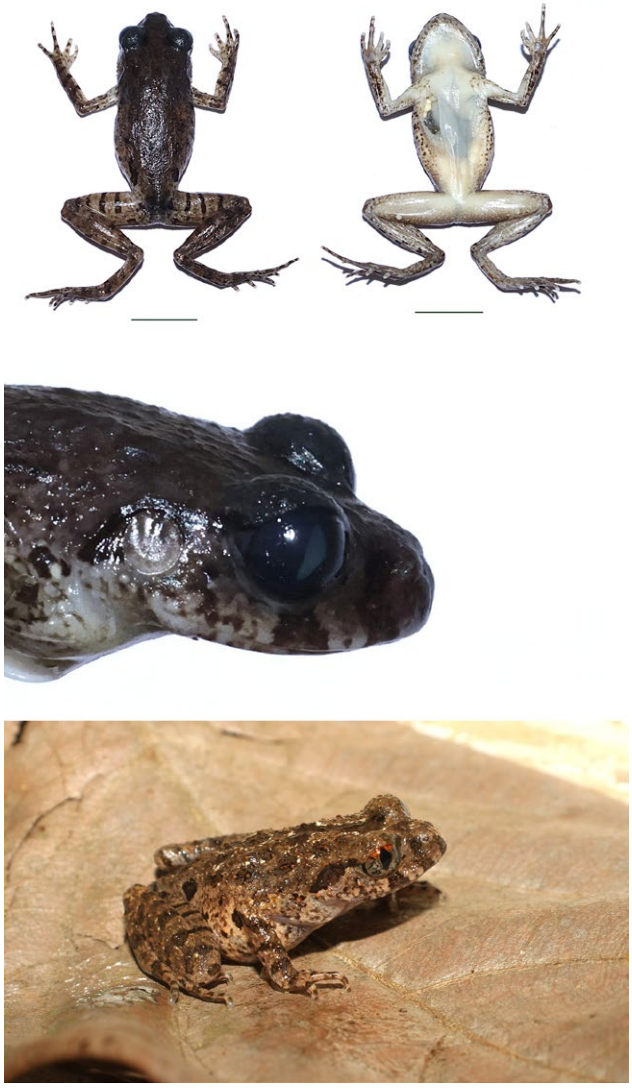


Fig. 2b. *Leptobrachella nokrekensis* syn. nov. (PU RSN 24) from Sakalgre, Garo hills.

DISCUSSION

With the results presented here, *Leptobrachella khasiorum*, previously thought to be Critically Endangered due to its narrow endemic distribution range, restricted to a small forested area in and around the Mawphlang Community Reserve, is now known to occupy a substantially wider range, including the known occurrence locations of *L. nokrekensis* syn. nov., across Nokrek National Park. In addition to its previously known occurrences in the Garo Hills, this species was recorded near Sakal Aduma (25.524°N, 90.342°E; 980 m asl) and near Tura Peak (25.515°N, 90.232°E; 820 m asl) during this study. In addition to this, Chandramouli et al. (2022) reported a specimen of *Leptobrachella* (referred to it as *Leptobrachella* cf. *khasiorum,* (SACON VA 115 – an unsexed subadult collected by P. Karthik), from Jirang (25.945°N, 91.567°E, 1280 m asl), approximately 50 km from Mawphlang in a straight line. It is likely that this specimen also belongs to the same species, as the location falls within the now known elevational range of *L. khasiorum* and is relatively close to Mawphlang. With these new records, the updated Extent of Occurrence for this species is 4,027 km^2 across an elevational range of 800 m asl to 1,600 m asl. In the locations where this

Table 1. Genetic divergences (uncorrected p-distance) between specimens identified as *Leptobrachella khasiorum*, *Leptobrachella nokrekensis*, and *Leptobrachella tamdil*.

	Location	1	2	3	4	5	6	7
L. nokrekensis PZ625865	Garo Hills							
L. khasiorum PZ625872	Khasi Hills	0.0026						
L. khasiorum KY022303	Khasi Hills	0.0131	0.0105					
L. tamdil ON500517	Mizoram	0.0236	0.0209	0.0105				
L. tamdil MW665130	Mizoram	0.0236	0.0209	0.0105	0.0157			
L. tamdil MW665131	Mizoram	0.0262	0.0236	0.0131	0.0183	0.0026		
L. tamdil MW665132	Mizoram	0.0236	0.0209	0.0105	0.0157	0.0000	0.0026	-

species was recorded during this study, it was found to be abundant. The species is also likely distributed across several other protected areas, in addition to Nokrek National Park and Mawphlang Sacred Forest. Based on this distributional information, the species qualifies for being downlisted from Critically Endangered under the IUCN Red List criteria (see IUCN 2012). This could still be an underestimation of the distribution range of this species and further survey efforts across these regions, as well as the clarification of the taxonomic status of *Leptobrachella tamdil* is required to accurately define the distribution and conservation status of *Leptobrachella khasiorum*.

In addition to these three species, Northeast India also harbours another Critically Endangered *Leptobrachella*, *Leptobrachella lateralis* (Anderson, 1871), which was originally thought to be from Bhamo but has now been neotypified from the Naga Hills. This species remains poorly documented, with no data on its phylogenetic position or natural history, and continues to be known with confidence only from the neotype locality, with no subsequent confirmed records elsewhere. We did not survey its range during the present study. Based on general biogeographic patterns, we infer that this species is unlikely to be closely related to the *Leptobrachella* populations of the Khasi, Garo, and Lushai Hills, as the Naga Hills are comparatively less connected to these ranges. Further surveys are therefore necessary to clarify its distribution and taxonomic status.

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Table 2. Morphometric measurements (in mm) of *Leptobrachella khasiorum* and *Leptobrachella nokrekensis* specimens examined in this study.

Voucher number	*PU RSN 23*	*PU RSN 24*	*PU RSN 34*	*PU RSN 32*	*PU RSN 33*
Location	Sakalgre Village, Garo Hills	Sakalgre Village, Garo Hills	Sakalgre Village, Garo Hills	Near Mawphlang Sacred Grove, Khasi Hills	Near Mawphlang Sacred Grove, Khasi Hills
Sex	Male	Female	Female	Male	Female
SVL	33.1	32.25	30.65	27.85	28.05
AGL	15.7	14.14	12.18	10.6	10.04
MBW	12.34	11.78	10.02	8.06	8.37
HW	11.06	11.3	10.85	9.31	9.54
HLD	11.31	11.2	10.35	10.03	9.26
NS	1.17	1.12	1.89	0.86	1.77
IN	2.34	2.66	2.34	2.91	2.63
UEW	3.38	3.04	3.02	2.9	2.94
ED	4.4	3.6	3.56	3.63	3.66
IO	3	3.82	3.43	2.8	3.1
EN	2.49	2.52	2.64	2.2	2.46
TYD	2.26	2.22	2.14	1.86	2
TE	1.26	1.32	1.1	1.15	1.02
UAL	6.16	5.34	5.76	4.53	4.26
FAL	8.51	7.26	8.44	6.32	6.49
PAL	7.62	7.66	6.62	7.11	7.25
THL	14.87	14.14	13.96	12.08	12.49
SL	14.33	15.02	14.36	12.96	12.45
FL	13.2	14.28	12.56	11.92	11.65

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Sexual dimorphism, feeding ecology, and reproductive traits in the grass snake (*Natrix natrix*) from the Ramsar site "Bardača Wetland" (Republic of Srpska, Bosnia and Herzegovina)

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Abstract. Through a capture-mark-recapture study, between 2011 and 2014, the first long-term investigations of the grass snake (*Natrix natrix*) were commenced in Bosnia and Herzegovina. A hundred-and-seventy-two adult individuals (96 ♀ : 76 ♂) have been captured and processed. Females reached larger overall body dimensions than males and had longer jaws than males of the same body length. On the other hand, tails were relatively longer in males compared to females. These results illustrate the typical sexual dimorphism in this species. Diet analysis revealed natural yearly variation in the qualitative composition of prey, which confirmed intra-population plasticity in the grass snake's diet. Also, their diet was strongly influenced by the drying of ponds. We found differences in the direction of prey swallowing depending on the prey type (frog or fish), which was conditioned by prey morphology. We also found intersexual differences in prey type: females consumed more diverse prey and ate green frogs (*Pelophylax* sp.) significantly more often. This finding results from sexual differences in head dimensions and illustrates differences in the energetic contents of different prey types. Clutch sizes ranged between eight and 28 eggs, and the correlation between female body size and clutch size was positive. These results are within the ranges of previously published findings. Semi-aquatic snakes are important elements of wetland ecosystems, and can indicate their condition. Long-term population studies of these animals can provide warning signals of wetland ecosystem disturbance and the need for immediate conservation actions.

Keywords. CMR study, morphometry, feeding frequency, prey type, clutch size, Bardača.

INTRODUCTION

Thanks to their high phenotypic plasticity (Madsen and Shine, 1993a) and population density (Ajtić et al., 2013), significant geographic variations in diet (Luiselli et al., 2005, 2007; Weiperth et al., 2014), and the specific reproductive biology (Madsen, 1983; Luiselli, 1996; Luiselli et al., 1997), snakes belonging to the genus *Natrix* are highly suitable for investigations of morphology and population ecology. Some natricine snakes were shown to be capable of adapting to human-modified landscapes, which can even positively influence these animals' abundance and certain aspects of morphology, but have adverse effects on others (Mészáros et al., 2023, 2024). Therefore, the significance of the studies of aquatic snakes is manifold.

Being gape-limited predators (unable to tear and chew their prey), snakes' bodily dimensions often strongly influence their diet (Arnold, 1993; Forsman, 1996). In numerous snake species, intersexual differences were

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found in the type and/or size of the consumed prey (Houston and Shine, 1993; Santos et al., 2000; Shetty and Shine, 2002), that are most often related to sexual body size dimorphism, SSD (Madsen, 1983; Shine, 1991a; Santos et al., 2000) and/or intersexual niche divergence (Houston and Shine, 1993). On the other hand, geographic differences in the type of consumed prey were most often explained by the differences in the availability of potential prey among different habitats/localities (Luiselli et al., 2005, 2007; Weiperth et al., 2014). Previous investigations related to *Natrix* species documented differences in the type and/or size of the consumed prey among the sexes, among age categories, and different populations (Filippi et al., 1996; Luiselli et al., 1997; 2005, 2007; Santos et al., 2000; Weiperth et al., 2014; Šukalo et al., 2014), but also intensive feeding on introduced prey species (Gregory and Isaac, 2004; Acipinar et al., 2006; Šukalo et al., 2014). Snakes belonging to the genus *Natrix* can persist in areas where the availability of their usual prey type drops, by switching to alternative prey, namely invasive fish, lizards or mammals (Luiselli et al., 2005; Šukalo et al., 2014). The ever-increasing anthropogenic influences were also shown to affect SSD in *N. natrix*, through the reduction of female body size (Bury and Zając, 2020). However, it is not completely clear how and how quickly the intra-population level changes occur: to determine this, long-term investigations are necessary.

According to Smith and Fretwell (1974), females can allocate the energy available for reproduction either to fewer, larger offspring or to more smaller offspring (Shine, 1988). In *Natrix* sp., the positive correlation between body size and clutch size was proven (Luiselli et al., 1997; Luiselli and Rugiero, 2005). However, this relationship is not straightforward, i.e., trade-offs between growth rate and reproduction output must be considered, under different/varying climatic conditions and prey (energy) availability (Shine, 1988; Luiselli et al., 1997). Clutch/offspring size is influenced by various factors, such as female body condition during ovulation (King, 1993), precipitation, prey availability (Bonnet et al., 2001), and genetic factors (Gregory and Larsen, 1993). Since reproductive success depends on environmental conditions, its variation at intra- and inter-population levels is expected. Also, because lifetime, not instantaneous, reproductive success is what matters in the evolution of various life-history traits (Shine, 1988), prolonged/permanent environmental disturbance should be considered in wildlife monitoring.

The grass snake is widely distributed in Europe (Arnold and Ovenden, 2002). It is often found in standing water habitats such as ponds and lakes, but also inhabits streams, rivers and meadows (Radovanović, 1951), and it can be found in forests far from water (Arnold and Ovenden, 2002). Sexual dimorphism in body size is female-biased (Madsen, 1983; Luiselli et al., 1997). Regarding diet, this species is an adaptable generalist preferring amphibians but consuming other vertebrates as well (Radovanović, 1951; Luiselli et al., 1997; Luiselli et al., 2005). This species' seasonal activity starts in March or April. Breeding usually occurs during April or May (Luiselli et al., 1997), when "mating balls" form in which one female mates with several males (Luiselli, 1996). Eggs are laid at the end of July, and the young hatch between late August and early September (Luiselli et al., 1997).

Various aspects of diet and reproductive biology of the grass snake (*Natrix natrix*) were thoroughly investigated in Italy (Luiselli and Rugiero, 1991; Filippi et al., 1996; Capula and Luiselli, 1997; Luiselli et al., 1997) and Sweden (Madsen, 1983, 1984, 1987; Madsen and Shine, 1993a, 1993b), but also in England (Gregory and Isaac, 2004) and the Middle East (Ahmadzadeh et al., 2011). Although the grass snake is considered the most common and widely distributed snake species in former Yugoslavia (Radovanović, 1951), investigations providing quantitative data on various aspects of its biology and ecology are scarce in ex-Yugoslavia and the entire Balkan Peninsula (Janev Hutinec and Mebert, 2011; Šukalo et al., 2014). This paper aimed to present the results of the first multi-year investigations into sexual dimorphism, diet, and reproductive biology of grass snakes in Bosnia and Herzegovina.

MATERIALS AND METHODS

Study sites

The Ramsar site "Bardača Wetland" is located in the north of Bosnia and Herzegovina, at the confluence of the Vrbas and Sava rivers. The total surface of the area is 3,500 ha, and it includes fishponds, flood meadows and forests, arable land and inhabited areas (Fig. 1). An integral part of the complex is the pond system "Bardača", comprising 11 lakes, with an area of 675 ha, located between 17°24′08″ and 17°28′11″ E, and between 45°05′16″ and 45°06′38″ N, with an altitude between 91.0 m and 93.8 m above sea level (Gašić and Dujaković, 2009). Already in 1969, thanks to the high diversity of wetland birds, Bardača was designated as an Important Bird Area (IBA) in Bosnia and Herzegovina, and in 2007, the Ramsar Secretariat designated it an internationally important wetland area (Panić and Nagradić, 2019). However, regardless of the above, at the beginning of 2012, eight of 11 lakes were completely dried up, which significantly degraded this ecosystem (Šukalo

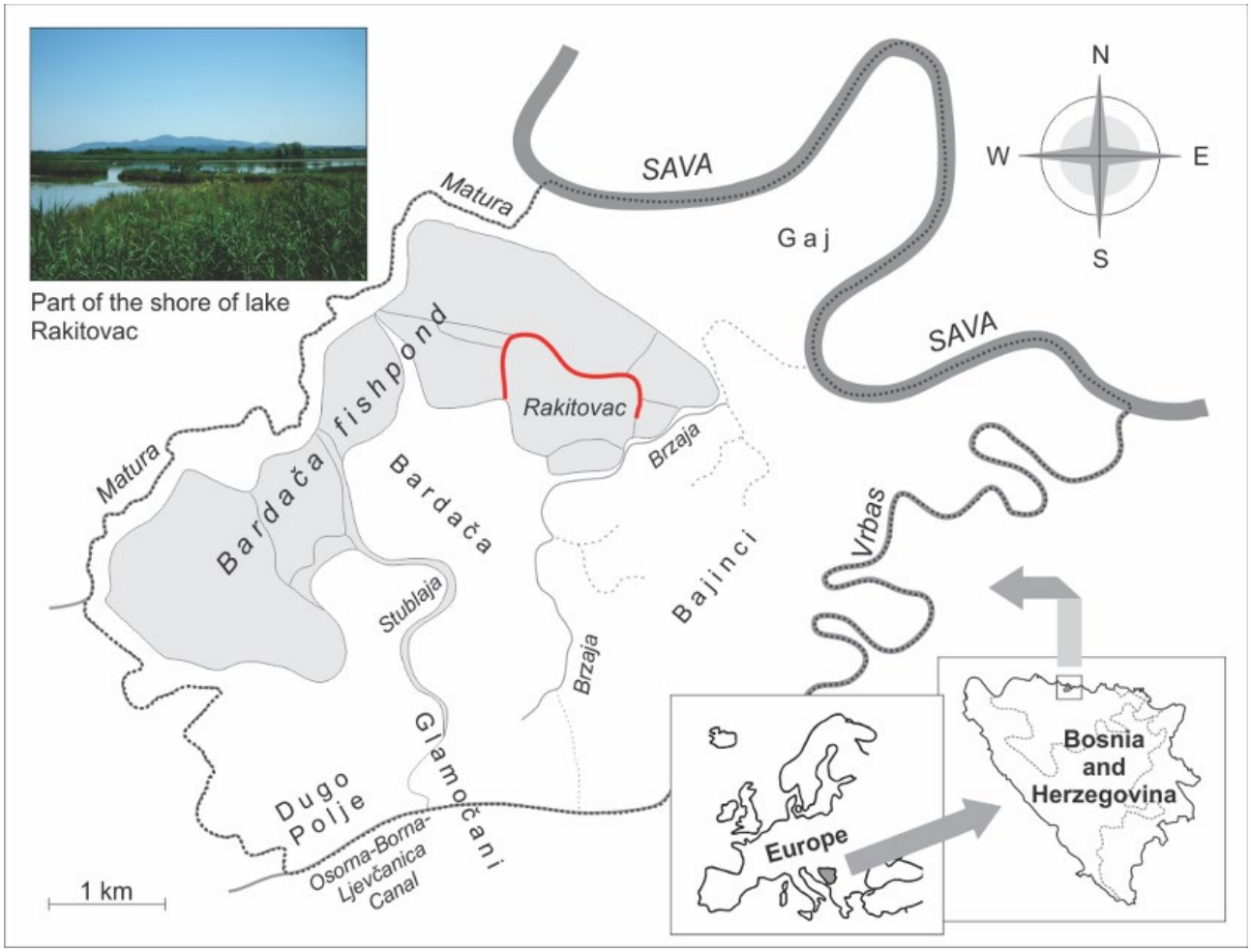


Fig. 1. Map of the Ramsar site "Bardača Wetland".

et al., 2014). In the last several years, only one or two lakes have been filled with water (Šukalo and Dmitrović, 2023). The ichthyofauna of the Bardača complex and natural watercourses (rivers Brzaja, Matura and Stublaja) is represented by 20 autochthonous and six allochthonous species (Radević, 2000; Vuković et al., 2008), among which two allochthonous species (*Ameiurus nebulosus* and *Carassius gibelio*) are the dominant fish in the natural watercourses (Vuković et al., 2008). The batrachofauna of "Bardača Wetland" is represented by nine species of amphibians, among which green frogs (*Pelophylax* sp.) are the most common and numerous amphibians in this area (Šukalo and Dmitrović, 2023).

Field procedure

Field investigations were undertaken between March 2011 and September 2014, along the part of the Rakitovac Lake shore (red line in Fig. 1). One to three persons spent 39 days working in the field, with an estimated sampling effort of approximately 208 hours per sampler. Annual distribution of fieldwork sessions was as follows: eight days in 2011 and 2012 each, 12 days in 2013, and 11 days in 2014. The research was conducted during spring (six days in March, 10 in April, and seven in May) and summer (11 days in June/July and five in September/October). The snakes were captured by hand or with a hand net attached to a long stick and kept in cotton bags until processing. Their gender was determined based on their external tail morphology (Feriche et al., 1993) and, when necessary, by squeezing out the hemipenes. Only adult individuals were analysed. The minimum body size at maturity for males was determined according to the smallest male found copulating; for females, the threshold was the length of the body of the smallest female in which growing follicles/eggs

were manually palpated. Body mass (BM) was measured with a digital scale (precision 1 g). Standard body length (snout-vent length, SVL) and tail length (TL) were measured with a tape meter (precision 1 mm), while head dimensions were measured with a calliper (precision 0.02 mm). Cranium length (CL) was measured from the tip of the snout to the posterior cranium edge. Interocular distance (IO) was measured between the outer edges of supraocular plates; post-parietal length (PPL) is the distance between the tip of the snout and the junction point of parietal plates with the frontal scale. Jaw length (JL) was measured from the tip of the snout to the posterior end of the upper jaw (Thorpe, 1975; King et al., 1999).

Every captured individual was manually palpated for the presence of food in its digestive system. Manual palpation was proven to be efficient in determining snakes' gut contents (Cruz, 2013). In addition, spontaneous regurgitation provided details regarding the qualitative composition of food and the direction of prey swallowing.

Amphibian prey items (green frogs) were determined to the genus level, but fishes were identifiable to the species level. We distinguished among "intact", "half-digested", and "digested" prey. "Intact" were the recently consumed prey items, with no or just minor signs of digestion. These were recognizable (fish or anuran) already at palpation, as was the direction of the ingestion. "Half-digested" were those partly processed by stomach enzymes (still palpable), and "digested" prey related to pulpy remains of food in the animals' digestive systems, which were not palpable. All three categories were included in the feeding frequency analysis.

The data regarding fecundity were obtained through abdominal palpation, i.e. palpating and counting the growing follicles/eggs through the females' body wall. During our field investigations, we found a communal nest with approximately 70 eggs of *N. natrix* in several clusters, which is quite common (Aubret et al., 2015). We randomly selected 11 eggs and measured their length, width, and weight, while the remaining eggs were left in the nest undamaged, so the incubation and hatching could be monitored. At the assessed hatching time, we caught eight hatchlings and measured their standard body length, tail length, and body mass.

To avoid the effects of pseudoreplication in the analyses of sex ratio, sexual dimorphism, and fecundity (within the same year), all individuals were marked by ventral scale clipping (Winne et al., 2006) and released at the places of capture. Regarding reproductive data, if a female was recaptured within the same reproductive season, only the first recorded value was included in the analysis to avoid pseudoreplication. However, if the same female was recaptured in two different reproductive seasons, both records were included because they represent independent reproductive events. Marking also prevented repeated morphometric measurements on the same individuals within a year. Diet data were treated as independent samples, regardless of marking. All measurements and data collection were performed on live individuals in the field within 1–2 hours following capture, i.e., the animals were not taken to the laboratory or otherwise removed from their habitat.

Statistical analyses

Statistical analyses were performed in Statistica 7.0 (StatSoft, 2004). For the basic presentation of variability, we used descriptive statistics. Values of $P < 0.05$ were set as a threshold for statistical significance. We checked the differences between the genders in morphometric traits with the analysis of variance (ANOVA), and to remove the influence of overall body size on its specific parts, we applied the analysis of covariance (ANCOVA). Pearson linear correlation and simple linear regression were used to determine the relationship between tail length and SVL, jaw length and SVL, and between egg number and SVL of gravid females. To estimate the relationship between prey weight and snake SVL, simple linear regression on log-transformed values was used. For the analysis of frequencies, χ^2 test and contingency tables were used.

RESULTS

Sexual dimorphism

In 4 years, we captured 172 adult individuals. Sex ratio among them was slightly skewed towards females: 96 ♀ vs. 76 ♂ ($\chi^2 = 1.17$, df = 1, n.s.). The analysis of variance found statistically highly significant ($P < 0{,}001$) higher values in females for all analysed morphological traits (Table 1).

The Analysis of Covariance (ANCOVA), with the standard body length (SVL) as a covariate, revealed statistically significant differences between the sexes only for the tail length, TL ($F = 17{,}3$; $P < 0{,}001$, longer in males) and jaw length, J ($F = 25{,}8$; $P < 0{,}001$, longer in females).

Fig. 2 shows the relationship between jaw length (J) and standard body length (SVL) of adult males and females (A), and the relationship between tail length (TL) and SVL (B).

Table 1. Average, maximum and minimum values and standard deviations of the measured characters, and the results of the Analysis of Variance (ANOVA) (N= number of individuals; mean = average value; SD = standard deviation; min = minimum value in the sample; max = maximum value in the sample; F = variance ratio; p = statistical significance). All values are given in millimetres (mm) except Body Mass, which is in grams (g). Trait abbreviations are given in the Material and Methods section.

Trait	Females			Males			ANOVA	
	N	mean ± SD	min – max	N	mean ± SD	min – max	F	p
SVL	96	733.0 ± 7.08	626.0 – 994.0	76	541.0 ± 3.67	485.0 – 640.0	353.43	0.000
TL	76	175.0 ± 1.64	133.0 – 221.0	61	156.0 ± 1.13	130.0 – 193.0	56.86	0.000
CL	95	22.1 ± 1.86	19.1 – 28.2	76	17.6 ± 0.96	15.8 – 20.0	247.39	0.000
PPS	94	19.5 ± 1.60	16.6 – 25.0	76	15.5 ± 0.86	13.9 – 17.9	284.91	0.000
IO	94	8.4 ± 0.68	7.0 – 10.8	76	6.8 ± 0.44	5.8 – 8.0	194.08	0.000
J	95	30.8 ± 2.95	26.0 – 41.8	76	21.9 ± 1.25	20.0 – 25.0	441.16	0.000
BM	93	147.1 ± 46.84	65.0 – 305.0	73	54.2 ± 12.49	38.0 – 84.0	222.45	0.000

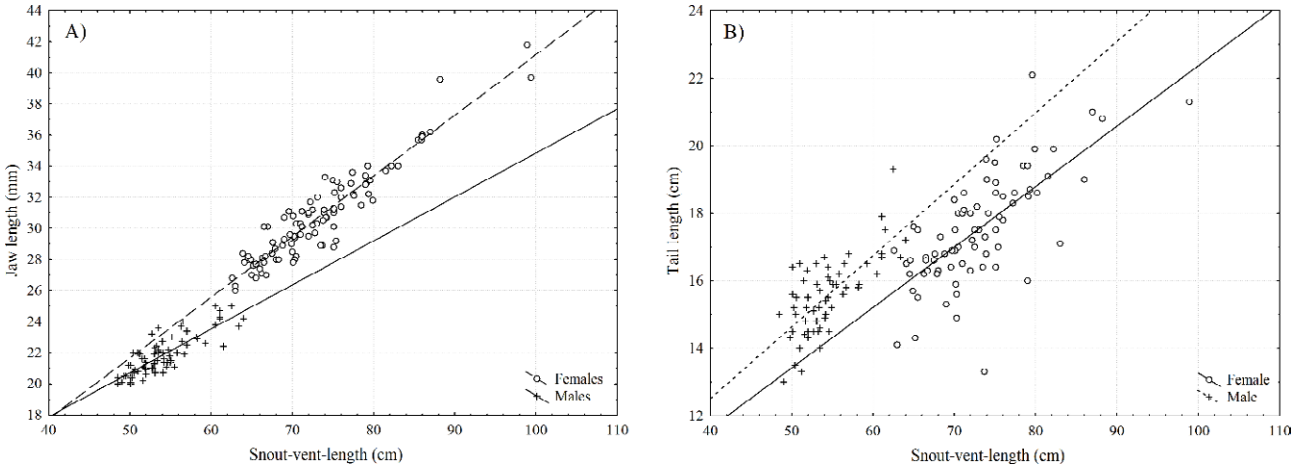


Fig. 2. Ratio of J vs SVL (A) and ratio of TL vs SVL (B) between males and females.

Diet

Of the 172 processed individuals, 111 (64.5%) had food in their digestive tracts. Of these, 39 individuals contained intact prey, 23 had half-digested prey, and 49 had fully digested prey. Feeding frequency did not differ between sexes, with 57.9% of males and 69.8% of females containing food in their digestive tract ($\chi^2 = 2.62$; df = 1; P = 0.105). Differences in feeding frequency among months when the snakes were collected were statistically significant for the complete sample (contingency table: $\chi^2 = 28.8$; df = 4; P < 0.001), and for the two sexes separately (females: $\chi^2 = 25.4$; df = 4; P < 0.001, and males: $\chi^2 = 12.8$; df = 4; P = 0.012). Females fed more frequently than males in all periods except June-July, when their feeding frequency was the lowest. On the other hand, the lowest feeding frequency in males was found in April and May (Fig. 3).

Identification of consumed prey was possible for 44 food items, while the remaining prey was too digested to be identified. In Bardača, the prey of adult grass snakes comprised six taxa – five fish species and green frogs from the genus *Pelophylax* (Table 2). Statistically significant differences were found in the type of consumed prey (frogs vs. fish) during the four years of analyses (contingency table: $\chi^2 = 11.6$; df = 3; P = 0.011). The prevailing prey during 2011, 2013, and 2014 were green frogs (*Pelophylax* sp.), while during 2012, the allochthonous invasive fish species (*Ameiurus nebulosus*) dominated the snakes' diet. Considering fishes, grass snakes significantly more often ($\chi^2 = 8.3$; df = 1; P = 0.004) consumed allochthonous species (*Carassius gibelio*, *Ameiurus nebulosus*, and *Pseudorasbora parva*) compared to autochthonous (*Cyprinus carpio* and *Silurus glanis*). Also, females consumed *Pelophylax* sp. frogs significantly more often than males ($\chi^2 = 3.97$; df = 1; P = 0.046). In

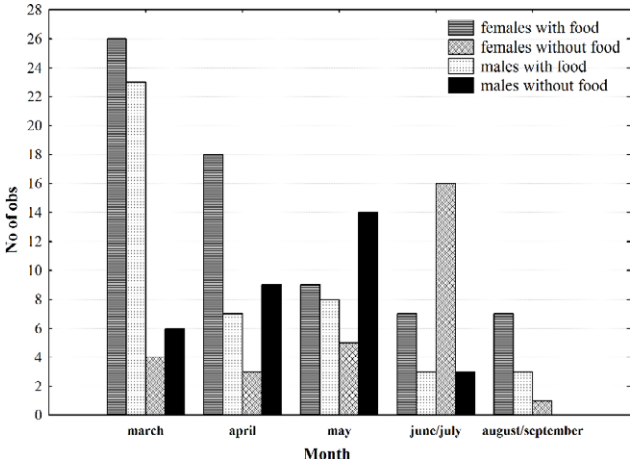


Fig. 3. Feeding frequency in adult grass snake males and females among months.

general, the diet of females was more diverse regarding types of prey (Table 2).

Seasonal (by month) diet analysis showed that females most often consumed green frogs during May, while males fed mostly on topmouth gudgeon (*Pseudorasbora parva*) (Fig. 4).

All individuals with intact prey (N = 39) regurgitated their stomach contents either before or during their processing in the field. Depending on the type of prey (frog vs. fish), swallowing direction differed significantly (χ^2 = 24.2; df = 1; p < 0.001): frogs were significantly more often swallowed hind-first (χ^2 = 4.26; df = 1; P = 0.039), and fish head-first (χ^2 = 8.17; df = 1; P = 0.004) (Fig. 5).

On several occasions, we observed grass snakes actively searching for prey, either frogs or fish.

In female grass snakes, a moderately strong linear correlation was found between their SVL and mass of the swallowed prey (r = 0.55; P = 0.003), while in males, a weak negative linear correlation was found between these two variables (r = -0.045; P = 0.902) (Fig. 6).

Table 2. Grass snake prey types in the analysed years and according to sex

Taxon	Year				Sex	
	2011	2012	2013	2014	Females	Males
Pelophylax sp.	5 (83%)	-	4 (50%)	6 (55%)	14	1
Carassius gibelio	-	2 (11%)	2 (25%)	-	4	-
Ameiurus nebulosus	-	17 (89%)	-	-	12	5
Cyprinus carpio	-	-	2 (25%)	-	2	-
Silurus glanis	-	-	-	1 (9%)	1	-
Pseudorasbora parva	1 (17%)	-	-	4 (36%)	0	5

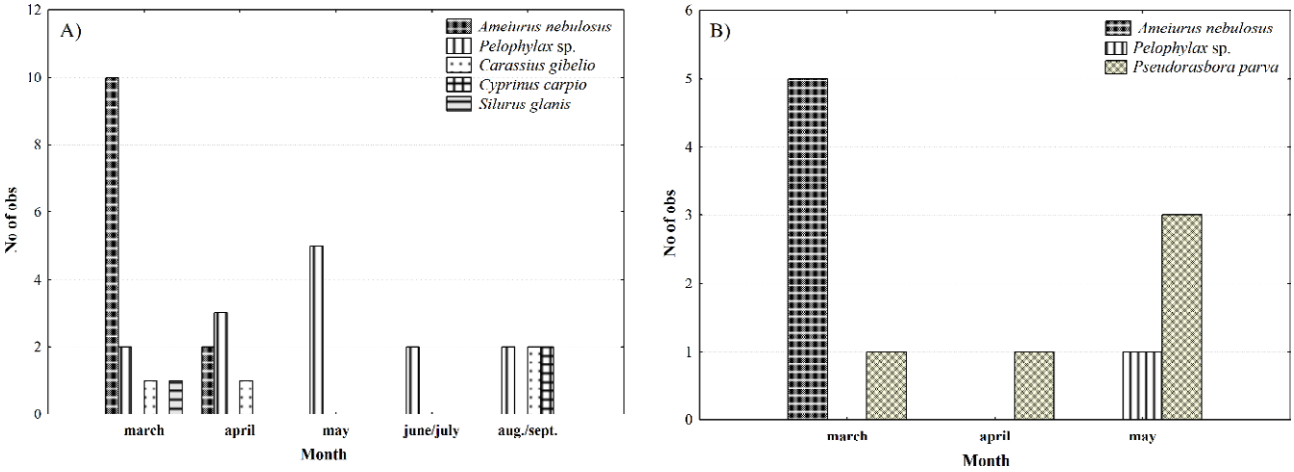


Fig. 4. Seasonal variations in the grass snake diet: females (A) and males (B)

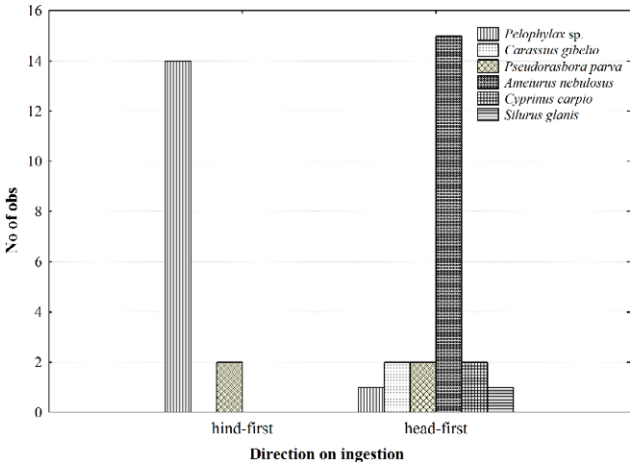


Fig. 5. Swallowing direction of different prey types

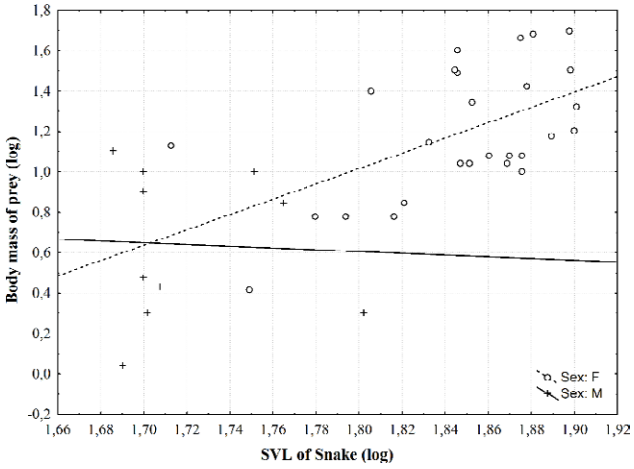


Fig. 6. Relationships between the grass snakes' standard body length and the mass of the consumed prey

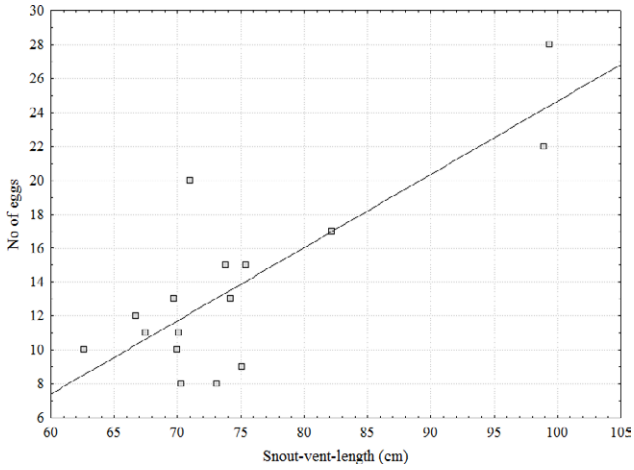


Fig. 7. Relationship between female standard body length and number of eggs

Reproductive biology

The standard body length of gravid females ranged from 62.6 cm to 99.4 cm (average 75.0 ± 10.4 cm). Clutch sizes varied between eight and 28 eggs (average 13.9 ± 5.54), and the correlation between female SVL and clutch size was strong (r = 0,809; P < 0,001) (Fig. 7).

Grass snake females in the gestational period were captured during 2013 and 2014, with no statistically significant differences in the frequency of reproduction between the two years (χ^2 = 0.12; df = 1; P = 0.729). In the given two years, 22 adult females were caught, 19 of which were gravid (86,4%). Among these, abdominal palpation revealed the presence of growing follicles/eggs in 16 individuals, while in three individuals, lateral skin folds were observed that indicated recent oviposition. In 2013, skin folds were observed in two females on July 13; this means that egg laying took place during the first half of July. In 2014, one female was captured on July 26 while laying her eggs in the communal nest (Fig. 8 A, B). This nest was under a pile of planks at a place where the previous year a pile of manure was placed. We counted more than 70 recently laid eggs. The average egg length was 32.7 ± 2.15 mm, average width 14.4 ± 0.31 mm (Fig. 8 C), and average weight 4.3 ± 0.43 g. We visited the nest several more times, and at the end of August, we found recently hatched snakes (Fig. 8 D). The average hatchlings' SVL was 16.1 ± 0.90 cm, total body length 20.2 ± 0.96 cm, and body weight 2.3 ± 0.35 g.

DISCUSSION

Sexual dimorphism

Descriptive statistics and ANOVA showed that grass snake females had significantly higher values of all analysed morphometric characters. This is in accord with the findings of Shine (1978, 1994), who argued that in most snake species with no male combat, females grow larger than males. Also, Capula and Luiselli (1997) found that in all three *Natrix* species (*N. natrix*, *N. tessellata* and *N. maura*), females were the larger sex. The results of ANCOVA showed sexual dimorphism among adults in tail length (TL) and jaw length (J). The relative tail length in males from Bardača was 22.3% of the total body length, and in females, 19.4%. The greater relative tail length in males is probably directly correlated with their reproductive anatomy, i.e. the need for additional space for hemipenes and the related muscles

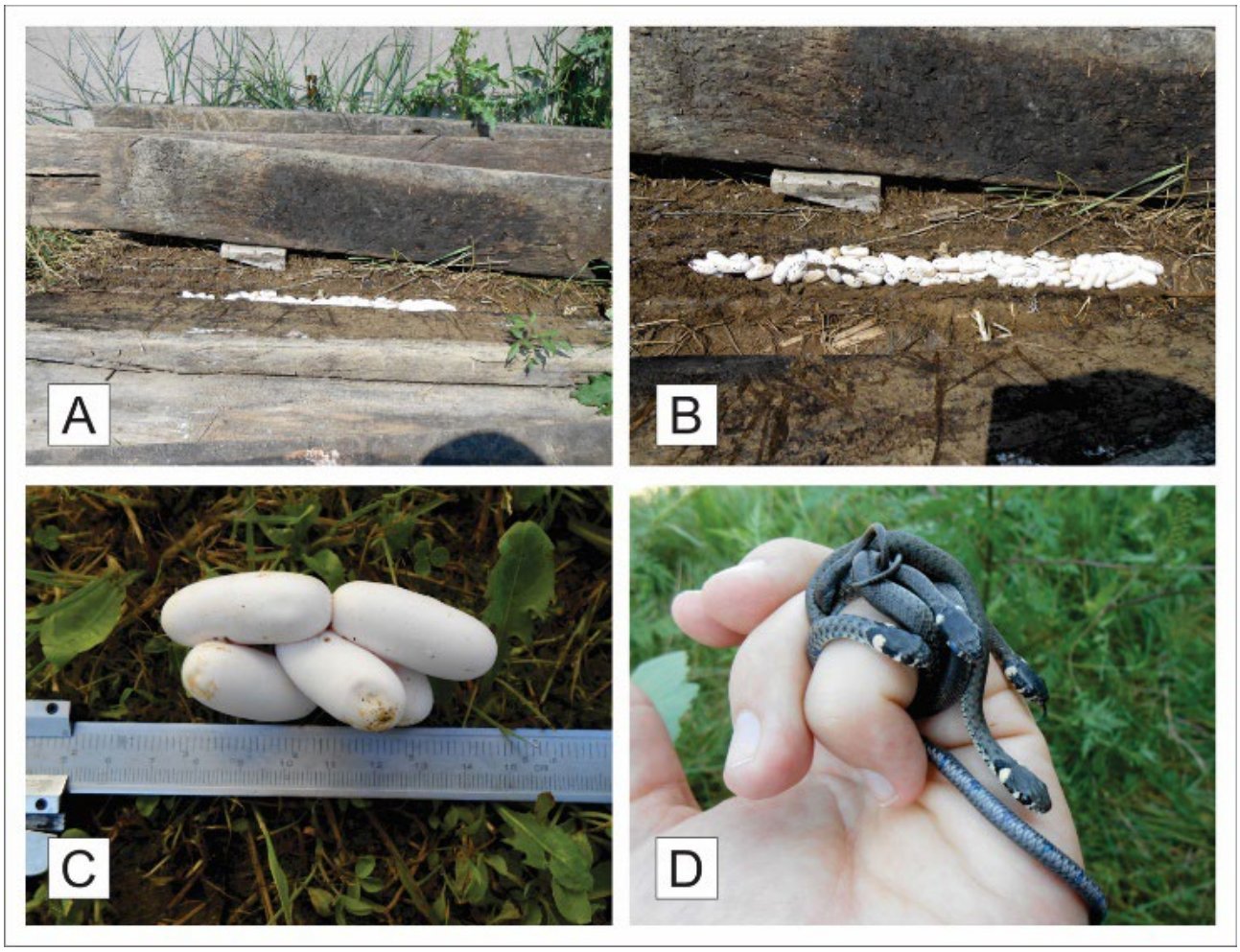


Fig. 8. Grass snakes' communal nest and the juveniles found during the 2014 fieldwork

at the base of their tails (King, 1989; Borczyk, 2007); relatively shorter tails in females most probably resulted from selection for larger body capacity (King, 1989) i.e. selection for fecundity (Shine, 1993, 1994). This type of sexual dimorphism in tail length is in accord with previous investigations into sexual dimorphism in the *Natrix* genus (Hailey and Davies, 1987; Feriche et al., 1993; Borczyk, 2007; Mebert, 2011). On the other hand, females had higher absolute and relative values of jaw length compared to males. This is in agreement with the results given by Shine (1991a), which indicated that in most analysed species of snakes for which sexual dimorphism in head size has been established, females are the sex with the larger head and longer jaws. Sexual dimorphism in trophic structures is often used as support to the hypothesis of "ecological divergence" between the sexes (Shine, 1989, 1991a). Since snakes are gape-limited predators, differences between the sexes in head dimensions can lower the intersexual food competition (Houston and Shine, 1993; Shetty and Shine, 2002). Also, since female snakes invest proportionately more into reproduction (Capula et al., 2011), their longer jaws can be explained by the "reproductive role" hypothesis. Longer jaws enable females the consumption of larger prey (Shine, 1991b) which enables increased input of energy which can be allocated to reproduction. For certain snake species, it was found that females can consume larger prey than males of the same size, i.e. that "despite ontogenetic constancy in head dimorphism, sex differences in maximum prey size are expected to increase in magnitude with increasing snake size" (King, 2002). Our results showed that females consume larger, but also more diverse prey than males, which is all in agreement with the above.

Diet

The frequency of food in the stomach for the entire sample from Bardača is notably higher (≈65%) compared to previous investigations of grass snakes' diet: in Italy, this was ≈ 42% (Filippi et al., 1996; Luiselli et al., 2005), while in England it was only ≈ 35% (Reading and Davies, 1996; Gregory and Isaac, 2004). These differences could have resulted from different methodologies, not the genuine ecological differences between the studied populations. Namely, in the population from England, the prey frequency analysis was done exclusively based on the prey obtained by forced regurgitation (Gregory and Isaac, 2004), while in Italy, in addition to regurgitation, the analysis of feeding frequency included dissections of individuals found dead in the field (Luiselli et al., 2005).

The analysis of seasonal feeding frequency showed that grass snakes in Bardača had two feeding peaks – in spring and during the second half of summer, while during the first half of summer (June–July), a notable drop was found in the presence of food in the stomach. The increased frequency of feeding during spring (March and April) could result from increased prey availability or increased need for more intense feeding after hibernation (Luiselli et al., 2005), while intense feeding during the second half of summer is most probably related to accumulating fat reserves necessary for hibernation. Findings in England (Gregory and Isaac, 2004) were similar to what we have found, while grass snakes in Italy (Luiselli et al., 2005) showed a similar pattern of increased feeding during summer and a drop in the first half of spring; contrary to what was found in Bardača and England, Italian grass snakes did not show an increase in feeding towards the end of summer. On the other hand, the opposite was found in some other investigations (Filippi et al., 1996; Reading and Davies, 1996), where the lowest feeding frequencies were found in early spring and the second half of summer. This was probably caused by different climatic characteristics that determine different prey availability in the given seasons (Luiselli et al., 2005). The lowest feeding frequency of adult grass snake females during June and July can be related to vitellogenesis. The previous investigations within the Natricinae subfamily showed that gravid females feed less frequently than non-gravid ones, i.e. snakes are traditionally considered anorexic during gestation (Gregory et al., 1999; Gregory and Isaac, 2004, but see Aldridge and Bufalino, 2003). Gestational anorexia can result from more frequent basking (precise thermoregulation is crucial for embryonal development) or physical restrictions – the development of eggs/embryos reduces the available space in the abdominal cavity and stomach (Gregory et al., 1999; Tuttle and Gregory, 2009). Also, given that pregnancy can impair snakes' mobility due to the weight of embryos/eggs (Shine, 1980), anorexia could result from difficulties in prey capturing. Regardless of the cause of gestational anorexia, feeding frequency increases after oviposition/parturition (Gregory et al., 1999; our results). On the other hand, our results indicated that males, like females, feed more frequently after hibernation (before mating). This contrasts with the results of investigations of seasonal feeding in *Thamnophis sirtalis parietalis* males that indicate less frequent feeding after hibernation when they direct time and energy into an active search for females (O'Donnell et al., 2004). Increased feeding frequency of grass snake males in Bardača after hibernation could be a consequence of the draining of numerous lakes (Šukalo et al., 2014), which probably led to the gathering of snakes of both sexes around several remaining lakes, so males did not have to search for females anymore. Also, since it was proven that in male grass snakes body size increases reproductive success during the so-called 'pseudofights' (Capula and Luiselli, 1997), increased feeding after hibernation and allocation of energy into growth can lead to increases in individual reproductive success. The lowest feeding frequency in adult males was found during May, which can be related to the mating period: mating was observed twice at the beginning of May.

The observed differences in qualitative composition of grass snakes' diet during the four years of research provide proof of intra-population differences in feeding and confirm this species' feeding plasticity and ability to switch to alternative prey in a very short time. Namely, the original feeding on green frogs *Pelophylax* sp. (≈ 85%) during 2011 was completely replaced by feeding on allochthonous fishes (*Ameiurus nebulosus* and *Carassius gibelio*) in 2012 (after the lakes were drained up during the 2011/2012 winter – Šukalo et al., 2014). However, during 2013 and 2014, green frogs became the dominant prey again (≈ 50%), and the brown bullhead was excluded from the snakes' diet. In natural aquatic ecosystems, the grass snakes' impact on allochthonous fishes is limited (Jones et al., 2009), but we assume it can be significant. Although we do not possess quantitative data regarding variations in prey abundance in Bardača during our investigation, some other snake studies did report increased consumption of invasive fish species. For example, *Carassius gibelio* accounted for more than 97% of prey biomass in *Natrix tessellata* (Acipinar et al., 2006), while the invasive round goby (*Neogobius melanostomus*) became the dominant prey (> 92%) in *Nerodia sipedon insularum* (King et al., 2006), which positively influenced growth and reproduction in these species. These findings

indicate that invasive fish species can become dominant and energetically profitable prey for aquatic snakes. On the other hand, more frequent consumption of allochthonous fishes compared to autochthonous species suggests that grass snakes can benefit fisheries because they often consume "fish weed".

Our swallowing-direction analysis found significant differences depending on the type of prey (frogs vs. fish). Fish were significantly more often swallowed head-first, while frogs were consumed starting with their rear legs. Head-first ingestion of fish was previously shown in dice snakes, *Natrix tessellata* (Ghira et al., 2009; Metzger et al., 2011). The reason for this is probably the fact that bony rays in fish fins are oriented from head towards tail, hence if a snake would start swallowing a fish tail-first, fin rays would damage the snake's digestive tract during ingestion (Ghira et al., 2009; Metzger et al., 2011) and if a fin is erected, it can pose additional resistance and make the act of swallowing more difficult. On the other hand, a fish's possibility to escape is lower if it is swallowed head-first, which is significant in swallowing larger individuals (Metzger et al., 2011). Also, the duration of ingestion is shorter if the fish is swallowed head-first (Ghira et al., 2009), thus, the snake's exposure to potential predators is shorter. The results provided by Mori (2005) indicate that the direction of prey swallowing depends on the place of the first bite, i.e., if the first bite to the prey was inflicted on its anterior part of the body, it is swallowed head-first. The place of the first bite can be correlated to the strategy a snake employs during capture (Ananjeva and Orlov, 1982; Metzger et al., 2011). Since grass snakes are actively foraging for prey (Ananjeva and Orlov, 1982; Janev Hutinec and Mebert, 2011; personal observations), they approach frogs from behind (both in the water and on land), hence the first bite and swallowing are from the rear body end. Another advantage of swallowing frogs from their rear end is their handling and immobilisation. Namely, when the frogs are caught by the head, they resist by hitting the ground with their hind limbs, and the ingestion, especially of larger frogs, from their hind limbs can help the snake to overcome the defensive movements of the frogs and reduce the risk of the prey escaping (Mori, 2005). Also, there is a possibility that snakes, by swallowing their prey from its larger end (fish from their head, frogs from the rear end), "estimate" its size and the possibility of ingestion thus saving energy they would potentially lose to regurgitate half-ingested prey. The energy-saving hypothesis in feeding is supported by laboratory investigations in two *Nerodia* species that showed that larger individuals spend more energy during ingestion of smaller prey (Hampton, 2018). On the other hand, laboratory studies of the dice (*Natrix tessellata*) showed that all analyzed individuals, regardless of body size, more effectively caught smaller fish (Bilcke et al., 2007). Therefore, future investigations should focus on these questions.

Our investigation indicated that grass snake females consume green frogs significantly more often than do males. This could result from physical constraints (Shine, 1991a; Forsman and Lindell, 1993), i.e. since snakes are gape-limited predators, and their head size determines the maximum size of prey they can ingest. Given that in grass snakes, female-biased sexual size dimorphism is present, males cannot swallow larger prey (such as green frogs) due to their smaller body and jaw sizes compared to females. On the other hand, active selection of larger prey items by adult females could result from higher energy demands (Shine, 1991a; Luiselli et al., 1997; Santos et al., 2000). The fact that female grass snakes more often consumed frogs during spring indicates the possibility that by actively choosing frogs, females provided additional energy necessary for vitellogenesis. This is supported by the fact that frogs consumed during spring were females full of eggs (approximately 64%), which are richer in energy per unit mass (Cummins and Wuycheck, 1971), and also less agile and easier to catch. Active choice of frogs by adult females during spring was recorded for the congener, *Natrix maura* (Santos et al., 2000). Also, in the field, females were more often observed basking on the shore, but were also more often caught far from water, while males were more often observed and caught in water. However, to discuss intersexual differences in the choice of habitat as a cause of sexual differences in feeding (Shetty and Shine, 2002), more thorough investigations are needed in the future.

Reproductive biology

Our investigations showed that the grass snakes in "Bardača" reproduce annually, like in populations in Italy (Luiselli et al., 1997) and Sweden (Madsen, 1983). Mating was observed on May 1, 2014. In one case, one female was surrounded by three males and in another by four – also in accord with findings from Italy and Sweden (Luiselli et al., 1997; Madsen, 1983). We found that in 2014, oviposition started on average 14 days later compared to 2013. Intra-population variations in the time of reproduction can be influenced by the variations in the end of hibernation (Rossman et al., 1996). Also, the beginning of vitellogenesis in numerous snake species is conditioned by attaining the minimum bodily reserves threshold (Bonnet et al., 2002), i.e. food availability was shown to directly influence the onset of vitellogenesis (Santos et al., 2005). Lower average air temper-

Table 3. Comparison of some reproductive traits of *Natrix natrix* from different localities

Reproductive trait	Bardača (this study)	Luiselli et al., 1997 (Italy)	Madsen, 1983 (Sweden)
Sexual maturity (total length)	79.5 cm	70.2 cm	68.0 cm
Number of eggs (mean value ± SD)	13.9 ± 5.4	9.2 ± 5.43	11.3 ± 4.96
Number of eggs (maximum)	28	24	24
Average body length of hatchlings	20.2 cm	20.4 cm	19.5 cm
Average body weight of hatchlings	2.3 g	3.4 g	3.0 g

atures, but also significantly higher precipitation (floods) that occurred during May of 2014 (https://rhmzrs.com/) probably caused the delay of vitellogenesis and, consequently, later oviposition. In Bardača, grass snakes reach sexual maturity at significantly larger total body lengths compared to other localities (Table 3). Predation pressure could be the possible cause of later sexual maturity here (numerous birds of prey – Gašić and Dujaković, 2009), which is confirmed by higher frequency of tail damages in adult individuals (18.8%) compared to subadults (5,3%; unpublished). Santos et al. (2011) considered the ontogenetic increase of tail damage a good indicator of predation pressure in the congeneric *Natrix maura*. In this way, delayed reproduction (lower immediate costs of reproduction) will be favoured in cases when high costs of reproduction reduce the chance of survival, and thus the possibility of subsequent reproduction (Madsen, 1987). Since the body size of grass snakes is positively correlated with the number of produced eggs (Madsen, 1983; Luiselli et al., 1997), which is confirmed by our results, allocation of energy into growth and delay of reproduction probably is an advantage in a habitat with pronounced predation pressure, like Bardača. Also, for snakes, it has been proven that improved nutrition – either directly or indirectly (longer seasonal activity) – can lead to faster growth and greater adult body length (Luiselli et al., 1997; Bronikowski and Arnold, 1999). Given our results, that show that grass snakes in Bardača actively feed since the second half of March until the end of September, which is longer compared to populations in Sweden and Italy (Madsen, 1983; Luiselli et al., 1997), longer feeding period could be one of the factors that influence the observed inter-population differences in the body length at maturity in females.

Our data also indicated inter-population differences in clutch size, i.e. higher average and maximum values in Bardača compared to populations in Italy and Sweden (Table 3). However, the analysis of covariance (with clutch size as a dependent variable, locality as factor and total body length as an independent variable), did not reveal statistically significant differences in numbers of eggs of grass snake populations from Bardača compared to those from Italy ($F_{1,32} = 0.61$; $P = 0.439$) and Sweden ($F_{1,29} = 3.33$; $P = 0.080$). On the other hand, the body weight of hatchlings in Bardača was noticeably lower compared to those from Italy and Sweden (Table 3). The results provided by King (1993) indicate the existence of a trade-off between the number and size of offspring but also that larger females and those in better condition produce smaller offspring in poorer condition, which corresponds with our results (Table 3). For successful eggs incubation snakes need warm and moist microhabitat that can be natural (Luiselli et al., 1997) or anthropogenic (Beebee and Griffiths, 2000). As natural grass snake nests, Luiselli et al. (1997) listed places under large rocks, moist mosses and rotting logs, and as anthropogenic manure piles, that were proven better for incubation than e.g. compost (Löwenborg et al., 2010). In Great Britain, most places where grass snakes laid their eggs were human-made habitats, i.e. compost, manure, haystacks, piles of sawdust, and bricks (Beebee and Griffiths, 2000) but they can be of half-natural origin, like rotting logs remaining after wood cutting (Baker, 2011). Therefore, where we found a communal grass snakes' nest in Bardača (under a pile of planks) presents a new type of human-made microhabitat that enables successful egg incubation.

CONCLUSION

Our investigation of the biology and ecology of grass snakes in Bardača is the first of its kind in Bosnia and Herzegovina. Being among the top predators in various ecosystems, snakes can be reliable indicators of the state of those ecosystems. Habitat fragmentation and destruction threaten all life (including humans) and have to be minimized by the education of all stakeholders and decision makers, among other things. On the other hand, snakes have learned to live close to people: we should learn to accept snakes as natural and beneficial components of ecosystems. Sadly, they are under more threats and pressure than investigation. Our team intends to change that by improving and spreading the knowledge

about these animals. Also, wetlands as such must be appreciated and effectively protected: while occupying only 6% of the Earth's land surface, wetlands support 40% of known plants and animals and provide numerous ecological services, but are declining at alarming rates, with more than one-fifth of the assessed wetlands being in a poor state (Convention on Wetlands, 2025). In that sense, investigations related to various components of wetland ecosystems should be continued. Our findings on snakes as predators and their prey should be properly disseminated and applied in future conservation plans.

