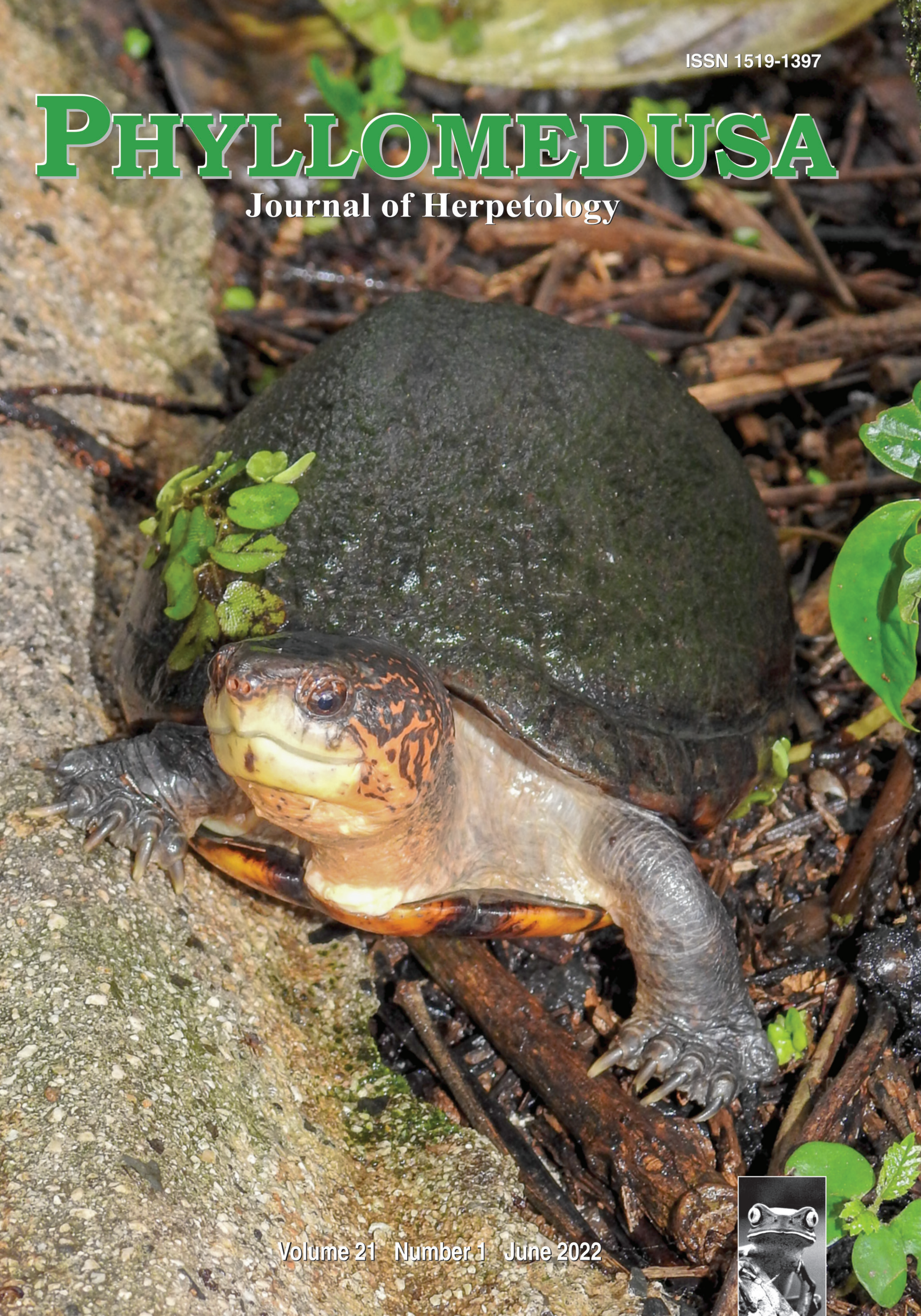

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Cover: A male *Kinosternon scorpioides* from
Palo Verde National Park, Costa Rica.
Photo: José Manuel Mora

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Terrestrial movements, activity patterns and habitat use by *Kinosternon scorpioides* (Testudines: Kinosternidae) in Palo Verde National Park, Costa Rica