ACKNOWLEDGEMENTS

In the period when this research was conducted, a list of protected reptile species did not exist in Bosnia and Herzegovina; therefore, we didn't need to obtain any permit for population studies of *Natrix natrix*. Researchers from Serbia participated in part of the research that was supported through Contract No. 451-03-136/2025-03/ 200178 (SN) and Contract No. 451-03-137/2025-03/ 200178 (LT) between the University of Belgrade, Faculty of Biology and the Ministry of Science, Technological Development and Innovation of Serbia.

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Assessing the climatic vulnerability of the *Micrurus sangilensis* (Niceforo Maria, 1942) under future scenarios

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Abstract. The vulnerable *Micrurus sangilensis* commonly known as the Santander coral snake distributes in dry and montane forests, ecosystems under severe anthropogenic pressure in northeastern Colombia. The habitat of this serpent is fragmented, and climate change may further intensify risks to the vegetation structure. We assessed whether the current distribution of the snake may be altered under different scenarios with climate change in the 2040-2060 years; aiming to recognize conservation priority areas. With ecological niche modeling we calculated current values of stability in the distribution range of the species, for the most conservative emission scenarios of Socio-Economic Pathways (SSP) 126, and 245; and the expected greater emissions 585 within five different global circulation models. We also escalated an index of vulnerability to land use change to 2050 in the remaining areas for the species, detecting prioritizing conservation zones. Our findings reveal a nearly 25% consistency of loss in the three SSP scenarios, while gaining stability varies between different GCMs. Over 37% of remaining suitable areas were categorized as highly vulnerable to land-use change, especially at elevations between 900 and 2000 m. We emphasize the need to integrate *M. sangilensis* habitats into Colombia's protected area network, restore degraded ecosystems, and establish ecological corridors to mitigate fragmentation. While the most vulnerable to changing areas appear to be the ones with critical requirements for conservation; We recommend targeting conservation efforts in areas of low to medium vulnerability to change, which are less likely to undergo significant modifications over the next 30–40 years.

Keywords. Biodiversity conservation, climate change, ecological niche modeling, fragmented landscapes, *Micrurus sangilensis.*

INTRODUCTION

The rapid changing patterns in climatic regimes have become a central concern in conservation sciences (Lovejoy, 2006; Young et al., 2011; Yu et al., 2014; Upadhyay, 2020; Fuentes et al., 2023). In the Americas, home to multiple biodiversity hotspots, ecosystems are increasingly at risk as altered climatic conditions force species to shift their historical distribution ranges toward new latitudinal and altitudinal zones in search of suitable habitat conditions (Allentoft and O'Brien, 2010; Bellard et al., 2012; Vicenzi et al., 2017; Archis et al., 2018). Furthermore, range-restricted and threatened species often lack the dispersal capacity to cope with these changes across fragmented landscapes with rapid shifting, among others, vegetation structures (Kwak and Freeman, 2010; Bestion et al., 2015; Upadhyay, 2020; Fuentes et al., 2023). While climate change is undoubtedly a major threat to

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already vulnerable ecosystems, rapid and drastic land-use changes may pose an even more immediate challenge, this compromises the structural integrity of habitats and increases barriers to species movement (Bellard et al., 2012; Kittel, 2013; Staudt, 2013; Bush et al., 2017; Cobos et al., 2018). These combined pressures underscore the urgent need for conservation strategies that enable the most threatened, range-restricted species to respond to both climate and land-use changes, particularly in fragmented landscapes (Heller and Zavaleta, 2009; Mawdsley et al., 2009; Rands et al., 2010).

In South America Andean ecosystems are important reservoirs of rich and complex biodiversity (Sarkar et al., 2009; Ramirez-Villegas et al., 2014; Bax and Francesconi, 2019), where inhabiting hundreds of endemic and range restricted species, some specialized to the typical habitats in ranges and associated environments; however, also among the most threatened ecosystems in the world (Young et al., 2011; Bax and Francesconi, 2019; Noh et al., 2020). Among many other groups affected by the changing landscape conditions in the Andes, reptiles exhibit high sensitivity to climate change and habitat loss, mainly in relation to their reproductive processes (Huey et al., 2009; Gamble, 2010). However, they should be given higher priority, particularly in the context of climate change impacts (Gumbs et al., 2018). Residing in delicate ecosystems already imperiled by deforestation and habitat degradation, many reptile species are categorized as threatened according to the IUCN Red List (Arredondo et al., 2015; Bolívar et al., 2016; Páez et al., 2016; Calderón et al., 2019; Hladki et al., 2019; Rainwater et al., 2022). They confront altered temperature and precipitation patterns, imperiling their ecological niches and reproductive cycles (Brown and Shine, 2006; Gamble, 2010). The ramifications extend to the intricate balance of their habitats, where some with restricted ranges and specialized ecological requirements are particularly susceptible to environmental perturbations (Holt, 1990; Moraes and Recchia, 2011).

Among reptiles, the family Elapidae, which includes coral snakes (*Micrurus*) have species with wide distributions such as (*Micrurus dumerilli* and *M. dissoleucus*), and a high tolerance for climatic regimes, as *M. mipartitus* inhabiting from 0 to near 2500 m (Rey-Suárez et al., 2016; Herrera-Lopera et al., 2018; Pitalua et al., 2018; Río-Soto et al., 2018). However, there are also some snakes with reduced distribution such as *M. sangilensis*, which is especially affected by the modification, degradation, and loss of natural habitat (Hladki et al., 2019; Flórez and Montoya-Cruz, 2023).

Micrurus sangilensis, commonly known as the Santander coral snake, is a triad-colored species distinguished by specific ring patterns and the absence of supracloacal keels. It differs from its close relatives, *M. dissoleucus* and *M. dumerilli*, by having 16–22 triads and a typical length of around 60 cm (Morales-Betancourt et al., 2015) (Fig. 1). Endemic to Colombia, its narrowed distribution within the departments of Cundinamarca, Boyacá, and Santander (Roze, 1996; Campbell et al., 2004; Caicedo-Portilla and Lynch, 2015) and more recently detected in Casanare (Flórez and Montoya-Cruz, 2023). This species limited to moderate elevations (800–2800 above sea level) along the Middle Magdalena River Basin, primarily inhabiting the vulnerable dry forests (Campbell et al., 2004). Currently classified as Vulnerable by the IUCN, its historical records show an estimated extent of occupation near 12000 km² (Caicedo-Portilla and Lynch, 2015; Hladki et al., 2019). Despite its conservation status, critical knowledge gaps remain regarding its population size, reproductive dynamics, and habitat requirements, which are essential knowledge when working for effective conservation planning (Van Teeffelen et al., 2012; Caicedo-Portilla and Lynch, 2015). Species like *M. sangilensis* may face acute risks not only from climate change but also from rapid structural changes in vegetation due to habitat degradation (Caicedo-Portilla and Lynch, 2015; Hladki et al., 2019; Flórez and Montoya-Cruz, 2023).



Fig. 1. Photograph of a living adult of *Micrurus sangilensis* with its characteristic coral pattern. Photo by: Elson Meneses-Pelayo.

Given the ongoing changes in land use and the potentially altered climatic regimes in rainfall and temperature within the degraded dry forests, typical habitat of the restricted distribution of *M. sangilensis*, it is necessary to develop management strategies predicting the potential shifts in the species' distribution under future climate scenarios. This research aims to assess the species' potential distribution under different Shared Socioeconomic Pathways (SSP) under climate change, through a vulnerability to change index for land use, using Eco-

logical Niche Modeling Methodologies (ENM) which are among the most widely used and effective tools for comparing and predicting historical and future scenarios (Peterson et al., 2011; Rangel and Loyola, 2012). By relating environmental and climatic variables with species occurrence data, ENM offers insights into species' range dynamics (Alvarado-Serrano and Knowles, 2014; Ramirez-Villegas et al., 2014; Mota-Vargas and Rojas-Soto, 2016; Moreno-Contreras et al., 2020; Sales et al., 2020). This assessment serves as a crucial resource for conservation planning strategies, highlighting areas for immediate and future protection efforts in landscapes increasingly fragmented by agriculture and urbanization.

MATERIALS AND METHODS

Occurrences and accessibility area (M)

We obtained records of *M. sangilensis* from the Global Biodiversity Information Facility database (GBIF https://doi.org/10.15468/dl.9cjqyy) and literature (Flórez and Montoya-Cruz, 2023), in total 59 Records were used for analyses after deleting duplicates and reviewing each locality consistency to the known species historical range, following the methodology based on Cobos and Bosch, (2018). The accessible area, commonly named as M (Soberon and Peterson, 2005), represents the geographic region that the species could have potentially occupied over relevant evolutionary time frames, based on its dispersal ability and known biogeographic barriers, this M is the spatial extent from which environmental variables are cropped to calibrate the model and generate predictions; by considering at least one occurrence in terrestrial ecoregions from The Nature Conservancy (Dinerstein et al., 2017), using the ArcMap 10.8 software (Fig. 2).

Environmental variables

We obtained climatic variables to characterize the climatic niche of the *M. sangilensis* from the WorldClim

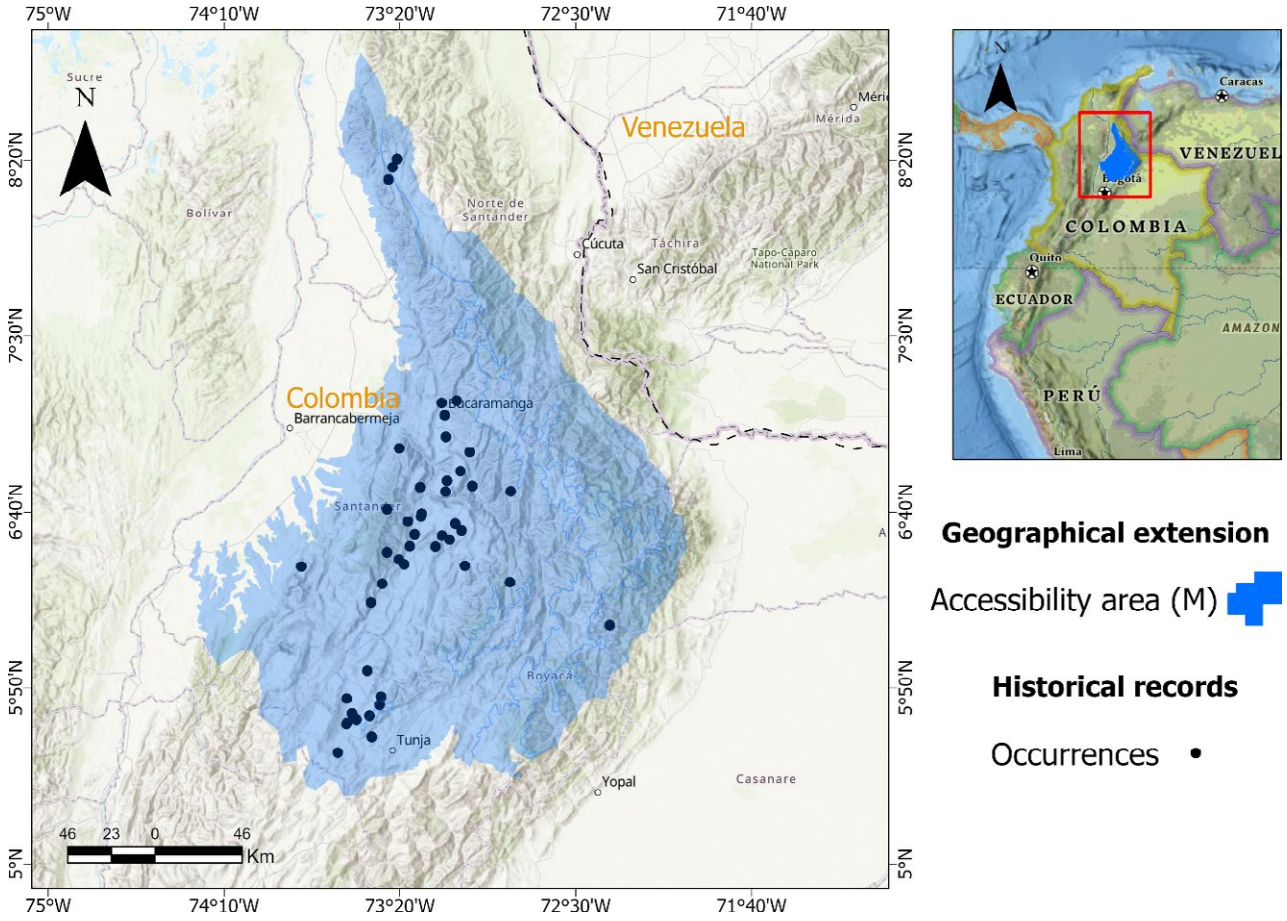


Fig. 2. Historically known distribution of *Micrurus sangilensis* and its generated accessible area (M).

2.1 database (Fick and Hijmans, 2017) at a 30 arc-second resolution (~1 km²). We exclusively used 15 biovariables, excluding bio 8 (mean temperature of wettest quarter), bio 9 (mean temperature of driest quarter), bio 18 (precipitation of warmest quarter), and bio 19 (precipitation of coldest quarter); these exclusions reduce redundancy and avoid potential collinearity, overfitting or misrepresenting the species' niche model (Escobar et al., 2014; Table 1 in supplementary material). Variables were masked and cropped to the extension of the accessible area of *M. sangilensis*. Because some highly correlated variables can be important for the biology of the species, we created different sets from the 15 biovariables for niche modeling evaluation (Cobos and Bosch, 2018; Echeverry-Cárdenas et al., 2021). The specific sets are shown in Table 2 in supplementary material.

Ecological niche modeling (ENM)

We randomly allocated 80% of the occurrence records for model training and the remaining 20% for evaluation, using the Maxent algorithm (Elith et al., 2011) implemented through the kuenm package in R (Cobos et al., 2019). We assessed multiple levels of model complexity following the approach of Warren and Seifert (2011) by varying the regularization multiplier from 0.1 to 1 in increments of 0.1, and then testing values of 2, 3, 4, and 5. Model selection was based on performance metrics provided by Kuenm, including the AUC ratio, omission rate, and AIC. The AUC (Area Under the Curve) ratio measures the model's ability to discriminate suitable versus unsuitable areas, while the AICc (corrected Akaike Information Criterion) evaluates model parsimony by balancing goodness-of-fit and complexity (Phillips et al. 2006; Pearson et al. 2007; Warren and Seifert 2011; Arango-Lozano et al., 2025). The selected model was projected without applying extrapolation modes (Extrapolation or Clamping in Maxent algorithm) onto the accessible area under future climate scenarios, specifically Shared Socioeconomic Pathways (SSPs) representing minimal and moderate climatic changes (SSP126 and SSP245), as well as the most extreme scenario (SSP585), a high-emissions scenario associated with continued fossil fuel use and the most severe climate change (Echeverry-Cárdenas et al., 2021; Arango-Lozano et al., 2025) for the cumulative years 2041-2060.

We used five Global Circulation Models (GCMs) for comparing results as: HadGEM3-GC31-LL (GCM1), IPSL-CM6A-LR (GCM2), ACCESS-CM2 (GCM3), EC-Earth3-Veg (GCM4) and UKESM1-0-LL (GCM5). Each GCM was chosen for its unique approach in simulating climate dynamics, using distinct datasets, including land cover, oceanic circulation, and atmospheric processes (Yu et al., 2014; Padhiary et al., 2020). The variety of GCMs enhances model robustness, enabling us to assess areas of agreement and divergence via a broader perspective on future climate variability (Reshmidevi et al., 2018; Padhiary et al., 2020). For example, HadGEM3-GC31-LL focuses on ocean-atmosphere interactions, while UKESM1-0-LL includes comprehensive land-use feedback (Reshmidevi et al., 2018; García-Franco et al., 2020). We reclassified models resulted in presence/absence maps using a consistent threshold across models (10 percentile training presence Cloghold in Maxent algorithm).

Gain, loss, and stability

We evaluated the current and future potential distribution by analyzing pixel counts (and then its scaled values to Km²). First, we calculated the percentage of pixels gained, lost, and stabilized in each scenario (SSP and GCM) relative to the current distribution. We then summarized the gained and stabilized pixels for each SSP, identifying areas that remained stable across future GCMs. To pinpoint regions of consistent stability, we focused on pixels that persisted in four or more GCMs, allowing us to observe the spatial assemblage of stable areas under future conditions across all five GCMs.

Vulnerability to change

Finally, to detect any potential vulnerable areas of habitat lost in the species potential distribution, we superpose the current and future (assembly pixels in four or more GCMs) ranges with a "Vulnerability of land cover to anthropogenic change raster" (Esri, 2024). This layer shows areas where natural vegetation such as forest could be converted to agriculture and urban lands by 2050, based on the predictions of human-induced land changes. Further, the layer includes regions susceptible to expansion of agricultural and urban footprints, excluding forecasts for unchanged land cover types like forests unless they are converted to agriculture or urban areas (available data in: https://livingatlas.arcgis.com/landcover-2050/).

The vulnerability data ranges from zero to one, indicating varying degrees of susceptibility to natural cover transformation. We classified the raster values into three distinct categories: low vulnerability (0.0-0.3), moderate vulnerability (0.3-0.7), and high vulnerability (0.7-1) for the species. Areas with high vulnerability values (0.7-1) are identified as the most critical zones for this snake.

These areas are also deemed most at risk of change in future climate scenarios (Arango-Lozano et al., 2025).

RESULTS

Ecological niche model selection

Out of 210 evaluated models, only 3 fulfilled the kuenm criteria, and to streamline niche model comparisons between scenarios, we selected the first exhibiting the lowest AIC and AUC results M_0.7_F_lq_Set 5. Set 5 consists of biovariables 2, 3, 4, 7, 16, and 17, representing a combination of precipitation and temperature conditions. (Table 2, Table 3 in supplementary material).

Gain, loss, and stability

For the current scenario of *M. sangilensis*, 19873 predicted pixels were identified, which translate into over 16879 km² of suitable environmental conditions (Fig. 4A). Building on this baseline, future projections indicate substantial variability in potential range across General Circulation Models (GCMs) and Shared Socioeconomic Pathways (SSPs), yet certain consistent patterns emerge that strengthen confidence in key findings. Across all scenarios, significant stability areas are consistently predicted, particularly in the lower-emission scenario (SSP126). For instance, stability remains high in models M2 and M4 under SSP126, with over 80% of predicted pixels showing little change (Fig. 3).

As emissions concentrations increase (SSP245 and SSP585), a marked rise in habitat gain is observed. Under SSP585, models such as M1 and M5 predict substantial habitat gains, with M5 showing a gain of 38.7% of pixels. This indicates possible new areas as climatic conditions shift, though this expansion comes with increased variability in predicted losses, particularly in M3 and M4 (Fig. 3). The appearance of these gained pixels under higher emissions scenarios reflects potential new suitable habitats as the climate changes. While the model variability shows the inherent uncertainties tied to different GCMs, the overall trends of habitat stability and gains under higher emissions scenarios are robust across models.

Vulnerability to change

We identified a mean reduction of at least 25% of suitable future areas for the species with respect to the current scenario. However, when contrasting the stability values with the vulnerability to change 2050 layer, we recognized that at least 37% of the remnant areas in the different future scenarios (each SPP) may be experiencing a high vulnerability, 30% a medium and almost just 23% a low vulnerability (Fig. 4). There was identified loss of areas in all elevation between 900 and 2000 m, Furthermore, these areas consistently show both lost in predicted presence and where not, are the ones with greater vulnerability values (0.7-1).

DISCUSSION

The predictions for *Micrurus sangilensis* are influenced by the inherent uncertainties associated with the different General Circulation Models (GCMs), each of which makes varying assumptions about climate processes (Reshmidevi et al., 2018; Padhiary et al., 2020).

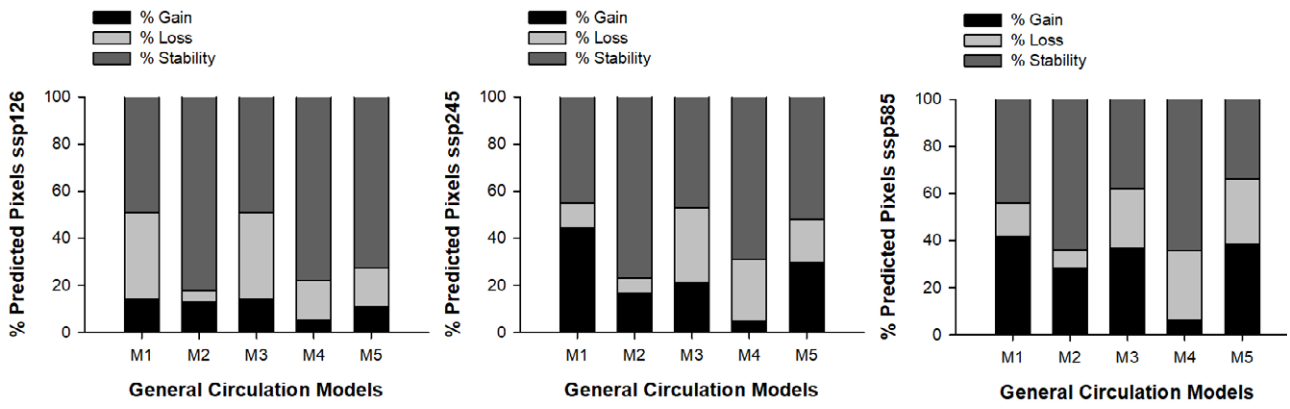


Fig. 3. Percentage of gained loss and stability of predicted pixels (occurrence likelihood = 1) under future scenarios for *Micrurus sangilensis*. Results are shown for different General Circulation Models (GCMs, labeled M1–M5) across the Shared Socioeconomic Pathways (SSPs: ssp126, ssp245, ssp585).

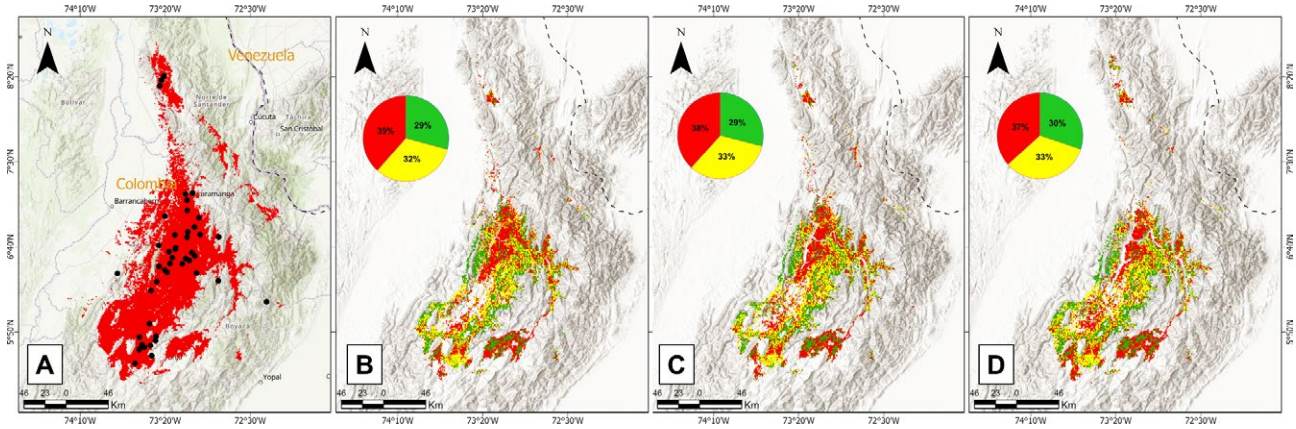


Fig. 4. Comparison of suitable conditions for *Micrurus sangilensis* across different scenarios. The subsequent maps depict the accessibility area (M) under various shared socioeconomic pathways and timeframes: (A) current conditions with occurrences (black dots), (B) assembly of consistent ideal areas in SSP126, (C) assembly of consistent ideal areas in SSP245, (D) assembly of consistent ideal areas in SSP585. Colored pixels indicating values of vulnerability to change in the land use: green = Low, yellow = Mid, red = High.

By narrowing our analysis to pixels that remained stable across four or more GCMs, we were able to highlight regions that are likely to maintain suitable conditions for *M. sangilensis* even under varying future climate scenarios. This method provided a clearer spatial picture of potential refugia and stable areas, ensuring that our findings reflect consistent patterns of stability rather than relying on any single model. The identification of these stable regions across multiple GCMs strengthens the forecasting future conditions for this coral snake.

Our study projects a significant contraction in the potential suitable habitat for *Micrurus sangilensis* due to climate change across all SSP scenarios. These findings are consistent with other studies on coral snakes, such as *M. lemniscatus*, where a similar vulnerability to habitat loss in lowland regions was observed (Terribile et al., 2018). For *M. fulvius*, a potential range shift into unsuitable areas was predicted, highlighting the species' inability to keep pace with changing climatic conditions (Archis et al., 2018). Similarly, *M. brasiliensis* faces the potential loss of 60% of its ideal habitat under future climate projections (Caten et al., 2017), further underscoring the widespread impact of climate change on coral snake distributions.

In the case of our study species *M. sangilensis*, our results indicate a potential loss of 25% of its ideal habitat by 2040–2060, with the species currently occupying degraded dry forest ecosystems, some of the most threatened habitats globally (Miles et al., 2006). Additionally, a 30% increase in the vulnerability of remaining suitable areas, particularly from land-use changes in forests (Galindo-Cruz et al., 2024). Within less than two decades, these critical habitats could undergo significant alterations, threatening the species' long-term survival (Hladki et al., 2019).

The broader implications of these results become clear when considering that future suitable habitats for this species may be out of reach. As observed in other species, the pace of climate-driven habitat change may exceed the distribution capabilities of an animal (Schloss et al., 2012; Bush and Hoskins, 2017; Sales et al., 2020). Furthermore, factors such as habitat fragmentation and population isolation could further limit the species' ability to migrate to newly suitable areas (Le Galliard et al., 2012; Bestion et al., 2015). As a result, even with new habitats emerging and stabilizing under future climatic conditions, the species may not be able to reach or colonize them effectively (Loarie et al., 2009; Le Galliard et al., 2012; Sales et al., 2020; Arango-Lozano et al., 2025).

The conservation implications of our results underscore the need for both immediate and long-term strategies. Areas of high vulnerability (with values between 0.7 and 1), as identified in this study, represent critical zones where habitat transformation is likely imminent. However, it is essential to recognize that focusing solely on these high-risk areas may not prevent transformation, as rapid environmental changes might already be underway (Global Forest Review, 2024). This introduces a crucial discussion point: should conservation efforts be prioritized in areas of highest vulnerability, or should a broader approach be taken, including areas with moderate vulnerability?

Given the lack of clear protocols for prioritizing conservation actions in snakes (Terribile et al., 2009; Andrade-Díaz et al., 2019), it might be strategic to include areas across the vulnerability spectrum.

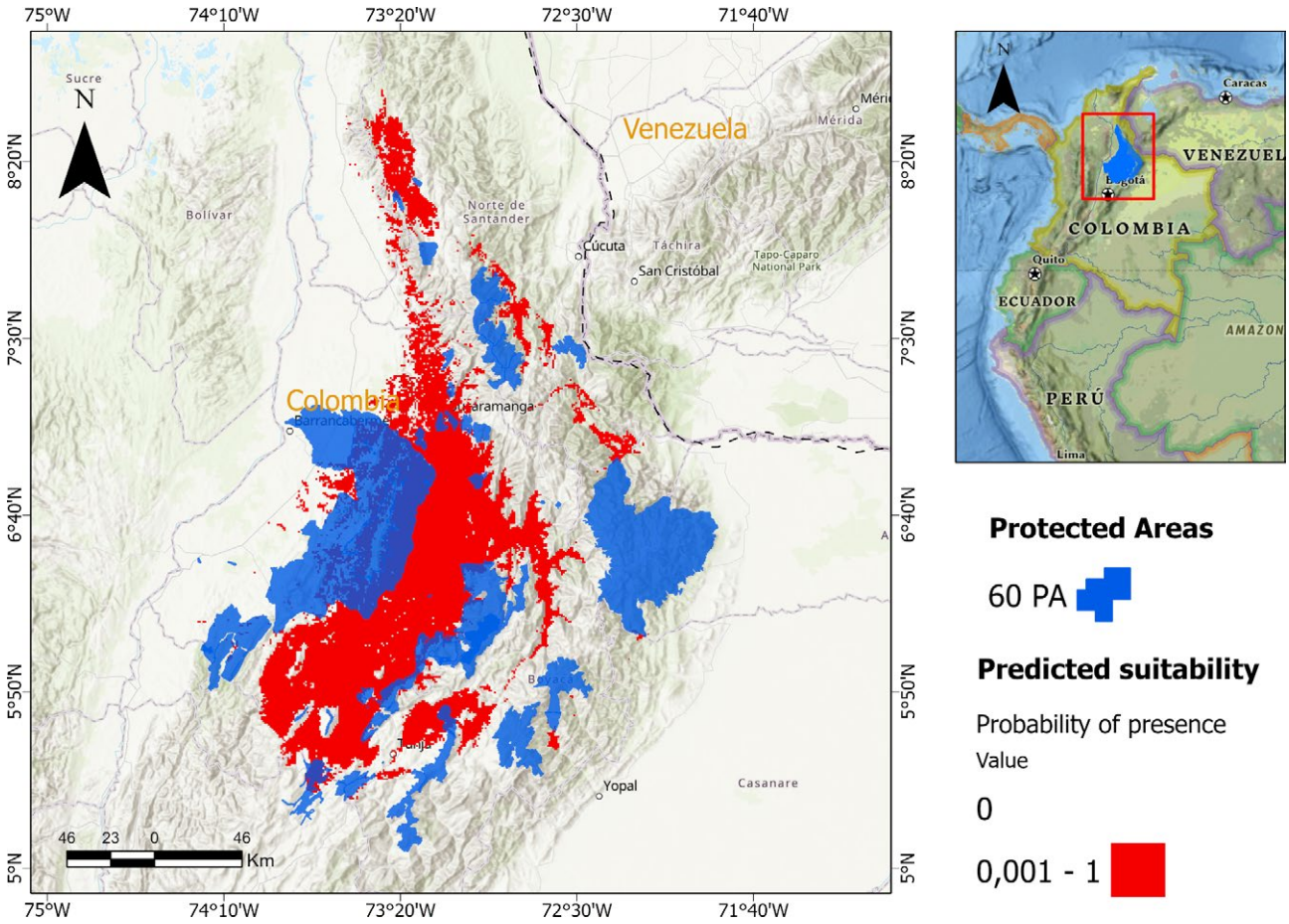


Fig. 5. Description of the sloping between the suitable areas for the distribution of *Micrurus sangilensis* and surrounding protected areas in the surrounding region of Santander Colombia.

For instance, regions with moderate vulnerability ('yellow zones') are less likely to undergo immediate change compared to high-risk areas ('red zones'), but they remain vital for habitat connectivity and the long-term survival of species like *Micrurus sangilensis*. Conservation efforts in these areas could help buffer against habitat fragmentation and provide refugia as the more vulnerable areas degrade.

In Colombia, existing conservation plans and frameworks provide an opportunity to safeguard the critical habitats for *Micrurus sangilensis*. The dry forests of Santander, where suitable areas for this species have been identified, are adjacent to over 60 different protected areas (Fig. 5). These areas include National Natural Parks, regional integrated management districts, national reserves, and forest reserves, all recognized under the National Register of Protected Areas (RUNAP). This network of protected zones represents a significant collective effort to preserve the country's biodiversity.

Although *M. sangilensis* may benefit from the protection offered by some of these existing areas, additional conservation strategies will be vital (Mi et al., 2023). Expanding the coverage of protected areas, restoring degraded habitats, and establishing ecological corridors to link fragmented landscapes are key steps to enhancing the resilience of this species under changing climate conditions (Terribile et al., 2009; Andrade-Díaz et al., 2019). Moreover, incorporating climate-adaptive conservation actions into these plans could help ensure that *M. sangilensis* can persist in its shrinking habitat. Ongoing monitoring of land-use changes and the potential threats posed by human activities will also be essential in aligning conservation efforts with the species' emerging vulnerabilities (Fordham et al., 2012; Mi et al., 2023). A collaborative, multidisciplinary approach is crucial to create effective conservation strategies, safeguard the species' habitat, and ensure the long-term survival of the Santander coral snake.

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SUPPLEMENTARY MATERIAL

The supplementary materials include three (3) tables describing the use of climatic variables and the results selected in this study ecological niche models, are available at https://oaj.fupress.net/index.php/ah/article/view/18167/15288. Additionally, we have made available online the resulted raster files with current and projected future species distributions at: OSF https://osf.io/h9srj/.

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Detection of CMTV-like ranavirus following a *Rana temporaria* mass mortality event in a northern Italian alpine lake

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Abstract. High-mountain lakes are vulnerable to climatic and anthropogenic stressors, and infectious disease may further exacerbate impacts on alpine communities, particularly during seasonal temperature peaks. In August 2024, a mass mortality event of common frogs (*Rana temporaria*) occurred in a high-altitude lake in the Cottian Alps (Piedmont, Italy). During a one-hour survey, 78 dead frogs were recorded; nine carcasses (all adult males) were sampled for diagnostic tests. Six showed ventral red discolouration and three of them had ulcerative lesions of the digits. Eight out of nine vitreous humour samples were culture-positive, with isolates including *Hafnia alvei*, *Acinetobacter guillouiae*, *Acinetobacter proteolyticus*, and *Serratia proteamaculans*. PCR screening of skin and pooled organs detected ranavirus in four out of nine frogs, while *Batrachochytrium dendrobatidis* and herpesvirus tested negative. Phylogenetic analysis of sequenced major capsid protein and DNA polymerase fragments grouped the virus within the CMTV-like clade, with high similarity to reference sequences. This represents the first geolocated detection of a CMTV-like ranavirus in free-ranging amphibians in Italy. Although the advanced state of decomposition precluded histopathological evaluation and causality cannot be conclusively established, the concordance between molecular detection and gross lesions consistent with ranaviral infection supports a plausible role of ranavirus in the observed die-off. Our findings highlight the need for targeted surveillance in Italy's alpine amphibians, including environmental DNA sampling and screening of non-native fish. Given ecological simplification and short reproductive seasons at high altitude, longitudinal monitoring is advisable to assess persistence, seasonality and potential spillover across life stages and sympatric species.

Keywords. *Acinetobacter*, CMTV, Cottian Alps, disease ecology, emerging infectious diseases, Germanasca Valley, outbreak, Ranavirus.

INTRODUCTION

High-mountain lakes are typically small, oligotrophic waterbodies located above the tree line, characterised by extended winters, brief ice-free periods, and extremely low nutrient levels. Their clear, cold waters support simplified biological communities, and the biodiversity of these ecosystems usually decreases with altitude, with communities composed of specialised and highly adapted taxa (Tiberti et al., 2014a; Pastorino and Prearo, 2020; Pastorino et al., 2024).

Despite their apparent isolation, alpine lakes across Europe face multiple anthropogenic stressors that can amplify ecosystem vulnerability: climate warming, especially at higher elevations, leads to increased water temperatures, reduced ice-cover duration, altered sea-

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sonal mixing regimes, and modified hydrological cycles (Råman Vinnå et al., 2021); pastoralism can drive nutrient enrichment and increased organic loading, particularly under high grazing pressure (Tiberti et al., 2014b); atmospheric transport introduces persistent organic pollutants and contaminants of emerging concern, resulting in chronic exposure risks to native biota (Machate et al., 2023; Pastorino et al., 2024); the intentional introduction of alien fish species, salmonids in particular, significantly alters indigenous amphibian and macroinvertebrate communities via direct predation (Tiberti and von Hardenberg, 2012), and may also pose risks by facilitating the spread of pathogens, including ranaviruses (Price et al., 2017); recreational tourism and water abstraction activities add pressure through habitat disturbance, direct contamination, and hydrological alterations (Pastorino and Prearo, 2020).

Although infectious diseases were long overlooked among the primary drivers of biodiversity loss, growing evidence indicates their significant role in wildlife population declines (Smith et al., 2006, 2009). Indeed, pathogens can trigger rapid population reductions or local extinctions, particularly when acting in synergy with climate change and other anthropogenic pressures (Fisher et al., 2012; Hoberg and Brooks, 2015; Di Nicola et al., 2025). Amphibians are exemplary in this context, having experienced some of the most dramatic disease-driven declines among vertebrates, notably linked to emerging pathogens such as the chytrid fungi (*Batrachochytrium dendrobatidis* and *B. salamandrivorans*) and ranaviruses, which have caused mass mortalities worldwide (e.g., Price et al., 2014, 2017; Fisher and Garner, 2020; Hartmann et al., 2022; Akçakaya et al., 2023; Luedtke et al., 2023; Schilliger et al., 2023). Such dynamics could be particularly relevant in alpine lake ecosystems, where amphibians often represent the dominant native vertebrate fauna, a status they would frequently hold exclusively if not for the presence of introduced fish (Catalan et al., 2017). In high-altitude habitats, the combination of environmental harshness, reduced ecosystem complexity, and short reproductive seasons may increase host vulnerability and limit population resilience following disease outbreaks. Moreover, climatic and anthropogenic pressures can interact with pathogens, potentially amplifying disease dynamics and impacts in amphibian populations at higher altitudes (Bosch et al., 2007; Knapp et al., 2011).

One of the most frequent amphibians in alpine lake ecosystems is the common frog, *Rana temporaria* Linnaeus, 1758, which is among the most widespread Palearctic amphibians, ranging from the Iberian Peninsula to western Siberia. In southern Europe, its distribution is largely confined to montane and submontane habitats, where it breeds in wetlands at elevations of up to 2800 metres in the Alps and Pyrenees (Tiberti and von Hardenberg, 2012; Di Nicola et al., 2021; Ilić et al., 2024). At higher altitudes, *R. temporaria* life cycle is shaped by short summers, requiring larval development to occur within a limited time window (Bison et al., 2021), which may increase its vulnerability to phenological disruptions and to population turnover caused by disease outbreaks.

Notably, ranaviruses (large double-stranded DNA viruses of the *Iridoviridae* family infecting ectothermic vertebrates; Jancovich et al., 2015), have been directly implicated in mass mortality events (MMEs) within *R. temporaria* populations inhabiting high-altitude lakes: Miaud et al. (2016) reported three outbreaks in the Mercantour National Park (French Alps), where molecular diagnostics confirmed the presence of a ranavirus (common midwife toad virus, CMTV) in all dead frogs, with near-complete die-offs of both larvae and adults. Similar MMEs involving montane amphibians were also documented in Spain, in the Cantabrian Mountains, where *R. temporaria* was recorded at several affected sites and CMTV-like ranavirus was detected in at least one individual (Price et al., 2014). In the Mercantour area, CMTV-like DNA was also detected throughout an activity season in live *R. temporaria* tadpoles, with infection peaking during a MME and persisting in survivors until metamorphosis; adults, in contrast, showed only transient infection and no substantial die-off was observed (Miaud et al., 2019). Long-term monitoring in the Cantabrian Mountains further indicated persistent negative effects of ranavirus outbreaks, although impacts on common frogs specifically appeared less severe compared to other sympatric amphibians (Bosch et al., 2021). In the French Pyrenees, a Frog Virus 3 (FV3)-like ranavirus was sporadically detected in dead amphibians, including *R. temporaria*, collected during chytridiomycosis-driven die-offs, but its contribution to mortality remains uncertain (Peñafiel-Ricaurte et al., 2025).

More broadly, ranaviruses have been detected in amphibians worldwide (e.g., Duffus et al., 2014; Hick et al., 2016; Ruggeri et al., 2019; Box et al., 2021; Brunner et al., 2021; Hartmann et al., 2022; Flechas et al., 2023; Herath et al., 2023; Lisachova et al., 2025). Outcomes range from subclinical infections to recurrent mass mortalities, with documented population-level declines and, in some systems, community-level change (Teacher et al., 2010; Price et al., 2014; Miaud et al., 2016, 2019; Rosa et al., 2017; Bosch et al., 2021; Hartmann et al., 2022). Outbreak probability and severity appear to be modulated by environmental and host factors, notably thermal anomalies (Thumsová et al., 2022), seasonal windows linked to aggregation at breeding sites and larval development

(North et al., 2015; Miaud et al., 2019), and local host density (North et al., 2015).

Clinically, ranavirus infections in anurans typically present systemic manifestations, with signs that may include lethargy, anorexia, neurological impairment such as loss of righting reflex and abnormal swimming behaviour in tadpoles, generalised oedema, hyperaemia, extensive haemorrhages, and ulcerative skin lesions often prominent in oral cavities and limb extremities (Forzán et al., 2017; Hartmann et al., 2022; Miller et al., 2025). Histologically, cases are commonly characterised by multisystemic necrosis and haemorrhage, often with basophilic intracytoplasmic inclusions in infected cells, and frequent involvement of the liver, kidneys, and spleen in fatal infections (Balseiro et al., 2009; Forzán et al., 2017; Bates et al., 2025; Miller et al., 2025).

Here, we investigated the possible aetiology of an MME affecting *R. temporaria* observed at an alpine lake in Piedmont, Italy. We aimed to assess whether ranavirus infection was implicated, based on the observed clinical signs, and to evaluate the involvement of other pathogens of concern, namely *Batrachochytrium dendrobatidis* (hereafter Bd) and herpesviruses. Where ranavirus was detected, we sought to contextualise the finding by sequencing and phylogenetically comparing the strain within the current European diversity.

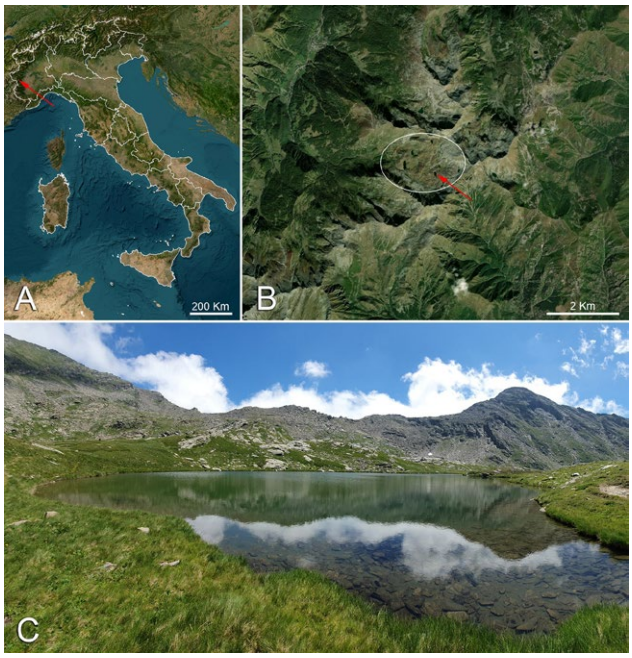


Fig. 1. Map of Italy with a red arrow indicating the area of the Conca dei Tredici Laghi (A); Satellite view of the territory with a red arrow indicating Lago Lungo within the Conca, outlined in white (B); View of Lago Lungo (C). Panels A and B were generated using QGIS 3.28.10 with ESRI satellite imagery.

MATERIALS AND METHODS

Study area and sampling

In August 2024, the *Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta* was alerted to a MME involving *Rana temporaria*. The event was observed in the waters of "*Lago Lungo*," a small alpine lake located within the municipality of Prali (Turin, Piedmont; coordinates: 44.8578, 07.0907; altitude: 2503 m a.s.l.), in the area known as the "*Conca dei Tredici Laghi*" (hereafter "*Conca*", from the Italian for "basin"). This high-altitude basin is a glacial cirque situated in the central sector of the Cottian Alps, within the upper Germanasca Valley (Fig. 1). It hosts a series of small glacial lakes formed by Quaternary glaciation and moraine deposition, representing an example of post-glacial alpine landscape dynamics (see Allasia et al., 2004; Nigrelli, 2005; Farina, 2008; Forno et al., 2011). Only two amphibian species occur in the *Conca* area: the common frog (*R. temporaria*) and the Lanza's salamander (*Salamandra lanzai* Nascetti, Andreone, Capula and Bullini, 1988), similarly to what has been reported for the nearby *Conca Cialancia* (Seglie, 2020). The former species reproduces in the lakes within the *Conca* (Di Nicola, pers. obs.), whereas Lanza's salamander typically gives birth to fully developed, terrestrial juveniles (Bergò and Andreone, 2002; Di Nicola et al., 2021).

A field survey was carried out in the *Conca* to assess the reported MME and to collect samples for pathogen screening. Dead common frogs were visually inspected, and a subset of the least decomposed individuals was collected and transported under refrigeration for further analysis.

Field inspection and gross pathology

An initial external examination to assess the general condition of the specimens was performed in situ shortly after sampling, to minimise further post-mortem alterations due to transport. Particular attention was paid to the skin and limbs, which were examined for external lesions and gross abnormalities, such as ulcerations, haemorrhages, necrosis, skin sloughing, oedema, and other deviations from normal appearance. Once in the laboratory, skin samples were collected from multiple body regions and pooled per individual for molecular analysis.

Subsequently, a detailed internal examination of the coelomic cavity and visceral organs was performed. Each

specimen was positioned in dorsal recumbency, and a sterile scalpel was used to make a ventral midline laparotomy from the intermandibular region to the cloaca. Major organs were inspected, sampled, and pooled per specimen for molecular analysis. Histopathological examinations were not performed due to the poor post-mortem condition of the carcasses.

Bacteriology

Bacteriological analysis was performed following standardised protocols (Pastorino et al., 2021). In detail, the vitreous humour was aseptically inoculated onto Columbia Agar (CBA) (Liofilchem®, Italy) supplemented with 5% sheep blood, after first swabbing the ocular surface with 70% ethanol. Plates were incubated at 22 ± 2 °C for 72 hours with daily evaluation. Dominant colonies were subcultured on CBA and incubated for an additional 24 hours at 22 ± 2°C. Bacterial identification was performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics Inc., Billerica, MA, USA).

Molecular pathogen screening

Bd and ranaviruses are among the most significant infectious agents currently threatening anuran populations in Europe (Price et al., 2014; Allain and Duffus, 2019; Fisher and Garner, 2020) and were therefore selected for the pathogen screening. Herpesviruses were also included, as they have been indicated as potential emerging pathogens of concern (Franklinos et al., 2018; Origgi et al., 2018; Allain and Duffus, 2019). Molecular analyses were performed on both skin samples and pooled target organs, following genomic DNA extraction using the ReliaPrep™ gDNA Tissue Miniprep System (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol.

Bd detection was carried out using a real-time TaqMan PCR protocol targeting the ITS-1/5.8S rDNA region (Table 1; Boyle et al., 2004; Meletiadis et al., 2025). The following thermal conditions were applied: 50°C for 2 min, 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. Primers and probe used were ITS1-3 Chytr, 5.8S Chytr, and Chytr MGB2, respectively. Genomic DNA from Bd-positive *Pelophylax* sp. skin was used as a positive control.

Herpesvirus detection followed a pan-herpesvirus nested PCR protocol with ten degenerate and deoxyinosine-substituted primers (Table 1 targeting a conserved region of the DNA polymerase gene (Ehlers et al., 1999; Franklinos et al., 2018; Bianchessi et al., 2024). The following thermal conditions were applied for both rounds of the nested PCR: initial activation at 95°C for 15 min, followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 46°C for 60 s, and extension at 72°C for 60 s, with a final elongation at 72°C for 10 min. Genomic DNA from a herpesvirus-positive lemur sample was used as a positive control.

Ranavirus detection was conducted using an end-point PCR protocol targeting the major capsid protein (MCP) gene, using primers OL T1_F and OL T2_R

Table 1. Primers used in diagnostic PCR reactions for Bd, herpesvirus and ranavirus. Degenerate and inosine-substituted equivalents are shown in bold (I = Deoxyinosine).

Target gene	Primer	Amplicon size (bp)	Nucleotide sequence (5' to 3')	Reference
ITS-1/5.8S rDNA	ITS1-3 Chytr	146	CCTTGATATAATACAGTGTGCCATATGTC	Boyle et al., 2004
	5.8S Chytr		AGCCAAGAGATCCGTTGTCAAA	
	Chytr MGB2		6FAM CGAGTCGAACAAAAT MGBNFQ	
DNA pol	DFA (step1 forward)	725	GAYTTYGC**N**AGYYT**N**TAYCC GAYTTYGC**I**^{a}AGYYT**I**^{a}TAYCC	Bianchessi et al., 2024
	ILK (step1 forward)	470	TCCTGGACAAGCAR**N**YSGC**N**MT**N**AA TCCTGGACAAGCAR**I**^{a}YSGC**I**^{a}MT**I**^{a}AA	
	KG1 (step1 reverse)		GTCTTGCTCACCAG**N**TC**N**CA**N**CCYTT GTCTTGCTCACCAG**I**^{a}TC**I**^{a}CA**I**^{a}CCYTT	
	TGV	225	TGTAACTCGGTGTAYGG**N**TTYAC**N**GG**N**GT TGTAACTCGGTGTAYGG**I**^{a}TTYAC**I**^{a}GG**I**^{a}GT	
	IYG		TGTAACTCGGTGTAYGG**I**^{a}TTYAC**I**^{a}GG**I**^{a}GT	
MCP	OLT-1	531	CACAGAGTCCGTRTC**I**^{a}CCRTA**I**^{a}AT GACTTGGCCACTTATGAC	Mao et al., 1997
	OLT-2R		GTCTCTGGAGAAGAAGAAT	

Table 2. Primers used in PCR reactions for ranavirus phylogenetic analysis. Degenerate equivalents are shown in bold. Nucleotide modifications relative to the original primer sequences are underlined. The column "Primer position" refers to the position of the primers on the FV3 genome (AY548484).

Target gene	Primer	Primer position	Amplicon size (bp)	Nucleotide sequence (5' to 3')	Reference
MCP	OLT-1	97387-97404	531	GACTTGGCCACTTATGAC	Mao et al., 1997
	OLT-2R	97917-97899		GTCTCTGGAGAAGAAGAAT	
	MCP-BF	97813-97830	548	ACCAGCGATCTCATCAAC	Ariel et al., 2010
	MCP-BR	98360-98341		AG**G**GCTGGCTCCAGGACCGT	
	MCP-5	98244-98263	622	CGCAGTCAAGGC**Y**TGATGT	Hyatt et al., 2000
	MCP-6Rb	98865-98843		TGCCATGTTAAGATTTGTCAGAG	This study
DNA pol	DNApol-F	67188-67208	560	GTGTA**Y**CAGTGGTTTTGCGAC	Holopainen et al., 2009
	DNApol-R	67747-67728		TCGTCTCCGGG**Y**CTGTCTTT	

(Table 1; Mao et al., 1997; Stöhr et al., 2013). The following thermal conditions were applied: activation of Taq polymerase at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 1 min; annealing at 50°C for 1 min; extension at 72°C for 1 min; and final elongation at 72°C for 10 min. A synthetic positive control amplicon of approximately 500 bp was included.

All DNA extracts were tested in technical duplicate across assays. No-template controls and extraction blanks were included in every run.

Sequencing and phylogenetic analysis

For Ranavirus-positive samples, Sanger sequencing was performed. Amplification products were checked by electrophoresis on a 2% agarose gel, purified using the ExtractMe DNA Clean-Up kit (Blirt, Gdańsk, Poland), and amplified with the BrilliantDye™ Terminator v3.1 Cycle Sequencing Kit (NimaGen, Nijmegen, The Netherlands). Sequencing reaction products were then purified using the DyeEx 2.0 Spin Kit (Qiagen, Hilden, Germany) and run on a SeqStudio™ Genetic Analyzer (Applied Biosystems, Waltham, MA, USA).

The phylogenetic analysis for ranavirus was performed by comparing the major part of the MCP gene in overlapping fragments (almost complete CDS and partial 3'UTR) and a partial sequence of the DNA polymerase gene (DNApol). The amplification, purification and sequencing were performed as previously described for the diagnostic PCR, using the primers listed in Table 2. All the primers were available in literature, except the reverse primer MCP-6Rb. The alignment between primer and ranavirus sequences showed that the MCP-6R primer developed by Hyatt et al. (2000) for the amplification of the last fragment of the MCP gene was localized in a region characterized by a two-nucleotide polymorphism. To avoid amplification failure, the new primer MCP-6Rb was developed in the 3'UTR region of the gene.

For each gene, the obtained sequences were aligned with similar regions from 24 complete genomes of the different ranavirus species presented in GenBank (Table S1), using Clustal W program on MEGA7 software. The sequences of MCP and DNA polymerase genes from Lymphocystis disease virus (LCDV) were used as an outgroup. Phylogenetic trees were constructed using MEGA7 software (Kumar et al., 2016) with the maximum likelihood method, using General Time Reversible model, uniform substitution type and invariable sites. All positions containing gaps and missing data were eliminated. The statistical robustness and reliability of the branching order were confirmed with bootstrap analysis using 1000 reiterations.

RESULTS

Field observations and gross clinical findings

During approximately one hour of field inspection, a total of 78 dead common frogs were recorded, all of which were adults except for two subadults. Of these, 73 were found on the bottom of *Lago Lungo* (generally within three metres of the shoreline; Fig. 2 A, B), three were found desiccated along the shore (Fig. 2 C), and two were observed in the outflow stream descending from the lake. Most carcasses exhibited advanced post-mortem decomposition, with several specimens reduced to skeletal remains. Moreover, limited lakebed visibility (about 5-6 meters from the shoreline) may have led to an underestimation of the total number of dead common frogs.

No dead frogs were observed in the other lakes of the *Conca*. In both *Lago Lungo* and the adjacent lakes, live adult frogs (n = 9) and tadpoles (n = 4; Fig. 2 D) were present and appeared clinically healthy upon visu-

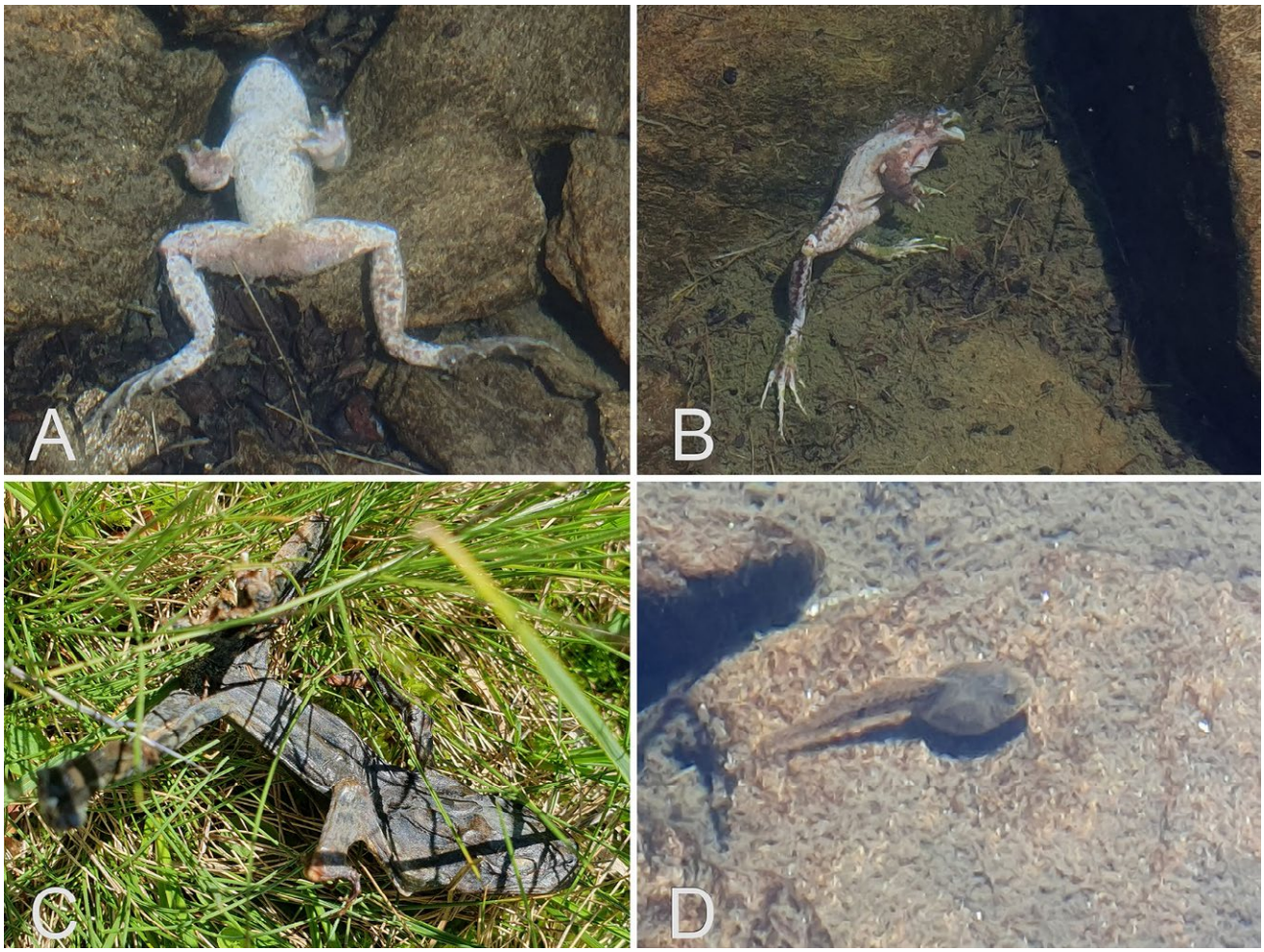


Fig. 2. Dead adult *Rana temporaria* specimens on the lakebed of Lago Lungo, showing visible post-mortem alterations (A and B); desiccated adult *R. temporaria* on the grass along the shoreline (C); live R. temporaria tadpole with no apparent clinical signs (D).

al inspection, with no external lesions or abnormalities detected.

Out of the 78 dead common frogs observed, nine specimens were collected for laboratory analysis; all were adult males. Six of these (specimens I, III, V, VII, VIII and IX) exhibited red discolouration consistent with hyperaemia or haemorrhage, either localised in the ventral thigh region or extending across the entire ventral surface of the hind limbs and/or the digits (Fig. 3). In three of these cases, ulcerative lesions were also present on the toes (I, III and VII). The remaining three frogs (specimens II, IV and VI) did not display any visible external clinical signs, apart from post-mortem alterations. At necropsy, all specimens showed post-mortem alterations, including partial liquefaction of internal organs in some cases, which limited thorough assessment. However, gastrointestinal haemorrhage was observed in three individuals.

Laboratory findings and phylogenetic analysis

Bacteriological cultures were positive in eight out of nine frogs. The isolated bacterial species were: *Hafnia alvei* (specimens I, IV, and V); *Acinetobacter guillouiae* (specimens III, VI and VIII); *Acinetobacter proteolyticus* (specimen IX); and *Serratia proteamaculans* (specimen VII) (Table 3).

All frogs tested negative for Bd and herpesvirus DNA in both skin and pooled target organ samples.Regarding ranavirus, skin samples from four frogs (specimens I, III, V and VI) tested positive using the diagnostic end-point PCR (Table 3). The obtained sequences were 98% identical to common midwife toad virus (CMTV) entries in the NCBI database. Based on this result, a phylogenetic analysis was conducted.

A 1437 bp fragment of the MCP gene was successfully amplified for two samples (specimens I and III),

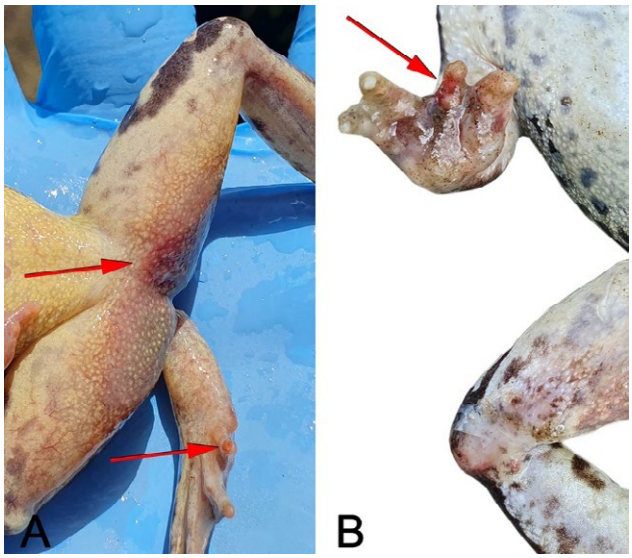


Fig. 3. Examples of external clinical signs in the collected frogs. Ventral thigh region, with red arrows indicating marked red discolouration, particularly at the base of the left thigh, and an ulceration on digit I of the right hind limb (A); close-up of a right forelimb showing red discolouration on digit II. The skin lesion on the stifle joint of the hind limb, present in several carcasses, may be attributable to post-mortem alteration (B).

while a 496 bp fragment of the DNA polymerase gene was obtained from all four PCR-positive individuals. The sequences showed no polymorphisms among the samples and the lengths corresponded to the similar CMTV-like sequences deposited in NCBI database. The obtained MCP and DNA polymerase gene sequences were deposited in GenBank under accession numbers PV990114 and PV990113, respectively.

The sequences of both genes were similar to CMTV-like sequences with a percentage over 99%, except for TorV-1 and CMTV-ES which similarity was 98% with our samples. The similarity with other ranaviruses ranged from 97-98 % but decreased below 80% with SCRV (Santee-Cooper ranavirus) and SGIV (Singapore grouper iridovirus)-like groups. This situation was evident in the phylogenetic analysis where the samples clustered with other sequences belonging to the CMTV-like group for both the genes (Figs 4 and 5). Considering the MCP gene, CMTV- and FV3-like groups clustered more closely together with high support (99%) in respect to EHNV-like (Epizootic haematopoietic necrosis virus) paraphyletic group, that includes ATV (Ambystoma tigrinum virus), EHNV, ENAR (European North Atlantic ranavirus) and SERV (Short-finned eel ranavirus). The most basally branching lineages were SCRV- and SGIV-like. The DNA polymerase gene sequence analysis showed a similar result (considering that the FV3-like group was paraphyletic, while EHNV-like was monophyletic) but with little support.

DISCUSSION

In the present study, we document the detection of a CMTV-like ranavirus following a summer MME affecting *Rana temporaria* in a high-altitude lake in the Cottian Alps, Piedmont, Italy. Ranaviral DNA was detected by PCR in four of nine carcasses and confirmed by Sanger sequencing of fragments of the MCP and DNA polymerase genes, with phylogenetic analysis placing these sequences within the CMTV-like clade. Several frogs, both among those tested and among additional carcasses observed in the field, showed clinical signs consistent with ranaviral disease, including ventral red discolouration consistent with hyperaemia/haemorrhage, as well as ulcerative and necrotic lesions affecting the digits.

Bacteriological analysis provided further insights into the condition of the frogs. Bacterial colonies were isolated from the vitreous humour in eight out of nine individu-

Table 3. Diagnostic overview of clinical signs, ranavirus detection and bacterial isolates in nine adult male *Rana temporaria* specimens.

Specimen	Clinical signs	Ranavirus PCR positive	*Hafnia alvei*	*Acinetobacter guillouiae*	*Acinetobacter proteolyticus*	*Serratia proteamaculans*
I	+	+	+	-	-	-
II	-	-	-	-	-	-
III	+	+	-	+	-	-
IV	-	-	+	-	-	-
V	+	+	+	-	-	-
VI	-	+	-	+	-	-
VII	+	-	-	-	-	+
VIII	+	-	-	+	-	-
IX	+	-	-	-	+	-

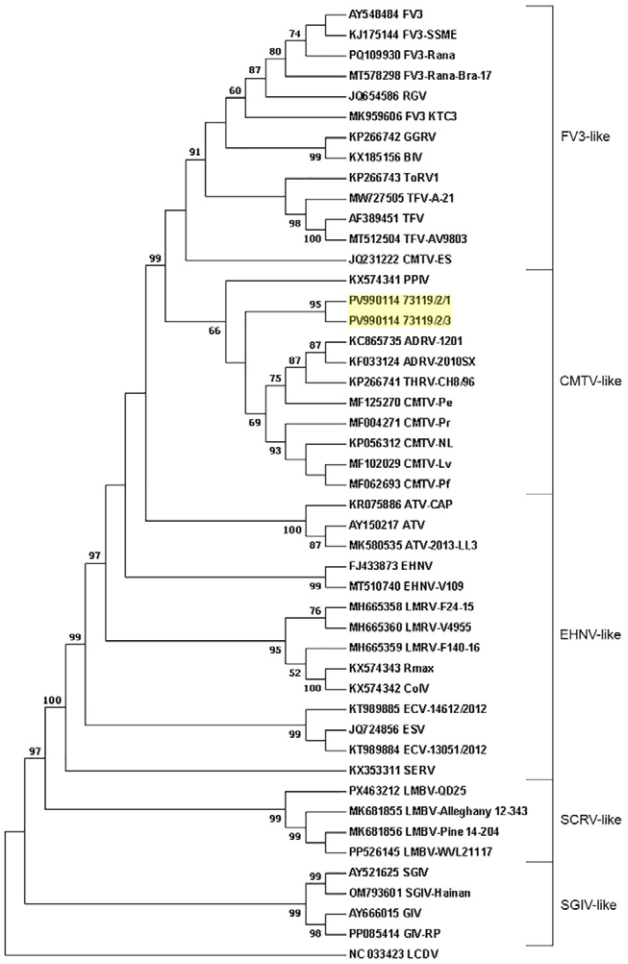


Fig. 4. Phylogenetic tree based on the MCP gene sequence (1437bp). Bootstrap values >50 are shown at the tree nodes. Lymphocystis disease virus (LCDV) was used as an outgroup. Classifications of the viruses into the different groups are indicated beside the square brackets. Samples 73119_2_1 and 73119_2_3 correspond to specimens I and III, respectively.

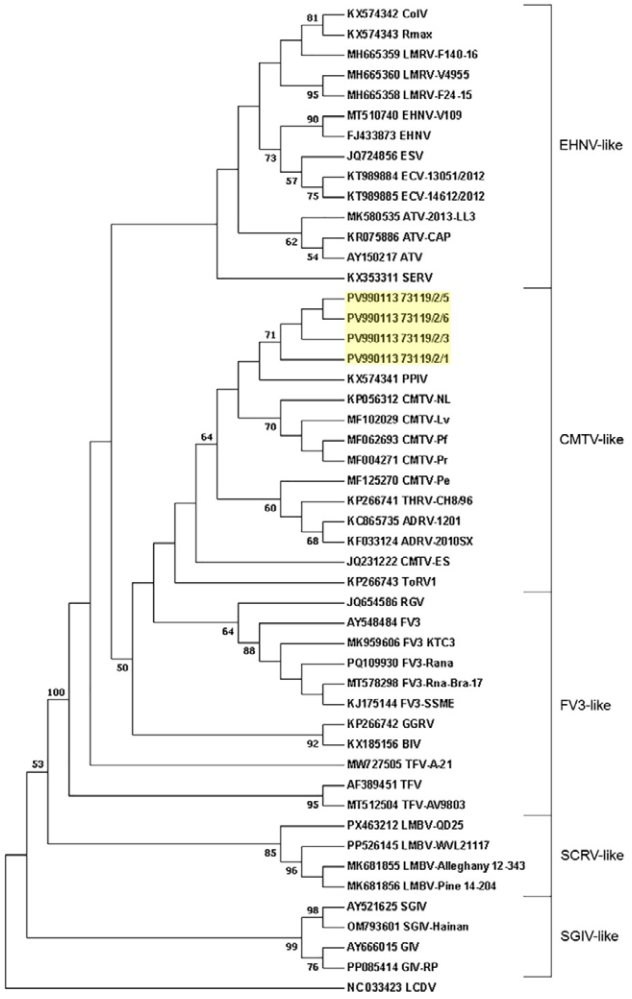


Fig. 5. Phylogenetic tree based on the DNA polymerase gene sequence (496bp). Bootstrap values >50 are shown at the tree nodes. Lymphocystis disease virus (LCDV) was used as an outgroup. Classifications of the viruses to the different groups are indicated beside the brackets. Samples 73119_2_1, 73119_2_3, 73119_2_5, and 73119_2_6 correspond to specimens I, III, V, and VI, respectively.

als; since this compartment is anatomically protected, the presence of bacteria is generally interpreted as evidence of systemic infection rather than external contamination (Hanna et al., 1990; Masli and Vega, 2011; Pigaiani et al., 2020). The identified bacteria, including *H. alvei*, *A. guillouiae*, *A. proteolyticus* and *S. proteamaculans*, are commonly found in the environment or in the gut, but have also been reported as opportunistic pathogens in immunocompromised or environmentally stressed animals (Mahlen, 2011; Padilla et al., 2015; Ionescu et al., 2022; Trinh and Nguyen, 2024). Of particular interest, *A. guillouiae* has recently been shown to cause systemic disease in amphibians, with experimental infections in *Quasipaa spinosa* resulting in tissue damage and mortality (Guo et al., 2025). Although the exact contribution of these bacteria to the mortality event cannot be determined, their recovery from an internal immune-privileged site supports the hypothesis of opportunistic invasion, possibly favoured by environmental stressors and host immunosuppression associated with viral infection. Anyway, findings from autolysing amphibian carcasses should be interpreted with caution (White and Dusek, 2015). Although histopathological analyses were not performed, the molecular evidence and observed external lesions support ranavirus infection as the most plausible primary cause of the MME.

To further characterise the outbreak, sequence data from two gene regions (MCP and DNA polymerase)

were obtained from ranavirus-positive *R. temporaria* samples. Both sequences showed high similarity and grouped unequivocally within the CMTV-like group.

Previous phylogeographic studies indicated that the genetic similarity of ranaviruses often reflects their geographic origin rather than host-specific relationships, demonstrating significant differences among European ranavirus strains compared to those from other regions globally (Stöhr et al., 2015). Specifically, CMTV-like ranaviruses are broadly distributed in continental Europe, with only limited detections in Asia and records confined to aquaculture facilities in North America (Claytor et al., 2017; Herath et al., 2023; Lisachova et al., 2025; Marschang et al., 2025). Available evidence indicates that CMTV-like ranaviruses are likely endemic to Europe, with the Iberian Peninsula emerging as a hotspot of genetic diversity. Multilocus analyses from multiple independent outbreaks in Spain over recent decades have identified novel Iberian genotypes and revealed phylogeographic patterns consistent with an ancestral Iberian origin followed by natural dispersal. Nonetheless, the precise origin remains unresolved (Thumsová et al., 2022).

From an evolutionary perspective, CMTV occupies an intermediate position among ranaviruses, retaining features characteristic of EHNV-like viruses but also acquiring unique genomic segments typical of FV3-like viruses (Mavian et al., 2012). The divergence between FV3 and CMTV lineages appears recent, as their separation cannot always be clearly resolved based solely on phylogenetic distance trees (Stöhr et al., 2015). Yu et al. (2021) tried to better examine the classification within *ranavirus* subgroups by a genomic phylogenetic analysis and a dot plot comparison and identified four genomic regions that consistently matched whole-genome analyses. They excluded the DNA polymerase gene due to recombination events and rejected the MCP gene for occasional inconsistencies despite a lack of recombination or substitution saturation. For example, they highlighted a discrepancy in the phylogenetic tree considering only the MCP gene sequences: the samples TorV1 and CMTV-ES were closely related to the FV3-like group, while they should belong to CMTV-like group based on genome analysis. Consistent with these observations, our phylogenetic analysis based on MCP gene fragments showed CMTV-ES and ToRV-1 grouped with the FV3-like viruses. Conversely, they clustered in the CMTV-like group considering DNA polymerase gene (even if with little support).

Overall, the phylogenetic analyses based on both DNA polymerase and MCP gene sequences conclusively placed our samples within the CMTV-like group, providing the first geolocated detection of CMTV-like ranavirus in free-ranging amphibians in Italy, expanding the documented range of this pathogen and highlighting its potential threat to alpine amphibian populations. The only other documented Italian instance was reported by Holopainen et al. (2009), who isolated Rana esculenta virus 282/I02 (REV 282/I02) from *Pelophylax esculentus* tadpoles collected from an unspecified locality in 2002 (see Ariel et al., 2017); the larvae developed disease and died shortly after transfer to captivity (Holopainen et al., 2009; Ariel et al., 2010, 2017). Subsequent genomic analyses placed REV 282/I02 within the CMTV-like clade (Ariel et al., 2017). In addition, online outreach articles report that ranavirus has been found in Italian territory, with ongoing investigations in the Maritime Alps in Piedmont (Aree Protette Alpi Marittime, 2019; Piemonte Parchi, 2019), but no peer-reviewed data from these surveys are currently available. Given the geographic proximity to the Mercantour outbreak sites in France (Miaud et al., 2016, 2019), it will be important to document any ranavirus detections that may occur in the Maritime Alps and to assess the phylogenetic placement of any strains involved, particularly with respect to the CMTV-like clade.

CMTV-like ranaviruses have been associated with multiple mortality events in Spain and France, in some cases even contributing to community-level declines (Price et al., 2014, Miaud et al., 2016, Thumsová et al., 2022). In Spain, one of the most recent outbreaks occurred in the northwest, affecting *Pleurodeles waltl* populations in small cattle ponds that typically dry in summer. Although Bd co-occurred at these sites, coinfection within individuals was rare. Ranavirus loads declined in the months following the outbreak, likely as a result of lower temperatures (Thumsová et al., 2024). By contrast, in the French Alps CMTV caused mass mortality of *R. temporaria* across several alpine lakes, affecting both larvae and adults, Bd was not detected, and experimental infections produced 100 % mortality in adults (Miaud et al., 2016). Our event shares the alpine setting and late-summer timing, but it was dominated by adult carcasses while tadpoles were observed alive, consistent with a context-dependent expression of CMTV impacts across systems.

Our samples were also screened for Bd and herpesviruses, with no positive detections. Although Bd was not detected at our site, Bd and ranaviruses can co-occur at the same localities, whereas coinfection within individuals is usually rare and infection with one pathogen does not reliably predict infection with the other (Bosch et al., 2020; Thumsová et al., 2024). This pattern is consistent with evidence that the two pathogens tend to peak under contrasting temperature and precipitation conditions, which may limit temporal overlap even where they co-occur geographically (Thumsová et al., 2025).

Anthropogenic activities such as wildlife trade and introduction of non-native species can facilitate ranavirus spread into novel ecosystems and host communities. In particular, fish introduced through aquaculture or stocking have been linked with amphibian outbreaks, and invasive fish can also disrupt pre-existing host-pathogen equilibria, increasing ranavirus prevalence and driving long-term declines even without introducing novel strains (Price et al., 2017; Rosa et al., 2022). In line with this, targeted screening of non-native fish species should be included in future monitoring at our study site, as they are present in the Conca for recreational fishing and may contribute to local transmission or amplification (Peñafiel-Ricaurte et al., 2025). In addition, environmental DNA-based surveillance from water samples may increase ranavirus detectability and support non-invasive monitoring, even when no overt die-off is observed (Miaud et al., 2019).

Although the drivers of ranavirus emergence in our study system remain unclear, climatic variability has consistently been linked to ranavirus dynamics in other regions. Warmer conditions have been associated with increased incidence and severity of outbreaks, and several studies have related ranavirus epizootics to ongoing global warming (Price et al., 2019; Thumsová et al., 2022). More recently, a large-scale study from the Iberian Peninsula suggested that increased ranavirus infection risk is not driven by rising temperatures alone, but rather by mismatches between local host adaptations and complex interactions between changing temperature and precipitation patterns (Thumsová et al., 2025). Accordingly, future work should integrate continuous water temperature logging, local precipitation metrics and basic physicochemical profiling (for example dissolved oxygen, pH, conductivity), and consider microhabitat features such as shading to better characterise conditions that may favour pathogen emergence. The surveyed area also hosts *Salamandra lanzai*, a live-bearing species endemic to the Cottian Alps and classified as "Vulnerable" in the Italian IUCN Red List of vertebrates (Rondinini et al., 2022); its inclusion in ranavirus surveillance programmes is advisable, given previous detections of ranavirus within the genus *Salamandra* (Vörös et al., 2020).

In summary, despite the limited number of specimens analysed and the lack of histopathology, our survey confirms the presence of a CMTV-like ranavirus in common frogs within the Italian Alpine range and provides the first geolocated detection of this lineage in free-ranging amphibians in Italy. These findings call for more detailed investigations to better characterise ranavirus circulation and clarify its role in mortality events, integrating host screening across taxa with targeted environmental monitoring.

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at https://oaj.fupress.net/index.php/ah/article/view/18382/15289.

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Home range and breeding ecology of *Phasmahyla cruzi* (Anura: Phyllomedusidae)

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Abstract. *Phasmahyla cruzi* is an endemic treefrog species from the Brazilian Atlantic Forest for which basic natural history information is still lacking. In this study, we monitored two populations over a 12-month period in Ubatuba, São Paulo, Brazil, to investigate the species' ecology, home range, and reproductive patterns. We used non-invasive photographic identification combined with minimum convex polygon (MCP) analysis to estimate home ranges. Based on the monthly sampling, we assessed the influence of abiotic factors (temperature and humidity) on activity patterns. We recorded 920 adult, 2,142 tadpole, and 22 egg clutch encounters. Males vocalized from September to April and showed territorial behaviour. Reproduction occurred through axillary amplexus, with oviposition on folded leaves suspended over streams. Mean home range for *P. cruzi* males was estimated at 460 m^2, with individuals in higher elevations using significantly larger areas. A positive relationship was found between humidity and temperature with both individual abundance and clutch frequency. This study presents the first detailed data on habitat use, reproduction, and spatial ecology of *P. cruzi*, providing essential information for conservation efforts targeting this poorly known species endemic to the Atlantic Rainforest.

Keywords. Anuran conservation, rainforest, reproductive biology, spatial ecology.

INTRODUCTION

Natural history studies rely on observational data collected *in situ* without experimental manipulation. They aim to search for and describe patterns through direct observation of the natural world. These studies are the baseline for test hypotheses in different branches of the natural sciences, such as ecology, conservation biology, and evolutionary biology (Nanglu et al., 2023).

Despite the rich diversity of anurans in the Atlantic Forest (Toledo et al., 2014), studies addressing seasonality, population size, and home range remain scarce. The breeding biology of anurans in this biome is relatively well documented, particularly for species within the families Bufonidae, Cycloramphidae, Hylidae, and Phyllomedusidae (e.g., Oliveira et al., 2012; Botelho et al., 2023; Pedrozo et al., 2023). In contrast, research on home range is considerably less common, with only a few published studies focusing on species from the families Hemiphractidae, Hylidae and Leptodactylidae (Tozetti and Toledo, 2005; Moser et al., 2019; Muscat et al., 2020; Moroti et al., 2022). Notwithstanding these contributions, many species still lack fundamental natural history data – information that is crucial for understanding ecological requirements and forming conservation strategies.

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Among the poorly known groups in the Atlantic Forest is the genus *Phasmahyla* Cruz, 1990, charismatic spotted leaf frogs from the family Phyllomedusidae. The monophyly of the clade is well supported by both molecular data (Faivovich et al., 2005, 2010) and possible morphological synapomorphies, such as: indistinct external vocal sacs, cream irises, and tadpoles with specialized oral discs, modified as a funnel-shaped structure (Bokermann and Sazima, 1978; Cruz, 1990; Altig and McDiarmid, 1999; Pereira et al., 2018). Currently, eight species of *Phasmahyla* are recognized: *P. cochranae* (Bokermann 1966); *P. cruzi* Carvalho-e-Silva, Silva & Carvalho-e-Silva, 2009; *P. exilis* (Cruz, 1980); *P. guttata* (Lutz, 1924); *P. jandaia* (Bokermann & Sazima, 1978); *P. spectabilis* Cruz, Feio & Nascimento, 2008; *P. timbo* Cruz, Napoli & Fonseca, 2008; and *P. lisbella* Pereira, Rocha, Folly, Silva & Santana, 2018. The genus is endemic to the Brazilian Atlantic Forest, being found in mountain streams from the south of the state of Bahia to the east of the state of Paraná, including the east of the state of Minas Gerais (Cruz, 1990; Cruz et al., 2008a, 2008b; Frost, 2010).

Phasmahyla cruzi is diagnosed from its congeners by its small body size (less than 5 cm), large eyes with palpebral membranes and pigmented reticulation covering them entirely and males with visible nuptial pad from the base of the first finger to the inner carpal tubercle (Carvalho-e-Silva et al., 2009). Although the species was described in 2009, basic aspects of its natural history are not available (Oliveira et al., 2012). We sampled a population of *P. cruzi* monthly over the course of a year, recording data on its breeding biology and home range. This type of basic natural history information is key for adequate species conservation status evaluation, contributing to amphibian conservation, a worldwide problem (Toledo et al., 2014).

MATERIALS AND METHODS

The study was conducted at the private reserve of the NGO Projeto Dacnis (23.46°S, 45.13°W; 15-500 m above sea level), a 136-hectare reserve in the Atlantic Forest in Ubatuba, state of São Paulo, Brazil. The area is mainly composed of swamp forests in flat regions and patches of primary and secondary dry forests on steep terrain. The climate in Ubatuba is characterized as humid subtropical, without marked seasonality (Rolim et al., 2007).

We conducted this study in two sites where we had previously recorded the species. Site 1 was in a forested area with shrub and tree vegetation and comprised streams with rapids, rocks, and sandy bottoms, 100 m above sea level (Fig. S1 a); site 2 was 250 a.s.l., in semi-open canopy along the sampling route, also containing streams with the same characteristics as site 1 (Fig. S1 b). The two sites were 350 meters apart from each other in a straight line. Three researchers sampled the populations three to four times per month from April 2023 to March 2024, totaling 46 visits of 3 h/night, resulting in 414 man-hours at site 1, and 47 visits of 3 h/night to site 2, which added up to 423 man-hours.

In nearly 14 years of daily studies and monitoring of the reserve, we did not obtain a single record of the species during the daytime (from 07:00 to 17:30). Therefore, to maximize our chances of encountering *P. cruzi*, our three-hour sampling period always happened between 18:30 and 23:00. During this time, we collected data on the species' natural history, more specifically, habitat use and reproductive period, with records of vocalization, amplexus, oviposition, and observations of adults, metamorphs and tadpoles in small pool areas around the stream.

To quantify the population size, we used a non-invasive mark-recapture methodology. All adult individuals encountered were captured, except those in amplexus, and were subsequently released at the same location. To identify each individual, we photographed the dorsal, right lateral, and left lateral regions of the abdomen, as well as the inguinal region. The images were taken with a Xiaomi Note 12s smartphone. Males were identified by the presence of the nuptial pad. To avoid potential ontogenetic changes in natural markings (Bardier et al., 2020), the census only considered fully metamorphosed adult frogs with a snout-vent length (SVL) longer than 3 cm for males and 4 cm for females (Carvalho-e-Silva et al., 2009).

The photographs were added to our image database, and after a more detailed analysis, we decided to use only the left side for individual identification purposes (Fig. 1) following Lima-Araujo et al. (2021). For better visualization, the photos were enlarged, highlighting the individual markings on the lateral abdomen and inguinal region (Lima-Araujo et al., 2021). Each image was assigned a code containing the area, date of record, individual ID, and number of captures (area_date_ID_capture). We then used the HotSpotter software (Nipko et al., 2020) to automate the recognition and matching of similar patterns. From the subset generated by HotSpotter for each individual, a human observer validated the correct match in the image database which was almost 100% correct.

In addition to recording natural history data, we also incorporated a method to evaluate the home range, similar to the one used by Moroti et al. (2022). To analyze the species' spatial distribution in the study area, we used plastic garden tags (3.2 width × 4.7 height, with a 15 cm tall base) throughout the monitoring period. Each tag was assigned a number and placed exactly where an



Fig. 1. Adult male *Phasmahyla cruzi* flank coloration and natural markings used for individual identification. All photographs are of the same individual, recaptured on April 4th 2023 (a); June 13th 2023 (b); December 5th 2023 (c); March 4th 2024 (d).

individual was sighted. If another frog was found within 100 cm of this mark, it was assigned to the same point. If it was found beyond this distance, a new numbered tag was installed at the new location. This procedure allowed us to map the home range of each individual. At the end of the study, we used a Garmin GPS (GPSMAP 64x) to record the coordinates of all the tags. With these data, we determined the home range of each individual.

To estimate male individuals' home ranges, we used the minimum convex polygon method (MCP 90%) through the adehabitatHR package (Calenge, 2011) in R environment (R Core Team, 2024). We chose MCP as it is a common method used to estimate home ranges with a low number of points per individual (Downs and Horner, 2008), and because it is the best method to represent herpetofauna home range size (Row and Blouin-Demers, 2006). As we didn't recapture any female enough times we only estimated the home area for male individuals recaptured more than five times (Moroti et al., 2022). Home range maps were built using QGis 3.34.12.

To assess the influence of temperature (°C) and humidity (%), we measured them *in situ* during each expedition using a thermometer-hygrometer with an accuracy of 1 °C (FEPRO-MUT600s). We tested if these abiotic variables were related to the number of captures of *P. cruzi*. To do this, we ran a Generalized Linear Model (GLM) using the number of captures as the response variable, and temperature and humidity as predictor variables. The family model was selected after inspecting the distributions of the response variables in the diagnostic plots generated in the *DHARMa* package in the R environment (Hartig, 2016). The family model that best explained the data was Negative Binomial.

We also tested if abiotic variables were related to the presence of egg masses of *P. cruzi*. The average monthly temperature, humidity, and monthly accumulated rainfall in Ubatuba were obtained from INMET (Instituto Nacional de Meteorologia (INMET, 2025). Rainfall and humidity showed higher correlation ($r > 0.7$), so we removed rainfall from the analysis. Once more we ran the Generalized Linear Model (GLM), this time using the number of egg masses found as the response variable, and temperature and humidity as predictor variables. The family model was selected after inspecting the distributions of the response variables in the diagnostic plots generated in the *DHARMa* package in the R environment (Hartig, 2016). The family model that best explained the data was Quasi-Poisson. We ran the GLM using the *glmmTMB* package (Brooks et al., 2017) in R environment (R Core Team, 2024).

We collected one adult male as the vocal voucher specimen and six tadpoles under the collecting permit (SISBio #51898-1) provided by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio). The specimens were euthanized, preserved in 10% formalin, and deposited in the zoological collection of the Museu de Diversidade Biológica (MDBio), Unicamp, Campinas, São Paulo, Brazil (ZUEC-AMP 26624 – adult male; ZUEC-AMP 26625 – tadpole lot). Calls were recorded with a TASCAM DR-40X and deposited at the Fonoteca Neotropical Jacques Viellard (FNJV), MDBio, Unicamp, Campinas, São Paulo, Brazil (FNJV 59118).

RESULTS

The species became active after dusk. Adult individuals were found on shrubby and arboreal vegetation near the stream bank (200 cm up from the stream was the maximum height of a frog found) or on foliage over the water. There were 158 encounters at site 1, of which 22 were males, and 3 females; 17 males and 2 females were recaptured. At site 2, we observed a larger population: 762 encounters, comprising 83 males and 35 females. Among these individuals, 66 males and 5 females were recaptured. No individuals observed at site 1 were encountered at site 2 or vice-versa. Furthermore, during monitoring of the studied area unrelated to this study, we

had three fortuitous encounters with *P. cruzi* in forested areas away from stream margins. They were all male adults, found on 9 December 2022 (23.45°S, 45.14°W, 196 m a.s.l.; 27 January 2023 (23.45°S, 45.14°W, 33 m a.s.l.); and on 5 June 2024 (23.46°S, 45.13°W, 34 m a.s.l.).

To determine the home range of *P. cruzi*, 54 points were added along the stream at site 1, while at site 2, 201 points were distributed along the stream. Considering more than five recaptures (Moroti et al., 2022), the frog's estimated home range was 461.07 ± 477 m^2. Site 1 showed an estimated home range of 64.2 ±130 m^2 (range: 5.5-454 m^2) considering data from 11 individuals and site 2 an estimated home range of 573.0 ± 481.3 m^2 (range: 15.5-2269.5 m^2) considering data from 39 individuals (Fig. 2) (Table S1).

We observed that males began vocalizing from September, when rain becomes more frequent in Ubatuba, until the end of April. They initiated vocalization after dusk, perched on vegetation on the bank or over the stream at heights ranging from 40 to 320 cm. During this period of activity, males exhibited territorial behavior, vocalizing and approaching in response to call playbacks from the researchers. We only employed this method as a tool to locate individuals that we heard but could not see.

We witnessed six amplexus in site 1 and 15 amplexus in site 2. The month with the highest number of amplexus (n = 7) was November 2023 (Fig. 3). The amplexus was axillary (Fig. 4a), with an approximate duration of two hours. Amplexus were observed on the ground, on rocks along the stream's edge, and in trees 200 cm tall overhanging the stream. Both males and females changed color during amplexus, becoming bright green after 30 minutes of observation (Fig. 5 a-b). This color change was also observed in individuals that were handled. During amplexus, both male and female collaborated to fold the leaf chosen for egg deposition (Fig. 6a). The female clung to the edges or petiole of the leaf with her arms and used her legs to bend it while releasing the eggs.

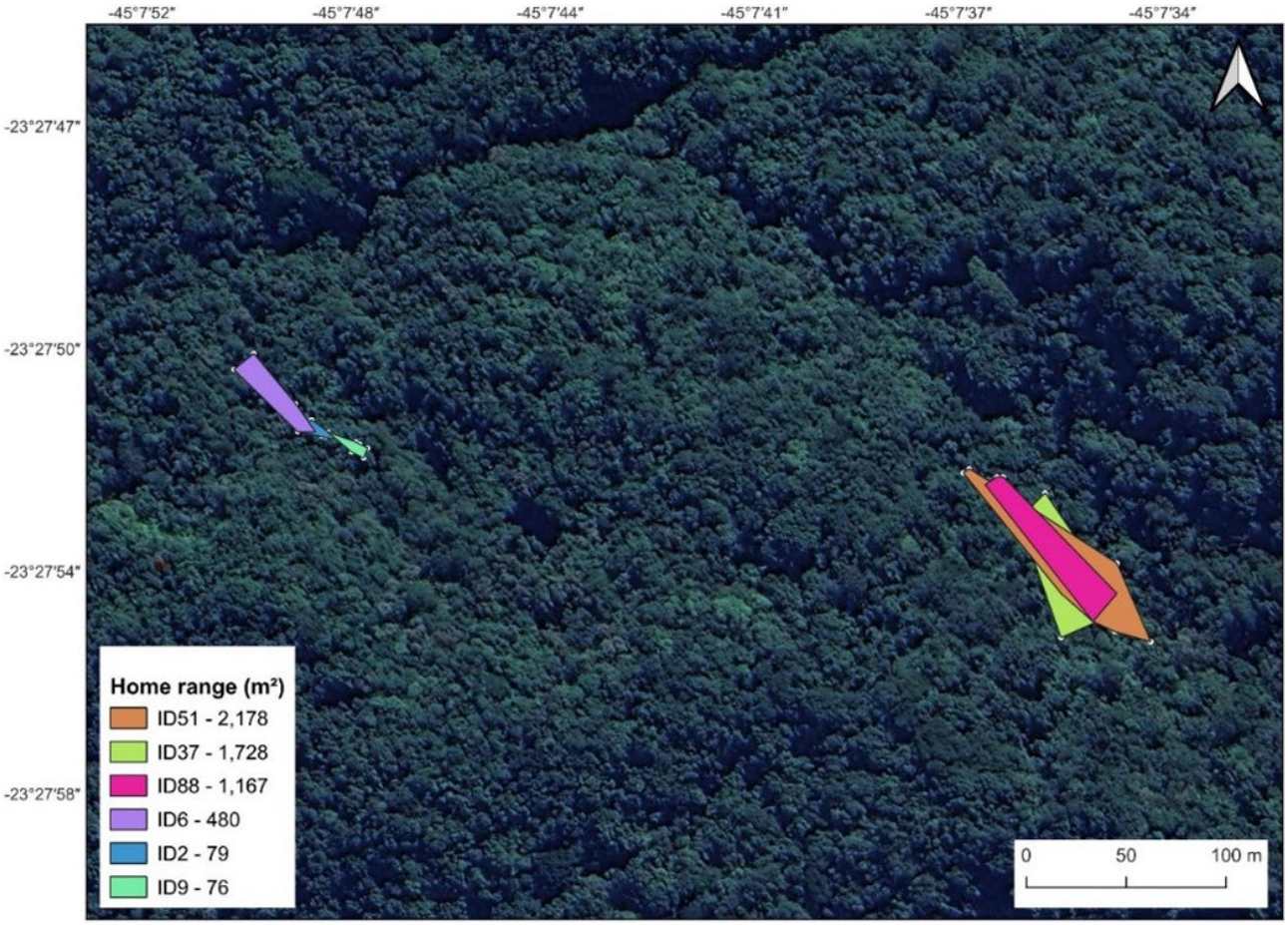


Fig. 2. Some home range estimates for *Phasmahyla cruzi* individuals in sites 1 and 2 at NGO Projeto Dacnis, Ubatuba, São Paulo, Brazil. ID51 = 25 encounters, ID37 = 24 encounters, ID88 = 12 encounters, ID6 = 14 encounters, ID2 = 19 encounters, and ID9 = 7 encounters.

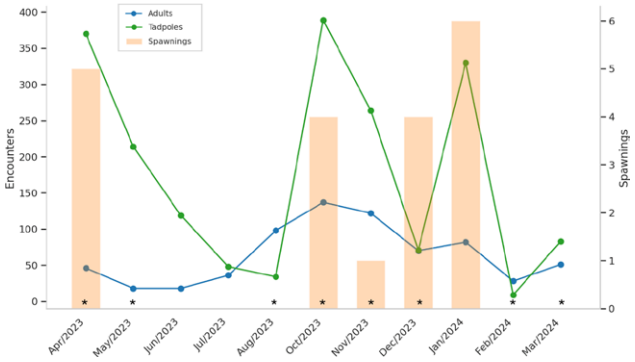


Fig. 3. Temporal distribution of adults (blue line), tadpoles (green line), amplexus (indicated with an asterisk) and spawnings (orange bars) of *Phasmahyla cruzi* between April 2023 and March 2024 at NGO Projeto Dacnis, Ubatuba, São Paulo, Brazil.

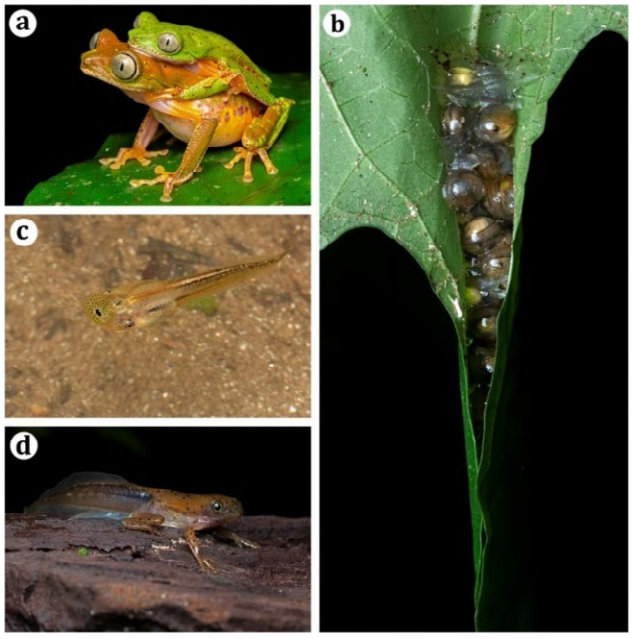


Fig. 4. *Phasmahyla cruzi* amplected couple (a); clutch in the final stage of development (b); tadpole (c); and a metamorph (d).

Meanwhile, the male remained attached to the female with his arms, alternating between pressing her abdominal region with his legs to stimulate egg release and using his legs to assist in folding the leaf. The egg masses likely had between 30 and 60 eggs; due to the shape of the deposition, precise counts were not possible (Fig. 6b). The fertilized eggs were protected by unfertilized gelatinous capsules positioned near the edge of the leaf. These capsules also played a role in keeping the leaf closed by adhering the two surfaces together.



Fig. 5. *Phasmahyla cruzi* amplected couple with reddish coloration at the beginning of amplexus (a); and the same couple with bright green coloration after 30 minutes of amplexus (b).

Spawnings were found on both the abaxial and adaxial surfaces of the leaves, at heights ranging from 20 to 180 cm. They were observed both on the water and on rocks with a 45° angle in relation to the water surface. Egg masses were observed on plants from the families Araceae, Fabaceae, Marantaceae, and Moraceae. No preference was noticeable for a specific type of vegetation, nor for the shape or size of the leaves. A total of 22 egg masses were found, with seven in site 1 and 15 in site 2. The month with the highest number of recorded egg masses was January 2024 (Fig. 3), with six egg masses (two in site 1 and four in site 2). We were unable to monitor any egg mass from deposition to hatching. They either failed to develop, were destroyed by heavy rains, preyed upon by harvestmen *Acutisoma discolor* (Fig. 6c), decayed (with the presence of mosquito larvae) (Fig. 6d), or contained only atresic eggs.

In April 2023 we identified a clutch of *P. cruzi* on the abaxial surface of a leaf of *Dorstenia* sp.; the gelatinous capsules kept the leaf folded and glued (Fig. 4b). The spawning was located 62 cm above a rock in the rapids, and 121 cm above the water. The larvae were in an advanced stage of development and hatched after heavy nocturnal rains between the third and fourth day of monitoring. We counted 41 tadpoles in an eddy below the spawn, organized in one shoal. One of the tadpoles was captured for measurement and was 16.8 mm long. Its larval development stage was at stage 25 according to Gosner (1960).

During the monitoring period, we recorded 865 tadpoles at site 1 and 1277 at site 2, totaling 2142 tadpoles.

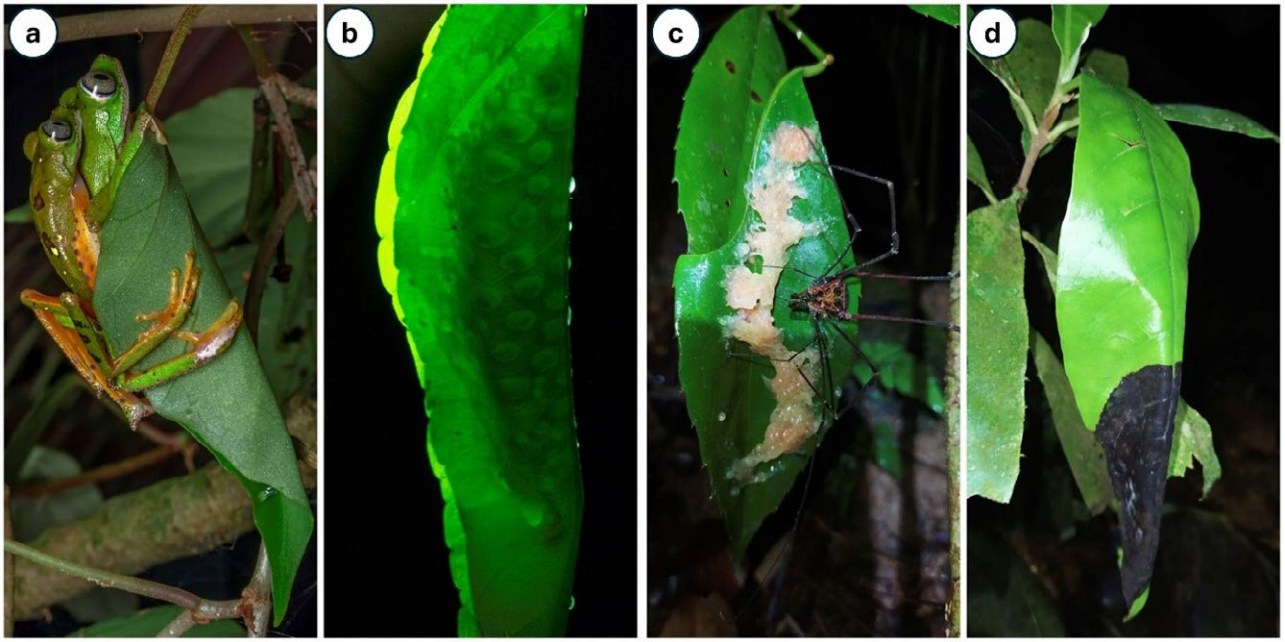


Fig. 6. *Phasmahyla cruzi* egg masses natural history observations: male and female closing the leaf together during the amplexus (a); egg masses deposited on a leaf (b); spawning being preyed upon by the harvestman *Acutisoma discolor* (c); and spawn in a state of decomposition (d).

The months with the highest numbers of occurrences were October 2023 (n = 389), followed by April of 2023 (n = 370), and January 2024 (n = 330). Tadpoles were observed throughout the year in various stages of development (Figs 4c and 4d). They were found in pools forming small beaches along the sides of rapids, which shared similar characteristics: sandy bottoms with polymodal granulometry, low organic matter, minimal suspended particles, and rocks of varying sizes both on the streambed and within the rapids. The pool depths ranged from 9.5 to 23 cm. During periods of strong water currents following heavy rainfall or when pools were disturbed by the passage of researchers, we observed tadpoles adhering to rocks using their umbelliform oral disk as a suction cup (ZUEC-VID 1045). Additionally, we frequently saw them feeding on larger particles, capturing and releasing them repeatedly until they were small enough to be ingested (ZUEC-VID 1046).

Through the abiotic data obtained in the field, we observed a positive relationship between the number of captures of *P. cruzi* with humidity ($z = 2.69$, $df = 7$, $p < 0.01$, pseudo-$r^2 = 0.50$), temperature ($z = 2.55$, $df = 7$, $p = 0.01$) and their interaction ($z = -2.54$, $df = 7$, $p = 0.01$) (Figure 7). We also identified a positive relationship between abundance of *P. cruzi* egg masses and humidity ($z = 2.2$, $df = 7$, $p = 0.02$, pseudo-$r^2 = 0.54$), temperature ($z = 2.18$, $df = 7$, $p = 0.02$, pseudo-$r^2 = 0.17$), and their interaction ($z = -2.16$, $df = 7$, $p = 0.03$) (Fig. 7).

DISCUSSION

The present study reveals important aspects about the natural history of *Phasmahyla cruzi*. Our data provide detailed insights into habitat use, reproductive activity, spatial distribution, and responses to abiotic variables, highlighting the importance of long-term studies.

Absence of diurnal records during 14 years of routine monitoring reinforces the strictly nocturnal activity of the species, corroborating previous observations in a congeneric species *Phasmahyla guttata* (Pereira et al., 2018). The selection of stream habitats with dense vegetation and semi-open canopy cover appears to be related to the species' reproductive strategy, which involves calling, amplexus, and oviposition on leaves near the marginal pools of small streams where tadpoles will develop, in a way that the larvae are less susceptible to be carried out due to strong currents. This preference is consistent with patterns observed in other Phyllomedusidae species, which utilize more protected aquatic environments for reproduction (Duellman and Trueb, 1994).

Deposition of eggs on different leaf surfaces (abaxial and adaxial), and at different heights (20-180 cm), suggests ecological plasticity, possibly as an adaptive strategy to minimize predation and avoid direct flooding. The use of plant species from different families (Araceae, Fabaceae, Marantaceae, and Moraceae) and the lack of preference for specific leaf shapes or sizes indicate low plant

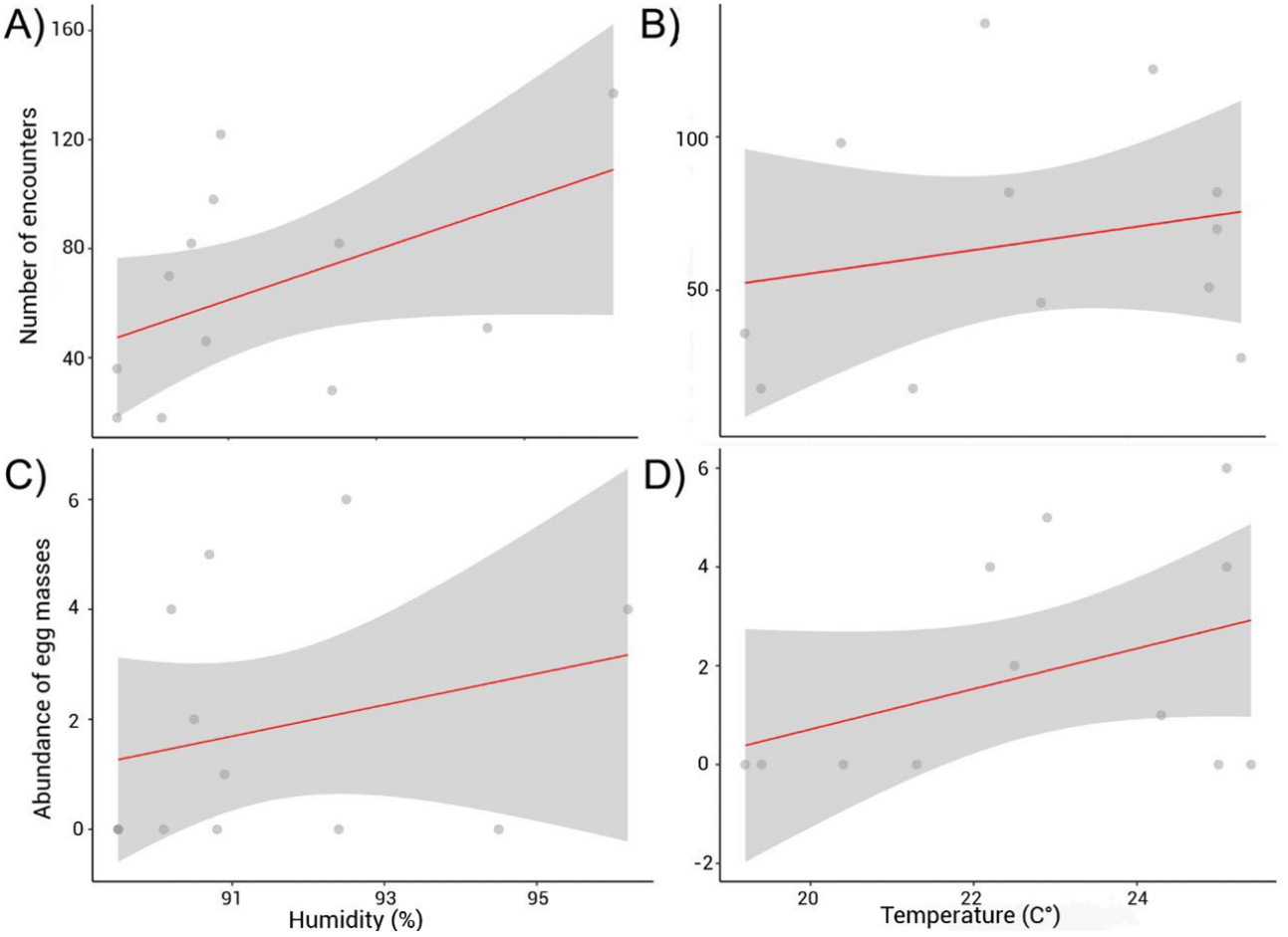


Fig. 7. Relationship between the number of encounters (A, B) and abundance of egg masses (C, D) of *Phasmahyla cruzi* with humidity and temperature at NGO Projeto Dacnis, Ubatuba, São Paulo, Brazil.

selectivity, which may be advantageous in densely vegetated and diverse environments such as tropical forests. Moreover, the higher concentration of egg masses recorded between September 2023 and January 2024 suggests potential reproductive seasonality, coinciding with the peak of the rainy season in the region. This is observed in the reproductive ecology of many Neotropical anuran species, which synchronize breeding with intense rainfall to increase tadpole survival chances by enhancing transport to suitable aquatic habitats, coinciding with other studies (e.g., Aichinger, 1987; Pedro and Feio, 2010; Silva et al., 2012). However, challenges during egg mass development – including destruction by heavy rainfall, predation by harvestmen, deterioration by mosquito larvae, and atresia – highlight the vulnerability of this life stage. These observations emphasize the critical influence of biotic and abiotic factors on anuran reproductive success and reinforce that early-stage mortality rates tend to be high – a common pattern in species with indirect development and type-III reproductive strategies (Crump, 1974; Wells, 2007).

The specific case documented in April 2023 in this study, involving the complete development of a *P. cruzi* clutch, highlights the function of gelatinous capsules in protecting and adhering the eggs to leaves and shows the relationship between heavy rains and hatching. The presence of 41 tadpoles, with one measured and classified at Gosner's stage 25, confirms that larval development reaches a critical threshold before release into the stream, suggesting a semi-independent developmental strategy that may be advantageous in unstable environments (Dias et al., 2018). In addition, the limited number of egg clutches (22 total) and their uneven distribution between both study sites may reflect microhabitat variations such as vegetation density, humidity, predator presence, or physicochemical characteristics of the site – factors that should be more deeply investigated in future studies.

The use of non-invasive photographic identification and the HotSpotter software proved effective for individual recognition, reducing animal stress and minimizing behavioral interference. This method has been widely applied in recent studies involving anurans and other vertebrates, offering an ethical and sustainable approach to monitoring natural populations (Muscat et al., 2020; Lima-Araujo et al., 2021; Moroti et al., 2022). Based on it, home range analysis revealed relatively small usage areas for recaptured males, fact that is documented in literature about territorial tropical anurans (Wells, 2007). The minimum convex polygon (MCP) method was appropriate, due to the limited number of individual recaptures, allowing for comparisons with other herpetofauna studies.

An important limitation of this study is the reduced number of records and recaptures of females. The considerably lower detectability of females may reflect sex-specific differences in behaviour, mobility, or habitat use, as females are often less conspicuous outside reproductive periods or may occupy microhabitats not routinely surveyed. Consequently, estimates of space use derived primarily from male data may underestimate the actual spatial requirements of the species. This potential bias should be considered when interpreting home range patterns and reinforces the need for future studies specifically designed to assess female movement, habitat use, and detectability in *P. cruzi*.

Generalized Linear Models indicated that both temperature and humidity influence activity and reproductive output in *P. cruzi*, as found in other studies (e.g., Oseen and Wassersug, 2002; Saenz et al., 2006). Adult activity, reflected by capture rates, showed a positive relationship with humidity and temperature. This pattern reinforces the dependence of anurans on humid and warm conditions due to their ectotherm habit and permeable skin (Duellman and Trueb, 1994). In addition, the abundance of egg masses was positively associated with both humidity and temperature, suggesting that these variables also influences the reproductive investment of this specie, also found for the majority of species in Atlantic Forest (Silva et al., 2012; Ceron et al., 2020).

The significant interaction between temperature and humidity indicates that the effect of temperature on adult activity and reproductive output is modulated by moisture availability. Higher temperatures appear to favour reproduction primarily when accompanied by high humidity, whereas warmer and drier conditions may limit reproductive success, potentially due to increased desiccation risk during embryonic development. This interaction highlights the importance of considering combined abiotic effects when evaluating reproductive patterns in anurans, particularly in forest environments subject to pronounced seasonal variation (Crump, 1974; Aichnger, 1987; Saenz et al., 2006).

Environmental conditions in this study were assessed at complementary spatial and temporal scales. Temperature and humidity recorded with handheld devices during field surveys reflect local environmental conditions at the stream level, whereas monthly averages obtained from a meteorological station represent broader macroclimatic trends and seasonal context. Although true microclimatic variables were not directly measured, the integration of these data provides a meaningful proxy for the environmental conditions influencing activity and reproduction. In forested riparian systems, broader climatic patterns strongly constrain the availability of suitable microhabitats, supporting our interpretation that the observed abiotic relationships reflect biologically relevant conditions experienced by *P. cruzi*.

Finally, the results of this study highlight the vulnerability of *P. cruzi* to habitat and abiotic changes. It also shows, at least to male of the species', a dependence on specific microhabitats, combined with low mobility and apparent fidelity to small home ranges, making the species particularly sensitive to environmental alterations. Females though need further research to be assessed. Therefore, we emphasize the importance of private reserves (Volenec and Dobson, 2020) such as Projeto Dacnis, for the conservation and maintenance of viable populations of *P. cruzi* and other endemic Atlantic Forest species.

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at https://oaj.fupress.net/index.php/ah/article/view/19377/15290.

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Dinner time in the Atlantic Forest: Trophic niche partitioning of two sympatric *Boana* species in the Southern Bahia State, Brazil

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Abstract. Understanding the ecological traits of species, such as dietary habits, is important for elucidating their natural history and ecological interactions. Thus, we investigated the trophic ecology and niche partitioning of two treefrog species (*Boana atlantica* and *B. semilineata*) in an Atlantic Forest fragment in southern Bahia, northeastern Brazil. In this study, we obtained the diet of 39 *B. atlantica* and 25 *B. semilineata* through stomach flushing. We recorded 72 prey items in these two hylids organized into 11 categories. The most consumed prey were plant material and Araneae. Although the niche breadth of *B. atlantica* was higher than that of *B. semilineata*, these hylids had a substantial trophic niche overlap (Pianka's niche overlap index = 0.89). Our findings provide novel insights into the feeding ecology of these endemic Atlantic Forest treefrogs, enhancing our understanding of their ecological roles and trophic resource partitioning in this biodiversity hotspot.

Keywords. Amphibians, diet composition, feeding habit, Hylidae.

INTRODUCTION

To coexist in an environment, a species needs specific resources and conditions that define its ecological niche (Hutchinson, 1957). Processes associated with foraging habitat selection, prey availability, and intraspecific and interspecific competition are crucial to understand the species' trophic niche (Moser et al., 2017). Beyond ecological interactions, this information is important for comprehending the natural history of the species, population fluctuations, and the effects of environmental change on populations and communities (Anderson et al., 1999; Santos et al., 2004; Richter-Boix et al., 2007).