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Abstract

Terrestrial movements, activity patterns and habitat use by *Kinosternon scorpioides* (Testudines: Kinosternidae) in Palo Verde National Park, Costa Rica. The Scorpion Mud turtle, *Kinosternon scorpioides,* is a widely distributed, semiaquatic species known to engage in regular terrestrial movement and terrestrial estivation. We studied terrestrial behavior in this species in Palo Verde National Park, northwestern Costa Rica. We determined terrestrial activity patterns by searching for active individuals during transects along 3.4 km of trails. We determined the distance that turtles traveled between the wetlands and estivation sites by fitting 10 terrestrially active individuals with a thread-bobbin tracking device attached to the rear of the carapace. We identified sex and marked and measured every turtle found active. We accumulated 92 observations of *K. scorpioides* active on land and we identified two behaviors: traveling and nesting. These turtles showed a bimodal activity pattern ($\chi^2 = 18.1$, $p < 0.02$, $df = 8$) with maximum activity during early morning hours (06:00 to 08:00 h) and twilight and early evening hours (16:00 to 20:00 h). We found 41 turtles estivating in deciduous dry forest at an average distance (± SE) of 156.4 ± 13.7 m (range 20.9–304.9 m) from the wetland border (water´s edge). Adults estivated at greater distances from the wetland than did juvenile turtles, and males estivated at greater distances than females. Turtles found estivating were in three different habitats: leaf litter (85%), rock cavities (10%), and tree buttresses (5%). *Kinosternon scorpioides* was observed to estivate for at least 84 days in PVNP during the dry season. We recorded several behavioral traits that may account for its ability to withstand desiccation during the estivation period. Our work underscores the need to include terrestrial habitats when considering the conservation needs of semiaquatic turtles.

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Keywords: Dry forest, Estivating sites, Freshwater turtles, Global climate change, Scorpion Mud Turtle.

Resumen

Movimientos terrestres, patrones de actividad y uso de hábitat por *Kinosternon scorpioides* (Testudines: Kinosternidae) en el Parque Nacional Palo Verde, Costa Rica. La tortuga candado, *Kinosternon scorpioides*, es una especie semiacuática ampliamente distribuida conocida por presentar movimientos terrestres regulares y estivación terrestre. Estudiamos el comportamiento terrestre de esta especie en el Parque Nacional Palo Verde, noroeste de Costa Rica. Determinamos sus patrones de actividad terrestre mediante la búsqueda de individuos activos en transectos a lo largo de 3,4 km de senderos. Determinamos la distancia que las tortugas viajaron entre el humedal y los sitios de estivación al seguir a 10 individuos activos en tierra a los que les pegamos un carrete de hilo en la parte posterior del caparazón. Identificamos el sexo y marcamos y medimos todas las tortugas que encontramos activas. Acumulamos 92 observaciones de *K. scorpioides* activas en tierra e identificamos dos comportamientos: viajar y anidar. Estas tortugas mostraron un patrón de actividad bimodal (χ^2 = 18,1, p < 0,02, gl = 8) con máxima actividad durante las primeras horas de la mañana (06:00–08:00 h) y el crepúsculo y las primeras horas de la noche (16:00–20:00 h). Encontramos 41 tortugas estivando en el bosque seco caducifolio a una distancia promedio (± SE) de 156.4 ± 13.7 m (20.9–304.9 m) del borde del humedal (borde del agua). Los adultos estivaron a mayor distancia del humedal que las tortugas juveniles y los machos estivaron a mayor distancia que las hembras. Las tortugas que encontramos estivando las encontramos en tres hábitats diferentes: hojarasca (85%), cavidades rocosas (10%) y raíces triangulares de árboles (5%). *Kinosternon scorpioides* estivó durante al menos 84 días en el Parque Nacional Palo Verde durante la estación seca. Registramos varios rasgos de comportamiento que pueden explicar su capacidad para resistir la desecación durante el período de estivación. Nuestro trabajo subraya la necesidad de incluir hábitats terrestres al considerar las necesidades de conservación de las tortugas semiacuáticas.

Palabras clave: Bosque seco, Cambio climático global, Sitios de estivación, Tortuga candado, Tortugas de agua dulce.