Anurans play an important role in maintaining environmental stability, participating in different food networks (Poulin et al., 2001; Egeter et al., 2015; Ceron et al., 2019), and contributing to the energy flow and nutrient cycling (Colón-Gaud et al., 2009; Huckembeck et al., 2014). In general, most anuran species are opportunistic and generalist predators consuming a great diversity of invertebrate prey (Solé and Rödder, 2009). However, amphibians' diets are influenced by various factors,

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such as prey availability, competition, foraging strategies, ontogeny, morphology, and environmental characteristics (Wells, 2010; Luría-Manzano and Ramírez-Bautista, 2019; Blanco-Torres et al., 2020; Moroti et al., 2021), making it important to study their trophic ecology. A recent review of Brazilian anuran dietary studies shows that, despite increasing research, only about 19% of species have published diet data, with many endemic and threatened taxa still lacking dietary information, particularly outside the Atlantic Forest ecoregion (Moser et al., 2025).

Anuran communities may be structured according to spatial, temporal, and trophic niches, where ecological factors such as competition and historical factors like phylogeny influence these niche dimensions (Leite-Filho et al., 2017; Caldas et al., 2019). Some studies investigated the trophic niche overlap of congeneric anurans (e.g., Oliveira et al., 2015; Moser et al., 2017; Araújo et al., 2023), including *Boana* species (Protázio et al., 2017; Moser et al., 2018). It is generally accepted that, in accordance with Gause's (1932) competitive exclusion principle, species must segregate along at least some niche axes, including trophic niche partitioning, to enable their successful coexistence in sympatry (Kneitel, 2019). Thus, understanding the partitioning of food resources is essential to comprehend species coexistence.

Boana atlantica (Caramaschi and Velosa, 1996) and *Boana semilineata* (Spix, 1824) are two endemic treefrogs from the Atlantic Forest that occur in sympatry from Alagoas to Bahia (Frost, 2025). *Boana semilineata* is known to have a generalist diet consisting primarily of coleopterans, acari, orthopterans, and plant fragments (Protázio et al., 2017). While some aspects of *B. atlantica*'s natural history are known, including its reproductive biology (Camurugi and Juncá, 2013) and advertisement call (Napoli and Cruz, 2005), its trophic ecology remains understudied. Thus, we aimed to (i) describe the diet of two treefrog species that occur in sympatry in a permanent pond within an Atlantic Forest fragment in southern Bahia, Brazil; (ii) test the trophic niche overlap between these hylids; and (iii) evaluate how body mass, body size, and mouth breadth influence prey volume in these anurans. We expected dietary differentiation between the two sympatric hylid species, resulting in low to moderate trophic niche overlap, and a positive relationship between anuran morphology and prey volume.

MATERIALS AND METHODS

Study area

This study was conducted in a permanent pond inside the Michelin Ecological Reserve (-13.82348° S, -39.17286° W, WGS84) in Bahia State, northeastern Brazil (Fig. 1). It encompasses a total area of 3.096 ha within the Atlantic Forest Biome, considered a Dense Ombrophilous Lowland Forest (Veloso et al., 1991), and characterized by the presence of Atlantic Forest fragments at various stages of regeneration (Flesher, 2014). It has an average precipitation of 2000 mm and daily temperatures between 18 °C and 30 °C, with rainfall throughout the year (Flesher, 2014). The area of the pond was ca. 450 m^2, with a maximum depth of 1.5 m, located in a forest fragment embedded within a rubber tree plantation area. Besides trees, the vegetation around the pond edge is composed of shrubs and herbaceous species.

Sampling design

We conducted fieldwork to search for specimens of *B. atlantica* and *B. semilineata* at a permanent pond during the night period (18 to 23 h) between March and May 2011, using auditory and visual searches (Heyer et al., 1994). Individuals were identified based on their advertisement calls, dorsal coloration patterns, and morphological traits (see Caramaschi and Velosa, 1996, for the diagnosis of *B. atlantica*, and Izecksohn and Carvalho, 2001, for the diagnosis of *B. semilineata*). We collected and transported the observed individuals in plastic bags to the lab in the Center for Biodiversity Studies of the Michelin Ecological Reserve. To avoid pseudoreplication, specimens were captured and released only at the end of each field campaign, ensuring that no individual was resampled within the same campaign. In the lab, the specimens were weighed on electronic scales and had their snout-vent length (SVL) and mouth breadth (MB) measured with a digital caliper with 0.01 mm precision. Subsequently, they were stomach flushed following the protocol proposed by Solé et al. (2005) and released into the pond, from which they had been collected.

Data analyses

In the laboratory, the stomach contents were separated, and the prey items were identified to the lowest possible taxonomic level (usually order) using the specialized literature (Rafael et al., 2024). Then, we calculated the frequency of occurrence (FO% = number of stomachs containing item i / total number of stomachs × 100), and the numeric abundance (N% = number of individuals of item i / total individuals of all items × 100). Since we did not calculate the volume of each prey item consumed, we used an adapted formula (IRI = (FO% + N%) / 2) for the index of relative importance originally proposed by Pinkas et al. (1971).

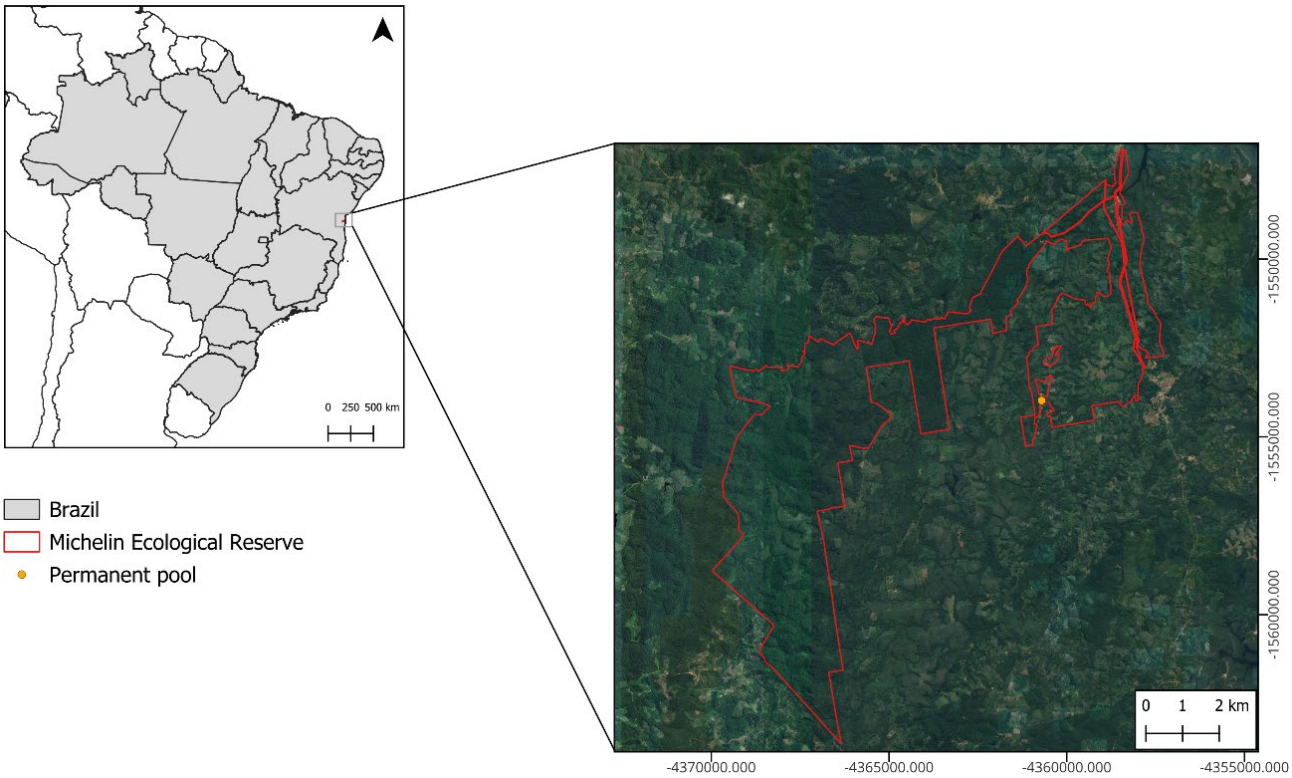


Fig. 1. Schematic map of the study site in the Michelin Ecological Reserve, Bahia State, northeastern Brazil.

Additionally, to measure the total volume of prey consumed by the *Boana* specimens, we placed each individual's stomach contents in an oven set to 60 °C to dry and stabilize the mass. We used a GEHAKA AG-200 scale (accuracy of 0.1 mg) to weigh the stomach contents. Collinearity among morphological predictors was evaluated using variance inflation factors (VIF). As all VIF values were below 2, no collinearity issues were detected, and all variables were retained for analysis (James et al., 2013). We then fitted generalized linear models (GLMs) to evaluate whether prey volume was influenced by body mass, snout–vent length (SVL), and mouth breadth (MB) in the two *Boana* species. We initially fitted a full model including all predictors simultaneously (Volume ~ Mass + SVL + MB), assuming a Gamma error distribution with a log-link function. Model adequacy was assessed through residual diagnostics appropriate for GLMs. In addition, we evaluated alternative models with reduced sets of predictors and compared model performance using Akaike's Information Criterion (AIC). Models with ΔAICc lower than 2 were interpreted as having the most substantial support (Burnham and Anderson, 2002).

We estimated the numerical trophic niche breadth (*Bnum*) of the two *Boana* species using the standardized Levins' index (Levins, 1968), which ranges from 0, indicating dietary specialization, to 1, indicating a generalist diet. Then, we used Pianka's niche overlap index (Pianka, 1973) to calculate the trophic niche overlap (*Ojk*) between *B. atlantica* and *B. semilineata*. We used a bipartite network graph as an exploratory tool to visualize prey diversity between the two hylid species, with interaction strength represented by the index of relative importance of each prey item for both *Boana* species. Statistical analyses were done using the R packages *bipartite* (Dormann et al., 2009), *ggraph* (Pedersen et al., 2022), *ggplot2* (Wickham, 2016), *igraph* (Csardi and Nepusz, 2006), *spaa* (Zhang, 2016), *tibble* (Müller and Wickham, 2023), *vegan* (Oksanen et al., 2019), and *usdm* (Naimi et al., 2014).

RESULTS

We found 64 anuran specimens (39 *B. atlantica* and 25 *B. semilineata*), including 48 with stomach contents (30 *B. atlantica* and 18 *B. semilineata*). We found 72 prey items grouped into 11 categories, with plant material (N = 23) and Araneae (N = 20) as the most consumed prey. Plant fragments were found in the stomach contents of 23 individuals, while Araneae appeared in 17.

Table 1. Absolute values and proportions (%) of frequency of occurrence (FO), abundance (N), and index of relative importance (IRI) of each prey category consumed in the diet of *Boana atlantica* and *Boana semilineata*.

	Boana atlantica					*Boana semilineata*				
	FO	FO%	N	N%	IRI	FO	FO%	N	N%	IRI
ARACHNIDA										
Acari	1	2.0	1	2.17	2.09	-	-	-	-	-
Araneae	14	28.0	12	26.1	27.0	6	27.3	5	23.8	25.5
HEXAPODA										
Blattaria	1	2.0	1	2.17	2.09	-	-	-	-	-
Coleoptera	6	12.0	6	13.0	12.5	-	-	-	-	-
Diptera	5	10.0	5	10.9	10.4	-	-	-	-	-
Hemiptera	1	2.0	1	2.17	2.09	-	-	-	-	-
Hymenoptera	1	2.0	1	2.17	2.09	1	4.55	1	4.76	4.65
Lepidoptera	2	4.0	2	4.35	4.17	1	4.55	1	4.76	4.65
Odonata	-	-	-	-	-	1	4.55	1	4.76	4.65
Orthoptera	6	12.0	4	8.70	10.3	3	13.6	3	14.2	14.0
OTHER ITEMS										
Plant material	13	26.0	13	28.3	27.1	10	45.5	10	47.6	46.5
TOTAL	50	100	46	100	100	22	100.0	21	100	100

We observed that *B. atlantica* consumed 10 prey categories (*Bnum* = 5.31), with plant material (IRI = 27.1) and Araneae (IRI = 27.0) being the most abundant and frequently encountered prey types. In contrast, Acari, Blattaria, Hemiptera, and Hymenoptera were consumed less frequently (IRI = 2.09), with only one individual recorded for each (Table 1). Additionally, we found no support for the influence of anuran weight (T = 0.559, P = 0.580), size (T = -0.584, P = 0.564), and mouth breadth (T = -0.460, P = 0.649) on the volume of the stomach contents.

Likewise, plant material (IRI = 46.5) and Araneae (IRI = 25.5) were the most prominent prey items in the diet of *B. semilineata*. This species consumed only six prey categories and had a narrower numerical trophic niche breadth (*Bnum* = 3.37). Hymenoptera, Lepidoptera, and Odonata were consumed less frequently, with only one individual recorded for each (Table 1).

We also observed that the volume of the stomach contents was not influenced by the anurans' weight (T = 1.194, P = 0.250), size (T = -1.160, P = 0.263), and mouth breadth (T = 0.978, P = 0.342). Model comparison based on AIC indicated that the null model best fit the data for both species. Models including morphological predictors (SVL, mouth breadth, or body mass), alone or combined, did not improve performance (ΔAIC > 2), indicating no effect of morphology on prey volume.

We observed that the two *Boana* species consumed different numbers of prey categories. *Boana semilineata* exhibited a more specialist dietary strategy (*Bnum* = 0.24), whereas *Boana atlantica* showed an intermediate to generalist strategy (*Bnum* = 0.43), indicating a broader trophic niche breadth. In addition, the two species exhibited a high trophic niche overlap (*Ojk* = 0.89), with five prey categories shared by both species (Fig. 2).

DISCUSSION

We observed that the *Boana* species studied had a generalist and opportunistic diet, which is a common pattern for most Neotropical anurans (Toft, 1981; Wells, 2010), including the genus *Boana* (e.g., Araújo et al., 2007; Moser et al., 2018; Rodrigues et al., 2023, Machado et al., 2024). The presence of prey with high mobility in the diet composition of these hylids, such as Araneae, Odonata, Orthoptera, and Diptera, may indicate a sit-and-wait strategy (Parmelee, 1999; Protázio et al., 2017). This foraging strategy pattern seems to be predominant for Hylidae species (e.g., Mahan and Johnson, 2007; Castro et al., 2016; Silva et al., 2021), including the genus *Boana* (e.g., Protázio et al., 2017; Sant'anna et al., 2022; Rodrigues et al., 2023).

Although some species, such as *Xenohyla truncata* and *Phrynoderma hexadactylum*, may exhibit an uncommon frugivory or herbivory pattern (Das, 1996; Oliveira-Nogueira et al., 2023), most studies suggest that anurans consume plant material incidentally while preying on invertebrates (Sabagh et al., 2010; Castro et al., 2016, Araújo et al., 2023). However, we observed that

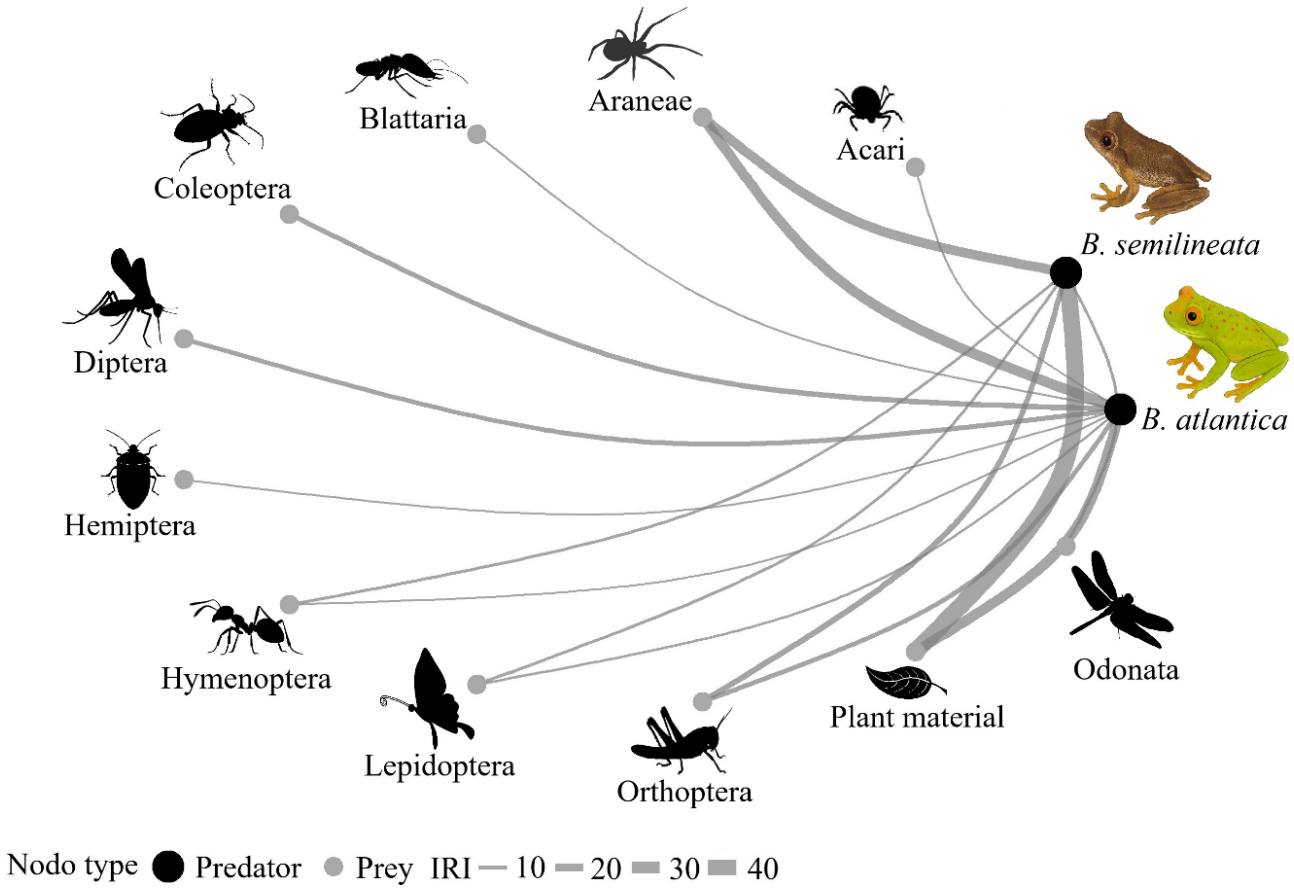


Fig. 2. Schematic graph of the prey items consumed by the two Boana species studied in the Michelin Ecological Reserve, Bahia State, northeastern Brazil.

the most consumed prey item by both *Boana* species was plant fragments. The consumption of plant material can aid in the elimination of intestinal parasites and provide an additional supply of water and nutrients, provided it is digestible (Anderson et al., 1999; Tupy et al., 2021). Additionally, Silva et al. (1989) demonstrated that during the dry season, when invertebrate populations diminished, *Xenohyla truncata* incorporated fruits and bromeliad seeds into its diet. While the ingestion of plant debris is frequently documented in *Boana* species (e.g., Moser et al., 2018; Tupy et al., 2021; Rodrigues et al., 2023), further research is necessary to elucidate the significance of plant fragments in the diet of these hylids.

Anuran–prey selection might be mediated by different traits, such as morphology, foraging strategy, prey mobility, and availability (Lima and Moreira, 1993; López et al., 2009; Blanco-Torres et al., 2020). However, the volume of stomach contents of *B. atlantica* and *B. semilineata* was not influenced by their weight, snout-vent length, and mouth breadth. Although some studies support the hypothesis that larger frogs tend to eat a larger prey volume (e.g., Rosa et al., 2011; Pacheco et al., 2017; Araújo et al., 2023), it is still an unclear issue because others do not support this relationship (e.g., Rodrigues et al., 2023; Ferreira et al., 2024). As a result, establishing a link between morphology and prey volume in anurans is difficult, particularly from an intraspecific perspective.

We observed that *B. atlantica* and *B. semilineata* had a high trophic niche overlap with opportunist characteristics. These species share five prey categories, including the prey items with the highest IRI such as plant material and spiders (see Fig. 2). Studies encompassing the anuran community (e.g., Protázio et al., 2015; Leite-Filho et al., 2017), as well as investigations focused just on two species (e.g., Sabagh et al., 2010; Araújo et al., 2023), indicate that generalist and opportunistic amphibians frequently share prey items within their diet. Likewise, coexisting *Boana* species have a substantial trophic niche overlap (Moser et al., 2018; Tupy et al., 2021; Sant'anna et al., 2022), which may be related to their opportunistic

trophic strategy. Given limited resources, species must segregate into certain niche axes to coexist (MacArthur and Levins, 1967; Gotelli, 2009). However, it is impossible to determine whether or not these species compete for trophic resources in the study area because we did not perform a prey availability analysis there. Thus, further studies are required to determine the mechanisms and processes that may allow *B. atlantica* and *B. semilineata* to coexist.

Overall, the studied hylids consume whatever is available in their surroundings, exhibiting a generalist dietary pattern and, as a result, a high degree of overlap in prey items. However, we cannot state that these species are truly competing for trophic resources. Our findings contribute to the understanding of the trophic ecology of two endemic *Boana* species from the Atlantic Forest. It is important to highlight the general lack of research on anuran diets, despite diet representing a fundamental aspect of natural history for understanding predator-prey interactions, hypothesis testing, and the assessment of conservation status (Moser et al., 2025). Consequently, natural history studies play a crucial role in informing conservation planning for species and their environments.

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Ecological aspects of *Coleodactylus meridionalis* (Squamata: Sphaerodactylidae) in an Atlantic rainforest fragment in Northeastern Brazil

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Abstract. Understanding the autecology of cryptic species is fundamental for developing effective conservation strategies, particularly in threatened biomes such as the Atlantic Forest. In this study, we investigated ecological aspects of *Coleodactylus meridionalis*, a small terrestrial lizard with a broad distribution, in an Atlantic Forest fragment. Specifically, we analyzed the usage of microhabitats, morphological characteristics, reproductive aspects, diet, and associated endoparasites. We conducted monthly seven-day expeditions from August 2014 to July 2015 in the Campo de Instrução Marechal Newton Cavalcante, Pernambuco, Brazil. We recorded spatial use by 285 individuals, 271 (95%) of which were found in the forest interior, predominantly in leaf litter microhabitats (90.5%; N = 258). Females were significantly larger than males and had proportionally higher head heights. The diet included 22 prey categories, with Isopoda being the most frequent and voluminous and Psocoptera the most numerous prey categories found in the stomachs. The population reproduces continuously throughout the year, with fixed clutches of a single egg. The macro endoparasites found was an acanthocephalan cystacanth, with a prevalence of 13.6% and a mean infection intensity of 1.5 ± 0.74, and a Brachycoeliidae trematode, with a prevalence of 1.9% and a mean intensity of 3 ± 1.9. This is the first record of parasites for *C. meridionalis*, both as a paratenic host for cystacanth and as a definitive host for Brachycoeliidae.

Keywords. Diet, lizard ecology, microhabitat use, parasitism, reproductive strategy, sexual dimorphism.

INTRODUCTION

Autecological studies provide essential baseline data for understanding the ecological requirements of species and guiding targeted, science-based conservation strategies (Hortal and Lobo, 2006; Tewksbury et al., 2014). This is particularly relevant for cryptic species or data-deficient taxa inhabiting historically disturbed biomes, such as the Atlantic Forest, one of the most threatened biodiversity *hotspots* in the world (Pinto and Voivodic, 2021). In this context, miniature lizards specialized to live in leaf-litter, such as those of the genus *Coleodactylus,* present significant knowledge gaps that must be filled to improve our understanding of their basic ecology.

Coleodactylus is a genus composed of five species: *Coleodactylus brachystoma, C. elizae, C. meridionalis, C. natalensis,* and *C. septentrionalis.* Among these, *C. meridionalis* is one of the smallest lizards in Brazil, reaching around 25 mm snout-vent length (Freire, 1999; Silva et al., 2015). This species is an active forager with a broad

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distribution, occurring in leaf litter or piles of woody debris microhabitats within the arboreal Caatinga, highland forest enclaves in the Caatinga morphoclimatic domain, Atlantic Forest fragments of northeastern Brazil, and areas of Cerrado vegetation (Feitosa et al., 2022; Uchôa et al., 2022; Roseno et al., 2024.). Despite its wide range distribution, data on the natural history and ecology of *C. meridionalis* remain limited, with specific data from areas of Caatinga, Cerrado, Restinga and highland enclaves. These studies have explored the defensive and foraging behavior, diet, microhabitat usage, sexual dimorphism, hatching, and neonate size of *C. meridionalis* (Colli et al., 2002; Werneck et al., 2009; Ribeiro et al., 2013; Silva et al., 2015; Feitosa et al., 2022; Gonçalves-Sousa et al., 2023; Roseno et al., 2024).

In the present study, we investigated the natural history and ecology of *C. meridionalis* in an Atlantic Forest fragment within the Aldeia-Beberibe Environmental Protection Area, Pernambuco, Brazil. Following the Hutchinsonian ecological niche concept, defined as a hypervolume n-dimensional (Hutchinson, 1957), we aimed to estimate key axes for this lizard. Specifically, we aimed to: (i) explore the spatial (microhabitat use) and trophic (diet) dimensions of the ecological niche; (ii) estimate the population density; (iii) analyze morphology (body size and sexual dimorphism); (iv) investigate reproductive aspects; and (v) identify the macro endoparasites that infect *C. meridionalis.*

MATERIALS AND METHODS

Study area

The study was conducted at the Marechal Newton Cavalcante Instruction Camp, a fragment of Atlantic Forest located between the Metropolitan Region of Recife and Zona da Mata Norte, Pernambuco state, northeastern Brazil. The area has 7,342 ha of forest and a constructed area that is part of Aldeia-Beberibe Environmental Protection Area and other forest fragments. Sampling was conducted in the municipalities of Araçoiaba (07°49'00.8"S; 35°06'08.6"W), Paudalho (07°49'54.3"S; 35°06'53.7"W) and Abreu e Lima (07°50'12.3"S; 35°05'29.8"W). There are two distinct seasons in the region. The dry season lasts from September to February, and the wet season between March and August. Monthly average temperature ranges from 21.8°C to 29.1°C, with an average annual rainfall of 2,458 mm (World Weather Information Service, 2013).

The vegetation is classified as Ombrophilous and Seasonal Semideciduous Forest (Veloso et al., 1991). Before 1944, this area belonged to 11 sugarcane mills; following expropriation, it began to regenerate (Guimarães et al., 2012). The area is characterized by herbaceous, shrub and forest canopy strata. It has a conserved forest area as well as various roads, a weir, several streams and exotic vegetation such as "dendê" (*Elaeis guineensis*), olive tree (*Syzygium cumini*), mango (*Mangifera indica*), jackfruit (*Artocarpus heterophyllus*) and bamboo trees.

Data collection

Between August 2014 and July 2015, we conducted monthly expeditions of seven days each to collect the lizards. In the sampled area, we installed 25 sets of Y-shaped pitfall traps, each containing four 20-liter buckets buried at 120° angles and interconnected by drift fences and spaced at least 15 meters apart (Cechin and Martins, 2000). We inspected these traps once a day and kept them open for six consecutive days. The accumulation of leaf litter inside the buckets simulated the adjacent microhabitats, provided shelter and maintained humidity, minimizing desiccation and predation risks for captured specimens. We also employed an active search method, in which three collectors searched in all potential microhabitats used by *C. meridionalis* (Fig. 1). For each active individual found, we recorded the time of activity and the microhabitat use. The active search was conducted for 6 hours daily over seven days in each expedition, divided into 3 hours in the day and 3 hours at night (19:00-22:00). To cover different activity peaks, the diurnal sampling alternated daily between a morning session (08:00-11:00) and an afternoon session (14:00-17:00). In this way, the total sampling effort of active searches was



Fig. 1. *Coleodactylus meridionalis* from a bamboo litter microhabitat, displaying characteristic orange markings, within an Atlantic Forest fragment in the Aldeia-Beberibe Environmental Protection Area, northeastern Brazil. Photo: José Vieira de Araújo-Neto.

1,512 h (504 per collector), whereas the total time the pitfall traps remained open was 43,200 h (25 pitfall sets × 24 h × 6 days × 12 months).

We euthanized the lizards with 2% lidocaine hydrochloride by peritoneal injection and used a digital caliper (0.01 mm accuracy) to measure the following variables: snout-vent length (SVL), head length (HeL), head width (HeW), head height (HeH), body width (BoW), and body height (BoH); forelimb length (FRL), hindlimb (HDL), and tail length (TL). Then, we fixed the collected lizards in 10% formalin, preserved in 70% alcohol and, subsequently, and deposited them at the Laboratory of Venomous Animals and Toxins of the Federal University of Pernambuco. All specimens were collected under federal authorization from SISBio / ICMBio (43750-1 and 43750-2).

Microhabitat use

In the field, we recorded the time of sighting, and the microhabitat used for each individual at the moment of first sight. These categories correspond to bamboo leaf litter, fallen trunk, leaf litter, and open ground. We separated the two types of leaf litter because they are visually and structurally distinct: the bamboo leaf litter is relatively homogeneous with light-colored, yellowish dry leaves, while the general leaf litter is heterogeneous and frequently dark-toned. We then used the inverse of the index of Simpson (1949) to calculate the microhabitat niche breadth and calculated the percentages of microhabitats used according to the occurrence of lizards.

Population density

To estimate the density of *C. meridionalis* in the studied area, we collected abundance data from 15 quadrants measuring 10 × 10 m (100 m^2). The minimum distance between these quadrants was 130 meters. Inside each quadrant, three collectors conducted an exhaustive search by systematically raking and removing the entire leaf-litter layer, starting from the edges, until the entire 100 m^2 area was cleared to the soil to maximize detection.

The average density was calculated by dividing the total number of individuals by the 15 quadrants. This value was then multiplied by 100 to standardize the density per hectare (ind/ha). We then used the 95% confidence interval to assess the spatial variability in the abundance of the studied lizard. This interval was calculated based on the sample mean, standard deviation, and standard error of the mean, considering the Student's t-distribution in the stats R-package (R Core Team, 2024).

Sexual dimorphism

Dimorphism analyses followed the model used by Mesquita et al. (2015), who tested dimorphism in *Anolis brasiliensis*. We log-transformed (base 10) the morphometric variables and selected the data for multivariate outliers with the mvoutlier R-package (Filzmoser et al., 2018). No males were considered outliers, but six females were considered outliers using a maximum limit for outlier detection of 0.001 and were not used in further analyses.

To partition total morphometric variation between size and shape variables, we defined Body Size as an isometric size variable (Rohlf and Bookstein, 1987). We calculated an isometric eigenvector defined a priori with values equal to $p^{-0.5}$, where p is the number of variables (Jolicoeur, 1963). The scores derived from this eigenvector, hereafter referred to as Body Size, were obtained by post-multiplying the n x p matrix of $\log_{10}$-transformed data, where n is the number of observations, by the p x 1 isometric eigenvector. To remove the effects of SVL from the log-transformed variables, we applied the method of Burnaby (1966), multiplying the n x p matrix of the $\log_{10}$-transformed data by p x p of the symmetric matrix L, defined as: $L = I_p - V(V^TV)^{-1}V^T$, where I$p$ is the p x p matrix, V is the Body Size eigenvector, and V^T is the transposed V matrix (Rohlf and Bookstein, 1987). The resulting size-adjusted variables were then considered shape variables. We assessed sexual dimorphism using analysis of variance (ANOVA) on the Body Size variable and logistic regression on shape variables. To evaluate the statistical significance of the complete model based on shape variables, we compared it against a single model constant (null) using a scaled difference chi-square test (Chambers and Hastie, 1992; Faraway, 2016). The importance of each variable was determined through single-term addition modeling (Chambers and Hastie, 1992).

Reproduction

The animals were sexed through direct analysis of the gonads. The presence or absence of oviductal eggs and/or vitellogenic follicles was verified to determine the status of reproductive activity of females, considering them sexually mature if they presented either of these two evidence, while those lacking both were considered immature. Sexually mature females were evaluated for the number, length and width of eggs, as well as the volume, number, and diameter of the largest vitellogenic follicle. To assess whether body size influences egg size, we used a parametric correlation of Pearson between SVL and egg volume. The eggs and vitellogenic follicles found were

used to infer the reproductive season, the number of eggs per clutch, and the frequency of oviposition per season.

The assessment of male sexual maturity relied on the presence of convoluted epididymis, identified under a stereomicroscope. We measured the testes (length and width) to estimate volume using the ellipsoid formula (Dunham, 1983): $V = 4/3\ \pi\ (w/2)^2\ (l/2)$, where w is the prey width and l is the prey length. To evaluate the influence of body size on testes volume, we performed a simple linear regression with SVL as the predictor and testes volume as the response variable using the stats R-package (R Core Team, 2024).

Diet

In the lab, we removed the stomachs of all lizards collected and analyzed them under a stereomicroscope to identify prey items up to the Order level. Prey items were analyzed for their frequency, number (abundance), and volume. The length and width of each prey item were measured using a digital caliper (± 0.1 mm accuracy), and its volume (in mm^3) was estimated using the ellipsoid formula. The importance index was calculated to determine the relative importance of each prey category within the total diet, using the formula: (F% + N% + V%)/3. Thus, we used the inverse of the index of Simpson (1949) to estimate the food niche breadth, both numerically and volumetrically.

To assess whether the size of the lizard influences the size of the prey it consumes, we used a multiple linear regression analysis with prey volume as the response variable and SVL, HeL, HeW, and HeH as predictors. To fit the volume data to a normal distribution, we applied a base-10 logarithmic transformation. Therefore, the final model was model_volume = lm (log_volume ~ SVL + HeL + HeH + HeW, data = morfo_volume), which resulted in residuals with a normal distribution (W = 0.99121, p-value = 0.4901).

Parasitism

We examined the liver, as well as the digestive, reproductive and respiratory systems in search of parasites. The parasites found were mounted on temporary slides in Hoyer medium and subsequently analyzed under light microscopy to be identified based on the specialized literature (e.g., Yamaguti, 1971; Schmidt, 1986) and by comparison with specimens deposited in the Parasitological Collection of the Regional University of Cariri.

Prevalence and mean intensity of parasite infection were calculated according to Bush et al. (1997). The index of discrepancy ranges from a minimum value of zero, indicating an equal distribution of parasites among hosts, to a maximum value of 1 (D = 1), indicating that all parasites are combined in a single host (Poulin, 1993). These indexes were calculated using Quantitative Parasitology software 3.0 (Reiczigel et al., 2019).

RESULTS

In total, 394 *C. meridionalis* specimens were found: 235 by manual capture, 89 by pitfall traps and 70 by visual identification (sighted but not collected).

Microhabitat use

We recorded microhabitat use by 285 individuals of *C. meridionalis*, including 269 adults and 16 juveniles. The distribution across microhabitats was leaf litter (90.52%; n = 258), bamboo leaf litter (5.96%; n = 17), fallen trunk (1.76%; n = 5) and open ground (1.76%; n = 5). The niche breadth based on microhabitat use was 1.214, revealing a high degree of specialization in leaf litter (Table 1).

Population density

The average density of *C. meridionalis* was 2.13 individuals per 100 m^2 quadrant, which corresponds to 213.33 individuals per hectare. The 95% confidence interval suggests that the average density per hectare may vary between 78 and 349 individuals. This density estimate (213 ind/ha) represents the local density within sampled microhabitats, which (based on our field records) were concentrated in patches of suitable leaf-litter microhabitats.

Table 1. Microhabitats used by *Coleodactylus meridionalis* in a fragment of the Atlantic Forest within the Aldeia-Beberibe Environmental Protection Area, northeastern Brazil. *n* = number of microhabitat use records (percentages).

Microhabitat	n (%)
Bamboo leaf litter	17 (5.96)
Leaf litter	258 (90.52)
Fallen trunk	5 (1.76)
Open ground	5 (1.76)
Total	285
Niche breadth	1.214

Table 2. Morphometric measurements (in mm) of adult *Coleodactylus meridionalis* from an Atlantic Forest fragment, Pernambuco, northeastern Brazil. SVL (snout-vent length), TL (tail length), HeL (head length), HeW (head width), HeH (head height), BoW (body width), BoH (body height), FRL (forelimb length), and HDL (hindlimb length).

Characters	Range (average ± standard deviation)	
	Females (N = 57)	Males (N = 59)
SVL	17.57 – 25.16 (22.62 ± 1.66)	18.35 – 23.73 (21.06 ± 1.14)
TL	12.23 – 21.00 (17.65 ± 2.4; N= 28)	10.29 – 20.70 (17.20 ± 2.7; N= 33)
HeL	3.11 – 6.16 (5.28 ± 0.51)	4.08 – 5.70 (4.99 ± 0.40)
HeW	2.17 – 3.99 (3.37 ± 0.36)	2.74 – 3.98 (3.25 ± 0.27)
HeH	1.06 – 3.27 (2.16 ± 0.36)	1.57 – 2.84 (2.16 ± 0.27)
BoW	2.57 – 6.21 (4.36 ± 0.74)	3.48 – 4.96 (4.05 ± 0.35)
BoH	1.79 – 4.19 (2.82 ± 0.52)	1.76 – 3.86 (2.64 ± 0.45)
FRL	4.42 – 7.59 (5.86 ± 0.64)	4.12 – 6.48 (5.45 ± 0.57)
HDL	5.38 – 9.14 (7.23 ± 0.80)	4.98 – 8.43 (6.99 ± 0.74)

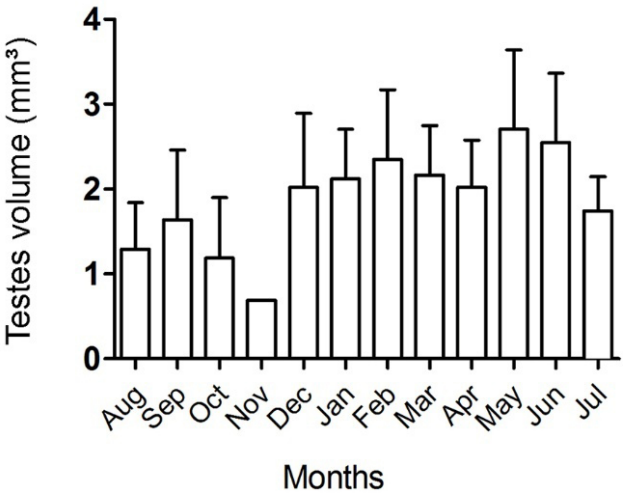


Fig. 2. Monthly variation in testes volume (mm^3) of adult male *Coleodactylus meridionalis* of an Atlantic Forest fragment within the Aldeia-Beberibe Environmental Protection Area, northeastern Brazil.

Sexual dimorphism

We analyzed the morphometric measurements of 116 *C. meridionalis* adult specimens, which 57 were females and 59 were males). We detected sexual dimorphism in the Body Size isometric variable ($F_{1,109} = 19.38$; $P < 0.001$), with females being larger than males (Table 2). However, model selection analysis showed no significant differences in individual variables between sexes (all $P > 0.05$).

Reproduction

We analyzed reproductive data of 192 *C. meridionalis* lizards (87 females, 78 males, and 27 juveniles), of which 153 (75 were females, 72 males and six juveniles) were collected. The smallest reproductively mature male measured 18.35 mm. Testes volume was significantly influenced by the SVL ($F = 13.244$; adjusted $R^2 = 0.1719$; $p = 0.0006$), with decreased volume between July and November (Fig. 2).

Coledactylus meridionalis presented a fixed clutch size of one egg. Females showed continuous reproduction throughout the year. However, no reproductively mature females were found in either April, or from August to October, possibly due to the low number of individuals collected during this period. The smallest reproductively mature female had an SVL of 17.57 mm. There was no significant correlation between egg volume and female SVL ($t = -1.549$, $df = 11$, $p = 0.1496$).

Three eggs of *C. meridionalis* were found at different times under leaf-litter, situated at the tree base. These eggs were collected with a portion of the substrate and stored at environmental temperature. One egg (length: 5.15 mm, width: 4.22 mm, weight: 0.051 g), collected in June, did not hatch. The remaining two eggs (length: 5.20 mm, width: 4.40 mm, weight: 0.53 g; length: 5.46 mm, width: 4.35 mm, weight: 0.57 g), found together in July, successfully hatched after 53 days. Post-hatching, the neonates measured: 1) SVL = 11.88 mm, TL = 8.50 mm, weight = 0.036 g; 2) SVL = 11.16 mm, TL = 9.20 mm, weight = 0.034 g). Juveniles were found throughout the year, except for April and November, with the highest numbers recorded in January (N = 5) and June (N = 7).

Diet

We analyzed the diet of all 154 *C. meridionalis* collected. Of these, ten specimens had empty stomachs and intestines, while seven presented unidentifiable content. We identified a total of 22 food categories, with an average of two prey categories per stomach (RANGE: 1-5).

The diet of *C. meridionalis* exhibited predominance in frequency with Isopoda (16.8%), Araneae (14.9%), and Collembola (10.2%); in number with Psocoptera (20.6%), Isoptera (18.97%), and Collembola (17.53%); and in volume with Isopoda (36.5%). According to the importance index, the most significant prey category was Isopoda (21.4%), followed by Psocoptera (12.1%) and Isoptera (11.5%) (Table 3). Among the six juvenile specimens examined, one had an empty digestive tract, one had uni-

Table 3. Diet composition of *Coleodactylus meridionalis* in an Atlantic Forest fragment within the Aldeia-Beberibe Environmental Protection Area, Pernambuco, northeastern Brazil. F = Frequency; N = number; V = Volume (in mm^3), with their respective percentages; I = importance index of prey categories.

Prey category	F	F%	N	N%	V	V%	I
Acari	6	2.80	7	1.42	0.56	0.09	1.44
Araneae	32	14.9	45	9.18	41.2	6.67	10.2
Blattodea							
Blattaria	4	1.80	4	0.81	64.4	10.4	4.37
Isoptera	18	8.40	93	18.97	45.4	7.35	11.5
Coleoptera	3	1.40	10	2.04	2.90	0.46	1.30
Collembola	22	10.2	86	17.5	15.2	2.46	10.0
Dermaptera	2	0.93	2	0.40	11.0	1.78	1.04
Diptera	7	3.27	10	2.04	9.66	1.56	2.29
Lizard shed skin	4	1.86	4	0.81	-	-	0.89
Gastropoda	2	0.93	2	0.40	18.8	3.05	1.46
Hemiptera							
Auchenorrhyncha	3	1.40	3	0.61	1.87	0.30	0.77
Heteroptera	1	0.46	1	0.20	0.25	0.04	0.23
Others	1	0.46	1	0.20	1.32	0.21	0.29
Hymenoptera							
Formicidae	6	2.80	10	2.04	5.16	0.83	1.89
Others	3	1.40	3	0.61	17.7	2.87	1.63
Isopoda	36	16.8	54	11.0	225.9	36.5	21.4
Insect eggs	4	1.80	5	1.02	1.22	0.19	1.02
Insect larvae	21	9.80	30	6.12	42.9	6.94	7.62
Insect pupa	2	0.93	2	0.40	33.1	5.36	2.23
Orthoptera	10	4.60	10	2.04	39.0	6.31	4.34
Pseudoscorpiones	6	2.80	7	1.42	2.45	0.39	1.54
Psocoptera	21	9.80	101	20.6	37.3	6.04	12.1
Trophic niche breadth			7.348		3.641		

dentifiable content, and four contained Diptera (n = 1) and Psocoptera (n = 2, n = 2, n = 16).

The diet of *C. meridionalis* was generalist, with a numerical food niche breadth of 7.348 and volumetric breadth of 3.641. No significant difference in the diet composition was found between adult males and females, either in number (ANOSIM, R = 0.0202; p = 0.055) or in volume (ANOSIM, R = 0.0078; p = 0.265). The multiple linear regression revealed that the size of the lizards influences the volume of prey ingested (F = 5.001; adjusted R^2 = 0.0982; p = 0.001). The two individual variables that showed statistical significance were head width (HeW: F = 8.472; p = 0.004) and head length (HeL: F = 5.411; p = 0.021).

Parasitism

Out of 154 specimens of *C. meridionalis* (75 females, 73 males and six juveniles), 21 specimens (16 females and five males) were infected by at least one parasite species (total prevalence of 13.6%). Twenty specimens were infected with cystacanth (Acanthocephala larvae) (N = 30) (Fig. 3A), with a prevalence of 13% and a mean infection intensity of 1.5 ± 0.74 (range 1 – 3). These cystacanths were found in seven infection sites: loosely in the body cavity (N = 9), adhered to the body wall (N = 7), adhered to the stomach (N = 7), adhered to the liver (N = 5), adhered to the testes (N = 2), adhered to follicles (N = 4), and subcutaneously (N = 1) (Table 4). Acanthocephala larvae adhered to the liver and stomach caused visible damage to host tissue due to their extreme adherence.

Additionally, three *C. meridionalis* were infected by nine adult Brachycoeliidae worms (Trematoda: Digenea) (Fig. 3B) found in the esophagus (N = 1) and stomach (N = 8), with a prevalence of 1.9% and a mean intensity of infection of 3 ± 1.9 (range 1 – 7) (Table 4). Only two lizards were infected by both parasites simultaneously. The discrepancy index for cystacanth was 0.895 and for Brachycoeliidae was 0.983. There was no significant difference in abundance observed between sexes (Z = 0.596; P = 0.116), between dry and wet seasons (Z = 839; P = 0.401) or when considering both sex and season (Z = 0.138; P = 0.891).

DISCUSSION

Coleodactylus meridionalis is a microhabitat specialist, predominantly and almost exclusively using leaf litter. Similar results have been found in other *C. meridionalis* populations (Silva et al., 2015; Gonçalves-Sousa et al., 2023) and congenerics such as *C. septentrionalis*, *C. brachystoma* and *C. natalensis* (Colli et al., 2002; Vitt et al., 2005; Sousa and Freire, 2011; Lisboa and Freire, 2012), suggesting phylogenetic conservatism in the microhabitat use. In a Cerrado area, *C. meridionalis* used the same number of microhabitat categories found by us, but with a more even distribution across two microhabitats (spatial niche breadth = 2.57).

The spatial niche specialization of *C. meridionalis,* with its preference for leaf litter, can be interpreted through the lens of microevolutionary optimization, particularly regarding fine-scale crypsis (Hendry and Kinnison, 2001). *Coleodactylus* spp. are known for using a low range of microhabitats (Present study; Vitt et al., 2005; Lisboa and Freire, 2012; Gonçalves-Sousa et al., 2023). The cryptic, coppery coloration of *C. meridionalis* (Vanzolini, 1957*)* favors camouflage within dark leaf litter, its preferred microhabitat (90.52% of individuals recorded in this study). However, in bamboo leaf litter, characterized by light-colored, yellowish dry leaves, *C. meridi-*

Table 4. Lizard species parasitized by cystacanths (Acanthocephala larvae) and Brachycoeliidae (Trematoda), with data on prevalence (P), mean intensity of infection (MII) ± standard error (SE), and infection sites. Cystacanth infection sites: (1) free in the body cavity, (2) subcutaneous, (3) attached to the stomach, (4) attached to the liver, (5) attached to the peritoneous, (6) attached to the testes, (7) attached to reproductive follicles, (8) attached to the intestine, (9) attached to the lung. Trematoda infection sites: inside the (A) esophagus, (B) stomach, (C) intestine. (-) no data available.

	Host species	P (in %)	MII ± SE	Infection site	Source
Cistacanth (Acanthocephala larvae)	*Ameiva ameiva*	35.70	1	-	Smales, 2007
	Ameiva ameiva	60	-	1	Macedo et al., 20016
	Anolis cristatellus	67	-	2	Nickol et al., 2006
	Coleodactylus meridionalis	13.6	1.5 ± 0.74	1:7	Present study
	Hemidactylus agrius	-	-	1	Anjos et al., 2011
	Hemidactylus mabouia	33.70	4.2 ± 7	3, 5, 8	Anjos et al., 2005
	Brasiliscincus agilis	90.90	15 ± 10.7	1, 3:5, 8-9	Vrcibradic et al., 2002
	Brasiliscincus agilis	57.10	6.3 ± 10.7	1, 3:5, 8-9	Vrcibradic et al., 2002
	Psychosaura macrorhyncha	90.90	35.5 ± 45.8	1, 3:5, 8-9	Vrcibradic et al., 2002
	Psychosaura macrorhyncha	7.10	3	-	Vrcibradic et al., 2002
	Anolis fuscoauratus	37.50	2.15 ± 2.2	1, 3:5, 7-8	Campos et al., 2016
	Tropidurus hispidus	3.50	-	-	Brito et al., 2014a
	Tropidurus hispidus	0.69	3.5	-	Brito et al., 2014b
	Tropidurus semitaeniatus	0.51	1	-	Brito et al., 2014b
	Tupinambis teguixin	14.30	1	-	Smales, 2007
Brachycoeliidae (Trematoda: Digenea)	*Anolis sagrei*	3	1	C	Goldberg & Bursey, 2000
	Cercosaura eigenmanni	7	1	C	Bursey & Goldberg, 2004
	Coleodactylus meridionalis	1.90	3 ± 1.9	A, B	Present study
	Diploglossus lessonae	-	-	C	Ávila & Silva, 2010
	Lepidophyma flavimaculatum	80	2.4 ± 1.8	-	Bursey et al., 2006
	Trachylepis atlantica	4	3	C	Ramalho et al., 2009
	Tropidurus torquatus	33.30	-	C	Rodrigues et al., 1990

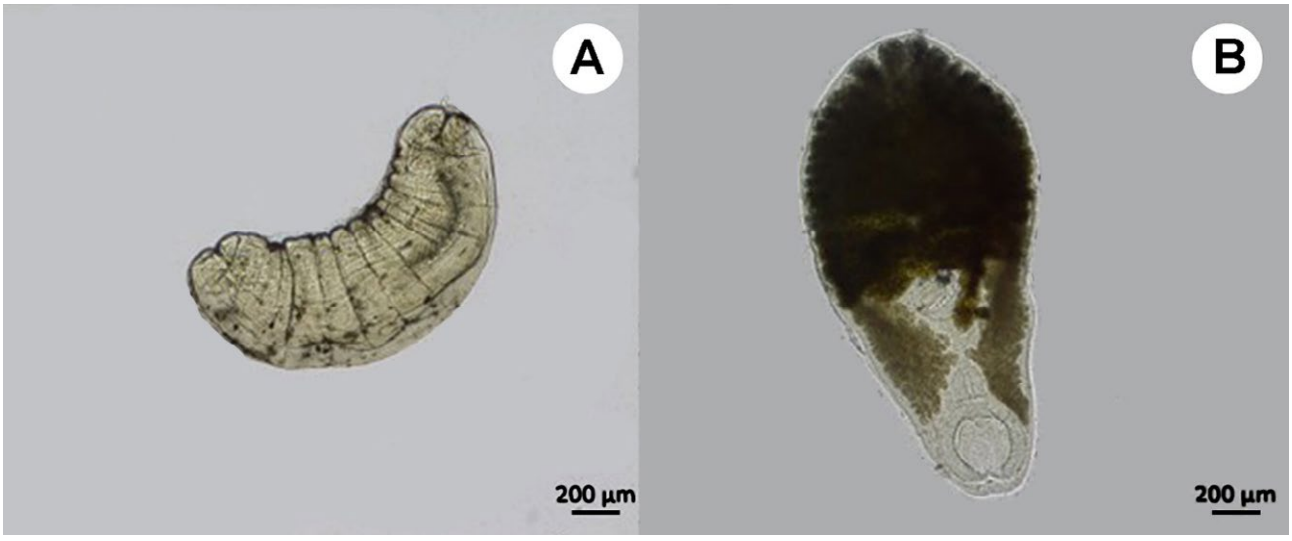


Fig. 3. Parasites found in *Coleodactylus meridionalis* of an Atlantic Forest fragment within the Aldeia-Beberibe Environmental Protection Area, northeastern Brazil. (A) Cystacanth (Acanthocephala); (B) Ventral view of Brachycoeliidae (Trematoda: Digenea).

onalis was less frequent (5.96%) and displayed intraspecific variation, exhibiting lighter, orange-toned coloration. This change improves camouflage in this lighter microhabitat, thereby reducing predation risk. This phenotypic alignment with the substrate underscores the role of selective pressures on coloration, where this crypsis enhances fitness and shapes the spatial distribution of the species at local spatial scales.

Although no single variable showed a significant difference when tested individually, the Body Size isometric variable was significant, with females larger than males. In contrast, Silva et al. (2015) found that males had significantly higher SVL than females in another population of *C. meridionalis* in a Humid Forest enclave within the Caatinga domain in northeastern Brazil. These characteristics may vary between different populations, although our data present a larger sample, which may better explain the morphological pattern of these lizards. Moreover, in other studies, mean SVL in *Coleodactylus* species was higher in females, including *C. meridionalis, C. elizae* and *C. natalensis* (Freire, 1999; Goncalves et al., 2012). These findings suggest that sexual dimorphism in *C. meridionalis* is a labile functional trait shaped by different local selective pressures across biomes.

Sexual dimorphism in lizards typically results from fecundity or sexual selective pressures, whose effects appear to geographically change across *C. meridionalis* populations. The females larger than males found in our study is often linked to fecundity selection (e.g., Andersson, 1994; Olsson et al., 2002), but the mechanism for this selection seems multifactorial in this miniaturized species. *Coleodactylus meridionalis* has a one-egg fixed clutch size, and our analysis found no significant correlation between egg volume and female SVL. This finding suggests that the advantage of larger female size is probably not associated with bigger offspring, but rather to improve energy storage, as in larger fat bodies. Greater energy reserves would be advantageous for sustaining the continuous reproductive effort observed throughout the year. On the other hand, *C. meridionalis* males larger than females found by Silva et al. (2015) suggests that sexual selection (Colli et al., 2003; Mesquita and Colli, 2003) may be dominant in that Caatinga population.

The diet of *C. meridionalis* consisted exclusively of arthropods, exhibiting a generalist feeding pattern that is best explained by high opportunistic food plasticity. In our study, the broad food niche dimension is similar to findings from two populations of Atlantic Forest (Teixeira et al., 2021), Cerrado (Werneck et al., 2009), and a population from Humid Forest enclave within the Caatinga domain (Silva et al., 2015), but contrasts with the specialist feeding habits reported in two other Atlantic Forest and Caatinga vegetation areas (Supporting Information S3 in Gonçalves-Sousa et al., 2023). This shifts across areas in the diet of *C. meridionalis*, even within the same biome or among close areas, reveals strong evidence of plasticity in prey selection. For example, the Order Isopoda was the main food category ingested in our study (importance index 21.4%) and in two other populations (35.71% and 34.1%; Teixeira et al., 2021) of the Atlantic Forest, and in a population from a semideciduous seasonal forest area (48.1% in frequency; Silva et al. 2015). On the other hand, Formicidae (44.1%) and Insect larvae (35.15%) were the main categories in other populations from the Atlantic Forest (Teixeira et al., 2021), whereas Isoptera (51%) and Siphonaptera (44.8%) were predominant in a Caatinga population from Cuité, Paraíba (Gonçalves-Sousa et al., 2023), and Araneae (80.8%) in another Caatinga population from Palmas de Monte Alto, Bahia (Gonçalves-Sousa et al., 2023). Additionally, in a Cerrado enclave in São Domingos, Goiás, Isoptera was the most important prey category (36.87%; Werneck et al., 2009). Therefore, it is plausible to infer that the diet of *C. meridionalis* is strongly influenced by local ecological factors, such as resource availability, with the availability of suitable sized food resources for this small lizard in each area determining the most significant categories in the diet composition.

We found that the size of the lizards, specifically head width and head length, significantly influenced the volume of prey ingested, with larger lizards consuming larger prey. This finding aligns with similar results found in some lizard species of the families Sphaerodactylidae, Teiidae and Tropiduridae (Van Sluys et al., 2004; Vitt et al., 2005; Sales et al., 2011). In some lizard species, such as the leiosaurid *Enyalius brasiliensis*, both SVL and head width strongly influence diet, as larger measurements correlate with a greater volume of prey consumed (Dorigo et al., 2014). In contrast, there are lizards that do not exhibit a significant correlation between body or head size with prey size. For instance, medium to large-sized lizards like the tropidurid *Plica plica* and the teiid *Ameivula ocellifera* have a generalist diet composition, but small prey items, such as ants and termites, plays a significant role in their diet (Vitt, 1991; Mesquita and Colli, 2003).

Lizards are susceptible to several macro endoparasites, such as Acanthocephala, Cestoda, Nematoda, Pentastomida, and Trematoda (e.g., Ávila and Silva, 2010; Ribeiro et al., 2012; Sousa et al., 2014). In the present study, we detected infections in *C. meridionalis* caused by a cystacanth of Acanthocephala and by a trematode from the Brachycoeliidae family. Acanthocephala can use lizards as paratenic or definitive hosts (Nickol et al., 2006; Smales, 2007).

Infection by cystacanths can occur through feeding organisms such as termites, which have been identified as intermediate hosts for Acanthocephala (Nickol et al., 2006; Amato et al., 2014) and are also part of the diet of *C. meridionalis* in the studied population. In *C. meridionalis,* cystacanth parasites showed low prevalence (13.6%). Nonetheless, this rate is still higher than those found in another population of *C. meridionalis* (6.2%), as well as in *Psychosaura macrorhyncha* (7.1%), *Tropidurus hispidus* (3.5% and 0.69%) and *T. semitaeniatus* (0.51%) (Vrcibradic et al., 2002; Brito et al., 2014a; 2014b; Teixeira et al., 2021). Six out of seven identified infection sites of cystacanth in *C. meridionalis* align with registers in other host species (Vrcibradic et al., 2002; Nickol et al., 2006; Macedo et al., 2016; Campos et al., 2021), except for attachment to the testes. The sites with highest infection rates were those attached to the stomach and to the body wall (both dorsal and ventral), a pattern commonly observed in other lizards (Vrcibradic et al., 2002; Anjos et al., 2005; Campos et al., 2021). Subcutaneous infection by Acanthocephala was rare, registered only once in our study and once for *Anolis cristatellus* (Nickol et al., 2006). Cystacanths were also found parasitizing *Anolis fuscoauratus* in the same sampled area of this study (Campos et al., 2021). Although we only encountered the larval stage, this finding highlights the high plasticity of this parasite, as it can infect lizards adapted to a wide range of microhabitats, including leaf litter and tree branches.

This is the first report of a trematode from the Brachycoeliidae family parasitizing *C. meridionalis*. This finding also represents the third record of parasitism in Sphaerodactylidae lizards within South America (Ávila and Silva, 2010). In Brazil, the family Brachycoeliidae (Trematoda) is reported as a Squamata parasite, mostly in lizards living in Caatinga regions and in the Archipelago of Fernando de Noronha (Ramalho et al., 2009; Ávila and Silva, 2010); in the Amazonia Forest region (Bursey and Goldberg, 2004); and in the southeastern Restinga area (Rodrigues et al., 1990). The prevalence of Brachycoeliidae infection was low in *C. meridionalis* (1.9%). A low prevalence of trematodes in lizards appears to be a common pattern, as several studied have found prevalences between 3% and 7% (Goldberg and Bursey, 2000; Ramalho et al., 2009; Teixeira et al., 2021). Contrarily, the Central America lizard *Lepidophyma flavimaculatum* showed prevalence of 80% (8/10 individuals), but with low infection intensity (2.4 ± 1.8; Bursey et al., 2006). Unlike *L. flavimaculatum,* where trematodes were almost uniformly, the discrepancy index (0.993) indicated that these parasites occur clustered in *C. meridionalis.* This finding is mainly due to the fact that 7 out of the 10 collected trematodes were found in just one host individual, suggesting that this parasite infection may occur casually or even accidentally in the studied area.

According to our revision, the most frequent Brachycoeliidae infection site is the intestine, aligning with preferred infection sites reported for other families of the Digenea subclass parasitizing lizards (Ávila and Silva, 2010). However, it is noteworthy that within this subclass, parasites from the same family can infect different sites, such as gallbladder, liver and intestines, with stomach as a potential infection site (Ávila and Silva, 2010). Considering that Brachycoeliidae was found inside the stomach and esophagus of *C. meridionalis*, the potential parasitic sites for this family extend beyond the intestine.

We concluded that *C. meridionalis* is a sexually dimorphic lizard that typically inhabit forested environments, mainly using leaf litter as its microhabitat. This microhabitat preference indicates phylogenetic conservatism. Contrarily, this lizard showed a generalist and opportunistic feeding habit, suggesting high food plasticity and adaptability according to the food resource availability in each area. Lastly, *C. meridionalis* is a paratenic host for cystacanth and a definitive host Brachycoeliidae species.

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Herpetofaunal diversity in the Upper Guinean rainforest: a baseline survey from the Dugbe region, Liberia

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Abstract. The Upper Guinean rainforest biome is a poorly studied, yet hyper-biodiverse region facing severe fragmentation due to ongoing habitat transformation. We conducted a herpetological baseline survey in the Dugbe region during 2021 using passive trapping and active searching. Our survey resulted in 1,145 herpetofauna observations, representing 71 taxa (38 amphibians and 33 reptiles), with active searching yielding 47.8% of the species richness. Nearly half (54.9%) of the documented species are West African endemics. Rarefaction/extrapolation sampling curves indicated incomplete overall sampling of herpetofauna diversity, though amphibian sampling completeness was high. Reptile diversity metrics revealed significant sampling deficiencies, largely explained by the high proportion of singleton observations (60.6% of reptile records). When compared to the IUCN predictive distribution maps, our survey documented 51.2% of the 123 species predicted for the region, with moderate overall Jaccard similarity (48.09%). Taxonomic groups showed varied patterns of congruence: amphibians displayed relatively high Jaccard similarity (59.26%), while reptiles showed lower similarity (40.26%). Notably, our survey documented substantially higher herpetofauna species richness than benchmark surveys from nearby areas, particularly for reptiles (33 species compared to 14 and 5 species in Krahn-Bassa proposed protected area and Grebo National Forest surveys, respectively). Several amphibian observations were not assignable to species using morphological characteristics alone, highlighting the need for further taxonomic research on West African herpetofauna.

Keywords. Reptiles, amphibians, West Africa, field survey, herpetology, tropical forest ecology.

INTRODUCTION

The Upper Guinean forests of West Africa are a global biodiversity hotspot with high faunal and floral diversity and endemism (Myers et al., 2000; Lewin et al., 2016; De Sousa et al., 2023; Ernst et al., 2025). These forests are important for amphibians, with high species richness and taxonomic diversity (Penner et al., 2011, 2019; Nneji et al., 2023). The region's reptiles are less well studied than amphibians, and there are significant gaps in our understanding of their diversity and distribution (Tolley et al., 2016); this is a globally observed pattern in the context of tropical forest herpetofauna (Deikumah et al., 2014; Tolley et al., 2016). The Upper Guinean forests which once spanned continuously across West Africa have been fragmented by deforestation, agri-

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cultural expansion, and mining (Sodhi et al., 2008, Hepner, 2025). Liberia still holds some of the largest remaining tracts of these forests, but habitat fragmentation and degradation have intensified in the last twenty years (De Sousa et al., 2023). This loss of habitat threatens the region's herpetofauna (Rödel et al., 2021) and baseline surveys are needed to document amphibian and reptile biodiversity, as loss or extinctions may be occurring without our knowledge.

Although there have been numerous studies on Liberian herpetofauna (e.g., Barbour and Loveridge, 1927; Loveridge, 1941; Angel, 1943a; Guibé and Lamotte, 1958), particularly in the Mount Nimba region (e.g., Xavier, 1978; Lamotte, 1998; Ineich, 2003; Rödel et al., 2009; Sandberger et al., 2010), large portions of the country remain poorly surveyed. Our understanding of herpetological species distributions is based on local surveys, species descriptions, and taxonomic revisions (e.g., Trape and Mané, 2006; Hillers and Rödel, 2007; Blackburn et al., 2008; Allen et al., 2019; Rödel and Glos, 2019). Comprehensive baseline surveys from understudied regions can address these knowledge gaps, and guide conservation efforts in response to ongoing habitat loss.

This study provides the first herpetological species inventory for the Dugbe region, located in southern Liberia (Fig. 1). The survey was conducted in 2021 by Flora Fauna and Man, Ecological Services Ltd. (FFMES) and the aim was to establish a baseline of herpetological diversity, identify species of conservation concern, and find potential novel or endemic species. To contextualise our findings, we compare them with surveys conducted approximately 100 km away in Grebo National Forest (Hillers and Rödel, 2007) and the proposed Krahn-Bassa proposed protected area (KBPPA) (Rödel and Glos, 2019). Both areas have similar ecological and biogeographical features to the Dugbe study area and our comparisons focus on species richness to provide an overview of the regional species diversity for this poorly studied area.

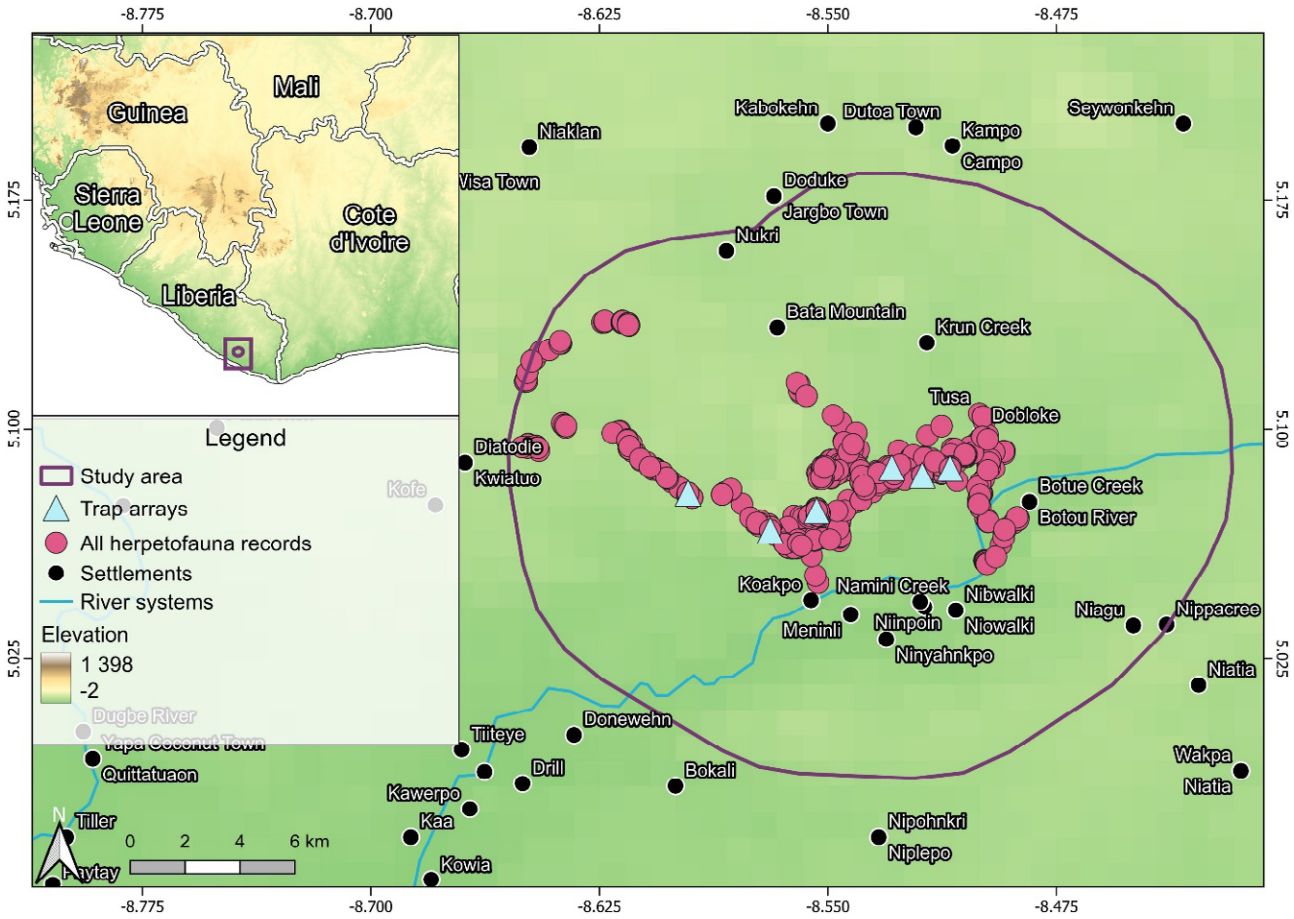


Fig. 1. Location of the Dugbe study area within Liberia and the locations of the herpetofauna observations and trap arrays within the study area.

The Dugbe study area is located within the Upper Guinean Rainforest biome, near to Sapo National Park and the Grand Kru–River Gee proposed protected area. Historically the landscape was dominated by primary closed-canopy rainforest (Cushing et al., 2016; Ernst et al., 2025). The landscape has high conservation significance and is currently characterised by a mosaic of natural and transformed habitat types with most of the forested areas in various stages of secondary succession. Over 14% of mature forest cover was lost between 2000 and 2018 due to ongoing habitat transformation and resource exploitation (De Sousa et al., 2023). Several villages are present within the study area and there is a high reliance on subsistence agriculture and artisanal mining. The study area consists of low hills with some rocky areas, and the elevation ranges between 10–170 m above sea level. The region is drained by three major rivers: the Bouto, Dugbe, and Geebo, as well as smaller streams.

METHODS

Field surveys

Surveys were conducted over two periods: an initial five-day scoping visit from 8-13 May 2021 during the wet season, and a follow-up 16-day survey from 20 October to 4 November 2021, during the transitional period between the wet and dry seasons. The first survey was undertaken by a single herpetologist (Marius Burger) and involved diurnal and nocturnal active searches to explore the terrain in preparation for a more detailed survey. The second survey was conducted by two herpetologists (Marius Burger and Ryan van Huyssteen) and included diurnal and nocturnal active searching and the use of passive trapping methods. Although the second season was planned for the transitional period between the wet and dry seasons, there was heavy rainfall for most of the survey period.

The sampling strategy followed a multistage stratified semi-random sampling (MSSRS) approach (Bourgeron et al., 2001, Mottram et al., 2025). The study area was divided into 576 grid cells of 1 km² each. Within each grid cell, the MSSRS approach was used to semi-randomly identify specific sampling sites through consideration of physiognomic and physiographic elements (Mottram et al., 2025). In addition to predefined points, opportunistic searches were conducted in microhabitats that are known to be ecological relevant for particular species (e.g., ponds, streams, rocky outcrops). In total we investigated 64 grid cells and sampled 133 sample points within those. Active searching involved looking for resting and active herpetofauna, investigating likely hiding places (holes, under logs and other cover, raking through leaf litter, etc.), and listening for vocalising frogs.

Acoustically detected amphibians were recorded as presence/absence data (i.e., single presence per calling event regardless of the number of individuals estimated to be calling). This was a methodological decision implemented to prevent potential bias in species accumulation analyses when pooling acoustic-detected species and visual-detected species. However, for all amphibians and reptiles directly observed during surveys, we maintained accurate count data reflecting the true number of individuals encountered.

The trapping effort consisted of six trap arrays that each had four 25-litre bucket pitfall traps and six double-ended funnel traps connected by drift fences assembled in a linear configuration (Fig. 2). Traps arrays were operational for 13 nights (78 trap array nights/780 trap nights), trapped specimens were recorded and removed daily.

Incidental observations, including photographs and specimens recorded by researchers of other taxonomic groups and mining personnel during the survey period were also included and documented. Most observations had a GPS accuracy of 5 m; however, several incidental records had a lower resolution of 5000 m.

Reptiles and amphibians were identified based on the keys provided by Chippaux (2006), Trape et al. (2012), Schiøtz (1999) and descriptions in Channing and Rödel (2019). In some instances, specialists were consulted for advice on identifications (see acknowledgements). Where possible observations were supported by photographic or sound-clip evidence and these were deposited as digital vouchers onto iNaturalist.org (Supplementary mate-

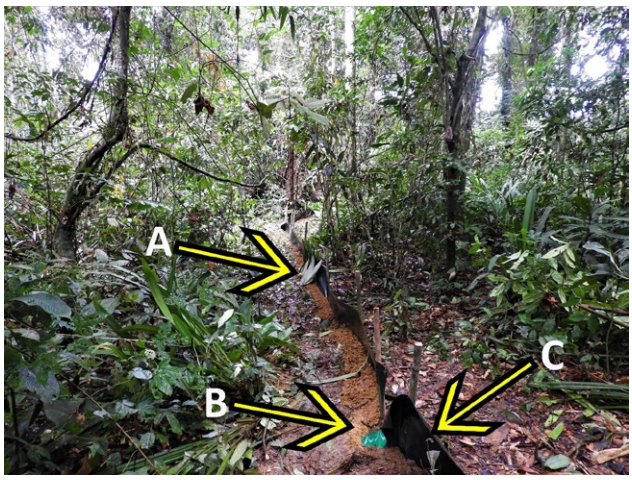


Fig. 2. One of six trap arrays installed for the Dugbe herpetofauna survey. The trap array is composed of six funnel traps (A) and four pitfall traps (B) that are installed along a 30-meter drift fence (C) in a linear fashion.

rial 1). Voucher specimens and genetic samples were collected for most species encountered and deposited at the American Museum of Natural History (AMNH) (Supplementary material 1). Amphibian taxonomy follows Frost (2025), and common names follow Channing and Rödel (2019), while reptile taxonomy is based on Uetz et al. (2025).

Survey evaluation

We used three methods to evaluate our survey effort: firstly, we used rarefaction/extrapolation (r/e) curves, secondly, we compared our survey to the IUCN (International Union for Conservation of Nature) predictive species lists for the study area which are based on expert-derived range maps, and finally we compared our survey species richness to two benchmark surveys that were conducted within 100 km of our study area. All herpetofauna observations made during the surveys, including incidental records, were included in our analyses. Amphibian taxa that were identified as recognisable taxonomic units, but not confidently assignable to currently known species, were also included in the sampling curves, diversity estimates, and similarity analyses.

Sampling curves. To evaluate survey effort in the Dugbe area, r/e curves were generated using the iNEXT package (Hsieh et al., 2016) in R (R Core Team, 2022) via the RStudio console (RStudio Team, 2022). The curves were plotted and extrapolated to twice the original survey effort, with observation sequences randomised 100 times using bootstrap resampling. Estimated species richness, along with standard error and 95% confidence intervals were calculated along with diversity indices (Species Richness, Shannon Diversity, and Simpson Diversity) for all herpetofauna, as well as amphibians, and reptiles independently.

IUCN predictive species lists. To compare our observed local diversity based on the field surveys, with the expected regional diversity, we extracted spatial distribution data for all amphibian and reptile species with ranges overlapping our study site using the IUCN Red List spatial database (IUCN, 2025). This predictive distribution list serves as a theoretical baseline of expected species richness in the region, enabling the evaluation of our field surveys performance, identifying potential sampling gaps, and contextualising our findings within the broader regional herpetofaunal assemblage.

To assess the similarity between our field survey results and the IUCN predicted species distribution, we calculated the Jaccard similarity index (J). This metric is based on presence/absence and measures the proportion of shared species compared to the total species pool (Magurran, 2004). The Jaccard index was calculated as $J = C/(A+B-C)$, where A is the number of species observed through our surveys, B is the number of species from the IUCN predicted distribution for the study area, and C is the number of species common to both lists. The index ranges from 0, (no overlap) to 1 (complete similarity) and was used to quantify the taxonomic overlap between our survey results and the predicted regional species assemblage based on IUCN distribution maps. For clarity, we report both the proportion of IUCN-predicted species we recorded in total (A/B) and the proportion of species our survey shared with IUCN predictions (C/B), which is the numerator of the Jaccard index.

Comparison to benchmark surveys. To ground our findings within the broader context of Liberian herpetofaunal research, we compared our survey results from the Dugbe study area with two benchmark surveys conducted in southeastern Liberia: KBPPA by Rödel and Glos (2019) and the Grebo National Forest survey by Hillers and Rödel (2007). These study sites offer appropriate benchmarks for comparison based on their proximity (both located within 100 km of our Dugbe) and similar habitat types. For each survey, we compared species richness for all herpetofauna, as well as amphibians and reptiles separately. As the focus and survey methods of Rödel and Glos (2019) and Hillers and Rödel (2007) studies differed from ours, the comparisons were restricted to species richness, providing an evaluation of our sampling effort and general insight into the regional herpetofaunal assemblage.

RESULTS

During the Dugbe survey, we documented 1145 individual amphibians and reptiles representing 71 distinct taxa (Table 1-2, Supplementary material 1). The assemblage comprised 38 amphibian species (982 individual observations) and 33 reptile species (156 individual observations). A comprehensive species inventory for the Dugbe study area is presented in Table 1 and Table 2, detailing amphibians and reptiles respectively, including common names, IUCN conservation status, observation frequencies, and West African endemism status. A selection of photos of recorded species is presented in Fig. 3-4.

Active searching proved the most productive survey method, contributing 985 observations (86%) and 34 unique species (47.8% of total richness). Trapping methods contributed 122 observations (10.6%) and four unique species (5.6%). Incidental records accounted for 38 observations (3.3%), including nine unique species (12.6%). Notably, 39 species (54.9%) documented in the Dugbe study area are West African endemics. The region-

Table 1. Amphibian species recorded during surveys in the Dugbe region, Liberia. IUCN status, number of observations, and West African endemism noted.

Taxon	Common name	IUCN	Observations	West African Endemic
Class: Amphibia				
Order: Anura				
Family: Arthroleptidae				
Arthroleptis poecilonotus complex	Mottled Squeaker complex	–	90	–
Astylosternus occidentalis	Western Night Frog	LC	8	Yes
Leptopelis macrotis	Large-eared Tree Frog	**NT**	2	Yes
Leptopelis occidentalis	Western Tree Frog	**NT**	2	Yes
Leptopelis spiritusnoctis	Ghostly Tree Frog	LC	4	Yes
Family: Bufonidae				
Sclerophrys maculata	Northern Flat-backed Toad	LC	50	No
Sclerophrys togoensis	Togo Toad	LC	4	Yes
Family: Conrauidae				
Conraua alleni	Allen's Giant Frog	LC	4	Yes
Family: Dicroglossidae				
Hoplobatrachus occipitalis	African Tiger Frog	LC	10	No
Family: Hyperoliidae				
Afrixalus dorsalis	Striped Spiny Reed Frog	LC	20	No
Afrixalus nigeriensis	Nigerian Spiny Reed Frog	LC	24	Yes
Hylambates boulengeri	Boulenger's Wot-wot	LC	20	Yes
Hyperolius chlorosteus	Large Green Reed Frog	LC	24	Yes
Hyperolius concolor	Uniform Reed Frog	LC	21	Yes
Hyperolius fusciventris	Dark-bellied Reed Frog	LC	45	Yes
Hyperolius guttulatus	Dotted Reed Frog	LC	12	No
Hyperolius picturatus	Variable Montane Reed Frog	LC	9	Yes
Hyperolius soror	Soror Reed Frog	LC	28	Yes
Family: Odontobatrachidae				
Odontobatrachus natator	Common Toothed Frog	LC	15	Yes
Family: Phrynobatrachidae				
Phrynobatrachus alleni complex	Allen's Puddle Frog complex	–	45	Yes
Phrynobatrachus fraterculus	Brother's Puddle Frog	LC	5	Yes
Phrynobatrachus guineensis	Guinea Puddle Frog	LC	10	Yes
Phrynobatrachus gutturosus	Guttural Puddle Frog	LC	7	Yes
Phrynobatrachus latifrons	Savanna Puddle Frog	LC	14	Yes
Phrynobatrachus liberiensis	Liberian Puddle Frog	LC	43	Yes
Phrynobatrachus phyllophilus	Leaf-loving Puddle Frog	LC	9	Yes
Phrynobatrachus plicatus	Ridged Puddle Frog	LC	7	Yes
Phrynobatrachus tokba	Tokba Puddle Frog	LC	221	Yes
Phrynobatrachus villiersi	Villiers' Puddle Frog	LC	9	Yes
Family: Pipidae				
Xenopus tropicalis	Tropical Clawed Frog	LC	88	Yes
Family: Ptychadenidae				
Ptychadena arnei	Schiøtz's Grass Frog	DD	5	Yes
Ptychadena cf. *bibroni*	Bibron's Grass Frog	LC	44	No
Ptychadena longirostris	Snouted Grass Frog	LC	23	Yes
Ptychadena pumilo	Western Dwarf Grass Frog	LC	13	No
Family: Pyxicephalidae				
Aubria subsigillata	Brown Fishing Frog	LC	4	No

(Continued)

Table 1. (Continued).

Taxon	Common name	IUCN	Observations	West African Endemic
Family: Ranidae				
Amnirana parva	Lesser White-lipped Frog	–	7	Yes
Family: Rhacophoridae				
Chiromantis rufescens	Western Foam-nest Frog	LC	24	No
Order: Gymnophiona				
Family: Dermophiidae				
Geotrypetes seraphini	Seraphin's Caecilian	LC	12	No

Table 2. Reptile species recorded during surveys in the Dugbe region, Liberia. IUCN status, number of observations, and West African Endemism noted.

Taxon	Common name	IUCN	Observations	West African Endemic
Class: Reptilia				
Order: Testudines				
Family: Testudinidae				
Kinixys erosa	Forest Hinge-back Tortoise	DD	1	No
Order: Squamata				
Family: Agamidae				
Agama africana	West African Agama	LC	50	Yes
Family: Atractaspididae				
Aparallactus modestus	Western Forest Centipede-eater	LC	1	No
Family: Boidae				
Calabaria reinhardtii	Calabar Ground Python	LC	1	No
Family: Chamaeleonidae				
Chamaeleo gracilis	Slender Chameleon	LC	1	No
Family: Colubridae				
Afronatrix anoscopus	African Brown Water Snake	LC	8	Yes
Dipsadoboa brevirostris	Short-headed Tree Snake	LC	1	Yes
Dipsadoboa underwoodi	Underwood's Tree Snake	LC	1	No
Hapsidophrys lineata	Black-lined Green Snake	LC	1	No
Hapsidophrys smaragdina	Emerald Snake	LC	1	No
Natriciteres variegata	Variable Marsh Snake	LC	1	No
Thelotornis kirtlandii	Forest Vine Snake	LC	2	No
Rhamnophis aethiopissa	Large-eyed Green Tree Snake	LC	1	No
Family: Elapidae				
Naja guineensis	Black Forest Cobra	NE	4	Yes
Pseudohaje nigra	Black Tree Cobra	LC	1	Yes
Family: Gekkonidae				
Hemidactylus fasciatus	Banded Leaf-toed Gecko	LC	3	No
Hemidactylus muriceus	Guinea Leaf-toed Gecko	LC	1	No
Family: Grayiidae				
Grayia smithii	Smith's African Water Snake	LC	2	No
Family: Lamprophiidae				
Bothrophthalmus lineatus	Red-black Striped Snake	LC	1	No
Gonionotophis klingi	Kling's File Snake	LC	1	Yes

(Continued)

Table 2. (Continued).

Taxon	Common name	IUCN	Observations	West African Endemic
Hormonotus modestus	Yellow Forest Snake	LC	1	No
Mehelya poensis	Equatorial File Snake	LC	1	No
Family: Scincidae				
Cophoscincopus durus	Keeled Water Skink	LC	9	Yes
Mochlus fernandi	Fernand's Fire Skink	LC	7	No
Trachylepis affinis	Senegal Skink	LC	29	No
Trachylepis maculilabris	Speckle-lipped Skink	LC	7	No
Trachylepis paucisquamis	Tropical Skink	LC	4	Yes
Family: Typhlopidae				
Afrotyphlops liberiensis	Guinea Blind Snake	NE	1	Yes
Family: Varanidae				
Varanus niloticus	Nile Monitor	LC	6	No
Family: Viperidae				
Atheris chlorechis	Green Bush Viper	LC	3	Yes
Bitis nasicornis	Rhinoceros Viper	NE	1	No
Bitis rhinoceros	West African Gaboon Viper	LC	1	Yes
Causus maculatus	Spotted Night Adder	LC	3	No

al distinctiveness is particularly pronounced among amphibians (28 species, 73.6% are endemic) compared to reptiles (11 species, 33.3%).

Sampling curves

The r/e curves (Fig. 5; Table 3) reveal incomplete sampling of overall herpetofauna diversity across the Dugbe study area. For combined herpetofauna, the model estimated total species richness at 116.09 (upper confidence limit: 160.53) compared to the 71 observed. Shannon diversity was 28.34 and predicted to be 29.78, while Simpson diversity was 15.30 and estimated to be 15.49. For amphibians, sampling completeness was high. The observed species richness of 38 and the estimated richness match (upper confidence limit: 40.61). This indicates that the amphibian diversity was adequately sampled. Shannon diversity was 20.26 and closely matched the estimated 20.66 and Simpson diversity was 11.91 and estimated to be 12.05 (Fig. 5; Table 3). In contrast, the species richness for reptiles of 33 was far less than the predicted species richness of 122.67 (confidence intervals: 33–256.05), indicating a significant sampling deficiency. The Shannon diversity of 12.69 was predicted to be 16.97, while Simpson diversity of 6.57 was estimated to be 6.81. There were a high proportion of reptile species being recorded only once, i.e. singletons (19 species; 57.57% of reptile records) (Fig. 5; Table 3).

IUCN species list comparison

When comparing the results of our Dugbe herpetofauna survey with the IUCN predictive distribution maps, we found notable discrepancies. Our survey documented 71 species, 63 of these were predicted by the IUCN predictive distribution maps. This represents 51.2% of the 123 species predicted by IUCN range maps for the region. Four species (*Afrixalus nigeriensis* Schiøtz, 1963; *Amnirana parva* (Griesbaum et al., 2023); *Hyperolius soror* (Chabanaud, 1921); *Leptopelis occidentalis* Schiøtz, 1967; and *Phrynobatrachus gutturosus* (Chabanaud, 1921)) are not predicted to occur in the study area by the IUCN, however they are predicted to occur nearby based on Channing and Rödel (2019) . Two reptile species (*Kinixys erosa* (Schweigger, 1812); and *Naja guineensis* Broadley et al., 2018) are not mapped by the IUCN. Considering our survey results in relation to the IUCN predicted list, we found a a moderate overall Jaccard similarity (48.09%) (Table 4).

Taxonomic groups showed differential patterns of congruence. For amphibians, 32 of the 38 observed species were predicted by IUCN (66.7% of 48 expected species). Amphibians showed a relatively high Jaccard Similarity Index (59.26%). In contrast, 31 of the 33 observed reptile species were predicted by the IUCN, representing just 41.3% of the 75 reptile species predicted by IUCN maps, with a correspondingly lower Jaccard similarity (40.26%) (Table 4).



Fig. 3. A) Seraphin's Caecilian (*Geotrypetes seraphini*); B) Mottled Squeaker complex (*Arthroleptis poecilonotus* complex); C) Western Night Frog (*Astylosternus occidentalis*); D) Large-eared Treefrog (*Leptopelis macrotis*); E) Western Treefrog (*Leptopelis occidentalis*); F) Ghostly Tree Frog (*Leptopelis spiritusnoctis*); G) Northern Flat-backed Toad Toad (*Sclerophrys maculata*); H) Togo Toad (*Sclerophrys togoensis*); I) Allen's Giant Frog (*Conraua alleni*); J) African Tiger Frog (*Hoplobatrachus occipitalis*); K) Brown Fishing Frog (*Aubria subsigillata*); L) Lesser White-lipped Frog (*Amnirana parva*); M) Schiøtz's Grass Frog (*Ptychadena arnei*); N) Bibron's Grass Frog (*Ptychadena* cf. *bibroni*); O) Snouted Grass Frog (*Ptychadena longirostris*); P) Western Dwarf Grass Frog (*Ptychadena pumilo*); Q) Striped Spiny Reed Frog (*Afrixalus dorsalis*); R) Nigerian Spiny Reed Frog (*Afrixalus nigeriensis*); S) Large Green Reed Frog (*Hyperolius chlorosteus*); T) Uniform Reed Frog (*Hyperolius concolor*); U) Dark-bellied Reed Frog (*Hyperolius fusciventris*); V) Dotted Reed Frog (*Hyperolius guttulatus*); W) Variable Montane Reed Frog (*Hyperolius picturatus*); X) Soror Reed Frog (*Hyperolius soror*); Y) Boulenger's Wot-wot (*Hylambates boulengeri*); Z) Allen's Puddle Frog complex (*Phrynobatrachus alleni* complex); AA) Brother's Puddle Frog (*Phrynobatrachus fraterculus*); AB) Guinea Puddle Frog (*Phrynobatrachus guineensis*); AC) Savanna Puddle Frog (*Phrynobatrachus latifrons*); AD) Liberia Puddle Frog (*Phrynobatrachus liberiensis*); AE) Leaf-loving Puddle Frog (*Phrynobatrachus phyllophilus*); AF) Ridged Puddle Frog (*Phrynobatrachus plicatus*). All photographs by Marius Burger and Ryan van Huyssteen.



Fig. 4. A) Tokba Puddle Frog (*Phrynobatrachus tokba*); B) Villiers' Puddle Frog (*Phrynobatrachus villiersi*); C) Allen's Puddle Frog complex (*Phrynobatrachus alleni* complex); D) Common Toothed Frog (*Odontobatrachus natator*); E) Tropical Clawed Frog (*Xenopus tropicalis*); F) Western Foam-nest Frog (*Chiromantis rufescens*); G) Banded Leaf-toed Gecko (*Hemidactylus fasciatus*); H) Guinea Leaf-toed Gecko (*Hemidactylus muriceus*); I) Keeled Water Skink (*Cophoscincopus durus*); J) Fire Skink (*Mochlus fernandi*); K) Senegal Skink (*Trachylepis affinis*); L) Speckle-lipped Skink (*Trachylepis maculilabris*); M) Tropical Skink (*Trachylepis paucisquamis*); N) West African Agama (*Agama africana*); O) Water Monitor (*Varanus niloticus*); P) Guinea Blind Snake (*Afrotyphlops liberiensis*); Q) Calabar Ground Python (*Calabaria reinhardtii*); R) Green Bush Viper (*Atheris chlorechis*); S) West African Gaboon Viper (*Bitis rhinoceros*); T) Spotted Night Adder (*Causus maculatus*); U) Western Forest Centipede-eater (*Aparallactus modestus*); V) Red-Black Striped Snake (*Bothrophthalmus lineatus*); W) Kling's File Snake (*Gonionotophis klingi*); X) Equatorial File Snake (*Mehelya poensis*); Y) Ugandan House Snake (*Hormonotus modestus*); Z) Black Forest Cobra (*Naja guineensis*); AA) Black Tree Cobra (*Pseudohaje nigra*); AB) African Brown Water Snake (*Afronatrix anoscopus*); AC) Short-headed Tree Snake (*Dipsadoboa brevirostris*); AD) Black-lined Green Snake (*Hapsidophrys lineata*); AE) Variable Marsh Snake (*Natriciteres variegata*); AF) Forest Vine Snake (*Thelotornis kirtlandii*). All photographs by Marius Burger and Ryan van Huyssteen.

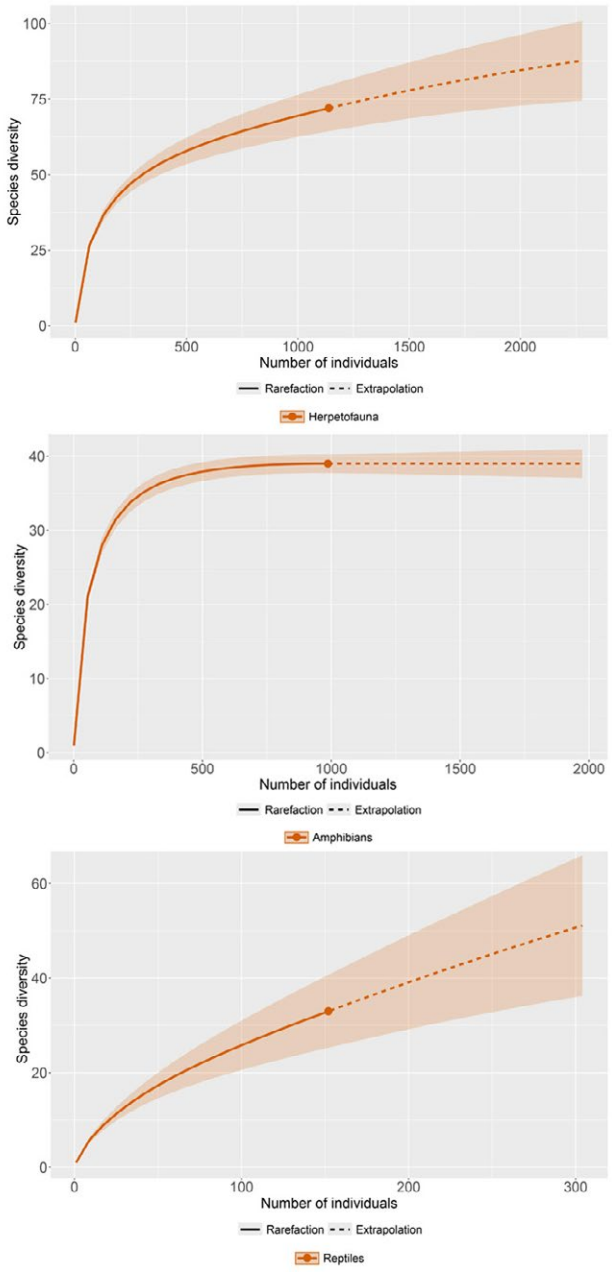


Fig. 5. The r/e sampling curves for all herpetofauna, amphibians and reptiles of the Dugbe region, solid line shows rarified survey effort and stippled line shows extrapolated results.

Comparison to benchmark

Our survey of the Dugbe region documented higher total herpetofauna species richness (71 species) compared to the KBPPA survey (48 species) and the Grebo National Forest survey (34 species). It is important to note that the two benchmark surveys employed different methods to our survey, lasted less days, and were both focused on amphibians rather than all herpetofauna. For amphibians, our survey recorded 38 species, compared to 34 species in the KBPPA survey and 29 species in the Grebo National Forest survey. Reptiles were recorded opportunistically by the benchmark studies, and therefore a pronounced difference can be observed in reptile diversity, where our survey documented 33 species, while the KBPPA and Grebo National Forest surveys recorded 14 and 5 species, respectively (Table 5).

Of the 48 species recorded in the KBPPA, 37 of these were also identified during our Dugbe survey, while there was overlap between 27 species found in Grebo. Together, the KBPPA and Grebo surveys documented 14 taxa which were not detected during our survey, including *Trionyx triunguis* (Forskål, 1775) and *Osteolaemus tetraspis* (Cope, 1861).

The comparisons between the benchmark studies are restricted to species richness as the focus and methods were different to ours. A comparative overview with the benchmark sites, KBPPA (Rödel and Glos, 2019) and Grebo National Forest (Hillers and Rödel, 2007), is available as a supplement which additionally serves as an evidence-based species list for the greater area (Supplementary material 2).

DISCUSSION

Our survey provides 1,145 herpetofauna observations comprising 71 species (38 amphibians and 33 reptiles) from a previously understudied region of Liberia. For all herpetofauna, 39 (54.9%) of the species recorded are endemic to West Africa. For frogs this endemism was 73.6% and for reptiles 33.33%. One of the most notable species in terms of endemism is *Odontobatrachus natator* (Boulenger, 1905) which belongs to West Africa's only endemic vertebrate family, the Odontobatrachidae (Barej et al., 2014). This high degree of endemism highlights the herpetological value of the study area, and the Upper Guinean forests in general (Myers et al., 2000; Ernst et al., 2025). Regional comparisons with nearby surveys (KBPPA and Grebo National Forest) contextualise our findings within the broader Upper Guinean forest landscape.

Four species of conservation concern were recorded: *Leptopelis macrotis* Schiøtz, 1967 (Near Threatened (NT)), *L. occidentalis* Schiøtz, 1967 (NT), *Bitis nasicornis* (Shaw & Nodder, 1792) (Vulnerable (VU)), and *Kinixys erosa* (Data Deficient (DD)). The *Leptopelis* species rely on primary forests and are threatened by human activities such as artisanal mining. *Bitis nasicornis* is affect-

Table 3. Diversity indices (Species Richness. Shannon Diversity, and Simpson Diversity) generated from r/e sampling curves.

Assemblage	Diversity	Observed	Estimator	s.e.	LCL	UCL
	Species richness	71.00	116.09	22.68	71.64	160.53
Herpetofauna	Shannon diversity	28.34	29.78	1.35	27.13	32.43
	Simpson diversity	15.30	15.49	1.07	13.38	17.60
	Species richness	38.00	38.00	1.33	38.00	40.61
Amphibians	Shannon diversity	20.26	20.66	0.74	19.21	22.11
	Simpson diversity	11.91	12.05	0.82	10.44	13.65
	Species richness	33.00	122.67	68.05	33.00	256.05
Reptiles	Shannon diversity	12.69	16.97	3.25	10.61	23.34
	Simpson diversity	6.57	6.81	1.11	4.64	8.98

Table 4. Jaccard Similarity between species richness (SR) recorded in the Dugbe field survey and IUCN predictive distributions for herpetofauna (total), amphibians, and reptiles. The observed richness includes eight taxa not mapped or predicted by the IUCN.

Metric	Herpetofauna	Amphibians	Reptiles
Dugbe SR	71	38	33
IUCN SR	123	48	75
Species in Common	63	32	31
Jaccard Similarity Index	0.48	0.59	0.40

Table 5. Species Richness Comparison of Dugbe survey to the KBPPA and Grebo NF surveys for all herpetofauna, amphibians, and reptiles.

Survey	Herps	Amphibians	Reptiles
Dugbe survey	71	38	33
Rödel & Glos 2019 (KBPPA)	48	34	14
Hillers & Rödel 2007 (Grebo NF)	34	29	5

ed by habitat loss and hunting pressure (Penner et al., 2021). It is important to note that the current conservation assessment for *K. erosa* is outdated, and preliminary assessments suggest it could be categorised as Endangered (EN) due to the impacts of deforestation and hunting (Luiselli and Diagne, 2014).

While sampling completeness metrics indicate comprehensive sampling for amphibians, reptile sampling was likely incomplete, with r/e curves skewed by a high proportion of singletons (60%). This difference in sampling success between amphibians and reptiles can be attributed to several factors: our surveys coincided with the wet season when amphibian breeding activity is at its peak. Amphibians are highly vocal during the breeding season and tend to aggregate at breeding sites, increasing encounter probability (Rödel and Ernst, 2004; Heyer et al., 2014). In terms of reptiles, while certain genera such as *Agama* and *Trachylepis* include relatively conspicuous species, reptiles are generally secretive, cryptic, and difficult to detect, particularly snakes (Durso and Seigel, 2015; Jordaan et al., 2021). For example, many reptile species exhibit secretive lifestyles (i.e., nocturnal, fossorial), shy behaviour, cryptic coloration, and are often sensitive to disturbances, all of which reduce detection probability during surveys. Additionally, high rainfall has been found to negatively correlate with reptile detectability in several studies (e.g., Eskew and Todd, 2017; Falaschi, 2021). The persistent heavy rainfall during our surveys likely contributed to the under sampling of reptiles in our study.

Based on morphology, species level identifications were problematic for some amphibian taxa, namely those in the *Arthroleptis poecilonotus* complex Peters, 1863, *Phrynobatrachus alleni* complex Parker, 1936, and several *Ptychadena* specimens remained unidentified. New taxa have been identified in these groups and are currently in the process of being described in the study area (Rödel et al., in prep.). Currently, without a clear taxonomic framework, undescribed species cannot be accurately classified under threat categories, impacting conservation efforts. There is a need for additional taxonomic studies on West African herpetofauna (Luiselli et al., 2019), particularly given the Upper Guinean forests' importance for amphibian diversification and the increasing anthropogenic pressures on these ecosystems (Penner et al., 2011, Rödel et al., 2021).

Our field survey documented fewer species than predicted by IUCN range maps, particularly for reptiles (33 observed vs. 75 predicted), compared to amphibians (39 observed vs. 48 predicted). Considering the limited survey duration, it is likely that many of the additional species predicted by the IUCN are present in the study area and were undetected. However, while IUCN interpreted distributions provide valuable tools for conservation planners and researchers, their accuracy varies significantly by region. In well-studied areas like South Africa

(Tolley et al. 2016), species ranges are well understood, resulting in highly reliable distribution data (Tolley et al., 2023). However, knowledge of herpetofauna distributions in Liberia remains largely in development. Therefore, it is possible that some of the IUCN distribution maps are inaccurate. Baseline surveys, such as ours, are required to inform and refine understanding of species distributions to improve the accuracy of future distribution maps.

Active searching yielded the most records (>80%) and approximately half of the unique species documented (47.8%). Passive trapping and incidental observations, accounted for only approximately 10% and 3% of records respectively, however, they contributed significantly to overall species richness by adding 5% and 11.5% of unique species. This highlights the importance of diversifying survey techniques to maximise detection during baseline surveys (Ribeiro-Júnior et al., 2008; McDiarmid et al., 2012).

The primary limitations of our survey were the climatic conditions and the challenges presented by taxonomic resolution. We recommend that additional future surveys in the region take place at the end of or at the start of the rain season, as that is when amphibian and reptile activity is at its greatest. To overcome taxonomic uncertainties, researchers should maintain collaborations with taxonomists specialising in West African herpetofauna, as this will improve future species inventories and our understanding of the region's herpetofaunal diversity.

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at https://oaj.fupress.net/index.php/ah/article/view/18279/15291.

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North of the wall: First record of *Salamandra atra aurorae* in the Sella Valley, South-eastern Alps

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Abstract. *Salamandra atra aurorae* is a rare and elusive subspecies endemic to a small area of the southeastern Italian Prealps, typically inhabiting mature mixed forests between 1200 and 1800 m a.s.l. Its detection is challenging due to cryptic behavior and dependence on favorable weather. Since its first record in Trentino in 2008, no new localities had been reported. In summer 2025, following a citizen report, field surveys confirmed a new occurrence in the Sella Valley, extending the known range of *S. a. aurorae* in Trentino. Two individuals were found at 1825 m and 1395 m a.s.l. New sites represent novel habitats for the subspecies: the uppermost on rocky scree and the lowest in a NNE-facing forest, both deviating from known preferences. Climatic niche analysis showed that these sites lie at the edge of the previously known climatic range, suggesting broader ecological tolerance. The detection of a gravid female at the forest site indicates the presence of a reproductive population, excluding animals in dispersal. The substantial topographical barriers separating the new sites from previously known localities suggest possible long-term isolation; however, the existence of narrow or discontinuous ecological corridors cannot be excluded. This discovery could have important conservation implications, as the detected individuals might be genetically distinct, warranting further genetic and phylogeographic studies. These findings highlight the need for targeted surveys and updated management strategies to protect this vulnerable taxon.

Keywords. Golden Alpine Salamander, endangered species, endemic taxon, distribution, climate niche, amphibian conservation.

The Golden Alpine Salamander, *Salamandra atra aurorae* (Trevisan, 1982), is a rare and elusive subspecies of Alpine Salamanders, endemic to a restricted area of the south-eastern Italian Prealps. It is considered as Endangered by the Italian IUCN assessment (Rondinini et al., 2022), and as a prioritary taxon by the "Habitat Directive" of the European Union. This subspecies inhabits forests between 1200 and 1800 m a.s.l. and has been found only in south-facing slopes (Beukema and Brakels, 2008; Romanazzi and Bonato, 2014). The typical habitat

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consists of mature mixed forests of silver fir and beech (Bonato and Fracasso, 2015; Romano et al., 2018), a pattern recently confirmed by species distribution models (Giachello et al., 2025). Habitat suitability depends on shelters, brushwood piles, and distance from open pasture (Romano et al., 2018), with dead wood also supporting key invertebrate prey (Centomo et al., 2023). This salamander is active from May to mid-October (Bonato et al., 2025), with surface activity closely linked to favorable meteorological conditions (Bonato and Fracasso, 1999; Bonato and Fracasso, 2015).

Its known distribution covers 31 km^2 on Sette Comuni and Vezzena plateaus (between Veneto region and the province of Trento, hereafter Trentino; Giachello et al., 2025). As reported for other Alpine Salamanders (Roner et al., 2022), *S. a. aurorae* is highly cryptic and difficult to detect under unsuitable weather conditions, even where density is high (Lefosse et al., 2016). As a result, knowledge of its fine-scale distribution remains incomplete.

In Trentino, the subspecies was first recorded in 2008, in an area extending from Sparavieri Valley to Postesina Valley (Beukema and Brakels, 2008), and further large-scale surveys on Vezzena plateau yielded no additional occurrences (Romano et al., 2018a). Here we report the first new locality records of *S. a. aurorae* in Trentino. We performed a multivariate analysis on the habitat and geographic characteristics to compare the newly identified sites with previously known records and evaluate their position within the established climatic niche of the subspecies.

In August 2025, we received a reliable, documented report including a photograph of *S. a. aurorae* in the Sella Valley, a small side valley located on the south-eastern edge of Trentino, reported by one of the authors (LD; Fig. 1). Of the three surveys carried out in the area in the following weeks, only one (28/08/2025), conducted under optimal conditions, was successful and led to the finding of a pregnant female in a second site. The coordinates of the two new sites were recorded using a Garmin GPSMAP 64s, with an accuracy of ±5 m.

To assess differences between the newly identified sites and those previously reported in the literature, we georeferenced records from Romanazzi and Bonato (2014) using a 1:25.000 IGM map (MASE, 2025) to locate the cited localities and the TINITALY Digital Elevation Model (Tarquini et al., 2007). Each occurrence was positioned at the reported locality and elevation and when multiple elevations were indicated for the same locality, they were treated as separate records. All these points spatially overlap with the occurrence areas shown in the original map from Romanazzi and Bonato (2014), which we digitized and georeferenced in QGIS. We sub-

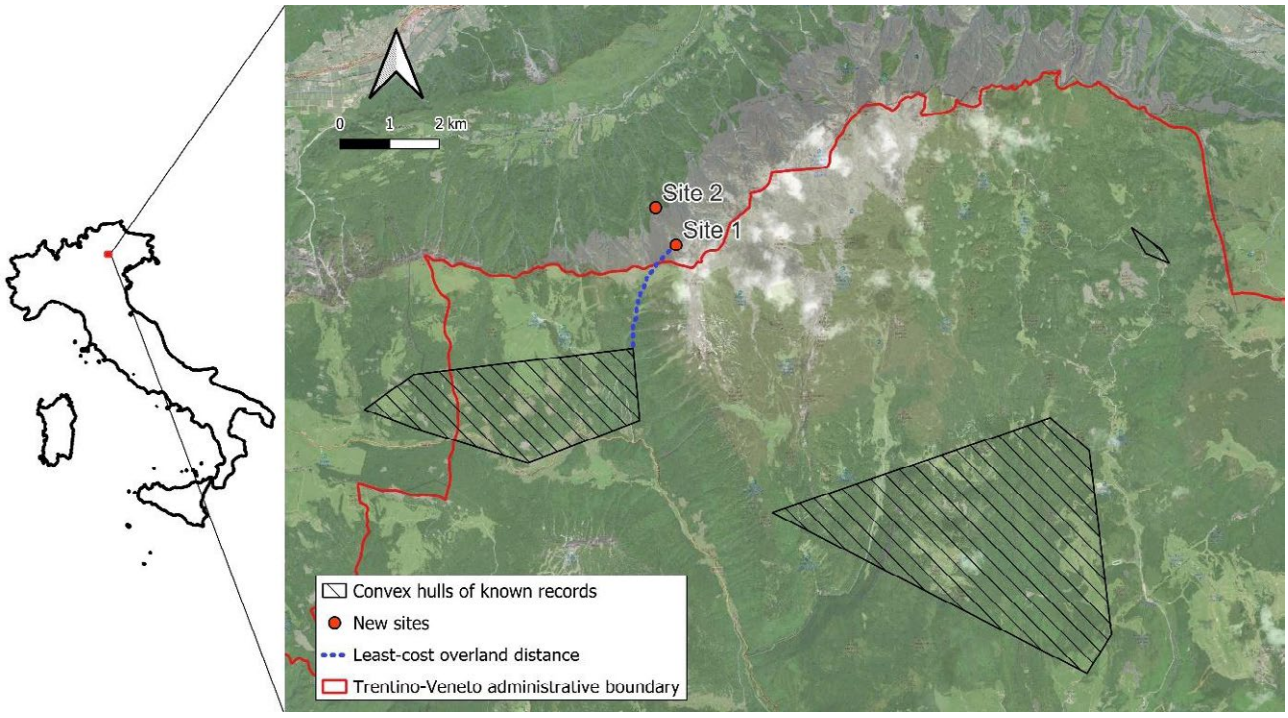


Fig. 1. Distribution map of *Salamandra atra aurorae*. Black polygons: literature sites; Red dots: new sites; Blue spotted line: least-cost overland distance; Red line: Trentino-Veneto boundary. Base map: Esri satellite imagery.

sequently added 17 representative presence localities from the Veneto region, provided directly by the authors of Giachello et al. (2025) together with presence data collected in Trentino during the monitoring conducted by MUSE – Science Museum between 2017 and 2025 (partially reported in Romano et al., 2018a). We used QGIS (v. 3.40.40) and GRASS GIS (v. 2.12.99) to calculate i) the elevation and the geographic aspect of the new sites, ii) the minimum Euclidean and least-cost overland distance between the new sites and the nearest previously known iii) the positive and negative elevation gain and the minimum pass elevation along the shortest overland distance. These metrics were calculated to provide a conservative estimate of the minimum topographical separation between new sites and nearest previously known localities. To characterize the habitat and forest composition around the new sites, we used a 40 meters buffer, considering the maximum known home range of the subspecies (mean 7.8 m; Bonato and Fracasso, 2003). Subsequently, we classified and quantified the habitat and forest types within the buffer based on a land use map of European Alps for the first (Marsoner et al., 2023), and a regional forest type layer for the second (PAT, 2021).

To evaluate the climatic position of the new sites relative to known localities of *S. a. aurorae*, we obtained fine-grained climate data for each occurrence location (n = 77). Climate data are usually available at a coarse spatial scale (e.g., 1 km grid), which is too broad to assess differences in micro-endemic species. To overcome this issue, we downscaled 19 bioclimatic variables (Karger et al., 2017) using the ClimateDT downscaling service (Marchi et al., 2024), which performs point-level downscaling of climatic variables and indices, averaging the values of the 19 bioclimatic variables over the last 20 years. Although downscaled data represent an approximation of true fine-scale conditions, which remain unknown and may differ from the values obtained by downscaling, this approach allows a finer spatial resolution than the raw data at 1 km resolution, and has been recognized as a successful technique for several taxa (Lenoir et al., 2017; King et al., 2025), including amphibians (Stickley and Fraterrigo, 2023; Costa et al., 2025).

To control for spatial autocorrelation and to reduce potential biases arising from uneven regional samples' availability (e.g., fewer records in Veneto more in Trentino) and spatially clustered occurrence data (Borcard et al., 2004; Legendre and Legendre, 2012; Borcard et al., 2018), we implemented a Principal Component Analysis (PCA) based on Moran's Eigenvector Maps (dbMEM; Borcard and Legendre, 2002; Dray et al., 2006), thereby reducing the risk that spatial clustering biases subsequent climatic niche analyses. Specifically, we first computed the residuals of a multivariate regression of the 19 bioclimatic variables on the dbMEM, derived from the geographic coordinates of the sites, using function '*dbmem*' in '*adespatial*' R package (Dray et al., 2018). These residuals, representing the spatially filtered climate variation, were then subjected to a standardized PCA, from which we extracted the scores for the first three principal components, which together explained 95.1% of the variance (PC1 = 77.3%; PC2 = 13.0%; PC3 = 4.8%). To evaluate if the new records displayed different climatic conditions from previously known locations, we calculated the climatic centroid of the 75 known sites representing the central tendency of the subspecies' realized niche in multivariate climatic space as the mean of the PCA scores (PC1–PC3). We then calculated the Mahalanobis distance, measuring how far each site is from the niche centroid, including the two new occurrences (Etherington, 2021), and compared these distances to those of known sites to assess whether the new sites represent novel climatic conditions.

Along the Sella Valley, two new occurrence sites were identified at 1825 m (Site 1) and 1395 m a.s.l. (Site 2) (Fig. 1). The uppermost site lies on a rocky scree, an atypical environment for the species, while the second site is situated within a typical forested habitat. These two sites are 600 meters apart in a straight line (± 10 m) and differ in altitude by 450 meters.

The aspect of Site 1 is west (258°), whereas Site 2 lies on a north-northeast-facing slope (21°). The nearest previously known site to those we report from the Sella Valley is located in Renzola Valley (Veneto region), at 1450 m a.s.l. (Fig. 1). The minimum Euclidean distance is 2260 m, whereas the least-cost overland distance is 2435 m (± 200 m). In the latter case, the positive and negative elevation gain are 595 m and 225 m, with a minimum pass elevation of 2045 m a.s.l. The habitat within a 40 m buffer, based on a land-use map of the European Alps, is composed as follows: i) Site 1 – mixed tree cover (57%), scrub and shrubland (24%), and managed grasslands/pastures (19%); ii) Site 2 – broadleaf tree cover (60-100% cover class; 83% of the buffer) and coniferous tree cover (60-100% class; 17% of the buffer). Within the same buffer, the forest types are: i) Site 1 – mountain pine and rhododendron scrub; ii) Site 2 – calcicolous silver fir-beech forest, mountain pine and rhododendron scrub.

Regarding the analysis of the subspecies' climatic niche, visual inspection of the PCA scatterplots (Fig. 2) indicates that the newly discovered sites are located toward the periphery of the previously characterized climatic space. The climatic centroid calculated from the 75 previously known sites was used as a reference representing the core niche of *S. a. aurorae*. Relative to

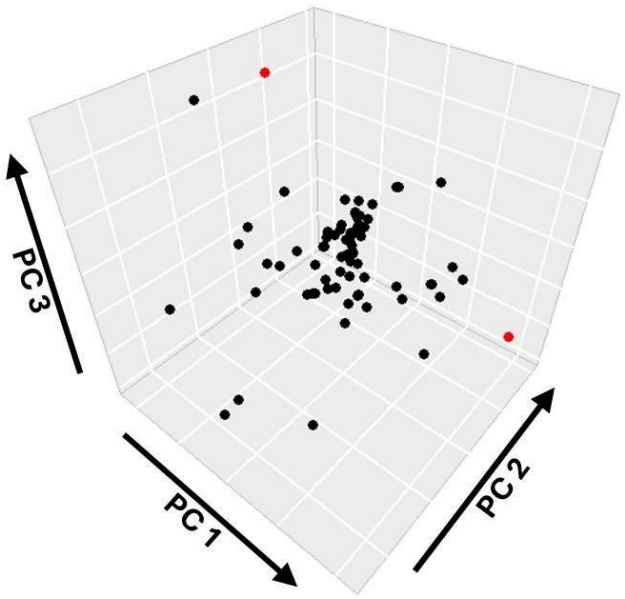


Fig. 2. 3D PCA ordination of the first three axes. Black dots: known occurrences; red dots: newly discovered occurrences.