Resumo

Movimentos terrestres, padrões de atividade e usio de hábitat em *Kinosternon scorpioides* (Testudines: Kinosternidae) no Parque Nacional de Palo Verde, Costa Rica. O cágado peito-de-mola, *Kinosternon scorpioides*, é uma espécie semiaquática amplamente distribuída, conhecida por se envolver em movimentos terrestres regulares e estivação terrestre. Estudamos o comportamento terrestre dessa espécie no Parque Nacional de Palo Verde, noroeste da Costa Rica. Determinamos padrões de atividade terrestre por meio da procura de indivíduos ativos durante os transectos ao longo de 3,4 km de trilhas. Determinamos a distância que as tartarugas percorriam entre as zonas úmidas e os locais de estivação dotando 10 indivíduos terrestres ativos com bobinas de rastreamento na porção posterior da carapaça. Identificamos o sexo e marcamos e medimos cada tartaruga encontrada em atividade. Acumulamos 92 observações de *K. scorpioides* ativas em terra e identificamos dois comportamentos: deslocamento e nidificação. Essas tartarugas mostraram um padrão de atividade bimodal (χ^2 = 18,1, p < 0,02, df = 8), com atividade máxima durante as primeiras horas da manhã (06:00–08:00 h) e o crepúsculo e as primeiras horas da noite (16:00–20:00 h). Encontramos 41 tartarugas estivando em floresta seca decídua a uma distância média (± SE) de 156,4 ± 13,7 m (intervalo de 20,9–304,9 m) da fronteira da zona úmida (borda da água). Os adultos estivaram a maiores distâncias da zona úmida do que os juvenis, e os machos estivaram a maiores distâncias do que as fêmeas. As tartarugas encontradas estivando encontravam-se em três diferentes habitats: serapilheira (85%), cavidades de rochas (10%) e raízes tabulares (5%). Observou-se que *K. scorpioides* estivou durante pelo menos 84 dias no Parque Nacional de Palo Verde durante a estação seca. Foram registradas várias características comportamentais que podem explicar sua capacidade de resistir ao dessecamento durante o período de estivação. Nosso trabalho ressalta a necessidade de incluir hábitats terrestres ao considerar as necessidades de conservação das tartarugas semiaquáticas.

Palavras-chave: Bosque seco, Cágados, Mudança climática global, Peito-de-mola, Sítios de estivação.

Introduction

Turtles are among the most threatened groups of vertebrates worldwide due to anthropogenic factors such as direct consumption for food, development, agriculture, and land and water pollution (Cox *et al*. 2022, Tan *et al*. 2022). Over half of all turtle species are included in at least one threat category (Rhodin *et al*. 2018, Stanford *et al*. 2020, TTWG 2021, Cox *et al*. 2022). In addition, rapid global change in climate patterns has the potential to further erode turtle populations (Lovich *et al*. 2018, Butler 2019). Increasing environmental temperatures are impacting spatial distribution, reproduction, physiological performance, behavior, and other life-history traits of ectothermic animals including turtles (Lovich *et al*. 2018, Berriozabal-Islas *et al*. 2020, Stanford *et al*. 2020). Climate change is likely to have severe consequences on semiaquatic species that rely on the health of multiple adjacent habitats in order to survive.

Studies of activity and movements of freshwater turtles can potentially reveal how variation in environmental parameters (i.e., weather, temperature) impact resource acquisition, and therefore an organism's life history (Rowe 2003, Rowe *et al*. 2009). Terrestrial activity of freshwater turtles has been widely documented and is known to be an important aspect of their ecology (Buhlmann and Gibbons 2001, Steen *et al.* 2012). Terrestrial behavior includes movements associated with nesting, hatchling emergence and transit from nests, dispersal of all life stages, movements to or from hibernating or estivating sites, and terrestrial migration in search of mates (Steen *et al.* 2012, Enders *et al*. 2021). Study of terrestrial movement patterns is a key aspect of chelonian conservation and survival (Burke and Gibbons 1995), but many species are still relatively understudied (Enders *et al*. 2021). The abundance and distribution of a particular species is often determined by the availability of habitat. This must include all necessary resources in different habitats (food, water, refuge, and nest sites) to ensure the species existence (Litvaitis *et al.* 1994). Aquatic or semiaquatic turtles that experience seasonal fluctuations of their aquatic habitat may be forced to use terrestrial habitats. Many organisms under these circumstances will either make terrestrial migrations to find other bodies of water or estivate until aquatic habitats are replenished (Ligon and Peterson 2002). Either way, the terrestrial habitat and its availability becomes an important component for the ecology and conservation of these organisms.

Mud turtles of the genus *Kinosternon* are small mostly semiaquatic turtles inhabiting a wide variety of natural (streams, rivers, lakes, lagoons, seasonal or perennial ponds and wetlands, and estuaries) and artificial aquatic environments (drainage ditches, irrigation canals, reservoirs, and water tanks) throughout their distribution (Iverson *et al*. 2013, Butler *et al.* 2016). They move overland for variable distances, approaching at least one km, in response to the seasonality of aquatic environments or in search of mates and/or nest sites (Cordero *et al*. 2012, Pérez-Pérez *et al.* 2017). *Kinosternon integrum* Le Conte, 1854 has been observed to estivate at distances > 560 m from the nearest lakeshore in Michoacán, Mexico (Aparicio *et al*. 2018). *Kinosternon subrubrum* Bonnaterre, 1789 in the mid-Atlantic region of the United States traveled distances of 903 and 887 m (Cordero *et al*. 2012). Even highly aquatic species such as *Kinosternon leucostomum* (Duméril, Bibron, and Duméril, 1851) can travel up to 600 m for nesting and estivating (Morales-Verdeja and Vogt 1997, Cordero and Swarth 2010). Multiple species in the genus rely on terrestrial activity. However, anthropogenic factors such as habitat fragmentation and urbanization cause loss of terrestrial connectivity that is required for populations of these species to survive or disperse to additional suitable habitat (Buhlmann and Gibbons 2001, Aparicio *et al*. 2018, Serrano *et al*. 2020).

The Scorpion Mud turtle *Kinosternon scorpioides* (Linnaeus, 1766) is the most widely distributed species in the genus. It is distributed from Mexico (Oaxaca in the west and Tamaulipas in the east), to southern Bolivia and northern Peru, Brazil and Argentina (Iverson 2010, Berry and Iverson 2011). It uses a variety of aquatic habitats throughout its range (Pritchard and Trebbau 1984, Ernst and Barbour 1989, Savage 2002). It is common on the Pacific slope of Costa Rica where it inhabits nearly any body of shallow water but prefers habitats with abundant aquatic vegetation (Acuña-Mesén 1998). It is found in marshes, permanent and temporary ponds, streams, rivers, and swamps (Acuña-Mesén 1998, Bedoya-Cañón *et al*. 2018). In Belize, *K. scorpioides* is known to move from drying ephemeral habitats through terrestrial environments into permanent streams as the dry season progresses (Moll and Moll 2004). It is also known to make substantial overland movements in Costa Rica (175–500 m from water), and to leave drying water bodies to estivate in adjacent terrestrial habitats (Teska 1976, Acuña-Mesén 1998, Savage 2002, Moll and Moll 2004). However, no research has been devoted specifically to the detailed study of terrestrial movements and terrestrial habits of this species. Because most climate models predict increases in temperature and seasonality in the tropics and other biomes, it is important to collect baseline data about the mechanisms by which organisms respond to present day seasonal droughts in order to predict their potential responses to climate change (Butterfield *et al*. 2018). The main objective of this research was to determine the temporal and spatial terrestrial movement patterns of *K. scorpiodes* in a protected dry forest in northwestern Costa Rica, and to examine the relationship of this activity to daily temperature and humidity changes.

Materials and Methods

We conducted our research in Palo Verde National Park (PVNP), an area of 19,800 ha in the northwestern lowlands of Guanacaste Province, Costa Rica (10°21' N, 85°21' W; Figure 1). At this site annual average temperature is 27°C, and annual precipitation is 2,296 mm (Castañeda and Mora 2010, Mora and Castañeda 2021). The dry season lasts from December to April and the rainy season from May to November (Mora 1989). A large variety of habitats are represented in PVNP: including primary and secondary deciduous dry forest, riparian forest, savannas, and wetlands (Hartshorn 1983, Mora 1989). A single wetland, Palo Verde Marsh or Palo Verde Lagoon covers 1,207 ha and accounts for 6.1% of the park's area (Trama *et al*. 2009). It is formed by a mosaic of aquatic and woody vegetation (Trama *et al*. 2009). During the rainy season, the lagoon's water level can rise to 1.5 m, but it gradually declines until it dries out completely at the end of the dry season in April or May (Trama *et al*. 2009).

We conducted our research from October 2003 at the onset of the rainy season to November 2004 at the end of the following rainy season. During these months we also monitored Guayacán waterhole, ca. 3 km west of the Hacienda Park Ranger Station. This natural waterhole varies in diameter from 4 m during the rainy season to 1 m during the driest months. As of 2001, it had apparently not gone completely dry for more than 25 years (Stoner 2001). It is the only source of water in the forest at our study area.