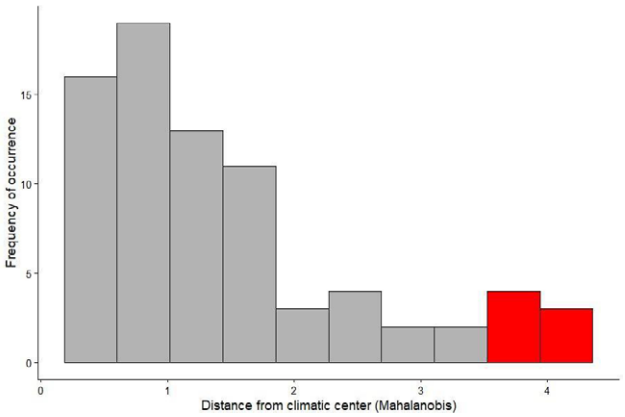


Fig. 3. Histogram of the Mahalanobis distances between each occurrence point and the climatic center of the subspecies based on 75 previous records. Bins including newly discovered occurrence locations are represented in red.

this centroid, the new sites exhibited relatively elevated Mahalanobis distances compared to the denser central cluster of occurrences, placing them within the subset of sites associated with more peripheral climatic conditions, although they do not represent the most extreme values observed (Fig. 3). Site 1, located at high elevation (1825 m), is slightly above the maximum elevation reported for this subspecies (1800 m a.s.l.; Bonato et al., 2025). Located on a rocky scree, it differs substantially from the typical habitat of the subspecies, dominated by mature mixed forests of silver fir and beech. The habitat of Site 1 resembles the environments occupied by *S. a. atra* and *S. a. pasubiensis*. Despite the vicinity to the nearest known site (Renzola Valley), steep topography and large elevation differences likely limits recent dispersal of *S. a. aurorae*, given its limited mobility and small home range (Bonato and Fracasso, 2003). Site 2 lies in optimal forest habitat, with the gravid female suggesting the existence of a reproductive population, and therefore a wider distribution in the Sella Valley than currently known.

The occurrence of the salamander in climatic conditions positioned toward the periphery of the previously characterized niche, though not representing the most extreme environments observed, suggests two non-mutually exclusive scenarios: (i) the presence of a marginal or partially isolated population occupying peripheral environmental conditions; or (ii) a population largely continuous with others, with the apparent geographic gaps reflecting false negatives due to low detectability rather than true absence.

From a conservation perspective, these findings are noteworthy. Given the extremely low genetic diversity reported for *S. a. aurorae* (Bonato et al., 2018), the newly detected individuals may represent an important resource for future conservation projects. Genetic analyses will therefore be essential to clarify if they represent a distinct lineage, their phylogenetic origin, and genetic relationships with neighbouring populations.

Moreover, the occurrence of a site in clearly atypical habitat expands current knowledge of the subspecies' ecological requirements and highlights the need to reconsider survey strategies, especially in peripheral or topographically complex regions. Overall, these findings suggest that the distribution of *S. a. aurorae* is likely fragmented and more widespread than previously documented, underscoring the need for targeted surveys in areas previously considered unsuitable.

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Validation of the post-hoc method to estimate snout-vent length in the order Caudata

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Abstract. Amphibians are the most endangered class of vertebrates, with a high rate of decline recorded since the 20th century. Even activities related to the study of these animals, for instance, handling them to collect individual biometric parameters, can have negative effects on amphibian health. A post hoc method for estimating snout-vent length from dorsal photographs has been developed to reduce handling time and stress for individuals, thereby improving precision and repeatability of measurements. However, to date, this methodology has been tested on only approximately 1% of known salamanders, thereby limiting its broad applicability. Here, we tested this method on a diverse sample of Caudata comprising 25 species across 5 families, characterized by diverse morphologies. The correlation between predicted SVL (estimated from dorsal photographs) and observed SVL (measured directly from ventral photographs) values was assessed using Linear Mixed Models. The results showed a significant correlation between observed and predicted SVL, with an average and constant discrepancy of approximately 1.6 mm. When considering the increase of SVL, there was a slight tendency to underestimate SVLe in newts, plethodontids, and proteids. Estimation errors slightly increased with the SVL. The error increased in larger newts, while decreased in larger plethodontids. Our study highlighted the reliability and applicability of adopting this methodology for data collection in all Caudata species.

Keywords. SVL, measure, post-hoc method, salamander, Urodela, photograph, dorsal.

Amphibians are the most endangered class of vertebrates, with a dramatically increased rate of decline recorded over recent decades (Blaustein et al., 1994; Houlahan et al., 2000; Stuart et al., 2004). Due to their physical and physiological sensitivity, even a well-intentioned human activity like handling them for research purposes can have negative effects on amphibians' health (Huber et al., 2019). Handling individuals is often essential in field data collection, allowing researchers to gather biometric parameters from captured individuals. However, if precautions are not taken, handling amphibians can facilitate the direct transfer of pathogens (Mendez et al., 2008; Phillott et al., 2010). A less appreciated, yet concerning effect of handling is the induction of high stress in wildlife (Huber et al., 2019). For example, individual handling can cause high stress that might contribute to altering their behaviour (Bliley and Woodley, 2012; Woodley and Porter, 2015) and physiology (Mostl and Palme, 2002; Caipang et al., 2014), having important cascading negative effects on their biology and consistently increasing their susceptibility to environmental threats (Bliley and Woodley, 2012; Woodley and Porter,

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2015; Karaer et al., 2023). Even brief manipulations can significantly alter their internal temperature (Lunghi et al., 2016) and compromise their immune defences, which increases their exposure to highly virulent pathogens (Raffel et al., 2006, Gabor et al., 2015).

Measuring amphibians in the field is not always trivial: their wet, slippery skin significantly reduces grip during handling, and this effect is further enhanced when individuals struggle (Lunghi et al., 2022). This can prolong the handling time and reduce measurement precision. Some approaches have been developed to potentially address these issues (Barzaghi et al., 2025). Tools like the "Mander Masher", the "Salamander stick" and the "Modified Salamander stick" helped researchers to increase the precision of recorded data (Wise and Buchanan, 1992; Walston and Mullin, 2005; Margenau et al., 2018); however, no concerns were raised about the possibility of also reducing individual stress. In recent years, the use of digital images has significantly improved data quality and animal safety (Mott et al., 2010; Speybroeck and Steenhoudt, 2017; Lunghi et al., 2020a). An example is the post-hoc estimation of the length, that involves using a digital camera to take photographs of individuals in the field, which are then processed in the lab to extrapolate multiple measurements using a standard reference (Lowe and McPeek, 2012; Lunghi et al., 2021; Cialente et al., 2025). With this technique, animal handling is limited to positioning the individual on a specific surface, with no further contact (Lunghi et al., 2021). This not only reduces handling time and the stress placed on individuals (Lunghi et al., 2016) but also lowers measurement error rate, enabling the collection of endless measurements for each individual (Lunghi et al., 2020a). The reduction of these issues is achieved because high-quality photos, even when taken dorsally, allow estimation of an important parameter hidden from view, for example the snout-vent length (SVL) (Mott et al., 2010; Lunghi et al., 2020a; Lunghi et al., 2022). The reliability of the post-hoc method to estimate SVL has been tested only on approximately 1% of the salamander species known to science (Mott et al., 2010; Lowe and McPeek, 2012; Lunghi et al., 2020a; Lunghi et al., 2022), and it requires further validation to be widely employed in studies on Caudata.

The aim of this study was to evaluate the reliability of the SVL post-hoc estimation on different species of Caudata, giving particular emphasis on testing the methodology on morphologically distinct groups of species. We constructed a dataset of high-quality photographs including 569 individuals belonging to 25 different species (on average, 22.76 ± 13.58 individuals per species). We identified five functional groups based on the general body shape of individuals: Amphiumids/Sirenids (including the genus *Amphiuma* and *Siren*), Newts (genus *Ichthyosaura*, *Lissotriton*, *Notophthalmus*, *Triturus*), Plethodontids (genus *Aneides*, *Desmognathus*, *Eurycea*, *Gyrinophilus*, *Plethodon*, *Pseudotriton*, *Stereochilus*), Salamanders (genus *Ambystoma* and *Salamandrina*), Proteids (genus *Necturus*). The photographs were obtained with the procedure described in (Lunghi et al., 2020b); captured individuals were placed next to a reference scale on a white flat horizontal surface and dorsal photographs were taken perpendicularly (Fig. 1A). Ventral photographs of the same individuals were also taken following the same procedure (Fig. 1B). Measurements were taken to the nearest mm using the program ImageJ (Troscianko and Stevens, 2015). First, SVL was measured by a single operator for each individual using a photograph of the ventral aspect. Then, for the same individuals, four operators estimated the SVL from the dorsal photographs (hereafter, SVLe), obtaining a repetition of four measurements for each individual. The landmark used to measure SVL is the cloaca, located between the hindlimbs and the tail. This area is demarcated in almost all salamander species by a conical frustum shape from a dorsal view, that narrows from the hind legs to the tail base (Fig. 1C). The posterior opening of the cloaca is placed among an imaginary line that delimits the end of the frustum from the base of the tail (Fig. 1C). In the genus *Salamandrina* additional landmarks can help to locate the cloaca. Vertebrae and ribs are well visible from the dorsal view, and the posterior opening of the cloaca corresponds perfectly to the intervertebral sulcus after the third caudosacral vertebra and ribs (Fig. 1D). In some species belonging to the Plethodontidae family (e.g., *Pseudotriton spp.*, *Gyrinophilus spp.*) the shape of the frustum is not always visible, due to the width of their robust tail. In these cases, the third fold behind the hindlimbs can be used as landmark to establish the correct position of the posterior end of the cloaca (Lunghi et al., 2020a) (Fig. 1E). Dorsal photographs were provided to the operators without any additional information to ensure unbiased measurements (MacCoun and Perlmutter, 2015).

We used Linear Mixed Models (LMMs) (package nlme; Pinheiro et al., 2016; R Development Core Team, 2023) to assess the potential relationship between SVL and SVLe. The SVLe was used as the dependent variable, while the mean-centered SVL was used as an independent variable. We added the morpho-group of the analysed species (hereafter: group) as a further independent variable to evaluate whether this trait may affect the precision of SVL estimation. The interaction between SVL and groups was added as an additional factor. The identity of operators and the species were assigned as random vari-

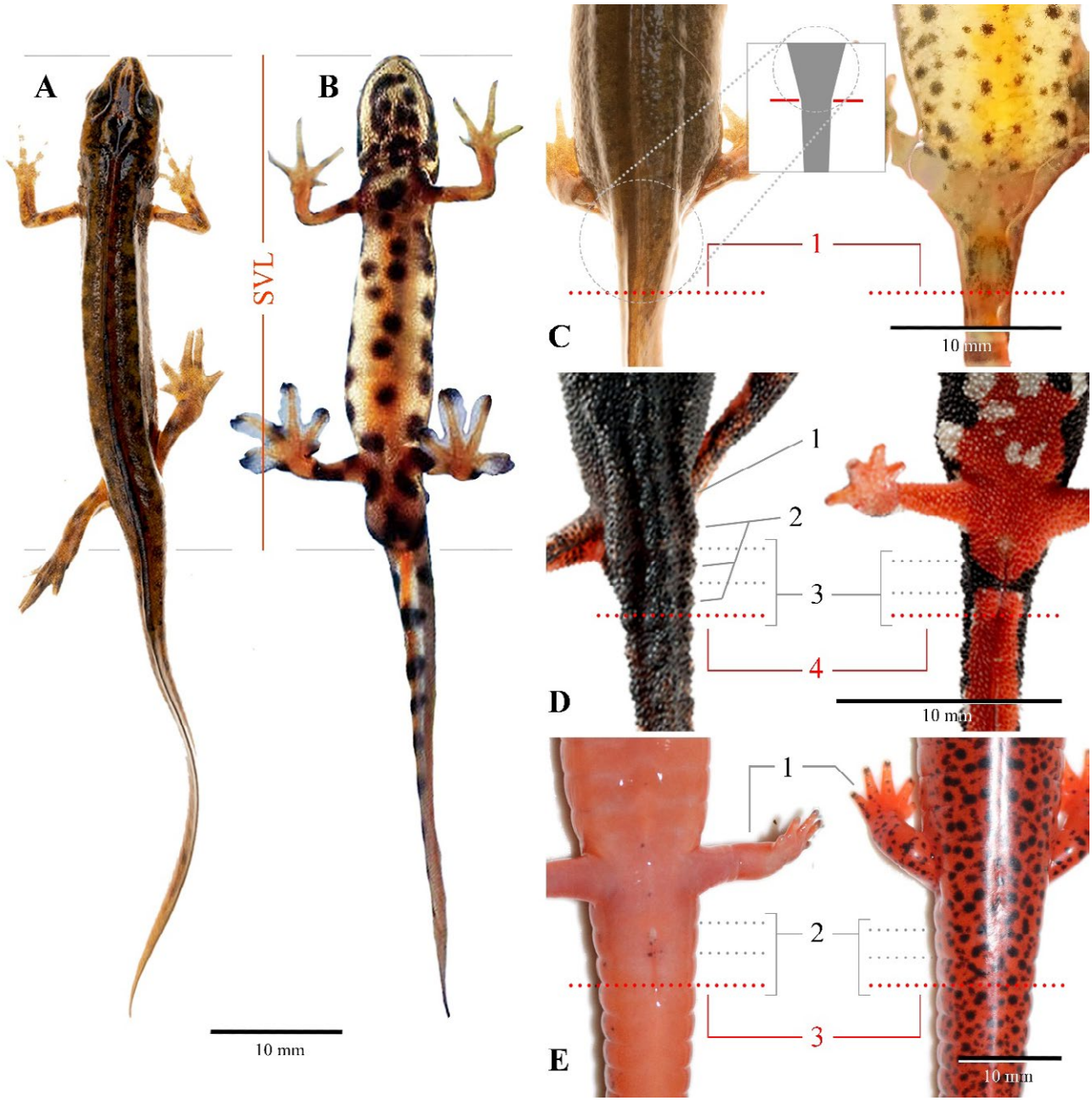


Fig. 1. An example of dorsal (A) and ventral (B) photographs used in this study to correlate predicted and observed snout-vent lengths (SVLe and SVL). In the photographs, the top and bottom ends are indicated to estimate (A) and measure (B) the SVL in a male of *Lissotriton vulgaris*. Methods of identification of the posterior cloacal edge in different Caudata groups. C) Landmark based on the frustum shape placed on *Lissotriton vulgaris*; 1. smaller base of the frustum / posterior end of the vent. D) Landmarks based on intervertebral sulci applied on *Salamandrina terdigitata*; 1. ilium and sacral rib; 2. caudosacral ribs (I, II, II); 3. Intervertebral sulci; 4. III sulcus / posterior end of the vent. E) Landmarks based on skin folds applied on *Pseudotriton ruber*; 1. Hindlimbs; 2. folds (I, II, III); 3. III fold / posterior end of the vent.

ables. Considering the possibility of non-constant variance of the error associated with both predictors, we fitted an extended model using a combination of a power variance function (SVL, continuous variable) and a variance identity structure (group, categorical variable). The comparison between the four models was performed using a likelihood ratio test (Lewis et al., 2011) and the Akaike information criteria (AIC). Likelihood ratio tests were

Table 1. Results of the likelihood ratio test comparing different models. The best model with the lowest AIC is in bold. Basic: predicted SVLe (dependent variable), observed SVL and groups (independent variables), species and operator identity (random variables). Full: addition of the power variance function (var_SVL) and the variance identity structure (var_Group). The last two models only include one of these functions that considers heteroscedasticity in the error distribution.

Model	df	AIC	BIC	logLik	Test	L.Ratio	p-value
basic	13	8993.433	9067.869	-4483.717			
full	**18**	**8144.837**	**8247.901**	**-4054.418**	**1 vs 2**	**858.597**	**<0.001**
var_SVL	14	8456.582	8536.743	-4214.291	2 vs 3	319.745	<0.001
var_Group	17	8493.228	8590567	-4229.614	3 vs 4	30.646	<0.001

also used to evaluate the significance of the best model terms. Additionally, marginal (R^2_m) and conditional (R^2_c) coefficients of determination were calculated (Bartoń, 2016). We then calculated the standardized squared residuals to explore the potential effects of observed SVL and groups. We performed an additional LMM to assess the model's goodness of fit and to examine the variability of squared residuals in relation to the fixed variables (SVL and groups). In this case, we used the squared residuals as the dependent variable, while the other fixed and random variables remained constant (see above: full model).

The results of the model comparison are shown in Table 1. The full model, including the power variance function and the variance identity structure, showed the lowest AIC (8144.837), suggesting that including heteroscedasticity significantly improved the model fit. We identified a significant correlation between the estimated (SVLe) and the centered SVL ($F_{1, 2171}$ = 272520.50, P < 0.001), while no effect was observed for the group ($F_{4, 20}$ = 1.76, P = 0.177) (Table 2). A significant effect was observed for the interaction between the SVL and the group ($F_{4,2171}$ = 11.25, P < 0.001). The slope of the regression line was approximately 1.00 (β = 1.003, P < 0.001), indicating that operators' estimates closely resembled the real SVL. However, we observed significant variability in the estimation accuracy between the studied groups of amphibians. In three of them, newts (β = –0.038, P = 0.018), plethodontids (β = –0.029, P < 0.001), and proteids (β = –0.038, P < 0.001), there was a slight tendency to underestimate body length in larger individuals (Fig. 2A). This pattern was not significant in Salamanders (β = –0.016, P = 0.171). Residual variability slightly increases with increasing body size (0.82). Considering the residual variability among groups, there was high heterogeneity in the estimation of SVL for newts (1.32), while for plethodontids the estimations were more consistent (0.68). The model explained the overall variance in the dependent variables accounting for 99.83% of the variance (R^2_m = 0.998). The inclusion of random effects further increased the overall model explanation to 99.99% (R^2_c = 0.999). The coefficient for SVL (0.978) indicates that the estimation of SVL was highly reliable, with a slight underestimation of the real values. The discrepancy between observed and predicted SVL (RMSE) was ~ 1.6 mm. The squared residuals were significantly correlated to SVL ($F_{1, 2171}$ = 58.78, P < 0.001) and to the interaction between SVL and group ($F_{4,21791}$ = 11.35, P < 0.001); no effect of the single variable group was observed ($F_{4, 20}$ = 2.47, P = 0.078). The estimation error generally increased in larger individuals (β = 0.087, P < 0.001). However, we observed specific patterns in two groups: when SVL increased, the error increased more quickly in newts compared to other groups (β = 0.124, P = 0.021), while the opposite occurred in plethodontids (β = -0.07, P = 0.004) (Fig. 2B). Residual error increases markedly with body size (1.6) and, among groups, newts showed the highest estimation error heterogeneity (2.30). The model explained a large proportion of the overall variance in the dependent variables (88.96%, R^2m = 0.889), and this explained variance increased to 99.99% (R^2_c = 0.999) after the inclusion of random effects.

Our study demonstrated the high reliability of this method for measuring SVL from dorsal photographs in Caudata species. Ventral SVL values were generally well retrieved by our dorsal measurements, regardless of the species analysed or the operator performing the measurements. The inclusion of morphologically distinct amphibian groups allowed us to test the robustness of this method across species and families characterized by different body shapes and sizes. Notably, the average estimated error remained remarkably low (1.6 mm), indicating a high level of measurement precision of this method as previously reported (Lunghi et al., 2020a). Some differences in accuracy were detected between groups. A slight increase in error estimation was observed in large newts, whereas higher precision was observed in larger plethodontids. However, these variations were minor and did not compromise the overall reliability of the approach.

Our study demonstrates that this method can be widely adopted to estimate SVL across all Caudata species. This can be particularly important in long-term monitoring, where populations are sampled repeatedly

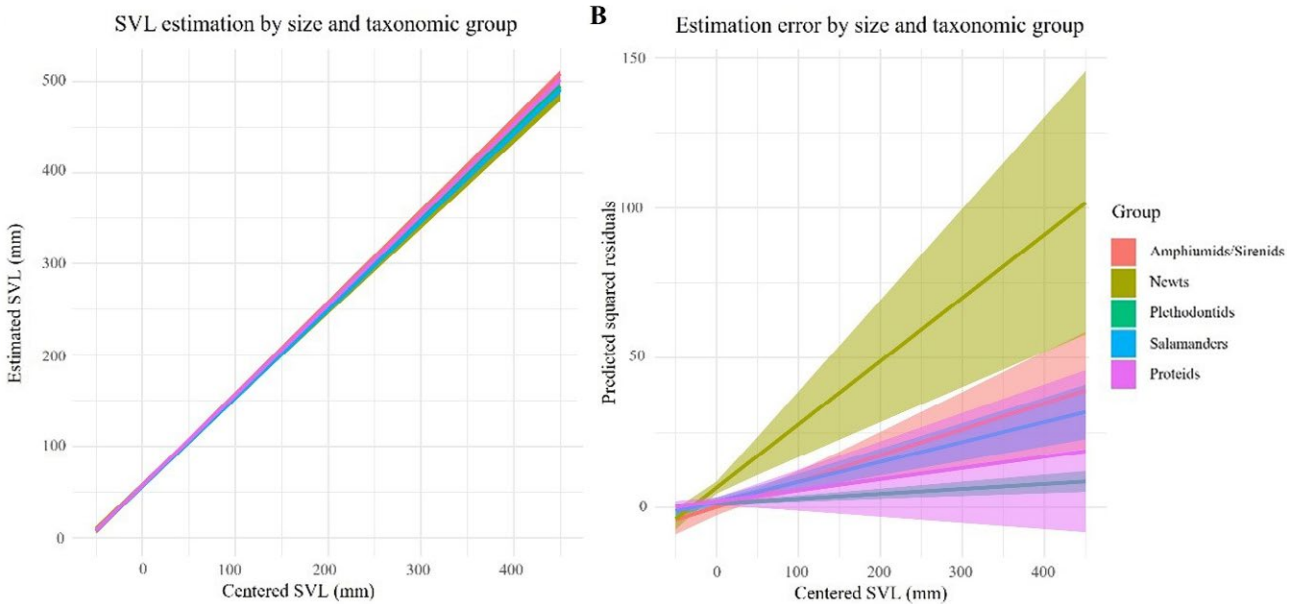


Fig. 2. Plots showing the LMM results for the correlation of A) estimated snout-vent length (SVLe) and B) Estimated error with the mean-centered SVL.

Table 2. Results of LMMs analysis performed on the best AIC model related to A) estimated SVLe, and B) distribution of error estimations. Significant factors are in bold.

	Value	Std.Error	DF	t-value	p-value
A) Model related to SVLe					
(Intercept)	56.710	0.828	2171	68.510	< 0.001
centered_SVL	1.003	0.004	2171	228.280	**< 0.001**
Group_Newts	1.274	1.038	20	1.230	0.234
Group_Plethodontids	-0.165	0.912	20	-0.181	0.858
Group_Proteids	-0.206	1.134	20	-0.181	0.858
Group_Salamanders	0.642	1.006	20	0.638	0.530
centered_SVL*Group_Newts	-0.038	0.016	2171	-2.360	**0.018**
centered_SVL*Group_Plethodontids	-0.029	0.005	2171	-5.663	**< 0.001**
centered_SVL*Group_Proteids	-0.038	0.006	2171	-6.188	**< 0.001**
centered_SVL*Group_Salamanders	-0.016	0.011	2171	-1.367	0.171
B) Model related to error estimations					
(Intercept)	0.208	1.392	2171	0.149	0.881
centered_SVL	0.087	0.024	2171	3.592	**< 0.001**
Group_Newts	6.581	1.738	20	3.787	**0.001**
Group_Plethodontids	1.295	1.432	20	0.904	0.377
Group_Proteids	1.869	1.591	20	1.174	0.254
Group_Salamanders	1.962	1.570	20	1.250	0.226
centered_SVL*Group_Newts	0.124	0.054	2171	2.310	**0.021**
centered_SVL*Group_Plethodontids	-0.070	0.024	2171	-2.880	**0.004**
centered_SVL*Group_Proteids	-0.020	0.026	2171	-0.778	0.437
centered_SVL*Group_Salamanders	-0.049	0.038	2171	-1.299	0.194

over extended periods, and where excessive, repeated handling could impose significant stress and adverse effects on population health. Another important feature of this method is its ease of application, especially in field data collection. It enables enhancing the contribution of citizen science by providing useful data using only a camera and a scale reference (e.g., a ruler). However, photos must be taken using appropriate precautions and methods (e.g., perpendicular framing, sufficient lighting) to obtain material of sufficient quality. Furthermore, attention must be paid to photographing only animals that are still and relaxed to obtain the best subjects for post-hoc measurements. Data obtained from species not included in our dataset (e.g., Asiatic species) can be used to further test its reliability and broad applicability. By using this approach, we have not only provided researchers with a tool to enhance data collection quality but also identified a method that likely reduces stress on salamanders collected during field studies.

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First record of attempted predation on the Scaled Dove (*Columbina squammata*) by the Red-tailed Boa (*Boa constrictor*) in a Restinga habitat of Northeastern Brazil

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Abstract. We report the first documented case of attempted predation on the scaled dove (*Columbina squammata*) by the Red-tailed Boa (*Boa constrictor*) in a restinga environment on the north coast of Paraíba, Brazil. This record contributes to the growing knowledge of the species' diverse diet and highlights the importance of opportunistic and arboreal foraging strategies in snake–bird interactions.

Keywords. Snake–bird interactions, feeding ecology, opportunistic predation.

The Red-tailed Boa (*Boa constrictor* Linnaeus, 1758) is a large-bodied member of the family Boidae, distributed in areas of the Neotropical region, specifically in South America. It occurs in Brazil, Argentina, Venezuela, Bolivia, Ecuador, French Guiana, Guyana, Paraguay, Peru, Suriname, and Trinidad and Tobago. In Brazil, it inhabits biomes such as the Amazon, Atlantic Forest, Cerrado, and Caatinga (Nogueira et al., 2019). It is a generalist predator, active both diurnally and nocturnally, with semi-arboreal habits and a constriction-based killing method. Its diet includes a broad array of vertebrates – birds, reptiles, amphibians, and small mammals – making it one of the most ecologically versatile snakes in the Americas (Marques et al., 2019; Henderson, 2023). Although the feeding breadth of *B. constrictor* is well established (Henderson, 2023), records of predation involving certain bird species have been frequently documented over time. Documenting new prey species is important to refine our understanding of the species' ecological role, especially in coastal ecosystems such as restingas, which are characterized by a mosaic of vegetation types and unique faunal interactions (Marques et al., 2015).

The scaled dove (*Columbina squammata* Lesson, 1831) is a small columbid bird with predominantly terrestrial foraging behavior, feeding on seeds and insects (León-Lleras, 2021). It occurs widely across South America and parts of the Caribbean (Estela et al., 2005). Despite its broad range, published records of its predation are limited to only two cases: by the green vine snake (*Oxybelis fulgidus*) (Miranda et al., 2013) and the red-legged seriema (*Cariama cristata*) (Moreira, 2017). Here, we document the first case of attempted predation on *C. squammata* by *B. constrictor*.

On 22 February 2024, at 15:26 h, we observed a juvenile *B. constrictor* (estimated total length: ~1.2 m) fall from a height of approximately 4 m from a black plum

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(*Syzygium cumini* (L.) Skeels) tree while constricting an adult *C. squammata*. The observation took place in the Barra do Rio Mamanguape Environmental Protection Area, North Coast of Paraíba, Brazil (6°46'2.32"S, 34°55'20.35"W; elevation: 7 m). The fall interrupted the constriction process, and during our photographic documentation, the snake released its prey and retreated into the nearby vegetation; however, it did not return to continue the interaction during the observation period. Although other *Columbina* species such as *C. minuta* and *C. talpacoti* have been reported in the diet of *B. constrictor* (Henderson, 2023), this is the first confirmed record of attempted predation on *C. squammata*. As an opportunistic ambush predator (Greene, 1997; Barbosa et al., 2022), *B. constrictor* is known to select ambush sites that maximize prey encounter rates, often near fruiting trees visited by birds (Rocha-Santos et al., 2014). While *C. squammata* is not reported to feed on *S. cumini* fruits, the tree may provide both shelter and vantage points for hunting. This observation aligns with studies showing that arboreal snakes, including boids, use perches in fruiting trees to exploit the predictable presence of avian prey (Lillywhite and Henderson, 1993; Shine and Li-Xin, 2002).

Our record underscores the importance of natural history observations in filling knowledge gaps about snake diets, even for well-studied species such as *B. constrictor*, which already has over 75 prey species documented (Henderson, 2023). Expanding the known prey list contributes to understanding the species' ecological impact, informs conservation planning for both predator and prey, and offers insights into predator-prey dynamics in restinga habitats.



Fig. 1. Juvenile *Boa constrictor* after falling from a *Syzygium cumini* tree while attempting to constrict a Columbina squammata. The red arrow indicates a feather in the snake's mouth.

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Human-mediated dispersals in the Aegean region: the case of *Anatololacerta pelasgiana* on Chalki Island (Greece: Dodecanese)

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Abstract. We report the first confirmed occurrence of *Anatololacerta pelasgiana* on Chalki Island (Dodecanese, Greece). The species was detected exclusively in and around the main settlement and port, within an area of ~0.9 km², suggesting an established population likely originating from a recent human-mediated introduction. To infer source affinity, we sequenced the mitochondrial cytochrome *b* gene from two individuals and compared these sequences with published ones from Aegean and adjacent Anatolian populations. Based on the analysed length of the cytochrome *b* fragment, the Chalki specimens share a widespread haplotype known from Rhodes, Kastellorizo, and Athens (Greece), as well as from southwestern Turkey. These results suggest repeated, transport-mediated introductions likely from nearby Rhodes as a recurrent driver of secondary colonization in the southeastern Aegean and underscore local trade links as pathways for small-island translocations, potentially relevant to other reptiles in the region.

Keywords. Lacertidae, Aegean Islands, lizard translocation.

Lizards of the genus *Anatololacerta* Arnold, Arribas and Carranza, 2007, are small lacertids present from western to southern Anatolia and many neighbouring islands. Currently, the genus comprises five species (Karakasi et al., 2021), i.e. *Anatololacerta anatolica* (Werner, 1900), *Anatololacerta danfordi* (Günther, 1876), *Anatololacerta ibrahimi* (Eiselt and Schmidtler, 1986), *Anatololacerta pelasgiana* (Mertens, 1959), and *Anatololacerta finikensis* (Eiselt and Schmidtler, 1986). Three representatives of the genus occur in Greece, which are found on Aegean islands: *A. anatolica* on Ikaria and Samos; *A. pelasgiana* on Rhodes, Symi and nearby islands; and *A. finikensis*, which is restricted to Psomi islet (Karakasi et al., 2021). *Anatololacerta pelasgiana* has the widest insular distribution among its sister species, occupying a broad range of different habitats (Şahin et al., 2022), and has already shown the potential to establish new introduced populations in other areas, as shown by the record from Athens in mainland Greece (Christopoulos et al., 2022).

During a series of field surveys to the Dodecanese archipelago, Greece, we visited the island of Chalki (36.230°N, 27.569°E) between 14 and 16 October 2025. Chalki is situated roughly 10 km from the island of Rhodes, which is part of the natural distribution of *A. pelasgiana*. The island hosts a notably poor herpetofaunal assemblage, comprising species that are widely distributed on nearby islands: *Hemidactylus turcicus* (Linnaeus, 1758), *Mediodactylus oertzeni* (Boettger, 1888), *Laudakia stellio* (Linnaeus, 1758), *Ablepharus kitaibelii* Bibron and Bory de Saint-Vincent, 1833, *Dolichophis jugularis* (Linnaeus, 1758), and *Zamenis situla* (Linnaeus, 1758) (Buttle, 1995; Grano and Cattaneo, 2015). However, *A. pelas-*

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giana has never been officially reported from the island despite its common presence in Rhodes and other islands and few previous herpetological investigations on the island (Buttle, 1995; Grano and Cattaneo, 2015).

Thus, we conducted random fieldwork along the main roads of the island. Due to its small size, surveys were conducted on foot. We visited both coastal and mountainous areas of the island. Across our survey visits, we recorded 39 individuals of *A. pelasgiana* of all ontogenetic stages (Fig. 1A-C) inhabiting the main village Chalki and the surrounding biotopes, within an area of approximately 0.9 km² (Fig. 2). Therefore, this record represents an additional reptile species for Chalki Island and likely results from recent human-mediated introduction.

To assess their genetic affinity, we captured two individuals from the main village of Chalki and collected tail-tip tissue samples for genetic analysis. Both individuals were released at their respective capture sites. We extracted total genomic DNA (E.Z.N.A.® Tissue DNA Kit), and amplified a fragment of the mitochondrial cytochrome *b* (cyt *b*) gene following the protocol of Karakasi et al. (2021) and using the primer pair (LgluLK/Lacertidae_*cytb*) described by Pavlicev and Mayer (2009) and Khan et al. (2021). The newly generated sequences ((GenBank accession number PZ147789-90; 693 bp)) were compared with publicly available datasets in GenBank using BLAST to verify genetic/species similarity (100% identity with sequences GQ142137 and KM434522 from Rhodes). To visualize the intraspecific genetic network, we further aligned newly generated sequences with 45 published sequences of *A. pelasgiana* from Aegean Greece and related Turkey mainland (Bellati et al., 2015; Kalaentzis et al., 2018; Karakasi et al., 2021; Christopoulos et al., 2022; Appendix 1). The final sequence alignment (47 sequences, 420-998 bp) was analysed with a haplotype-network approach (alignment length 1143 bp) in Hapsolutely (Vences et al., 2024). To provide species distribution overview in the region, we compiled distribution data from Broggi (2006), Cattaneo (2006), Bader et al. (2009), Grano et al. (2015, 2018), and Karakasi et al. (2021), presented in Appendix 2. Distribution maps were prepared

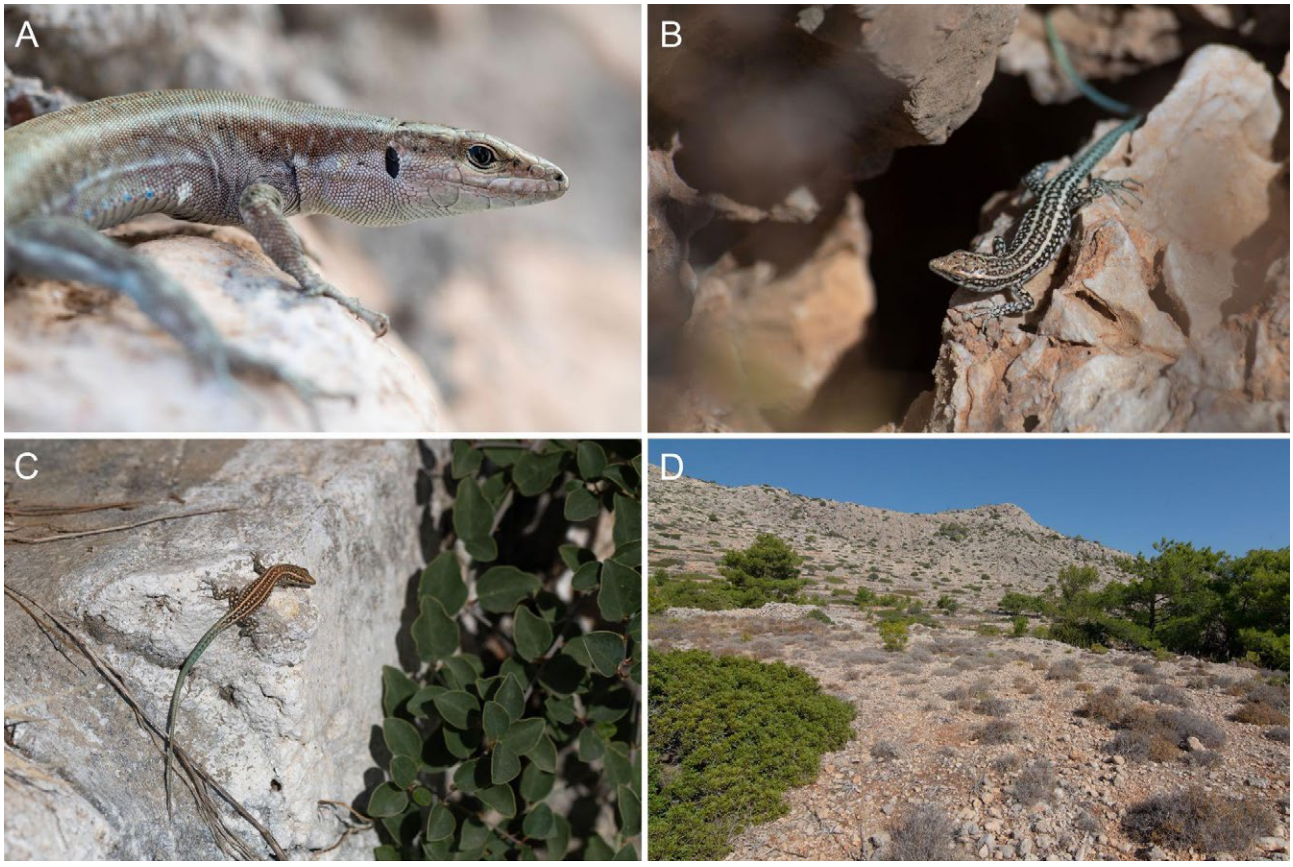


Fig. 1. Adult female (A) and young individuals (B, C) found basking at the main Chalki village. Dry-stone walls within phrygana shrubland provide habitat for the naturalised population of *Anatololacerta pelasgiana* in the island (D).

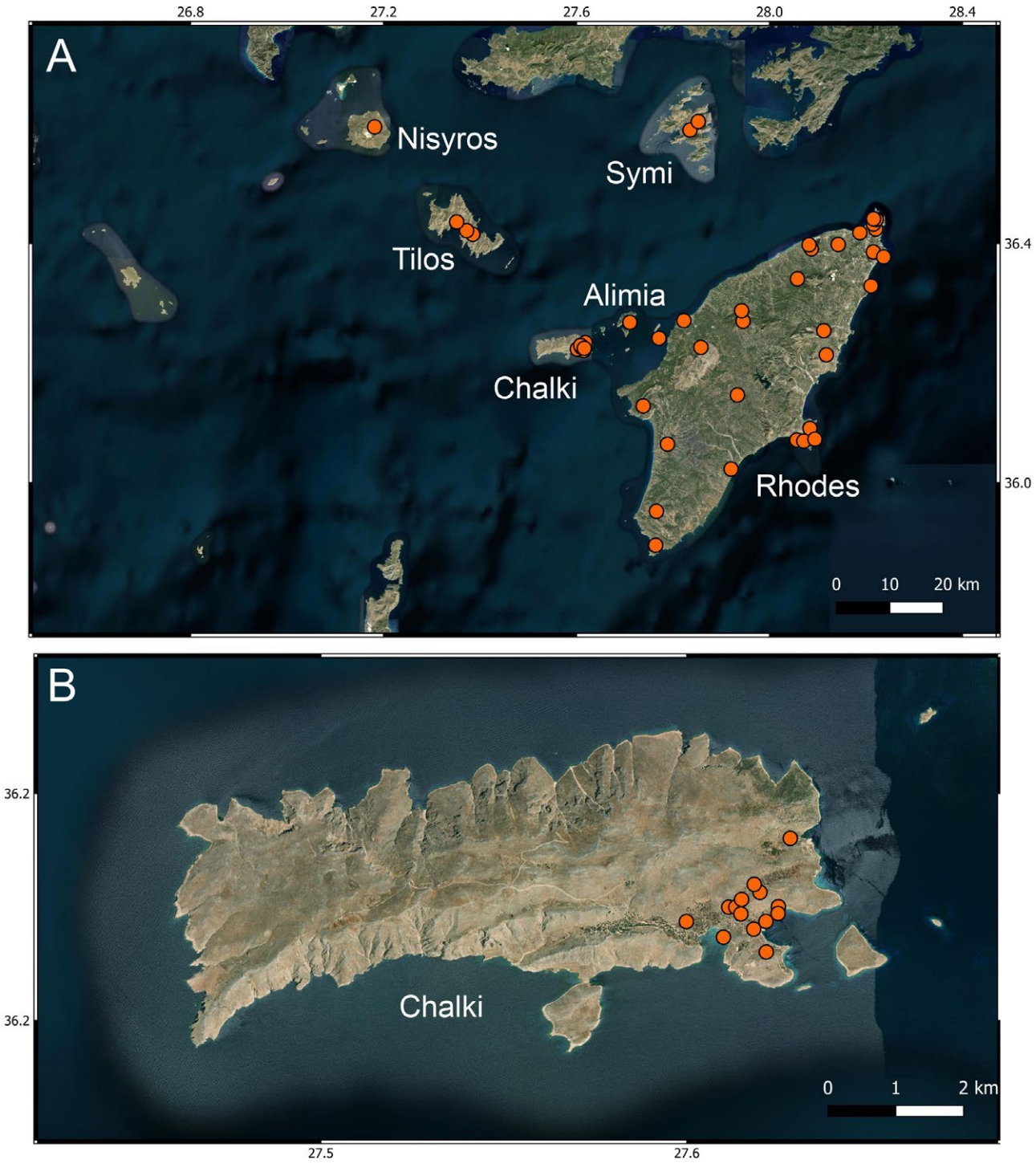


Fig. 2. The known distribution of *Anatololacerta pelasgiana* in Greece (A) and details on herein reported records from Chalki Island (B).

using QGIS 3.44 Solothurn (QGIS Development Team 2026; https://qgis.org/).

Based on the cyt *b* dataset, the Chalki population shares a haplotype present in populations from Rhodes and Kastellorizo (Greece), as well as Marmaris and Tavas (Turkey). Notably, the introduced Athens population also falls within this haplotype (Fig. 3).

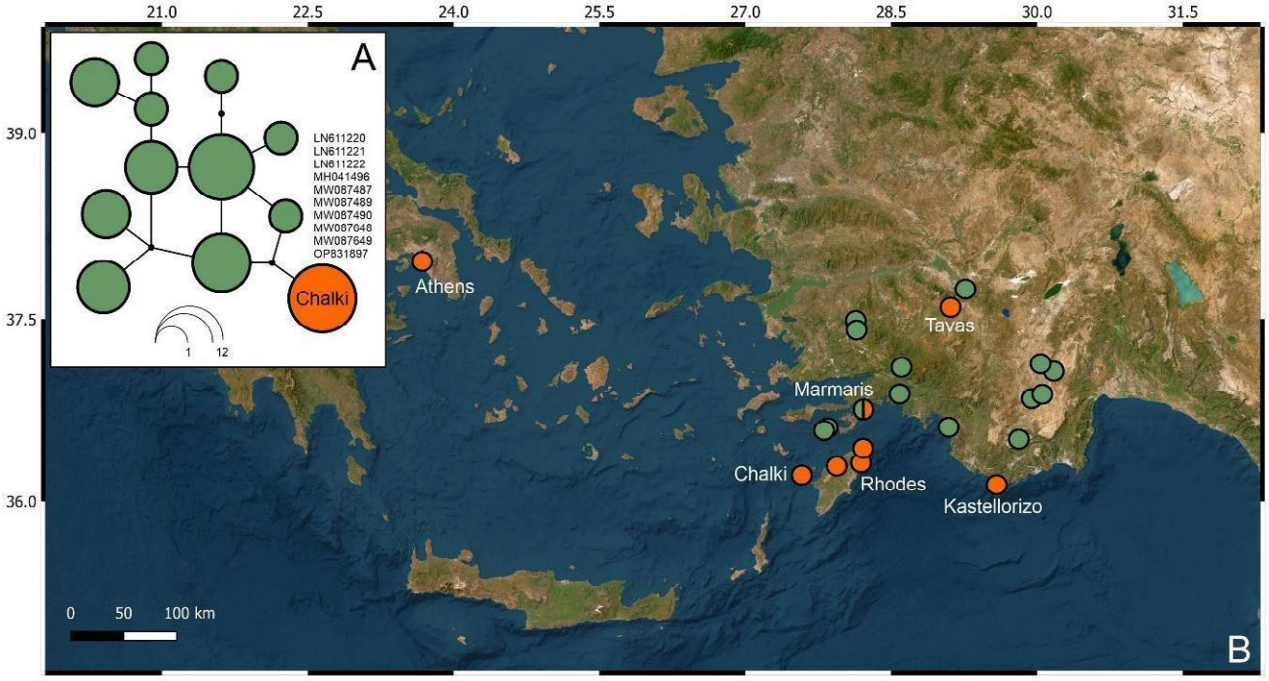


Fig. 3. Haplotype network of *Anatololacerta pelasgiana* (A) and geographic distribution of Chalki Island–affiliated haplotype (B). Numbers in panel A indicate GenBank accession numbers of sampled populations that are genetically concordant with the Chalki population.

In the Aegean region, *A. pelasgiana* is naturally distributed in the Dodecanese archipelago, where it inhabits the islands of Nisyros, Symi, Tilos, Rhodes, and Alimia, as well as the islets of Seskli, Pentanisos, and Strogyli (Bader et al., 2009; Cattaneo et al., 2020). In addition to its native range, the species has recently established populations on Kasos and Kastellorizo, two Dodecanese islands where it had not previously been recorded (Kornilios and Thanou, 2016; Kalaentzis et al., 2018). Furthermore, Christopoulos et al. (2022) reported the first colonization within the metropolitan area of Athens in mainland Greece. Interestingly, Grano et al. (2018) suggested that the population found on Tilos Island likely resulted from secondary human activities as the species is restricted to a few established sites. However, this hypothesis has not yet been tested using genetic data. These naturalized populations found on Kasos, Kastellorizo and Athens showed proximity to individuals from Rhodes, suggesting this particular island may have originated the introduced populations (Kornilios and Thanou, 2016; Kalaentzis et al., 2018; Christopoulos et al., 2022; and references therein). We confirm that these populations share the same haplotype that colonized other areas.

Small Dodecanese islands in the vicinity of Rhodes are strongly connected through local trade and frequent transport, which likely facilitates the spread of *A. pelasgiana* among neighbouring islands. On Chalki, the relatively broad area in which we recorded *A. pelasgiana* suggests an already established population. Nevertheless, *A. pelasgiana* has not been reported from Chalki in the most recent herpetological surveys by other researchers (Buttle, 1995; Grano and Cattaneo, 2015, 2017), possibly indicating a rare case of early and recent colonization. If so, the presence of juveniles further supports the rapid establishment of the population after introduction.

Despite this, the population appears spatially restricted and has not expanded across the island beyond the vicinity of the settlements and port area. We detected *A. pelasgiana* only in suitable habitats near or within human-modified environments, primarily rocky shrublands with scattered trees (Fig. 1D). Most individuals were observed in phrygana scrub associated with short, dry-stone walls and ground-level microhabitats (e.g. fallen logs, rocky shorelines). This pattern is consistent with our additional surveys farther east on the island, where *A. pelasgiana* was not detected, suggesting that these suitable areas may be colonized in the future.

Similar cases have also been documented in other native lacertids within the Aegean area and broader Greece. Among others, recent examples include the Peloponnese endemic *Podarcis peloponnesiacus* (Bibron and Bory de Saint-Vincent, 1833), which has established a population on the Attic Peninsula (Hedman et al., 2017), the widely distributed *Podarcis muralis* (Laurenti, 1768),

now reported from Athens and Corfu Island in the Ionian Sea (Hill and Mayer, 2004; Karameta and Pafilis, 2017), the Balkan endemic *Algyroides nigropunctatus* (Duméril and Bibron, 1839), introduced to Athens, Crete, and the Peloponnese (Deimezis-Tsikoutas et al., 2020; De la Cruz et al., 2024; Prondzynska et al., 2025), as well as *Podarcis erhardii livadiacus* (Werner, 1902), introduced to a small Cycladic island (Adamopoulou et al., 2025).

Considering the Chalki herpetofaunal composition, coexistence with the endemic Dodecanese *M. oertzeni* and the Juniper Skink *A. kitaibelii*, may lead to interspecific competition, as the species may also exhibit potential predation toward smaller lizards (Bader et al., 2009). Nevertheless, this note documents an additional translocation event of *A. pelasgiana* within the Dodecanese archipelago, resulting from human-mediated activities that are historically and recently reported from the Aegean (e.g. Kornilios and Thanou, 2016; Kalaentzis et al., 2018).

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at: https://oaj.fupress.net/index.php/ah/article/view/19819/15292.

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Study of the anatomy and histology of the female reproductive system of the Asian snake-eyed skink, *Ablepharus pannonicus* (Sauria: Scincidae)

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Abstract. This study examines and contrasts the anatomy and histology of the female reproductive system in *Ablepharus pannonicus*, a member of the Scincidae family, across the spring and autumn seasons. Female specimens of *A. pannonicus* were collected from the northern slopes of the Shorkhah Dizah village (34°23'49.1"N 46°03'15.1"E), located 125 km west of Kermanshah Province in western Iran. After anaesthesia, the specimens were dissected in the laboratory, and their reproductive systems were processed for tissue analysis, using serial sections stained with Hematoxylin and Eosin for detailed histological examination. The findings indicate that during the spring, the ovaries of *A. pannonicus* contain all stages of follicular development, including primordial, mature, and ovulation-ready follicles. In contrast, the autumn samples predominantly exhibit primordial or preovulatory follicles, with a notable absence of mature follicles. These observations suggest a seasonal reproductive pattern in female *A. pannonicus*, characterized by significant morphological changes and heightened activity within the ovaries and genital tracts during spring. As the ambient temperature drops and the reproductive season concludes, gonadal size and activity decrease, leading to the absence of reproductive activity in autumn. It is hypothesized that during this period, the animals engage in feeding and fat storage in preparation for hibernation.

Keywords. Reproductive cycle, *Ablepharus pannonicus*, reproductive system, ovary.

The exploration of reproductive diversity among lizards offers a rich tapestry of biological phenomena for scientific inquiry. These reptiles display a remarkable array of reproductive strategies: from oviparity to viviparity, prolific to sparse offspring production, and varied levels of parental investment. Some species are known to lay eggs immediately post-shell formation, while others retain shelled eggs for prolonged periods before laying. Remarkably, viviparity has independently evolved numerous times within squamates, and certain species even exhibit parthenogenesis. These reproductive variations offer a glimpse into the captivating complexity of lizard reproductive ecology (Rheubert et al., 2014).

Lizards typically exhibit one of three reproductive cycles: constant, associated, or dissociated. The constant cycle is characterized by nearly year-long gonadal activity. In contrast, the associated and dissociated cycles feature a discontinuous mating season. The associated cycle is marked by a surge in gonadal activity just before the mating season, synchronously in both sexes, obviating the need for sperm storage due to its ready availability (Censky, 1995; Huang, 1997). Conversely, the dissociated cycle is defined by diminished gonadal activity during the mating season, with a peak in the non-mating period. Notably, male gonadal activity is briefer compared to females, necessitating the storage of sperm within the female reproductive tract for subsequent fertilization (Torki, 2006).

The oviductal structure in lizards exhibits a conserved morphology, characterized by three distinct

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regions identifiable across species. These regions, extending from the caudal to the cranial end, comprise the nonglandular uterus – commonly referred to as the vagina – the glandular uterus, typically known as the uterus, and the infundibulum (Siegel et al., 2011). Female lizards possess bilateral oval ovaries anchored to the dorsal body wall via a slender mesovarium. The ovarian cortex is surrounded by the tunica albuginea, and is lined by a simple squamous epithelium (Klosterman, 1983; Guraya, 1989). Oogenesis occurs within the germinal beds situated dorsally on the ovary, proximal to the mesovarium. These beds harbor proliferating oogonia, undifferentiated somatic cells, oocytes encased by a few somatic cells, and primary follicles (Jones and Guillette, 1982; Klosterman, 1983). The ovarian follicles, serving as the functional units of the ovary, are organized into a hierarchical structure, including primordial, primary, secondary, previtellogenic, vitellogenic, and preovulatory follicles. This follicular hierarchy is a well-defined feature of reptilian ovarian architecture (Guraya, 1989; Etches and Petitte, 1990).

The skink *Ablepharus pannonicus* (see Fig. S1) inhabits a range of environments including semi-desert and desert areas, steppe habitats, and mountainous terrains. Its geographical distribution extends from the Middle East to Central and South Asia, covering countries such as Iraq, Iran, Jordan, Syria, Afghanistan, Pakistan, India, Kyrgyzstan, and Uzbekistan (Karamiani, 2018). Despite the breadth of studies on various aspects of this species, there is a notable gap in the literature regarding the anatomical and histological characterization of the female reproductive system. Addressing this lack of information, the present study provides the first detailed description of the anatomy and histology of the female reproductive system of *A. pannonicus*, offering insights into a previously unexplored aspect of this widely distributed lizard.

This research was conducted in the natural surroundings of the village of Shorkhah Dizah (34°23'49.1"N 46°03'15.1"E), located 125 km west of the city of Kermanshah, in Kermanshah Province, western Iran. Sampling was conducted in the spring (April–May) and autumn (October–November) of 2021, with four adult female specimens of *A. pannonicus* collected in each season. These two sampling periods were selected to capture contrasting reproductive stages: spring representing peak ovarian activity and autumn reflecting a regressed state, consistent with seasonal reproductive patterns reported in scincid lizards (Sever and Hopkins, 2004; Vergilov et al., 2018). According to long-term climate normals (1981–2010) reported by the Iran Meteorological Organization (IRIMO), average temperatures in the study area range from 15-22 °C in spring and 9-17 °C in autumn. Rainfall is moderate in spring but substantially lower in autumn (Iran Meteorological Organization, 2021).

Upon arrival at the laboratory, the specimens underwent anaesthesia using diethyl ether-soaked cotton. Subsequently, we euthanised them through intracoelomic injection of sodium pentobarbital, following the animal care guidelines approved by Southeastern Louisiana University Animal Care and Use Committee (Rheubert et al., 2020). Next, we meticulously recorded morphometric parameters, including snout–vent length (SVL) and body mass. A longitudinal incision was made on the ventral surface of the specimens, allowing for the removal of the digestive tract. This surgical approach exposed the reproductive system, which we then photographed for subsequent analysis. The entire oviductal tract was carefully dissected and weighed using a digital analytical balance. Using this data, we calculated the gonadosomatic index (GSI), providing a quantitative measure of reproductive investment (Jacobson, 2007). The GSI was calculated as GSI = (ovary weight / body weight) × 100, reflecting the proportional reproductive investment of each individual. After the oviductal tract was removed from the lizard's body, the samples were immediately placed in 10% formalin for 72 h. Subsequently, the samples were dehydrated in a series of ethanol solutions: 60% for 60 min, 70% for 30 min, 80% for 30 min, and 96% for 120 min. For clearing, the samples were placed in three containers containing xylene, each for 30 min. The samples were then embedded in paraffin in an incubator set at 58°C. In this step, three containers of paraffin were used, and the capsules containing the tissue samples were placed in each container for 120 min to allow the paraffin to replace the xylene in the tissue. L-shaped metal molds, known as Leuckhard molds, were used for molding. The mold was adjusted to the appropriate size, and molten paraffin was then poured into the mold. The samples were subsequently placed vertically inside it. In the next step, the blocks were separated from the mold, resulting in a molded block for each individual. A rotary microtome (CUT SLEE 4060) was used to obtain transversal and longitudinal sections from paraffin blocks containing the entire oviductal tract. Sections were prepared from multiple regions along the tract, and representative slides were selected for histological analysis. The sections were stained using hematoxylin-eosin staining (Suvarna et al., 2018).

The female reproductive system in *Ablepharus pannonicus* comprises a pair of ovaries and an oviduct. The oviducts traverse past the kidneys and terminate in the cloaca (Fig. 1A, B). The ovaries are situated within the abdominal cavity as whitish glandular structures, attached to the dorsal wall by the mesovarium. The

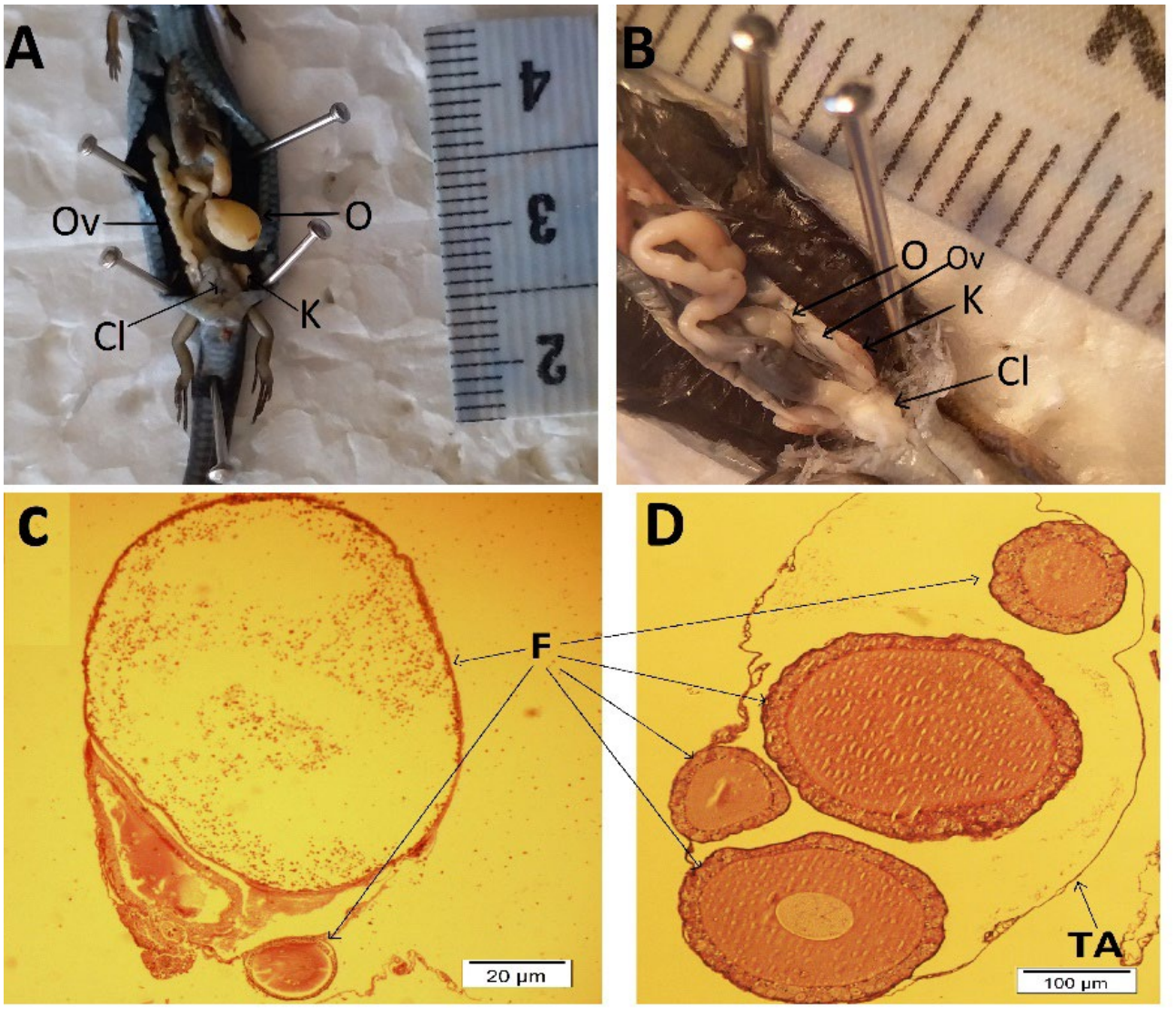


Fig. 1. Gross anatomy and ovarian histology of the female reproductive system of *Ablepharus pannonicus* in two seasons. (A, B) Dissected views of the female reproductive system in spring (A) and autumn (B), showing the position of the ovaries and oviducts relative to the kidneys and cloaca. (C) Section from a spring-collected individual showing an advanced follicle (likely preovulatory) and developing follicles (presumed vitellogenic). (D) Section from an autumn-collected individual illustrating follicles at various earlier stages of development, including presumed early, intermediate, and previtellogenic follicles. Sections stained with hematoxylin and eosin (H&E). Abbreviations: O, ovary; Ov, oviduct; K, kidney; Cl, cloaca; F, follicle; TA, tunica albuginea.

ovarian surface exhibits small and large protrusions due to the presence of follicles at various stages of maturity. Each ovary is enveloped by a thin covering known as the tunica albuginea. During the spring, all types of follicles (especially vitellogenic and preovulatory follicles) are observable in the ovarian sections (Fig. 1C). However, in autumn samples, mature follicles are scarce, and the predominant follicles are in the primary, secondary, or previtellogenic stage (Fig. 1D).

Microscopic examination of the previtellogenic follicle wall reveals at least two distinct cell types – large and small cells (see Fig. S2). The ovarian and body weight data, and gonadosomatic index data collected from each season are presented in Table S1. No notable structural differences were observed in the oviductal anatomy or histology between spring and autumn specimens. Moving along the oviduct, the most anterior portion is referred to as the infundibulum. The epithelial cells lining its proximal region exhibit a squamous to cuboidal

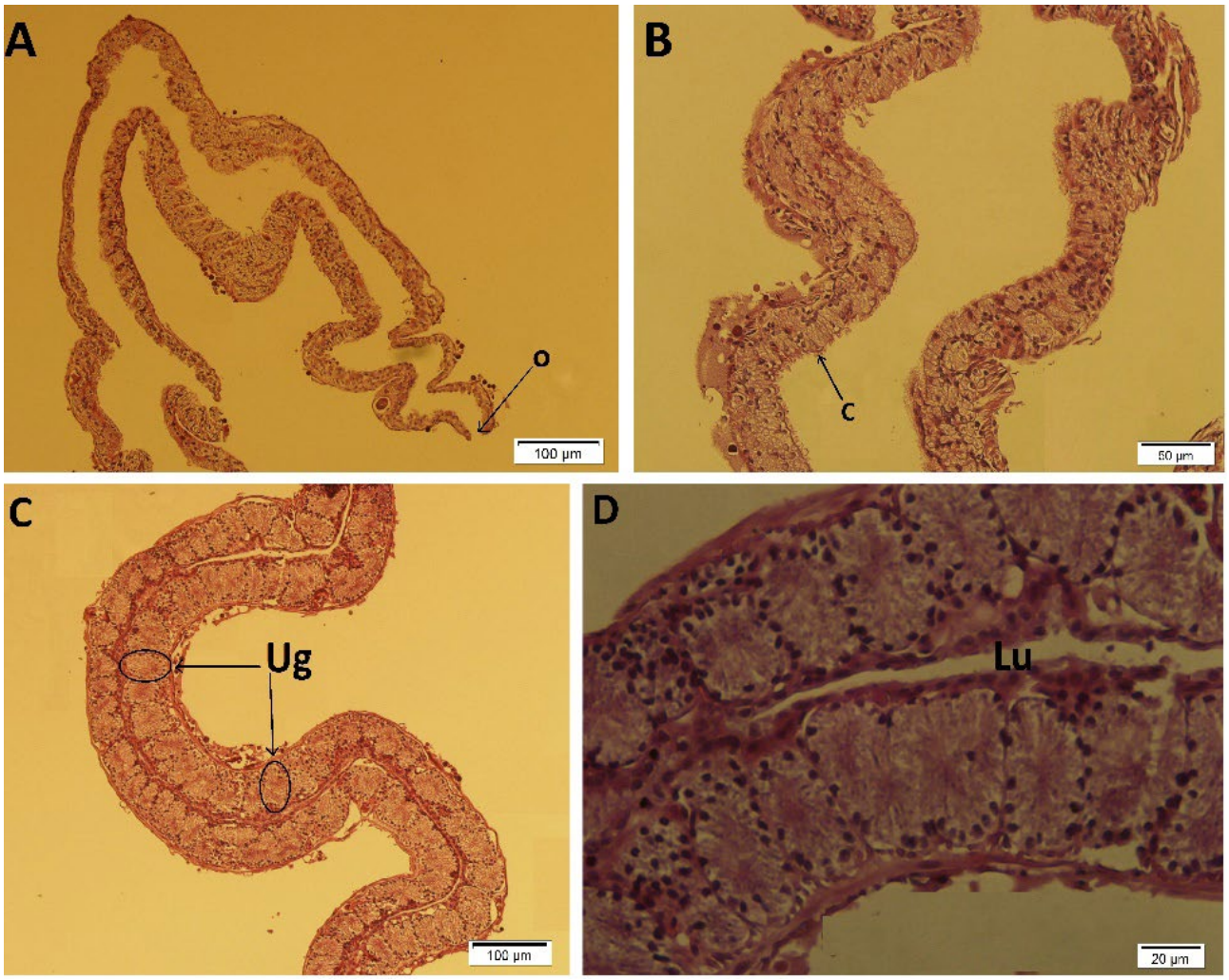


Fig. 2. Longitudinal histological sections of the infundibulum and glandular uterus in *Ablepharus pannonicus*. (A) Low magnification view showing the entire infundibulum from the anterior ostium to the posterior connection with the uterus. (B) Higher magnification of the posterior region of the infundibulum, revealing epithelial cilia. (C) Low magnification longitudinal section of the glandular uterus, showing the distribution of uterine glands within the uterine wall. (D) Higher magnification view of the glandular uterus, illustrating the acinar morphology of the uterine glands. Sections stained with hematoxylin and eosin (H&E). Abbreviations: O, ostium; C, cilia; Ug, uterine gland; Lu, lumen.

morphology. Toward the distal region, the cellular height increases, culminating in a cylindrical form. Notably, certain cells in the distal infundibulum display abundant cilia (Fig. 2A, B).

The glandular uterus constitutes the central segment of the oviduct in *A. pannonicus*. Its wall is densely populated with mucous glands, which are acinar in configuration and embedded within the tissue. The predominantly columnar cells of these glands play a crucial role in eggshell formation through their secretions (Fig. 2C, D).

The most posterior region of the oviduct is the non-glandular uterus, connecting the glandular uterus to the urodeum of the cloaca. In this area, numerous crypts are present, their abundance increasing as one approaches the cloacal urodeum (see Fig. 3 and Fig.S3).

Unlike the glandular uterus, this region lacks secretory glands, allowing for clear demarcation (see Fig. 4). The number of Germinal Beds (GBs) per ovary exhibits both intra- and inter-specific variation, ranging from one to six among lizard species (Jones et al., 1979; Radder et al., 2008). Studies have posited a correlation between the number of GBs per ovary, clutch size, and breeding frequency. Jones and Guillette (1982) identified a pattern linking the number of GBs to clutch size. Typically, species that undergo monoallochronic ovulation – ovulating a single egg alternately from each ovary, as seen in

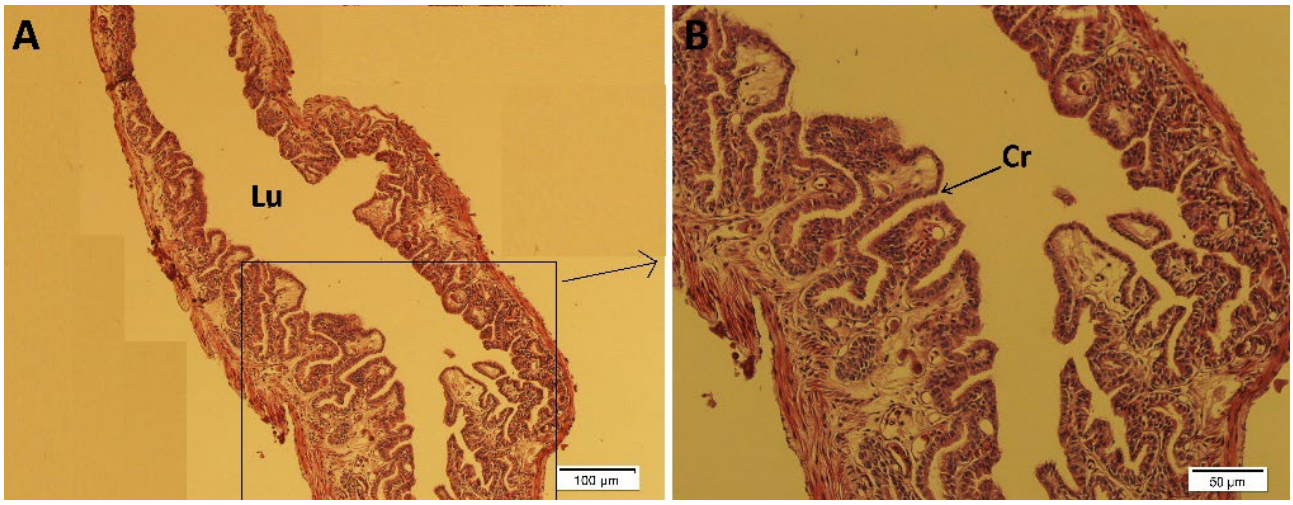


Fig. 3. Longitudinal section of the non-glandular uterus (vagina) of *A. pannonicus* collected in spring. (A) Low magnification showing overall tissue structure and distribution of epithelial crypts. (B) Higher magnification illustrating the detailed morphology of crypts. Sections stained with hematoxylin and eosin (H&E). Abbreviations: Lu: lumen, Cr: crypt.

the Dactyloidae family (Jones et al., 1979) – and those with low fixed clutch sizes and monoautochronic ovulation – releasing a single egg simultaneously from both ovaries, such as in Gekkota (Jones and Summers, 1984) and Gymnophtalmidae (Vitt, 1982) – possess one GB per ovary. Conversely, species with high fecundity, characterized by polyautochronic ovulation and multiple large clutches, exhibit two or more spatially distinct GBs in each ovary (Radder et al., 2008). In all *A. pannonicus* specimens examined during the spring, GBs were indistinct. However, the presence of a single follicle poised for ovulation in both ovaries suggests a monoallochronic ovulation pattern.

Within the walls of previtellogenic follicles in certain lizards, granulosa cells of varying types are discernible. For instance, in *Ablepharus kitaibelii's* previtellogenic follicles, three granulosa cell types – small, large, and pyriform – are identifiable (Vergilov et al., 2018). In this study, two cell types – large and small – were observed in *A. pannonicus's* previtellogenic follicle walls, yet pyriform cells were absent.

In species like *Eumeces egregius* (Schaefer and Roeding, 1973) and *Scincella lateralis* (Sever and Hopkins, 2004), the non-glandular uterus contains numerous ciliated crypts functioning as sperm storage sites. Examination of the non-glandular uterus of *A. pannonicus* revealed numerous crypts, similar to those reported in the species mentioned above. However, no sperm was detected in specimens collected during either spring or autumn. This absence may reflect phylogenetic differences among scincid lizards or limitations of our sample size, rather than conclusively indicating that the crypts are not used for sperm storage.

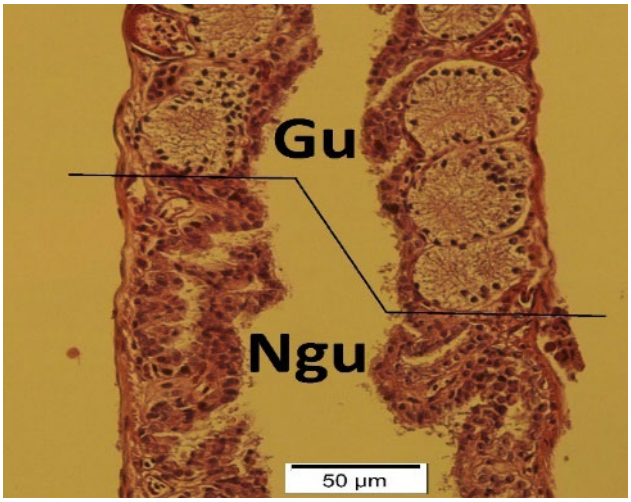


Fig. 4. Longitudinal section illustrating the junction between the glandular (Gu) and non-glandular (Ngu) regions of the uterus in *A. pannonicus*. The boundary between the two is marked by a black line. Section stained with hematoxylin and eosin (H&E). Abbreviations: Gu: glandular uterus, Ngu: non-glandular uterus.

Glandular uterine glands play a pivotal role in egg membrane formation via their secretions. These glands exhibit considerable morphological diversity across lizard species. In *Plestiodon obsoletus*, the glands are tubular (Guillette Jr. et al., 1989), while in *Chalcides chalcides*, they are tubular with simple cuboidal epithelium (Blackburn et al., 1998), and in *Chalcides ocellatus*, they

are simple alveolar (Corso et al., 2000). In these species, secretions are expelled through an end duct to the uterus's glandular lining. The glands in the glandular uterine wall of *A. pannonicus* were acinar with columnar epithelial cells in specimens from both spring and autumn. While no seasonal structural differences were noted, potential variations in functional activity cannot be ruled out.

The infundibulum, featuring numerous crypts in lizards like *Acanthodactylus scutellatus* and *Hemidactylus turcicus*, is recognized as a sperm storage site (Bou-Resli et al., 1981; Eckstut et al., 2009). However, comprehensive documentation of sperm storage in this region among skinks is lacking. In *A. pannonicus*, as in other skinks, sperm was not detected in the infundibulum.

These findings suggest that reproduction in female *A. pannonicus* is seasonal, as indicated by significant morphological changes in the gonads and increased activity in the genital ducts during spring. This conclusion is further supported by a marked increase in the gonadosomatic index (GSI) observed in spring-collected specimens compared to those collected in autumn, reflecting a higher reproductive investment during the active breeding season. As the ambient temperature declines, a noticeable seasonal reduction in both gonadal size and GSI is evident, corresponding with the cessation of reproductive activity. Consequently, this lizard exhibits no signs of sexual activity during the autumn season. This seasonal variation in gonadal condition and reproductive readiness aligns with regional climatic patterns, where average daily temperatures range from 15–22 °C in spring and decrease to 9–17 °C in autumn in the mountainous areas south of Kermanshah Province.

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at https://oaj.fupress.net/index.php/ah/article/view/16392/15293.

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First report of caudal display in the longtail whiptail *Aurivela longicauda* (Squamata: Teiidae)

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Abstract. Squamate tail movements has multiple functions, including intraspecific communication, defense, autotomy, aggression, and feeding. Here we report for the first time the occurrence of unusual caudal behavior in Longtail Whiptail (*Aurivela longicauda*, Teiidae), an endemic lizard distributed in the Monte Desert region in Patagonia, Argentina. The observed specimen was buried underground, with only the reddish part of the tail was sticking out of the sand. The tail was extended and subvertically oriented, and the individual was wagging and curling the tail laterally, doing a slow tail wag with intermittent "flailing", resembling the movement that earthworms do out of the ground. Knowing that the *Aurivela* species are almost exclusively insectivores, with a particular predilection for gregarious insects (e.g., termites and ants), it is not improbable that tail having worm-like coloration and movement may act as a lure to attract prey for consumption.

Keywords. Tail-wagging, caudal behavior, Teiidae, Patagonia, Argentina.

Squamate tail movements has multiple functions, including intraspecific communication, defense, autotomy, aggression, and feeding (Greene, 1973; Arnold, 1984, 1988; Foster and Martin, 2008; Carvalho, 2016; Peters and Ramos, 2022). Among the latter, tail movements may be used to distract prey by shifting their attention away from the head and toward the caudal end of the predator (Hasson et al., 1989; Foster, 2006; Braun and Baird, 2018), a behavior that is somewhat frequent in snakes (Heatwole and Davison, 1976; Hagman et al., 2008; Reiserer and Schuett, 2008) but very uncommon in lizards (Murray et al., 1991; Pernetta et al., 2005; Foster, 2006; Foster and Martin, 2008; Braun and Baird, 2018).

Here, we report the occurrence of an unusual tail behavior in Longtail Whiptail (*Aurivela longicauda*; Bell, 1843). This teiid lizard is endemic to the Monte Desert region in Argentina and is distributed along arid and semiarid dunes, scrublands, and areas with scattered vegetation along central and southern provinces in the country (Cei, 1993; Scolaro, 2005).

The observation here reported was made incidentally during fieldwork close to General Roca city, at Río Negro province (Patagonia, Argentina). On 9 October 2024 (spring season) at 10:50 h we observed an adult male individual of the species *Aurivela longicauda* buried in the sand of a flatland with few grasses and scattered bushes under direct sunshine (39°26'16"S; 67°17'54.4"W). Temperatures were around 22-28°C and the sky was mostly clear. No other individuals of this species or predators were detected in the area near the observation.

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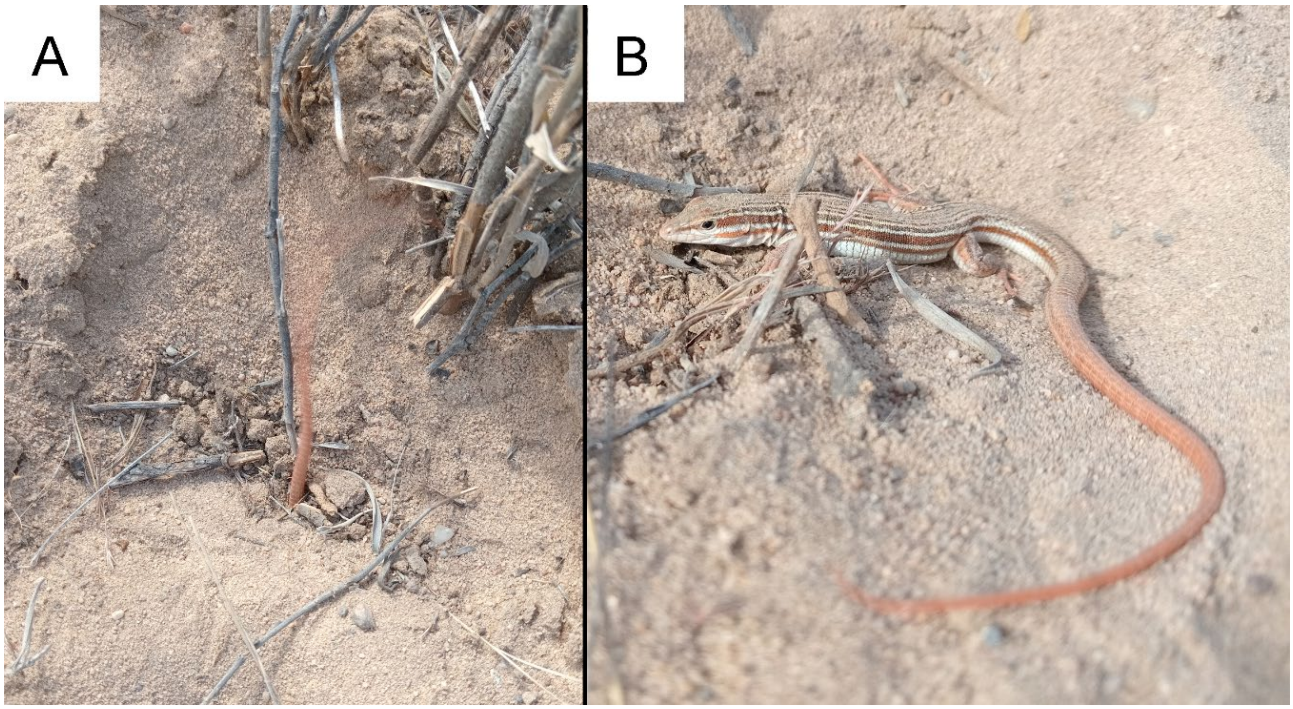


Fig. 1. Adult male of Longtail Whiptail (*Aurivela longicauda*) wagging the tail, buried in the sand (A) and the same individual above the ground (B).

The lizard was buried underground, in a concave semicircular area of 15 cm in diameter, and only the reddish part of the tail was sticking out of the sand (Fig. 1A). The tail was extended and subvertically oriented and the individual was wagging and curling the tail laterally, doing a slow tail wag with intermittent "flailing", resembling the movement that earthworms do out of the ground. No other part of the lizard was visible. Tail movement time interval lasted for 5 minutes of observation. Although numerous ants were moving in the vicinity of the lizard, this individual was not observed capturing or consuming arthropods, as it was partially buried (with its head covered by sand) during the tail movement. Subsequently, as we approached, it detected our presence, uncovered itself (Fig. 1B), remained alert, and then slipped away into the bushy vegetation.

Tail movements in lizards are poorly known and remain poorly reported in literature (e.g., Hasson et al., 1989; Foster, 2006; Foster and Martin, 2008; Telemeco et al., 2011; Braun and Baird, 2018; Peters and Ramos, 2022). The function of these movements is still uncertain, and our knowledge of this mode of signaling in lizards is almost limited by a general lack of basic natural history knowledge (Ramos and Peters, 2016). Future works should aim to determine the purpose, if any (Tinbergen, 1952), that these reported caudal movements serve.

In spite of that, some ambiguous evidence suggests that caudal movements in *A. longicauda* may be related to prey capture. Tail movement here reported for *A. longicauda* is more similar to squamate caudal movements associated with prey distraction (Mullin, 1999; Foster, 2006) than to movements associated with defensive behaviors (Foster, 2006; Foster and Martin, 2008). In fact, Collared Lizards of the genus *Sceloporus*, elevate the stiffened tail and slowly waves it laterally, which directs the attention of prey (in this case flies) away from the lizard head and mouth which are held very close to the ground (Foster and Martin, 2008; Braun and Baird, 2018). This behavior is very similar to that reported here for *A. longicauda*.

Many lizard species from several unrelated families have tails with conspicuous coloration, that contrasts with the usually cryptic body-color (Murali et al., 2018). In many cases, it may be possible that the tail coloration enhances its distractive effect so that predator or prey attention is more likely to be directed towards the tail rather than to the head and body (Arnold, 1984). Among these taxa, *A. longicauda* is characterized by its long, pink-colored tail, present in both males and females that contrasts with striped body pattern (Cei, 1993). The gymnophthalmid *Vanzosaura multiscutata* is very similar in color pattern to *A. longicauda* (Cei, 1993), and it has

been described to move its red tail in a worm-like pattern when poked (Carvalho, 2016). This behavior closely resembles that reported here for *A. longicauda*, and it is plausible that it may be used in a similar way.

Considering that *Aurivela* species are almost exclusively insectivores, with a particular predilection for gregarious insects, such termites and ants (Gallardo et al., 2019), our hypothesis is that a tail having worm-like coloration and movement may act as a lure to attract these preferred prey items. Although we did not directly observe prey capture associated with this behavior, it is plausible that such a mechanism may be present in the species. Notably, a large number of ants were observed in the immediate surroundings during our observations.

It should also be noted that we cannot rule out the possibility that the observed caudal movements serve some form of intraspecific communication (e.g., mate attraction). This hypothesis is difficult to substantiate, as no other individuals of *A. longicauda* were observed in the vicinity exhibiting or responding to this unusual tail behavior.

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Negative impacts of cultural monument restoration on the Common Wall Lizard population

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Abstract. Some animals successfully inhabit anthropogenic environments but are often exposed to negative impacts such as habitat destruction, disturbance, and pollution. Lizards frequently occupy stone walls, abandoned buildings, and cultural monuments, including fortresses, where crevices provide important sites for hiding, thermoregulation, and hibernation. However, urbanisation and restoration activities can negatively affect animal populations by restricting movement and dispersal and by reducing reproductive success, growth, and survival, potentially leading to population declines or local extinctions. The main objective of this study was to assess the impact of cultural monument restoration on *Podarcis muralis* populations by analysing body condition index and population structure. The study was conducted at Kalemegdan Fortress, located in the urban centre of Belgrade, Serbia. This three-year-long research showed that at the locality Kalemegdan 1, the fortress restoration caused local population extinction due to habitat destruction and the killing of lizards, with no evidence of lizards migrating to the neighbouring locality – Kalemegdan 2. Interestingly, lizards from Kalemegdan 1 consistently exhibited a higher body condition index than those from Kalemegdan 2, thus suggesting more favourable hibernation conditions at the former locality. However, body condition index values at Kalemegdan 1 showed a declining trend over the study period, likely as a consequence of restoration activities. Overall, the results indicate that restoration of historic fortresses can result in habitat loss and population declines in urban lizards. Given that urban populations are often small and fragmented, the absence of appropriate mitigation measures during restoration may increase the risk of local extinction.

Keywords. Kalemegdan Fortress, urban population, *Podarcis muralis*, body condition index, capture–mark–recapture, habitat destruction

INTRODUCTION

Urbanisation is a major human-driven cause of habitat fragmentation, alteration, and loss (Scanes, 2018; Li et al., 2022). These changes negatively affect animal populations by restricting movement and dispersal, and by reducing reproductive success, growth, and survival, which can ultimately result in local or regional population declines or extinctions (Baur and Erhardt, 1995; Gonzalez-Suarez and Revilla, 2014; Powers and Jetz, 2019; Lal and Nadim, 2021).

In anthropogenic environments, animals often lack optimal conditions for nutrition, reproduction and thermoregulation, and are frequently exposed to pollution

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and disturbance (Preeti et al., 2018). Nevertheless, some species persist in anthropogenic habitats (Bateman and Fleming, 2012; Garcia et al., 2017; Putman and Tippie, 2020), even thrive in the centres of large cities, where they may occupy river banks, parks, stone walls, ruins, or cultural monuments (Mollov, 2011; Pulev and Sakelarieva, 2013; Simbula et al., 2019). However, the ability to cope with urban environments varies markedly among taxa, and reptiles are among the most vulnerable groups in anthropogenic landscapes (Cox et al., 2022). Reptiles are ectotherms and their body temperature depends on the environmental temperature (Vitt and Caldwell, 2014). They require specific habitat structures (rocks, rubble, crevices) for hiding, thermoregulation, egg laying and hibernation (Downes and Shine, 1998; Smith and Ballinger, 2001; Goller et al., 2014). Due to their specific requirements for thermoregulation and nutrition, reptiles are susceptible to changes in habitat structure, thermal heterogeneity and refugia availability (Doherty et al., 2020; Cordier et al., 2021). Reptile populations in urban environments are under additional pressure because they are often small and isolated, with limited dispersal abilities (Wall et al., 2024). Lizards and snakes frequently inhabit stone walls, abandoned buildings, and cultural monuments such as fortresses, because the crevices within these structures are important for shelter, thermoregulation and hibernation (Scali et al., 2013). Plugging these shelters can result in immediate mortality, especially if it occurs during hibernation. Although the conservation of cultural heritage is essential for preserving historical identity and supporting tourism, restoration activities often involve intensive habitat modification, such as removing vegetation and fallen leaves, as well as plugging holes and shelters. This creates a potential conflict between cultural heritage conservation and biodiversity protection, particularly for taxa that inhabit such places.

One reliable way to assess the impacts of human-induced environmental change and to implement mitigation measures promptly is the systematic monitoring of wildlife populations using multiple variables and parameters. Capture–mark–recapture (CMR) studies are widely used to monitor trends in population growth or decline (Sandercock et al., 2020). At the individual level, the body condition index (BCI) reflects energy reserves and overall physiological status, representing the ratio of an individual's body mass to body length (Jakob et al., 1996; Peig and Green, 2009). Individuals with higher BCI values have greater energy reserves, thus increasing their chances of surviving hibernation and achieving higher reproductive success (Shine et al., 2001; Madsen et al., 2023). Individuals inhabiting urban environments may have lower BCI values due to factors such as disturbance, reduced food availability and environmental pollution (Gallego-Carmona et al., 2016; Lazić et al., 2017; Giery et al., 2025). Used together, the BCI and CMR methods provide a robust framework for evaluating both population-level and individual-level responses to environmental disturbance.

Despite the widespread presence of reptiles in urban cultural monuments, the ecological consequences of monument restoration are poorly documented, and environmental impact assessments rarely consider herpetofauna (O'Sullivan et al., 2023). Given the important ecological roles of lizards and snakes as both predators and prey in urban ecosystems, the loss or decline of these populations may cause cascading effects on local food webs (Brum et al., 2023). Therefore, understanding how restoration activities affect reptile populations is essential for developing management strategies that reconcile cultural heritage preservation with biodiversity conservation.

The main objective of this study was to evaluate the impact of cultural monument restoration on *Podarcis muralis* populations inhabiting the cultural monument. The specific objectives were to quantify changes in the Common Wall Lizard abundance, BCI and habitat use caused by the restoration of cultural monuments, and to provide conservation recommendations for future restoration projects.

MATERIALS AND METHODS

Study site

The study was conducted at Kalemegdan, located in the urban centre of Belgrade, the capital of Serbia (44°49'38''N, 20°27'5''E). Kalemegdan, also known as the Belgrade Fortress, was declared a cultural monument of exceptional importance in 1979. In addition, the part of Kalemegdan known as "Kalemegdanski rt" was declared a Natural Monument in 2021. This dual protection (cultural-historical and natural) highlights the significance of Kalemegdan as a unique cultural-historical and natural entity within Kalemegdan Park. Kalemegdan consists of a park and an old fortress (Fig. 1). Before restoration, the fortress contained numerous fissures, cavities, and stone crevices that provided suitable habitats for lizards and snakes. The surrounding area includes urban green spaces, such as landscaped lawns and tree lines intersected by pedestrian walkways. The complex offers various facilities, including a zoo, an amusement park, sports fields, coffee shops, a museum and kiosks. Numerous events are held there, and a large number of tourists are constantly present.



Fig. 1. A detailed overview of the analysed localities Kalemegdan 1 (green polygon) and Kalemegdan 2 (red polygon).

The analysed locality, Kalemegdan 1, is bordered on one side by a parking area, a street and the zoo's enclosures, and on the other side, the fortress is surrounded by a park with a landscaped lawn intersected by walking paths (Figs 1-2). The area of this locality is approximately 2280 m². The fortress walls, about 4-7 m high, are constructed of brick and massive stone, with numerous cracks and dents that provide ideal hiding places for lizards, snakes and other animals (Fig. 2). The habitat is characterised by low vegetation, as well as plants growing on the walls, which offer basking and shelter opportunities for various species. The second analysed locality, Kalemegdan 2, is located approximately 130 m from Kalemegdan 1 (Fig. 1). Kalemegdan 2 is characterised by lower walls (1.5-2 m) than Kalemegdan 1 and by the presence of ruins of older fortifications (Fig. 2). The area of this locality is about 1950 m².

Description of restoration activities

In the second half of 2023, the parking lot located directly next to the studied locality Kalemegdan 1 was reconstructed. During this period, lizards avoided the fortress wall facing the car park due to disturbance from workers and heavy machinery; significantly fewer active lizards were observed on that side of the wall compared to the period before the works. Restoration of the fortress wall at Kalemegdan 1 began in April 2024, and according to the plan, it is scheduled to continue until March 2026. Throughout 2024 and especially in 2025, the restoration involved major structural interventions (Fig. 2B-C; Fig. 3). Activities included filling cavities, crevices and holes in the stone walls, removing loose stones and vegetation, maintaining a continuous human presence and using heavy machinery. During this period, lizards hibernated and hid in holes, where they became trapped. These actions also eliminated microhabitats previously used by reptiles for shelter, foraging, egg laying and thermoregulation.

Analysed species

The Common Wall Lizard, *Podarcis muralis* (Laurenti, 1768), is a medium-sized lacertid lizard, with a body length of about 20 cm, a snout-vent length (SVL) of up to 7.5 cm and a tail that can be twice as long as the body (Speybroeck et al., 2016). Sexual dimorphism is pronounced, with males being larger than females (Gruschwitz and Böhme, 1986). The Common Wall Lizard inhabits various habitats but prefers sunny, rocky areas with low vegetation (Arnold and Ovenden, 2002). It has successfully adapted to highly urbanised areas,

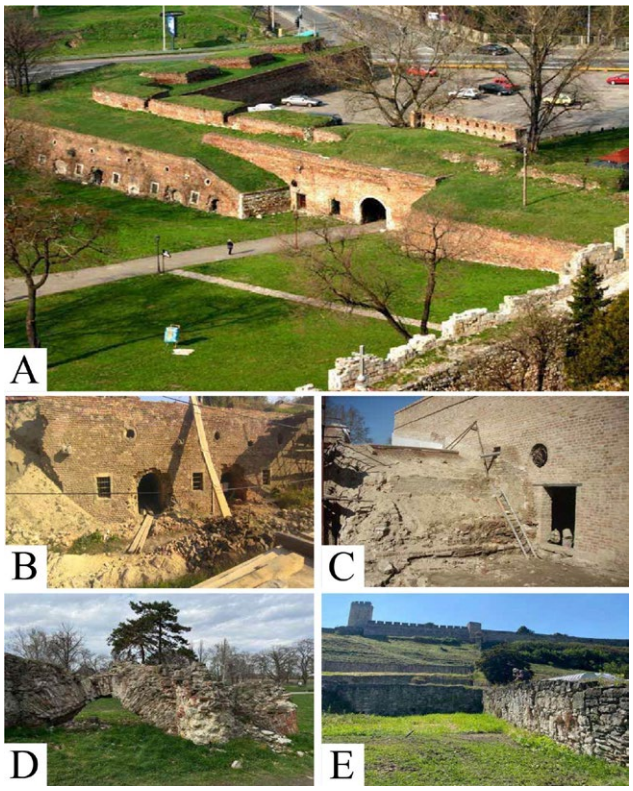


Fig. 2. A) Kalemegdan 1 before restoration (the image was downloaded from the website https://www.beogradskatvrdjava.co.rs/glarij aslika/%d0%b2%d0%b8%d0%b4%d0%b8%d0%bd-%d0%ba%d0% b0%d0%bf%d0%b8%d1%98%d0%b0/?lang=en), B) Kalemegdan 1 during restoration, C) Kalemegdan 1 during restoration, D) Kalemegdan 2 before restoration and E) Kalemegdan 2 before restoration (The author of this photo is Dr. Katarina Breka).

including large cities and villages, often inhabiting stone walls and buildings (Böhme et al., 2009; Speybroeck et al., 2016). It is a diurnal species, but limited nocturnal activity was observed under artificial light (Carretero et al., 2012). In the northern part of its range, the Common Wall Lizards are active from February to November, while hibernating during winter (Speybroeck et al., 2016). However, activity can also occur in winter on sunny and warm days (Gvozdenović Nikolić et al., 2024; Sakelarieva et al., 2025). This species exhibits homing behaviour (Scali et al., 2013) and males display territorial behaviour (Avery, 1978). The Common Wall Lizard is not globally endangered and is classified as a species of Least Concern (LC) according to the IUCN criteria (Bowles, 2024). However, at the European level, the species is protected under the Bern Convention (Appendix II) and included in Annex IV of the European Habitat Directive. In Serbia, it is not protected.

Fieldwork and capture-mark-recapture

At the Kalemegdan 1 locality, a CMR study was conducted from October 2022 to October 2024 (before the restoration of the old fortress) to establish baseline data on the size and demographic parameters of the Common Wall Lizard population. During eight seasons, lizards were captured in suitable weather and daylight conditions, using a standard noose technique. Each individual was marked using the heat-branding method, in which the unique combinations of ventral scales were burned (Ekner et al., 2011). During each sampling period, Kalemegdan 1 was thoroughly surveyed. We inspected the fortress wall several times in detail, including depressions and holes, and captured active lizards. The intervals among sessions were not uniform, but this was accounted for in the population size estimation. For each capture, the following data were recorded: sex, snout-vent length (SVL), body mass and location.

During the restoration of the fortress, locality Kalemegdan 1 was surveyed, when possible, as access to the site was limited by construction works. Adjacent sections of the fortress that had not undergone restoration were also surveyed to detect potential dispersal of marked individuals. At locality Kalemegdan 2, near Kalemegdan 1, where the fortress has not been restored, individuals of the Common Wall Lizard were observed, and the CMR study continued during 2025. Lizards were captured during five sessions from March to October 2025. All individuals were checked for marks applied during the pre-restoration CMR study.

Captured lizards were transported to the laboratory at the Institute for Biological Research "Siniša Stanković" – National Institute of the Republic of Serbia, where they were measured and marked, and then returned to the place of capture.

Statistical analysis

Population size was estimated using the openp() function in the Rcapture package in R (Rivest and Baillargeon, 2022; R Core Team, 2025), applying an open-population Jolly-Seber parameterisation. Population size was estimated separately for both study localities (Kalemegdan 1 and Kalemegdan 2) using pooled sex data to increase the robustness of the estimates. For each locality, the total population size (N) and associated 95% confidence intervals (CI) were calculated based on the observed capture histories. Habitat suitability, recapture rates, and sex ratio were compared between localities and between the pre- and post-restoration periods at Kalemegdan 1.



Fig. 3. Historical view of the Kalemegdan 1 locality from October 2022 to May 2025. Source: Google Earth Pro.

Residuals from the linear regression of logarithmic body mass on logarithmic body length (SVL) were used as a measure of BCI. Body condition index values between the localities were compared separately for October and March using independent-samples t-tests. For this analysis, only individuals from the Kalemegdan 1 locality captured in October 2022 and March 2023 were included; other individuals from this locality were excluded because they were affected by reconstruction works on the parking lot and fortress walls. Differences in BCI among years were evaluated separately for each sampling month (October and March) and sex using pairwise independent-samples t-tests. To account for multiple comparisons, p-values were adjusted using the Bonferroni correction.

RESULTS

Capture-mark-recapture

At the Kalemegdan 1 locality, during eight sessions, we captured 372 lizards. Of these, 44 individuals were recaptured after marking, with some individuals captured once, twice or three times, resulting in a total of 51 recapture records (Table 1), with an overall recapture rate of 11.82%. According to the Jolly-Seber model,

Table 1. Number of captured and recaptured *Podarcis muralis* individuals by locality and sex

Locality	Capture/Recapture	Sex	N
Kalemegdan 1	C	F	198
		M	174
	R	F	19
		M	32
Kalemegdan 2	C	F	93
		M	85
	R	F	8
		M	11

the estimated population size at Kalemegdan 1 was 1211 individuals (95% CI: 1179-1244). At Kalemegdan 2, during five sessions from March to October 2025, 178 individuals were marked. Of these, 17 were recaptured after marking, with two individuals recaptured twice, resulting in a total of 19 recapture records (Table 1), with an overall recapture rate of 9.64%. The estimated population size at Kalemegdan 2 was 780 lizards (95% CI: 752-808). No lizards recaptured at Kalemegdan 2 had been previously marked at Kalemegdan 1.

At both localities, more females than males were caught (198 vs 174 and 93 vs 85 at Kalemegdan 1 and Kalemegdan 2, respectively), but males were recaptured more frequently than females (18.39% vs 9.60% and 12.94% vs 8.60% at Kalemegdan 1 and Kalemegdan 2, respectively) (Table 1).

Habitat suitability

Both analysed localities were suitable habitats for the Common Wall Lizard, as the walls of the Kalemegdan Fortress contained numerous cracks and plants where the lizards could feed, hide and thermoregulate. Even before the restoration, these sites were exposed to anthropogenic pressure due to grass cutting and the presence of many tourists. However, the reconstruction of the fortress walls at the Kalemegdan 1 locality in 2024 and 2025 resulted in habitat destruction; consequently, no lizards were found at this locality in 2025.

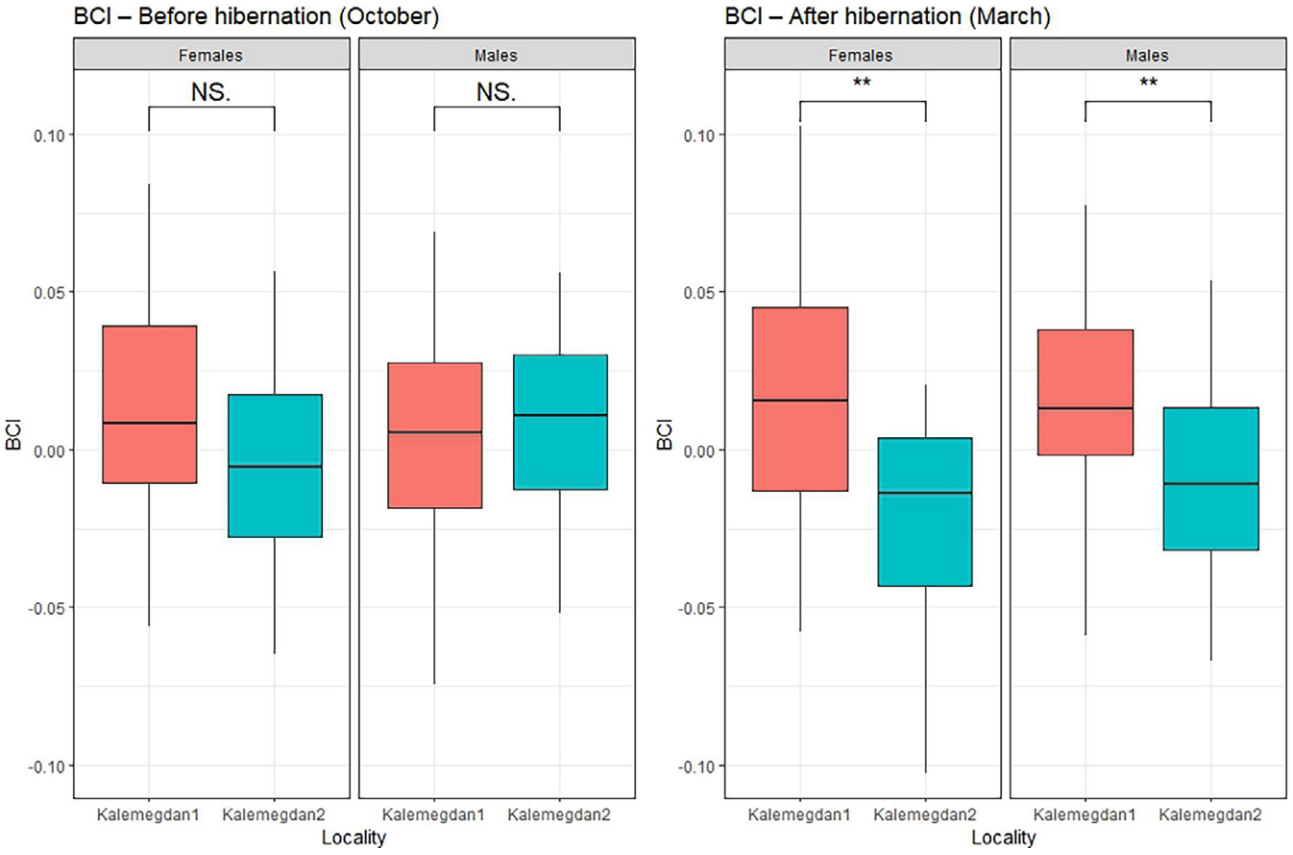


Fig. 4. Body condition indices for *Podarcis muralis* individuals at Kalemegdan 1 and Kalemegdan 2 localities: before hibernation (October) and after hibernation (March).

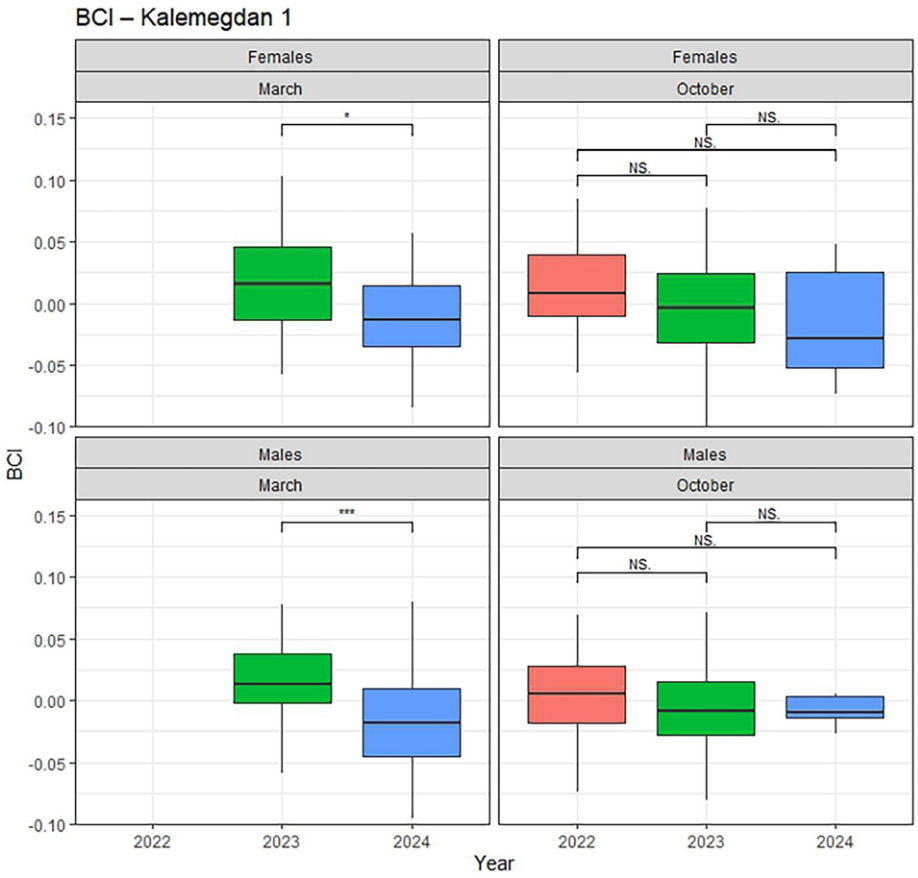


Fig. 5. Body condition indices for *Podarcis muralis* individuals from the locality Kalemegdan 1 across years, shown separately for each sex and month.

Body condition index

Before hibernation (in October), no significant differences in BCI were found between localities for either females (P = 0.434) or males (P = 0.413) (Fig. 4). After hibernation (in March), both female and male lizards from Kalemegdan 1 exhibited significantly higher BCI than those from Kalemegdan 2 (P = 0.003 and P = 0.002, respectively) (Fig. 4).

Body condition index values in lizards from Kalemegdan 1 showed a decreasing trend over the years. In March, this decrease was statistically significant in both females (P = 0.012) and males (P < 0.001), while in October, the overall decreasing trend in BCI values over the years was not statistically significant in either sex after correction for multiple comparisons (Fig. 5).

DISCUSSION

Our results highlight a rarely addressed conservation issue: cultural monument restoration can cause biodiversity loss when ecological considerations are excluded from planning. This study shows that the restoration of the cultural monument Kalemegdan led to the local collapse of a previously stable lizard population. Before restoration, Kalemegdan 1 supported approximately 1211 individuals, with high recapture rate (11.8%). However, no marked individuals were detected among 178 lizards captured in nearby unmodified area (Kalemegdan 2), indicating near-total mortality rather than dispersal.

Reptiles are particularly vulnerable to such impacts due to their dependence on microstructural habitat features, which are often targeted for removal during restoration (O'Sullivan et al., 2023). Historic structures serve as artificial habitats for many animals, yet restoration guidelines rarely consider reptile fauna (Todd et al., 2010). Plugging holes and crevices likely caused direct mortality, as many lizards and snakes were hibernat-

ing within stone walls when reconstruction began. Disturbance during hibernation is a major threat because reduced metabolic rates and body temperatures limit escape capacity (Dubiner et al., 2023). Despite the presence of suitable habitat in nearby unmodified sections of the fortress, no marked individuals were found after restoration. This is consistent with previous findings that many lizard species exhibit strong site fidelity, small home ranges, and limited movement between habitat patches (Scali et al., 2013; Spikmans and Bosman, 2015; Williams et al., 2021). Additionally, urban landscapes further exacerbate isolation due to barriers such as roads, buildings, and human activity (Beninde et al., 2016). One side of Kalemegdan faces the Sava and Danube rivers, while the other side is bordered by a highly urbanised part of the city. The entire complex is surrounded by busy streets, thus the lizards have nowhere to migrate. Although limited dispersal to surrounding habitats cannot be excluded, it was probably minimal.

In addition to habitat destruction and lizard mortality, the disturbance by workers and machinery on the site can indirectly affect BCI, reproduction and survival rates through decreased activity due to hiding (Putman et al., 2024). Increased human presence leads to increased stress and mortality of animals (Josserand et al., 2017; Lazić et al., 2017). Structural complexity is crucial for reptile survival; thus, the loss of crevices eliminates thermal refugia, hiding places, and overwintering sites, as well as reduces basking, sheltering, and egg-laying areas for lizards (Elbahi et al., 2023). If they managed to mate during the spring, the females had fewer suitable places to lay eggs than before the restoration, and it is very likely that those eggs were also buried and destroyed during the season.

Results showed that the restoration activities at Kalemegdan 1 negatively affected the BCI of lizards, during the post-hibernation period. While no significant differences in BCI between localities were detected before hibernation, both females and males from Kalemegdan 1 had significantly higher BCI than those from Kalemegdan 2 after hibernation. As lizards do not feed during hibernation, post-hibernation BCI reflects the cumulative effects of refuge quality, thermal stability and disturbance experienced during the overwintering period. Differences in BCI between localities observed after hibernation may reflect differences in hibernation conditions. The absence of significant differences in BCI between localities during October suggests that lizards are able to partially compensate for early-season energetic deficits later in the active season.

The values of the body condition index of *Podarcis muralis* individuals at Kalemegdan 1 declined over successive years, indicating a progressive habitat deterioration. Significantly lower spring BCI values in 2024 compared to 2023 at Kalemegdan 1 probably reflects restoration activities beginning in late 2023. As a saxicolous species, *Podarcis muralis*, strongly depends on wall microstructures for thermoregulation and hibernation; their loss possibly increased energetic costs and overwinter mortality. Additionally, post-hibernation energy reserves can have cascading effects on survival, growth and reproduction (Naulleau and Bonnet, 1996). A downward trend in the condition index was also recorded for the pre-hibernation period. Although not statistically significant, it is most probably a consequence of locality reconstruction. Structural modification of the fortress walls progressively reduced habitat quality for lizards by reducing available food. Vegetation removal affects the diversity and abundance of insects, which are the main food source for the Common Wall Lizard (Speybroeck et al., 2016). The Common Wall Lizards exhibit territorial behaviour, and the loss or alteration of habitat can increase aggressive interactions and displacement (Avery, 1978). These social interactions may elevate energy expenditure and stress levels, further reducing body condition. Additionally, the loss of refugia probably elevated predation risk, further contributing to reduced condition and survival.

Conservation implications

Effective conservation plans can mitigate the impacts of anthropogenic activities on urban populations by reducing habitat fragmentation and by building and maintaining bio corridors (Bozhilova and Frissen, 2025). Integrated heritage–biodiversity management is necessary for mutual benefit. There are good examples of how the negative effects of reconstruction can be mitigated (Spikmans and Bosman, 2015).

Conservation measures in similar cases should include: conducting pre-restoration reptile population surveys to identify critical refugia and hibernation sites; implementing artificial refugia, such as rock piles or crevice blocks, in nearby areas before and after restoration; planning restoration activities seasonally, avoiding work during hibernation and breeding periods; using phased restoration – closing holes progressively rather than sealing entire walls at once – to allow animals time to relocate gradually; conducting continuous long-term monitoring of reptile populations; and ensuring coordination between conservation agencies and cultural heritage authorities to comply with wildlife protection legislation.

Further directions

Other animals also inhabit this locality, and *Podarcis muralis* is only one component of the ecosystem. For example, *Dolichophis caspius* (Gmelin, 1789) also inhabits Kalemegdan and was probably under considerable pressure during the restoration because it also had been using holes in the wall for shelter and hibernation. *Dolichophis caspius* feeds on lizards and small rodents, and the sudden decline in lizard abundance likely reduced food availability. Such trophic disruptions can destabilise urban ecosystems, where predator–prey dynamics are already simplified compared to natural environments (Straka et al., 2025). Given that the current habitat of Kalemegdan 1 has been greatly altered, with shelters and much of the green areas concreted, it is necessary to monitor the state of the population at Kalemegdan 2 and whether parts of lizard and other reptile populations may re-settle at the Kalemegdan 1 locality.

Conclusions

In many countries, including Serbia, there is no practice of conducting detailed assessments of the impact of the construction or restoration of facilities on reptile populations. Based on the results of this study, we have concluded that restoration of cultural monuments, old fortresses, and stone walls can cause loss of reptile habitat and a decline in reptile populations. Urban lizard populations are small and fragmented, so if appropriate mitigation measures are not implemented during restoration (such as construction of migration corridors, artificial shelters or translocation), local populations may decline or become extinct. Culturally important structures are also ecological assets; therefore, integrated planning that explicitly incorporates biodiversity considerations is essential to preserve both cultural heritage and urban wildlife.

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Life history of the Anji salamander (*Chordata*, Amphibia, Caudata, Hynobiidae) from fertilized eggs to subadults

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Abstract. Anji salamander *(Hynobius amjiensis*) is a rare species endemic to China, living in peat moss swamp at an altitude of 1,300 meters on Longwang Mountain in Anji County, Zhejiang Province. Anji salamander larvae live in natural puddles in the swamp, and the adults spend most of their time in the humus under the peat moss, only briefly visiting the puddles during the breeding period to spawn and then disappear back into the swamp. Therefore, the life history of Anji salamander is poorly understood. In this study, we analyzed the developmental characteristics, behaviors and life habits of Anji salamander embryos from fertilized eggs to subadults. Following previous studies on the development of small salamanders and amphibians, we divided Anji salamander development into six stages, including embryonic development, balance bar, limb development, pre-metamorphosis, metamorphosis, and subadult development, with 34 developmental nodes. The period before the shedding of the balance bar was a relatively sensitive period. The metamorphosis period, especially the late metamorphosis period, was the most sensitive period from larvae to subadults, during which the small salamanders undergo complex physiological and biochemical changes such as relatively long fasting time, respiratory changes, and transition from aquatic to terrestrial life. These data fill in some of the gaps in the life history of Anji salamander from fertilized eggs to subadults, and are valuable for its conservation and future endeavor on artificial breeding.

Keywords. Anji salamander, *Hynobius amjiensis*, life history, metamorphosis, molt, subadult.