During the dry season, as the lagoon water level diminishes, mud turtles are commonly seen moving on land (Acuña-Mesén 1990). To determine daily terrestrial activity patterns, during the dry season of 2004 (February to May) we searched for active turtles by walking three transects. Transect A was a dirt road between the MINAE station and "Puerto Chamorro" on the Tempisque River (2000 m); transect B was a dirt road between the OTS station and the MINAE station (900 m); and transect C was a trail leading east from the MINAE station (500 m). All transects were oriented parallel to the ecotone between the wetland and the dry forest (Figure 1). Surveys were conducted between 06:00 and 24:00 h, and observations were divided into nine

Figure 1. Study site at Palo Verde National Park in northwestern Costa Rica. 1 = Puerto Chamorro, 2 = MINAE station, 3 = OTS station, A = trail of 2000 m, B = trail of 900 m, C = trail of 500 m.

periods of 2 hours each (i.e., from 06:00 to 08:00 h; from 08:01 to 10:00 h … from 22:01 to 24:00 h). We walked transects during 4–5 periods each day chosen randomly but making sure all nine time periods were covered three times each week. We searched for active mud turtles for 57 days (16 in February, 15 in March, 14 in April, and 12 in May). During these days, we actively searched for a total of 233 person-hours. To determine variation in terrestrial movements throughout the day we kept records of the sampling effort and the number of active turtles found for each 2 hours period. The frequency of active turtles was given by the number of individuals found moving per unit search time during each of the nine periods. We individually identified sex and marked and measured each turtle captured. We also recorded the direction each turtle was traveling. Measurements included curved carapace length (CCL, type D of Iverson and Lewis 2018) and curved carapace width (CCW) in mm to the nearest 1 mm taken with a flexible measuring tape, and body mass to the nearest 0.1 g (using either a 300 g or a 1,000 g capacity Pesola spring balances). Turtle activity was correlated with ambient temperature and humidity using data from the OTS facility weather station. The station automatically records readings every 30 min, which allowed us to construct a profile of temperature and humidity from 06:00 to 24:00 h during the study months.

To determine the distance that turtles travel from the wetland to the estivation sites, we fitted 10 individuals found active on land with a thread-bobbin tracking device, which we attached to the rear of the carapace (Wilson 1998). Turtles equipped with thread bobbins were released within a few hours, at their capture location. We attached the free end of the thread to the vegetation, which allowed the thread to unwind as the turtle walked away. Twenty-four hours later we followed the thread until we found the turtle. We also searched for estivating turtles in the leaf litter using a hand rake, and between tree buttresses and rock crevices. Searches for estivating turtles were carried out to a maximum of 350 m from the wetland. We marked each site where an estivating turtle was found and determined the geographic location (using a Garmin Geko 201 GPS). GPS points were plotted on an Ikonos satellite image of PVNP and the straight-line distance from the wetland (defined as the water's edge) to each GPS point (estivating site in the dry forest) was measured in m using ArcGIS 3.2 (Esri Inc. 2002). All turtles equipped with thread bobbins moved mostly in straight lines (south to north) from the wetland to the estivating sites in the forest. Thus, a straight-line movement was assumed for all turtles found estivating.

We recorded data for each turtle found estivating including microhabitat or refuge selected. We measured the temperature of 13 estivation sites, and humidity in five sites. We took readings inside and outside estivating refuges during the hottest time of the day (11:00 to 14:00 h). To measure air temperature and humidity we used a digital hygro-thermometer (model 445582 Extech Instruments), and for soil temperature we used a digital thermometer (model HI-145-30 Hanna Instruments).

Turtles were assigned to 10 mm size-class categories, but for some analyses were assigned only to age class: juvenile or adult. To determine age class, we used the size of the smallest known mature female of *K. scorpioides* (Savage 2002): 122 mm straight-line carapace length (CSL). Since we used CCL in our study, we measured the CSL and the CCL of 103 mud turtles from PVNP (Castañeda and Mora 2010), and found the mean difference to be 22.9 mm. Thus, we considered all individuals < 140 mm CCL to be juveniles, and all measuring ≥ 141 mm to be adults.

We tested the relationship between activity and humidity, activity and temperature, and turtle size and distance moved from the wetland, using a Pearson correlation ($\alpha = 0.05$). We tested for differences in distances from the wetland among sex and age of turtles with the Mann-Whitney U test ($\alpha = 0.05$) in SYSTAT 9 for Windows (Systat 9 1999). Measurements and all other calculations are given as mean ± SE.

Results

During this study, we observed a total of 92 *Kinosternon scorpioides* active on land during both dry and rainy seasons, and two behaviors were identified: traveling and nesting. We did not observe feeding or social behavior on land. All turtles reacted to observers by closing their plastron when we approached. After five to 10 min of handling (for measurements and marking) most turtles emptied their urinary bladder and liberated a strong musk odor.

During the dry season of 2004, we found 76 mud turtles actively moving on land between

February and May; 18 were juveniles and 58 were adults of both sexes (Figure 2). Turtles were already leaving the wetland and entering the forest to estivate by early February. All turtles we observed from February to April were moving from the wetland towards the forest (south to north). During February we followed 12 females moving from the wetland to the forest where they nested. After nesting they returned to the wetland. We found actively nesting females between 07:00 and 10:00 h and between 15:00 and 23:00 h (Mora and Castañeda 2021). During April, we found three turtles moving on land (south to north) after a single isolated heavy rain event. During May we found only one turtle moving north (towards the forest), but from the last week of May onwards, all active turtles were moving back from the forest to the wetland (north to south). During the dry season we recorded the highest terrestrial activity in March and the lowest in April (Figure 3). During this dry season (2004) we found only two *K. scorpioides* in Guayacán waterhole before it was completely dry by the end of March.

During the rainy season of 2004 (October and November), we found 16 *K. scorpioides* active on land. Fourteen of these individuals were females (four were nesting). The two males found on land were less than 1 m from the water; females were farther from water (some more than 200 m).

Kinosternon scorpioides in PVNP showed a bimodal daily activity pattern ($\chi^2 = 18.1$, $p < 0.02$, $df = 8$; Figure 4), with maximum activity during early morning hours (06:00 to 08:00 h) and twilight and early evening hours (16:00 to 20:00 h). Their daily activity pattern was positively correlated with relative humidity but not significantly ($r^2 = 0.33$, $p = 0.11$, $N = 12$) and barely negatively correlated with ambient temperature ($r^2 = 0.37$, $p = 0.08$, $N = 12$) during the day (from 06:00 to 24:00 h). During night hours (from 18:00 to 24:00) turtle activity diminished even though ambient temperature continued to drop and humidity continued to increase (Figure 5).

We found 41 estivating turtles in the deciduous dry forest north of the wetland (Figure 1) at an average distance of 156.4 ± 13.7 m (range 20.9 to 304.9 m) from the wetland border (water´s edge). Adults ($N = 28$) were found at a significantly greater distance from the water (183.2 ± 15.0 m) than juveniles ($N = 3$) (98.7 ± 21.3 m) ($U = 274.5$, $p = 0.009$). The average distance traveled by males (190.7 ± 25.6 m, $N = 13$) was not significantly different from that traveled by females (163.2 ± 18.4 m, $N = 19$) ($U = 94.5$, $p = 0.26$). Most juveniles were found within the first 150 m from the wetland, females were more evenly distributed from 0 to 300 m, and males tended to be found at the farthest distances (Figure 6). The largest number of turtles was found between 50 and 100 m from the wetland, and only one individual was found between 300 and 350 m. Estivating turtles ranged in size from 85 to 214 mm CCL (Figure 2), from 77 to 189 mm CCW, and from 59 to 870 g. There was a direct but very weak relationship between turtle size and distance from the wetland to estivating sites (CCL: $r^2 = 0.20$, $p = 0.003$; CCW: $r^2 = 0.18$, $p = 0.005$; weight: $r^2 = 0.15$, $p = 0.009$).

Turtles found estivating were in three different types of habitats: leaf litter, rock cavities and between tree buttresses. Thirty-five turtles (85%) estivated under leaf litter; these individuals constructed a shallow form in the soil (about 5 cm deep) that covered the plastron and the lower half of the carapace, while the rest of the shell was covered by leaves. Four turtles (10%) were found estivating inside natural limestone cavities, and two (5%) were found between tree buttresses. When measured during the hottest time of the day (11:00 to 14:00 h), the estivation sites maintained lower temperatures and higher humidity than the environment external to the refuge. Average temperature was 29.6 ± 0.5°C and 34.3 ± 0.6°C inside and outside refuges respectively. Average humidity was 59.9 ± 6.2% and 41.1 ± 3.8% inside and outside refuges, respectively.