INTRODUCTION

Anji salamander, first discovered in 1991 in Longwang Mountain, Anji County, Zhejiang Province (Gu et al., 1999), and subsequently named *Hynobius amjiensis*, belongs to the class Amphibia, order Caudata, family Hynobiidae, genus *Hynobius*. It is a critically endangered (CR) species in China, one of only two families of Hynobiidae in Zhejiang Province (Yang et al., 2016). Previous studies showed that Anji salamander lives in peat moss swamps at an altitude of about 1300 m (Zhang et al., 2010a), and only enters the swamp puddles to spawn during the breeding season from late November to March each year (Liu et al., 2016). Anji salamander lives in peat moss swamps during the non-breeding season. This cryptic habitat preference contributed to

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its recent discovery (Zhang et al., 2010a). Anji salamander has strict requirements for the quality of its living environment, and the scarcity of its spawning sites and intraspecific competition have led to a very low survival rate of Anji salamander larvae. Therefore, fewer than 1% of hatchlings typically survive to the subadult stange and successfully emerge onto land (Gu et al., 1999; Liu et al., 2016). In recent years, with global warming, the habitat suitable for its survival has been decreasing (Pan et al., 2020). We work in the reserve to explore ways to improve the survival rate through artificial breeding of hatchlings into subadults and then release them back to the land (Chen et al., 2018a). During this process, we also carried out a study on the life history of Anji salamander in order to provide valuable reference data for subsequent conservation works.

Life history refers to the adaptive evolution of morphological, physiological, and behavioral patterns under long-term natural selection in which animals develop their own unique life responses based on their genetic characteristics as well as environmental pressures (Williams, 1966; Begon et al., 1990; Nettle et al., 2019). Life history traits generally involve biological characteristics such as growth, development, and reproduction, and are specifically described as growth patterns, body size, age at sexual maturity, longevity, and clutch size (Stearns, 1977). Amphibians are more vulnerable to habitat loss and fragmentation than other terrestrial animal groups, due to their specialized amphibious life history, relatively weak terrestrial migration, and high dependence on habitat (Tan et al., 2023). With environmental degradation such as reduced precipitation, diseases, and habitat loss, amphibians are facing tremendous survival challenges. The study of internal and external factors such as amphibian life history, habitat, etc., is of great significance to amphibian conservation efforts (Meletiadis et al., 2023; Pottier et al., 2025).

Some studies have shown that the life history of the Hynobiidae is unique compared to other amphibians, Mi et al. (2007) categorized the embryonic development of *Hynobius guabangshanensis* into 21 developmental stages. The developmental period of *Hynobius leechii* from fertilized egg to subadult is divided into 27 developmental stages. They suggested that the long period of time after metamorphosis without emerging onto land would cause the death of *Hynobius leechii*, and that *Hynobius leechii* completing metamorphosis may die due to foraging difficulties. Therefore, special protection methods can be used to improve the survival rate of subadults during this developmental period (Ma et al., 1994). The development of *Hynobius maoershanensis* is divided into four stages: hatching stage, forelimb bud stage, hindlimb bud stage, and metamorphosis stage. After the metamorphosis stage, *Hynobius maoershanensis* has a strong crawling ability, but it still cannot survive in a dry environment. This has significant relevance for artificial breeding. Therefore, during metamorphosis, the developmental status should be closely monitored and it should be placed in a safer environment to prevent unwanted mortality due to climbing out of the culture tank (Ning et al., 2021). Data on the Anji salamander development is limited to observational studies on the staging of developmental stages and developmental processes from fertilized egg to embryo (Cao et al., 2024; Qiu et al., 2024).

In this study, we illustrated the complete life history of Anji salamander from fertilized eggs to subadults. We also analyzed the developmental characteristics of each developmental stage and the ontogenetic habitat shifts of Anji salamander, advancing our understanding of the physiological behavior of Anji salamander, contributing additional data to conserve *Hynobius* species.

MATERIALS AND METHODS

Objects of study

In order to reduce the number of Anji salamander larvae that are unable to come ashore due to cannibalism and to increase the larvae survival rate, we selected the last batch of spawned eggs to be raised until the subadults and released in our sanctuary. The reason for this is that most of the larvae hatched from the last batch of spawned eggs in nature become the target of predation by larvae hatched earlier, and very few of them can survive.

Embryo incubation and animal breeding

Fertilized eggs were brought into a glass tank (500 × 300 × 160 mm), waiting for them to come out of the membrane, and then separated from the live bait and fed after 3 days, usually opening on the 3rd-5th day. The water environment is set according to the data detected in the natural puddle in the reference habitat, maintained at 4-10 °C before hatching, and 8-15 °C after hatching out of the membrane (the dissolved oxygen was maintained at 7.700-7.900mg/L, the pH value maintained at 6.9-7.0, the phosphate maintained at 0.045 ± 0.005mg/L, and the zinc maintained at 0.05 ± 0.01mg/L), and the full-spectrum light was maintained for 12 hours each day. A quiet environment was maintained.

Staging criteria

In this study, the staging criteria for Anji salamander were based on other species of Hynobiidae, such as the growth and developmental stages of *Hynobius leechii* (Ma et al. 1994), the postembryonic developmental characteristics of *Hynobius guabangshanensis* (Mi et al., 2007), and the developmental stages of *Hynobius maoershanensis* (Ning et al., 2021). Together with the developmental characteristics of the Anji salamander itself, we divided Anji salamander development from fertilized egg to sub-adult into six stages, namely embryonic development stage, balance bar stage, limb development stage, pre-metamorphosis stage, metamorphosis stage, and subadults, with a total of 34 developmental nodes. The first stage, the embryonic development stage, spans from egg fertilization to hatching. The second stage, the balance bar stage, is marked by the appearance and subsequent disappearance of the balance bar. The third stage, the limb development stage, involves the growth of limbs, including the appearance of the limbs, and the development of fingers and toes. The fourth to six stages are pre-metamorphosis stage, metamorphosis stage, and subadult stage, respectively.

Data acquisition

We used a soft ruler to measure the body length from the tip of the head to the tail, and a Hored Portable Water Quality Detector (HD-S15) to measure the water quality indicators. Images were taken using a ZEISS microscope or HUAWEI nova11. Pattern diagrams were drawn based on the pictures of the samples and incorporating the main characteristics of the development in each period using the Honor magicpad2 on the GoPaint 3.1.2 software, Photoshop 2024 was used for photo layout, and Origin 2024 for statistical charts.

RESULTS

Fertilized egg and embryonic stages of Anji salamander

Adults Anji salamander pair up for spawning in natural puddles in peat moss swamps from November through March each year. Fertilized egg particles (defined as 0h, Fig. 1 A1) were found in egg capsules. The fertilized egg then began the cell division phase, a period of increasing cell ploidy, and at 16 ± 3 h (average ± standard deviation) of embryonic development, as the number of cell divisions increased, more and more cells formed clusters of cells known as multicellular phases (Fig. 1 A2). At 132 ± 4 h of embryonic development, as the embryo continued to develop, cells continued to divide and increase, the blastoderm cells were rolled into the embryo to form the dorsal lip, and the embryo was irregularly round with an uneven surface, known as the protoganglionic embryonic stage (Fig. 1 A3). At 164 ± 4 h of embryonic development, a shallow neural groove appeared in the embryo. The sides of the shallow neural groove were relatively flat, and the edges had not yet fully formed the neural folds, which is called the neural plate stage (Fig. 1 A4). At 176 ± 3 h of embryonic devel-

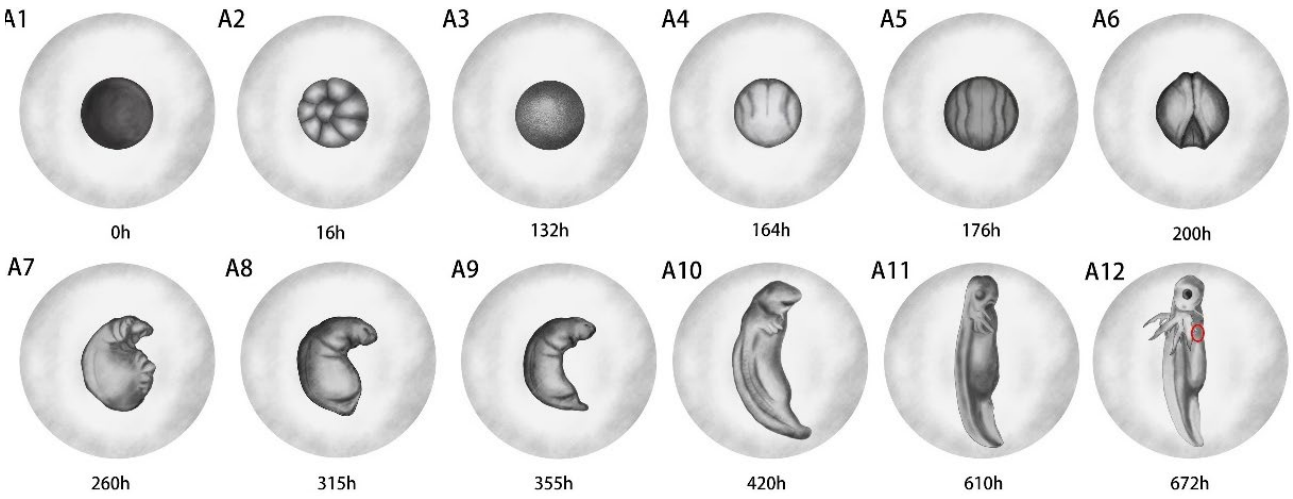


Fig. 1. Embryonic development in Anji salamander. (A1) Fertilized egg; (A2) Multicellular stage; (A3) Gastrula stage; (A4) Neural plate stage; (A5) Neural fold stage; (A6) Neural groove stage; (A7) Neural tube stage; (A8) Early tail bud stage; (A9) Late tail bud stage; (A10) Early external gill stage; (A11) Late external gill stage; (A12) Incubation stage. Red circles mark forelimb buds.

opment, the folds on either side of the neural groove continued to develop into irregular folds known as the neural fold stage (Fig. 1 A5). At 200 ± 3 h of embryonic development, the embryo elongated in the direction of the neural plate, the neural folds converged inward, and the sulcus became deeper, referred to as the neural sulcus stage (Fig. 1 A6). At 260 ± 5 h of embryonic development, the gradual fusion of the embryonic neural folds and the subsequent formation of the embryonic neural tube occurred. At this time, the embryo formed a primitive head and distinct somites, which is called the neural tube stage (Fig. 1 A7).

At 315 ± 3 h of embryonic development, the embryo elongated along the neural tube toward both ends, tail buds appeared, and both the primitive head and tail buds curved ventrally, with a more pronounced expansion of the abdomen, referred to as the early tail bud stage (Fig. 1 A8). The embryo's body size increased, and there was a slight depression on both sides of the head. At 355 ± 5 h of embryonic development, the abdominal expansion became markedly smaller, and the tail bud contour was clear, referred to as the late tail bud stage (Fig. 1 A9). At 420 ± 5 h of embryonic development, the embryo was larger and more extended, the larval body was largely formed, and three primitive external gills and a balance bar appeared on both sides of the head, referred to as the early external gill stage (Fig. 1 A10).

At 610 ± 3 h of embryonic development, the embryonic head organ development was essentially complete. The eye was basically formed, and smaller gill filaments were present on the three pairs of gills, referred to as the late external gill stage (Fig. 1 A11). At 672 ± 3 h of embryonic development, as the embryo continued to develop, the hatchling's morphology matured, the gill filaments on the three external gills became abundant, the balance rods were clearly visible, forelimb buds were forming, and the hatchling was occasionally seen to move within the egg, referred to as the incubation period (Fig. 1 A12). At 694 ± 48 h of embryonic development, larvae broke through the egg membrane and hatched out, usually with a small amount of yolk remaining. Most lay on their sides at the bottom of the water and are referred to as out-of-membrane larvae (Fig. 2 B1).

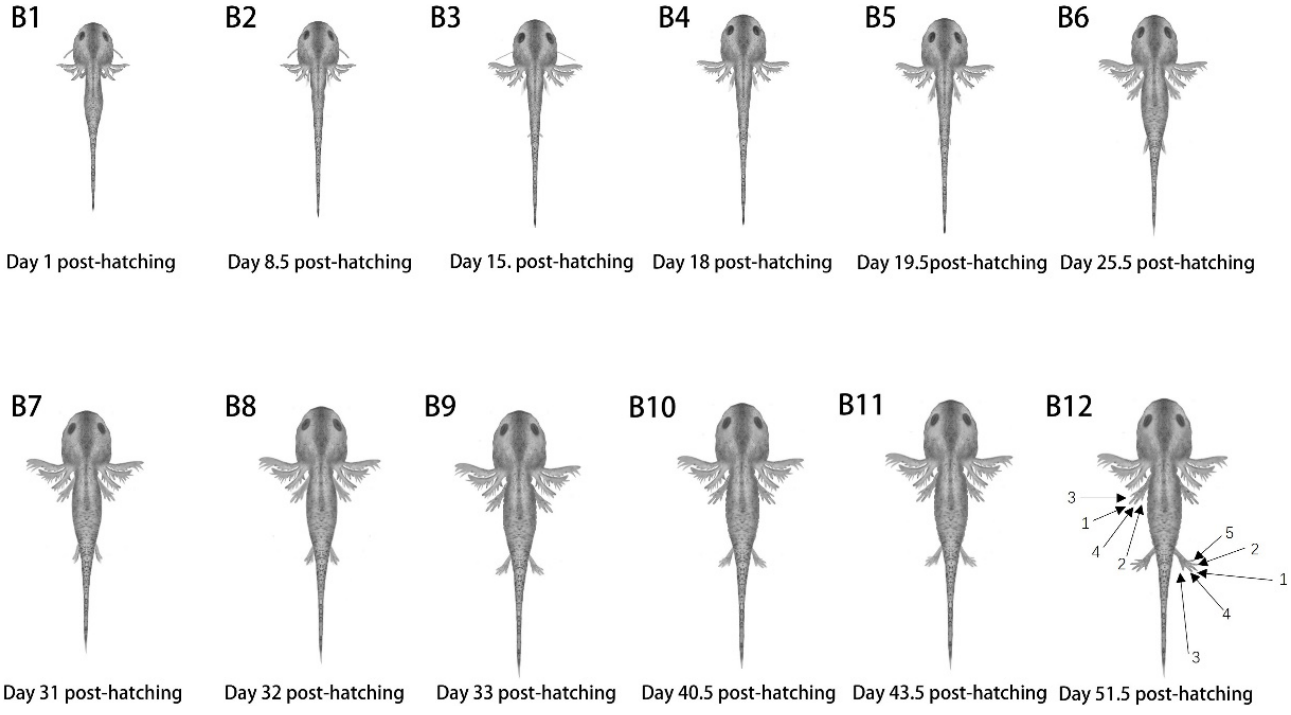


Fig. 2. Development of the limbs in Anji salamander. Development of the limbs in Anji salamander. (B1) Hatchlings of larva; (B2) Differentiation of forelimb forearm; (B3) Differentiation of forelimb 1st finger; (B4) Differentiation of forelimb 2nd finger and hindlimb buds; (B5) Differentiation of forelimb 3rd finger and hindlimb forearm; (B6) Differentiation of forelimb 4th finger and hindlimb 1st toe; (B7) Webbing between the fingers of the forelimbs and differentiation of the hindlimb 2nd toe; (B8) Differentiation of hindlimb 3rd toe; (B9) Completion of forelimb development and differentiation of hindlimb 4th toe; (B10) Differentiation of hindlimb 5th toe; (B11) Webbing between the toes of the hindlimbs; (B12) No webbing of the toes of the hindlimb. The numbers in the Fig. indicate the order of development of the different fingers/toes from top to bottom.

Anji salamander balance bar stage

We define the day of emergence of hatch as post-hatching day 1. Balance bars began to appear and develop in the early stages of the external gills. Small bar-like tissues appeared on both sides, and the stage from the appearance of the balance bars to their complete shedding is referred to as the balance bar stage. The development of the balance bars on both sides of the head was completed in the embryo 2-3 days before exsanguination (Fig. 1 A10). After the membrane was removed, the balance bars gradually changed from a short, thick state to a long, slender form. This transformation essentially stopped around the 17.5 ± 2.5 day post-hatching, reaching a length of approximately 2.00 ± 0.50 mm, and disappeared by the 19 ± 2 day post-hatching (Fig. 3). The balance bars of Anji salamander disappeared in an asymmetric shedding pattern, and the balance bars were shed with either the left or right side of the bars shed first, and the other side shedding within the next 24 h. Moreover, the entire stage in which the balance bar exists is accompanied by the development of the forelimbs, and the hindlimbs began to develop later in the development of the balance bar.

On day 5 ± 2 post-hatching, the larvae began feeding, and if there was no external food as an opener, Anji salamander might resort to intraspecific predation. The swallowing process typically involved biting the head or other body parts, with the prey being gradually ingested. The digestive time varies depending on the size of the food, and some of the digestive time in the mouth could reach up to 16 hours or even longer. After hunting for a companion, Anji salamander hide in a quiet corner and slowly enjoyed a meal on its own. However, if it was attacked or otherwise disturbed, it would assess the risk of abandoning the food or taking it and running away. During this stage, Anji salamander was relatively active, preferring dark and quiet water or even hiding in the gaps between rocks or in the mud underwater, occasionally swimming out of the water. Individuals at this stage were small, flexible, and preferred to be alone. They exhibited agitation during balance bar shedding and gradually settled down afterwards.

Anji salamander limb developmental stage

The process of limb development, including the 4 fingers on the forelimbs and the 5 toes on the hindlimbs, from their emergence to complete development, is referred to as the limb developmental stage of Anji salamander. As early as the embryonic stage, the forelimb bud began to develop (Fig. 1 A12). On day 8.5 ± 1.5 post-hatching, the forelimb bud developed into the forearm (Fig. 2 B2). On day 15.5 ± 1.5 post-hatching, the first forelimb finger began to differentiate, but the longest clear skin was followed by the development of the fourth finger (Fig. 2 B3). On day 18.5 ± 1.5 post-hatching, the second finger of the forelimb began to differentiate (Fig. 2 B4). On day 18 ± 2 post-hatching, the hindlimb bud also began to form (Fig. 2 B4). On day 19.5 ± 1.5 post-hatching, the forelimb's third finger and the hindlimb's forearm began to develop (Fig. 2 B5). On day 20.5 ± 1.5 post-hatching, the fourth finger of the forelimb began to differentiate, and capillaries started to appear within the longest transparent skin layer. On day 25.5 ± 1.5 post- hatching, the rudimentary form of the four fingers was formed, but there were still web-like connections between each finger (Fig. 2 B6).

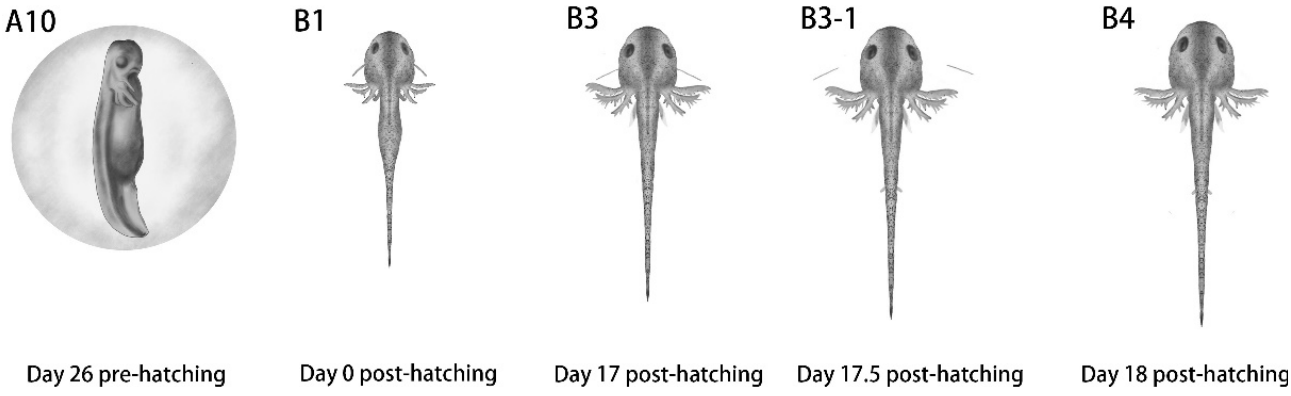


Fig. 3. Balance bar stage development in Anji salamander. (A10) Balance bar emerges during embryonic development, indicated by the red arrow. (B1) Balancing bars at the time of exiting the membrane; (B3) Balance bars get longer; (B3-1) Balance bars falling off; (B4) Balance bars disappear.

On day 30 ± 1 post- hatching, the first toe of the hind limb began to differentiate (Fig. 2 B6). On day 31 ± 1 post- hatching, the second toe of the hind limb began to differentiate (Fig. 2 B7). On day 32 ± 1 post-hatching, the third toe of the hind limb began to differentiate, and at the same time, the skin of the fourth toe had also formed, but it had not yet undergone differentiation from capillaries (Fig. 2 B8). On day 33 ± 1 post-hatching, the fourth toe of the hind limb began to differentiate (Fig. 2 B9). On day 36.5 ± 1.5 post-hatching, the forelimbs had fully developed, with the fourth toe having formed and there were no web-like connection between the fourth toe and the other toes (Fig. 2 B9). On day 40.5 ± 1.5 post-hatching, the hind limb began to differentiate and the fifth toe emerged (Fig. 2 B10). On day 43.5 ± 1.5 post-hatching, the rudimentary form of the hind limb's five toes was formed, although there were still web connections between the five toes of the hind limb (Fig. 2 B11). On day 51.5 ± 1.5 post-hatching, limb development was completed. At this point, the hind limbs had developed the fifth toe and there was no web connection between the five toes (Fig. 2 B12).

On day 34 ± 1 post-hatching, the ventral costal fovea begins to appear (Fig. 4 B9). On day 49 ± 1 post-hatching, the ventral costal fovea gradually became deeper (Fig. 4 B10). The four fingers of the forelimbs of Anji salamander developed in the following sequence from top to bottom: 3, 1, 4, 2 (Fig. 2 B12). That is, the finger at the top of the picture developed as the third one, followed by the first, fourth and second fingers in sequence. The sequential development order of the five toes of the hind limbs from top to bottom was 3, 4, 1, 2, 5 (Fig. 2 B12).

The behavioral characteristics during this stage were mainly characterized by using live prey as the main food source, with an increase in movement ability. They could swim, or move on the bottom of the water, or reach the

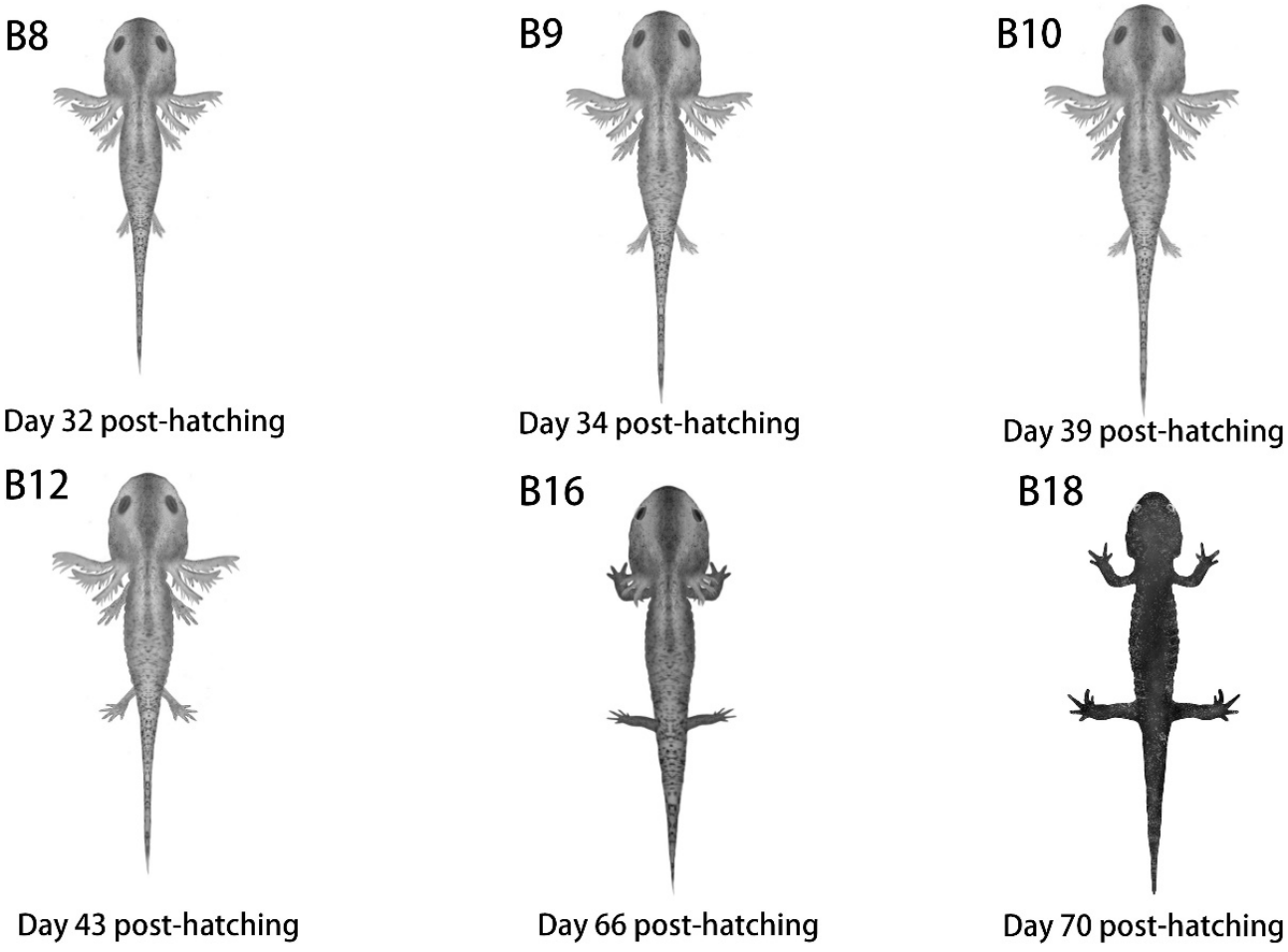


Fig. 4. Ventral costal fovea formation in Anji salamander. (B8) Day 32 post-hatching, the ventral costal fovea have not yet formed; (B9) Day 34 post-hatching , the ventral costal fovea begin to form; (B10) Day 39 post-hatching; (B11) Day 43 post-hatching; (B16) Day 66 post-hatching, the ventral costal fovea is basically shaped; (B18) Day 70 post-hatching, the ventral costal fovea are obvious.

surface. The growth rate was relatively fast. During this stage, if the larval Anji salamander lived in groups and touched each other's bodies, they would engage in mutual attacks and biting, especially on the limbs and external gills. By this stage, the limbs, the four pairs of fingers on the front limbs and the five pairs of toes on the hind limbs had fully developed, with the body length reaching 50.0 ± 5.0 mm.

Anji salamander pre-metamorphosis stage

The stage from the completion of the development of the limbs and fingers of the Anji salamander to 12 ± 3 days before the metamorphosis stage is referred to as the pre-metamorphosis stage. During this stage, the gill filaments of Anji salamander turned red, the blood vessels became abundant, the body size increased larger and longer, the limbs became strong and powerful, the body became plump, with a body length of 55.0 ± 5.0 mm. The dorsal fin gradually disappeared, the ventral costal fovea deepened, and the eyeballs slightly protruded.

The behavioral characteristics during this stage are mainly characterized by a significant increase in food intake, frequent movement at the bottom of the water and in the upper water layer, and sometimes extending their heads close to the water surface to exhale bubbles. They prefer to stay in dark corners alone, had less activity during the day, and were relatively more active at night.

Anji salamander metamorphosis stage

The external gills began to retract inward until they completely disappeared. The breathing method changed from gill respiration to using skin and lungs for breathing, and the lifestyle shifted from aquatic to semi-aquatic. This process is what we call the metamorphosis period. During the metamorphic stage, the gill filaments of Anji salamander gradually became thicker and shorter, and

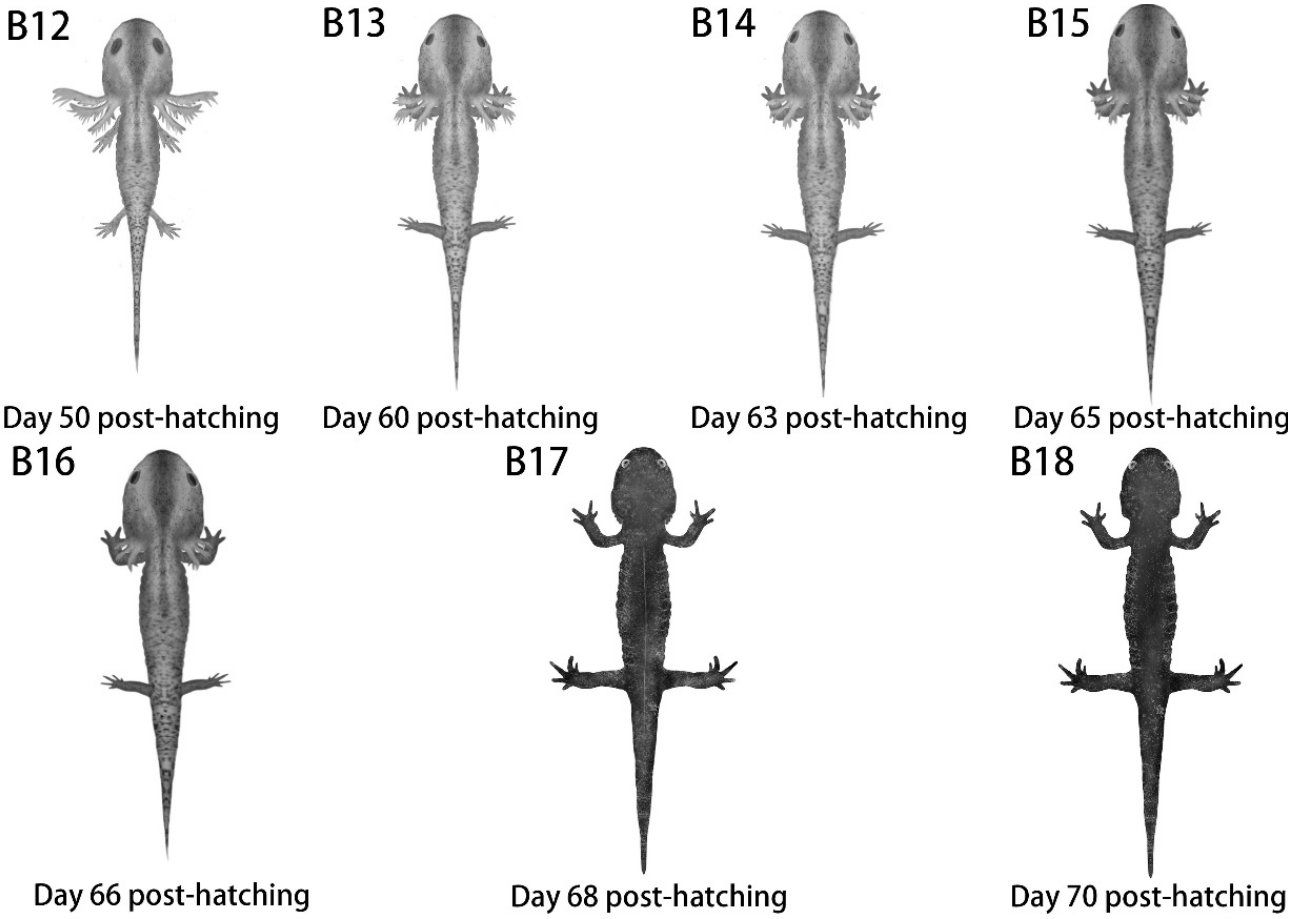


Fig. 5. Metamorphosis in Anji salamander. (B12) Day 50 post-hatching; (B13) Day 60 post-hatching; (B14) Day 63 post-hatching; (B15) Day 65 post-hatching; (B16) Day 66 post-hatching; (B17) Day 68 post-hatching; (B18) Day 70 post-hatching.

the external gills also became shorter (Fig. 5), and before the external gills had completely disappeared, Anji salamander began to undergo its first molting process of life (Fig. 6). Subsequently, the skin on the upper part of the gill cover and the skin on the neck gradually merged and covered the area where the gills had been exposed, forming a neck fold. Eventually, the outer gills completely disappeared. During this stage, the head of Anji salamander became more pointed, its body color darkened, white spots appeared on its body surface. There was a significant protrusion of the eyeball when compared to the larval eyeball, and the ventral costal fovea continued to deepen.

The behavioral characteristics of Anji salamander during its metamorphosis stage were that it spent a long time hiding in darker places, was sensitive to light and sound, had a darker body color and more body spots, with enhanced color-changing and concealment abilities. During metamorphosis stage, there was almost no phenomenon of mutual attacks or cannibalism. After the external gills disappeared, Anji salamander began to engage in long-distance crawling, leaving the water puddles and searching for new habitats and food. Its dietary characteristics also underwent significant changes. From the early stage of metamorphosis, its appetite significantly weakened and even led to prolonged fasting. It took approximately 42 ± 3 days of fasting before it could resume normal feeding.

Anji salamander subadult stage

We refer the period from the end of metamorphosis to sexual maturity as subadult stage. During this stage, the color of Anji salamander was significantly different from that of the larvae. The color of the subadult is darker and has white dot-like spots compared to the larvae. The head of the larva was sharper, the eyes were more prominent and had fully formed ventral costal fovea (Fig. 5 B12, B18). During the subadult stage, the body length and weight of the salamander gradually increased, and it underwent frequent molting. The aging skin usually fell off from the head to tail, forming a complete "shell" (Fig. 6C). Under the microscope, the shed skin maintained the body's shape (Fig. 6 C, C-1), and the pigment spots on the skin might also be clearly observed (Fig. 6 C-2). The entire exfoliation process took approximately 12 hours.

In terms of behavior, Anji salamander at this stage seemed to prefer living in groups, especially shortly after their abnormal development. There was almost no phenomenon of cannibalism. Groups of three or four can be seen gathering together. However, they had territorial awareness. If their territory was invaded by outsiders, there might be biting behavior, mostly targeting the limbs and tail. During this stage, although they could live away from water, they still required a high level of humidity. If they left the water environment and the humidity was insufficient, they might suffer from dehydration, leading to skin wrinkling, tail curling, and in severe cases, it could even endanger their lives.

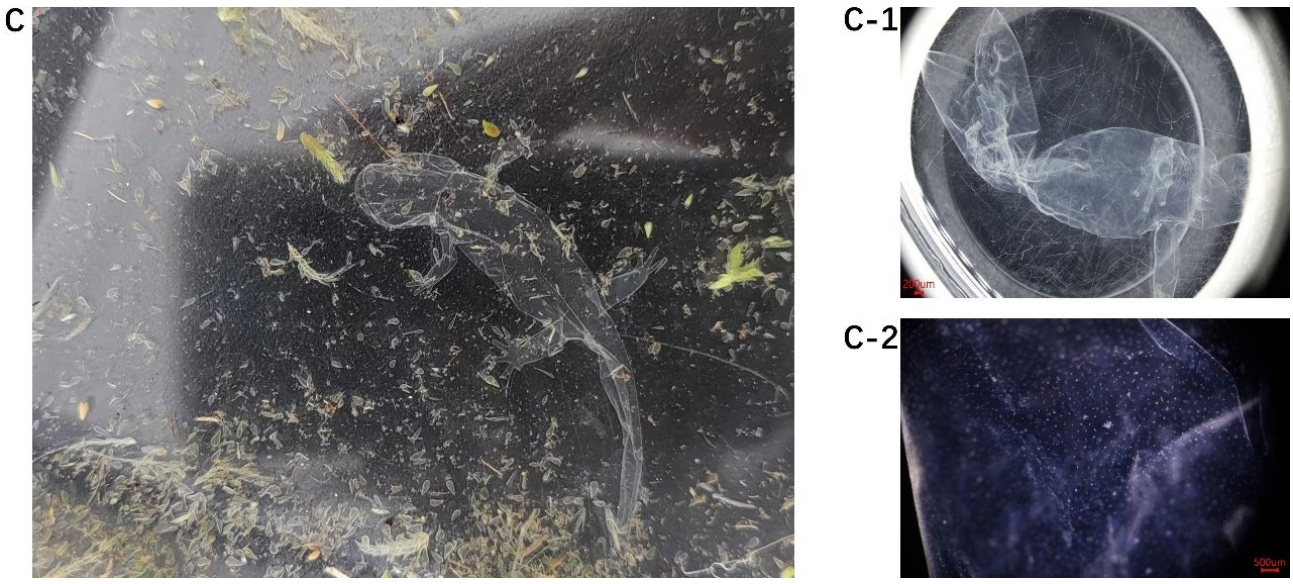


Fig. 6. Molting of Anji salamander. (C) Complete molted skin; (C-1) Molted skin magnified 6.3 times; (C-2) Spots remaining on the skin, magnified 50 times.

General characteristics of the development from fertilized egg to subadult stage of Anji salamander

In aquatic environments at 4-15 °C, the embryonic development period of Anji salamander lasted for 30 ± 5 days. The higher the temperature, the faster the development. From the fertilized egg to the subadult stage, there were a total of six major developmental stages: embryonic development stage, balance bar stage, limb development stage, pre-metamorphosis stage, metamorphic stage, and subadult stage. The balance bar stage required 18 ± 3 days, the limb development stage 50 ± 5 days, the pre-metamorphosis stage 10 ± 5 days, the metamorphosis stage 10±5 days, and after the metamorphosis is completed, it took 30 ± 5 days to develop into subadult stage (Fig. 7).

There were 34 developmental nodes, which are Fertilized egg, Multicellular stage, Gastrula stage, Neural plate stage, Neural fold stage, Neural groove stage, Neural tube stage, Early tail bud stage, Late tail bud stage, Early external gill stage, Late external gill stage, Incubation stage, Hatching stage, Forelimb forearm stage, Forelimb first finger stage, Forelimb second finger stage, Hindlimb buds stage, Balance bar falling off stage, Forelimb third finger stage, Forelimb fourth finger stage, Forelimb four - finger initial stage, Hind limb first finger stage, Hind limb second finger stage, Hind limb third finger stage, Hind limb fourth finger stage, Forelimb four-finger mature stage, Hind limb fifth finger stage, Ventral costal fovea appearance stage, Hindlimb five - toe initial stage, Ventral costal fovea deepening stage, Hindlimb five - toe mature stage, Pre metamorphosis stage, Metamorphosis stage, and Subadult stage (Fig. 8). The changes and characteristics of the 34 developmental nodes throughout the entire development process were presented in Table 1 and Table 2.

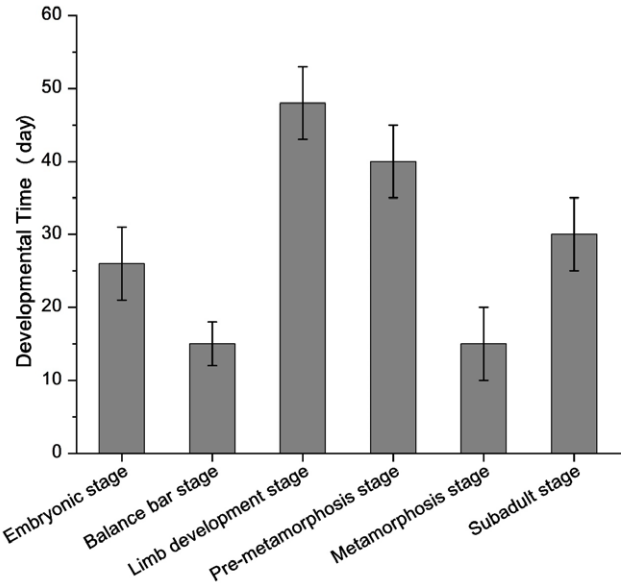


Fig. 7. Column graphs comparing the development time of each stage of the egg capsule of the Anji salamander in this study.

DISCUSSION

Embryonic development of Anji salamander

The egg capsule of Anji salamander could develop normally under normal conditions without any infection, except for a few unfertilized eggs. The unfertilized eggs would gradually disintegrate, and the cells eventually died as the eggs became concave. The embryonic development process of Anji salamander is extremely similar to that of other Hynobiidae species, including other vertebrates. For instance, *Hynobius leechii*, *Hynobius guabangshanensis*, Hynobiid Salamander, *Onychodactylus japonicus*, and fish all undergo the developmental process of fertilized eggs, cleavage, blastula, gastrula, organogenesis, and finally membrane formation (Iwasawa and Kera, 1980; Iwasawa and Yamashita, 1991; Ma et al., 1994; Mi et al., 2008; Mousavi et al., 2022). The difference is that the forelimbs of Anji salamander were merely a raised bud-like structure before hatching, while those of the Chinese salamander have already developed into a bifurcated shape and have formed the rudimentary form of two fingers before hatching.

The development time varies even among different species and within the same species. During the embryonic development stage, the development time we measured is slightly different from two recent reports (Cao et al., 2024; Qiu et al., 2024). This might be due to environmental factors, such as light exposure, water temperature, oxygen saturation, etc., resulting in different developmental speeds. Under natural conditions, the development speed of the eggs laid by Anji salamander varied at different times. Generally, eggs incubated in warmer water developed more rapidly.

The balance bar of Anji salamander

In other species of *Hynobius*, the presence of balance bars has been observed during a similar stage (Zhang et al., 1987b; Ma et al., 1994; Mi et al., 2008). Furthermore, Ning et al. (2021) also discovered on *Hynobius maoershanensis* that the balance bar will fall off (Ning et al., 2021). In this study, we not only reported the times of the appearance and disappearance of the balance bar but also the fact that the balance bar fell out in an asymmetric manner. This asymmetric detachment might imply that

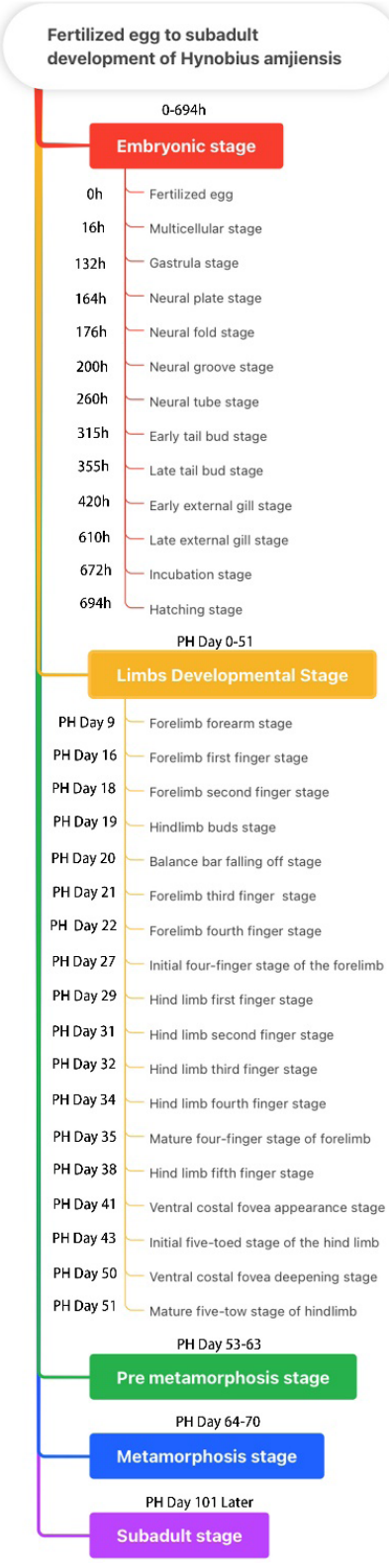


Fig. 8. Developmental tree from fertilized egg to subadult in Anji salamander. Complete developmental nodes from fertilized egg to subadult are illustrated.

the development of the balance bars on the left and right sides was not synchronous.

So far, there have been no relevant studies on the function of the balance bar in salamanders. We hypothesize that the balance bars of Anji salamander are similar to the lateral lines and antennae of other vertebrates, which serve the functions of navigation, detecting and sensing changes in the aquatic environment, intra-species communication to assist in locomotor equilibrium, and predation, etc. Lateral line exists in fish, primarily composed of the superficial and optic nerve mounds (Omura et al., 2014). The functions of the lateral line in fishes include sensing changes in the water flow and pressure (Mogdans, 2019), as well as prey detection, spatial localization, predator avoidance, flocking behavior, intraspecific communication, and station keeping (Klein and Bleckmann, 2015). Catfish have tentacles which are sensitive to changes in water flow and help catfish navigate, sense and hunt in dark environments (Biedenbach, 1971; Muka et al., 2010). Lateral line is also present in axolotls (Northcutt et al., 1994). We found Anji salamander larvae were able to sense food beyond the distance of the balance bar and slowly swim towards the prey, quickly biting it when the bar was about to touch or touching. At the same time, if another salamander leaned into another salamander, they were both able to quickly avoid each other without touching their bodies.

Patterns of limb development in the Anji salamander

There are two patterns of vertebrate limb development. One pattern is preaxial dominance, where structures close to the medial side of the body (i.e., radial or thumb side) preferentially form or differentiate during the development of an animal's limbs, such as the radius develops before the ulna in forelimb development, and the 2nd finger develops before the 4th finger (Royle and Young, 2021). The other is postaxial dominance, where structures close to the lateral side of the body (i.e., ulnar or pinky side) preferentially form during the development of an animal's limbs (Shubin, 1986). The limb development of Anji salamander follows a preaxial dominance developmental pattern, similar to that of many other Caudata (Fröbisch et al., 2007). In terms of the morphology of the limbs, except for the *Salamandrella keyserlingii* (Zhang et al., 1993c) and *Batrachuperus pinchonii* (Jia et al., 2018) which have 4 fingers on the forelimbs and 4 toes on the hindlimbs, most species of Hynobiidae have 4 fingers on the forelimbs and 5 toes on the hindlimbs (Ma et al., 1994; Mi et al., 2007; Ning et al., 2021). The preaxial dominance developmental pattern may reflect its long-term environmental adaptation.

Table 1. Embryonic development of *Hynobius amjiensis.*

Number	Developmental stage	Distinguishing Characteristics	Time of Emergence /h	Growth temperature/°C
1	Fertilized egg	The fertilized eggs are spherical, with a grayish-black color	0	6.5 ± 3.5
2	Multicellular stage	Cells continuously divide and the number of cells increases	16 ± 3	6.5 ± 3.5
3	Gastrula stage	The embryo is irregular and round, the embryo surface is smooth, and the blastoderm cells are involved into the embryo, with bulges or depressions on the edge	132 ± 4	6.5 ± 3.5
4	Neural plate stage	Embryo exhibits slight folding, shallow ditch appears	164 ± 4	6.5 ± 3.5
5	Neural fold stage	Embryonic folds became significantly opened	176 ± 3	6.5 ± 3.5
6	Neural groove stage	Embryonic folds were significantly elevated and converged to the center	200 ± 3	6.5 ± 3.5
7	Neural tube stage	Embryo is spindle shaped	260 ± 5	6.5 ± 3.5
8	Early tail bud stage	Embryonic head rudiments appear	315 ± 3	6.5 ± 3.5
9	Late tail bud stage	Embryonic eye sacs, gill arch, and tails are well characterized	355 ± 5h	6.5 ± 3.5
10	Early external gill stage	Embryo appear with primitive outer gills and balance bars	420 ± 5	6.5 ± 3.5
11	Late external gill stage	Embryonic eye formation with three outer gills and gill filament development	610 ± 3	6.5 ± 3.5
12	Incubation stage	Embryonic development is basically complete, with a pair of balance rods and forelimb buds	672 ± 3	6.5 ± 3.5
13	Hatching stage	The larvae lie on their side under the water with a small amount of yolk residue	694 ± 48	6.5 ± 3.5

It may also be closely related to the evolutionary process of 4-toed to 5-toed hindlimbs in the Hynobiidae (Ledbetter et al., 2019; Jia et al., 2022; Urošević et al., 2024). It has been shown that the four fingers of the forelimbs of amphibians with Caudata are adapted to aquatic life, which helps to reduce the resistance when swimming, and the forelimbs also have the role of maintaining the balance and stability of the body, while the five toes of the hindlimbs are adapted to terrestrial life and used to support the body and propulsion. These forelimbs and hindlimbs characteristics are highly compatible with their amphibious lifestyle (Urošević et al., 2024).

We observed that the front four limbs of Anji salamander gathered to form a shovel-like grubbing away soil or peat moss, similar to digging a burrow, a process in which the back five fingers grasped the ground to support the body. We hypothesize that the 4-fingered front and back salamanders and the 4-fingered front and 5-fingered back salamanders evolved different numbers of fingers due to differences in their lifestyles, such as some salamanders need to dig burrows, while others do not; some live in static waters, while others inhabit dynamic waters. The corresponding number of fingers allows them to better adapt to the resistance of water currents in their environments. Further data are needed to validate or refute this hypothesis.

The limb function of *Hynobius amjiensis* may serve the same purposes of assisted locomotion and maintenance of balance as many vertebrates (Molnar et al., 2021; Smith et al., 2021; Chen et al., 2025b). In the larval stage, when there are individual small salamanders with bitten limbs, their balance and locomotor ability will be reduced, and their swimming speed will be slowed down or even flipped on its side, so the limbs in the larval stage may play an important role in their locomotion and balance. The development of forelimb buds in *Hynobius amjiensis* larvae prior to membrane the emergence (Fig. 1 A12) may be an attempt to adapt as quickly as possible to body the balance in the water after emergence from the membrane. Following the disappearance of the balance bars, both hind limbs developed rapidly (Fig. 3 B4), which allowed the body to continue to have "four symmetrical supports", which may have been important for the maintenance of the body's locomotor equilibrium. After the development of the four limbs is completed, *Hynobius amjiensis* larvae often crawl underwater, probably to develop the support and propulsion function of the hind limbs, which lays the foundation for the metamorphosis to crawl long distances to the shore in search of food and habitats. It has been suggested that the forelimbs of tetrapods mainly play a braking role and the hindlimbs mainly play a propulsive role (Kawano and Blob, 2013). Furthermore, we observed in the field that *Hynobius amjiensis* may have crawled out of underwater burrows to pair-lay their eggs (Appendix 1). We hypothesized that their more pointed heads and stout limbs may have the ability to excavate the burrows, but we need further studies to confirm our speculations.

Table 2. Development of Larva to subadult of *Hynobius amjiensis.*

Number	Developmental stage	Distinguishing Characteristics	Time of Emergence /Day	Growth temperature/°C
1	Forelimb forearm stage	Larval forelimbs become longer and there is no differentiation of fingers	8 ± 2	12 ± 3
2	Forelimb first finger stage	Larval forelimbs fingers form a triangle	13.5 ± 1.5	12 ± 3
3	Forelimb second finger stage	Larval forelimbs fingers are bifurcated, emergence of hindlimb buds and the shedding of balance bars	18.5 ± 1.5	12 ± 3
4	Hindlimb buds stage	A pair of transparent and spotless buds appeared in the hindlimb	18 ± 2	12 ± 3
5	Balance bar falling off stage	The balance bar on both sides of the mouth became thinner and disappeared in the form of falling off	19 ± 2	12 ± 3
6	Forelimb third finger stage	Larval forelimb fingers are trifurcat	19.5 ± 1.5	12 ± 3
7	Forelimb fourth finger stage	Larval forelimb fingers are basically formed, there are webbed connections between the fingers	20.5 ± 1.5	12 ± 3
8	Forelimb four - finger initial stage	Larval forelimbs with four fingers basically formed and webbed connections between fingers	21.5 ± 1.5	13 ± 3
9	Hind limb first finger stage	Larva hind limb bud is triangular	30 ± 1	13 ± 3
10	Hind limb second finger stage	Larval hind limbs toes are quad,but only two toes developed, and the excess was cutis	31 ± 1	13 ± 3
11	Hind limb third finger stage	Larval hind limbs toes are quad, but only three toes developed, and the excess was cutis	32 ± 1	13 ± 3
12	Hind limb fourth finger stage	Larval hind limbs are four to four forked	33 ± 1	13 ± 3
13	Forelimb four-finger mature stage	Larval forelimb fingers are fully developed, and there is no web connection between the fingers	36.5 ± 1.5	13 ± 3
14	Hind limb fifth finger stage	Larval hind limbs have pentamerous toes	40.5 ± 1.5	13 ± 3
15	Ventral costal fovea appearance stage	Larval appear as indistinct depressions on both sides of the abdomen near the head	42 ± 1	13 ± 3
16	Hindlimb five - toe initial stage	Larval five toes of the hind limbs of juveniles are largely formed, but are connected by webbing between the toes	43.5 ± 1.5	13 ± 3
17	Ventral costal fovea deepening stage	Larvae have more defined depressions on both sides of the abdomen and develop towards the sides of the abdomen near the hind limbs	44 ± 1	13 ± 3
18	Hindlimb five - toe mature stage	Five toes of the hind limb form but there are webbed connections between the toes	51.5 ± 1.5	13 ± 3
19	Pre metamorphosis stage	The bulge of the larva's eyeball, the body color becomes darker, and the caudal fin becomes smaller	58 ± 5	14 ± 1
20	Metamorphosis stage	The three outer gills of the larva gradually shorten, the body color deepens, and a small amount of white spots appear	67 ± 4	14 ± 1
21	Subadult stage	No outer gills, increased white spots, neck folds	106 ± 5	14 ± 1

Molting of Anji salamander

This is the first report of the molting phenomenon in Anji salamander. Molting is a natural process of growth and development in vertebrates, influenced by environmental and hormonal factors (Jorgensen et al., 1960; Heimeier and Shi, 2010). Extensive studies have shown that the molting process of amphibians is regulated by thyroid hormones (Dowling et al., 1966; Tata, 2006). It has been found that the level of thyroid hormone secretion in amphibians increases during metamorphosis (Denver, 2021; Tanizaki et al., 2021). The first molt occurred during the metamorphosis stage, and it is possible that hormones were also involved in the development of molt and metamorphosis in Anji salamander.

Amphibian molting behavior is necessary for their metamorphosis. The main component of molting is the cuticle of the skin surface, and the presence of molting indicates some keratinization of the skin (Heimeier and Shi, 2010; Akat et al., 2023). The skin of amphibians serves as an important organ for their respiration and gas exchange (Tattersall, 2007), and molting might regulate

respiration and reduce water loss to some extent (Torri et al., 2020). It has also been implied that amphibians in the growth phase molt as a result of their body growth becoming increasingly tighter with their cuticle, and the old cuticle skin needs to be shed to accommodate growth (Lu, 2018). Molting can also defend against pathogenic microorganisms on the old skin, removing pathogenic microorganisms that have accumulated on the old skin over time (Ohmer et al., 2017). In this study, we found that Anji salamander molted more frequently after completing metamorphosis, so the molt of Anji salamander might be more closely related to their growth, development, immunity and other physiological processes.

Risks faced during metamorphosis and development of Anji salamander

Amphibian metamorphosis enables amphibians to cross the formidable ecological barrier between aquatic and terrestrial environments by coordinating morphological remodeling with physiological functional transitions, a process critically important for their subsequent survival. Studies have indicated that during amphibian metamorphosis, the degeneration of external gills may exhibit temporal mismatch with pulmonary developmental processes. Furthermore, the gradual functional decline in gill respiratory capacity throughout metamorphic progression can potentially lead to hypoxic stress and associated mortality during gill resorption (Burggren, 1982; 1994). Amphibians use their skin to assist respiration before and after gill shedding. However, after metamorphosis, the use of skin for respiration needs to be carried out at a more suitable humidity; otherwise, dry environments may cause respiratory distress, dehydration, and disruption of skin microflora, increasing the risk of infection with pathogenic microorganisms (Akat et al., 2023; Buttimer et al., 2024; Riddell et al., 2024). Amphibians can experience long period of cessation of feeding during metamorphosis due to multiple reasons, including hypothalamic regulation and reorganization of the digestive system (Kuzmin, 1997; Bender et al., 2018; Zhu et al., 2020).

Anji salamanders spend the vast majority of their metamorphosis to subadult in a state of relative feeding cessation, and have to face threats such as hypoxia from respiratory shifts after emerging onto land, significant energy expenditure from crawling long distances, and elevated habitat temperatures, and even failing to survive once they mistakenly enter a dry area with water-starved and wrinkled skin and frizzled tails for 12-24 hours. Studies have also been shown that some small salamanders maintain a larval form and are unable to complete metamorphosis (Okamiya et al., 2021). Additionally, skin fusion of the head and neck following gill internalization might greatly burden the immune and endocrine systems. Pathogen invasion at this stage could pose a significant survival threat. Therefore, we believe that the metamorphosis stage is a high-risk stage from fertilized eggs to subadults in Anji salamander.

CONCLUSIONS

In this study, we delineated the six main developmental processes of Anji salamander (Fig. 8) encompassing the whole developmental process, including 34 developmental nodes (Tables 1 and 2), and described the life habits of different developmental periods, filling gaps in the life history of Anji salamander. It is worth noting that Anji salamander is particularly vulnerable during the balance bar shedding stage and throughout metamorphosis, which is informative for conservation. For example, we in the reserve may consider the need to keep the habitat as free from external disturbance as possible before and after the shedding of the balance bar and during the process of metamorphosis. In addition, the subadult stage of Anji salamander has a relatively high dependence on air humidity and water. Therefore, if there is an extreme drought before or after the Anji salamander comes ashore, we can take the necessary intervention to ensure adequate water supply in the reserve. In conclusion, the knowledge of the life history of Anji salamander from larvae to subadults is useful for its conservation and future captive breeding.

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