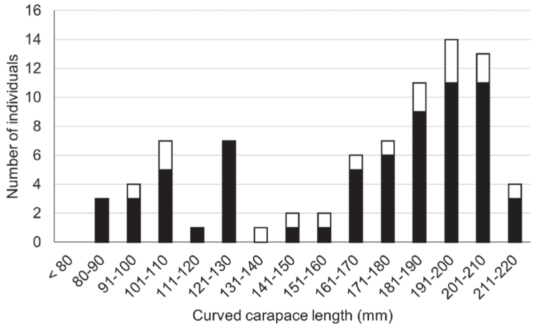

Figure 2. Population size structure of Scorpion Mud turtles (*Kinosternon scorpioides*) (*N* = 76) found on land during the dry season of 2004 (February to May), in Palo Verde National Park northwestern Costa Rica. Black bars = females, white bars = males

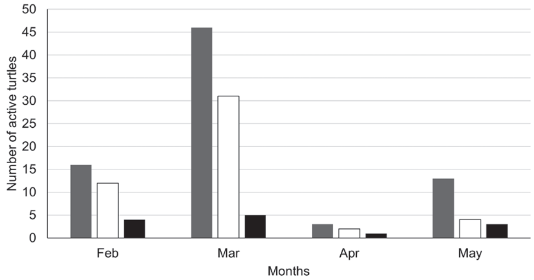

Figure 3. Number of Scorpion Mud turtles (*Kinosternon scorpioides*) active on land from February to May 2004 in Palo Verde National Park, northwestern Costa Rica. White bars = females, black bars = males, gray bars = total

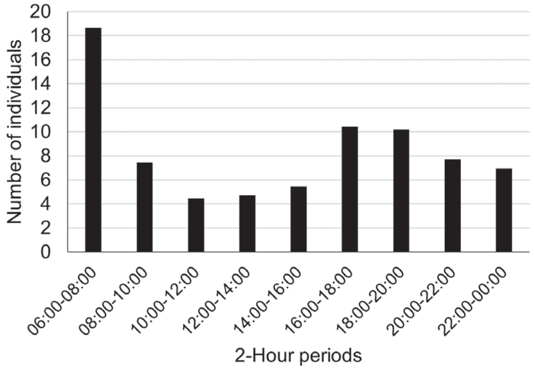

Figure 4. Number of individuals of Scorpion Mud turtles (*Kinosternon scorpioides*) active during each 2-hour sampling period in Palo Verde National Park, northwestern Costa Rica.

Discussion

In PVNP, during the dry season, *Kinosternon scorpioides* engages in terrestrial activity apparently for only two purposes, either to estivate or to nest. Feeding and social behavior were never observed in this species while on land. Nesting seems to be the main purpose of the terrestrial activity observed during the rainy season. Turtles that abandoned the wetland during February would have to spend 80 days or more estivating in the forest. Moreover, since the dry season in PVNP begins in December, it is possible that some individuals migrated as early as January. The seasonal drying of wetlands is well-known to force aquatic turtles to move to other wetlands, e.g., *Clemmys guttata* (Schneider, 1792) (Rowe *et al*. 2013), and *Sternotherus odoratus* (Latreille, 1802) (Seburn and Burns 2021). Because no other water source is available for *K. scorpioides* in PVNP, the turtles that migrated to the forest do not have access to water (the waterhole dried out by March) and must estivate until they return to the wetland in May or during an occasional rain in April. The distance from estivation sites to the wetland were only weakly related to individual size (Figure 6), as was found for *Kinosternon leucostomum* in Mexico (Morales-Verdeja and Vogt 1997).

Many individuals of *K. scorpioides* have been found buried under mud and dry aquatic plants in the wetland during April and May (Castañeda and Mora 2010) suggesting that not all turtles leave the wetland and enter the forest to estivate (Acuña-Mesén 1990). Although, the dry season in PVNP starts in December, in a normal year the wetland holds water until March and some small, scattered puddles remain even until the first half of April. Thus, turtles that remain in the wetland would be able to significantly shorten the inactivity and deprivation period, probably to only about 30 days or even less. Why some individuals of *K. scorpioides* start to migrate to the forest of PVNP during February (not counting nesting

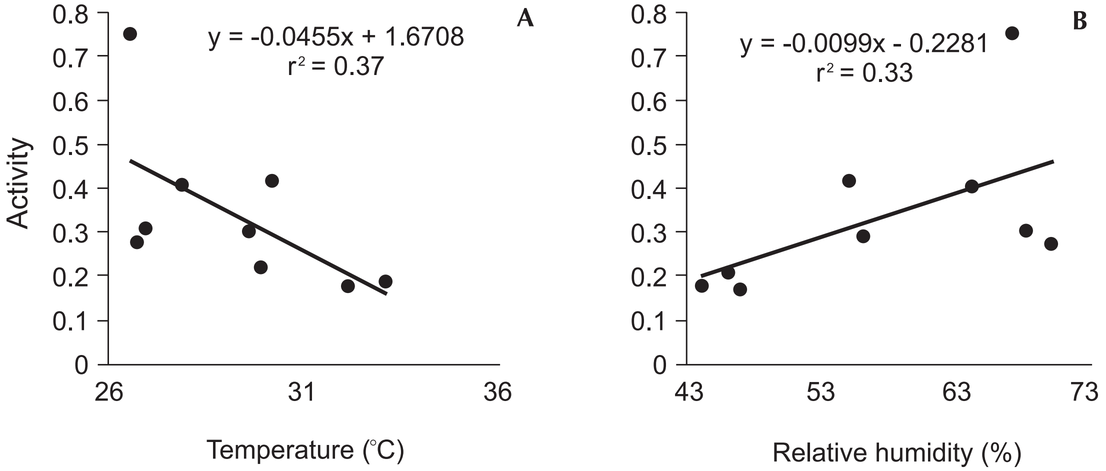

Figure 5. Scorpion Mud turtles (*Kinosternon scorpioides*) daily activity pattern (number of individuals found moving per 2-hr unit) correlated to ambient temperature (**A**), and to ambient relative humidity (**B**) in Palo Verde National Park, northwestern Costa Rica.

females) when there is still plenty of water in the lagoon, and what factors determine which individuals stay in the wetland and which migrate to the forest, remain unknown.

Because of the physiological constraints of ectothermy, environmental parameters such as temperature and humidity have an important effect on the activity patterns of turtles. Terrestrial movements by *Kinosternon integrum* in Mexico clearly relate to seasonality and especially rainfall (Pérez-Pérez *et al*. 2017). Individuals of *Kinosternon baurii* (Garman, 1891) in Florida spend a significant amount of time on land when aquatic areas dry out (Stemle *et al*. 2019). Apparently, heavy rainfall initiates mass migration in this species and extreme temperature inhibits terrestrial movement (Wygoda 1979). As was observed in this study, seasonal terrestrial movement may involve nesting as well as estivation. Seasonal drying of a lake and the nesting season induced terrestrial activity in *K. leucostomum* in Veracruz, Mexico (Morales-Verdeja and Vogt 1997). In Nebraska, female *K. flavescens* moved to nest sites, excavated a body cavity, and dug an additional nest cavity, and then often remained buried with the eggs for up to 38 days (Iverson 1990). *Kinosternon subrubrum* also remain buried after nesting up to 29 days until the next rainstorm (Burke *et al*. 1994). Several of these mud turtles used estivation sites within forests to afford

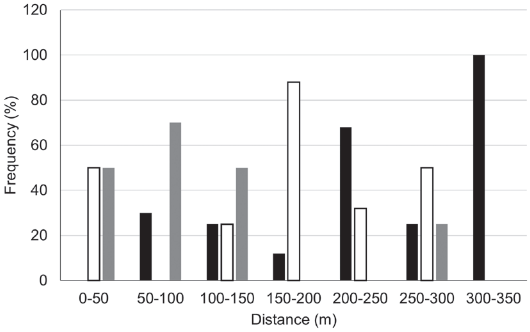

Figure 6. Frequency expressed as 100% at each distance category of estivating individuals of Scorpion Mud turtles (*Kinosternon scorpioides*) by sex, and age, in Palo Verde National Park, northwestern Costa Rica. White bars = females, black bars = males, gray bars = juveniles

protection from high temperature, dehydration and predation (Wygoda 1979, Morales-Verdeja and Vogt 1997).

During the dry season in PVNP, the average daily temperature exhibited a range of 6.5°C, changing from a low of 26.7°C in early morning and late afternoon hours to 33.2°C during midday hours. However, environmental temperatures in the study area were relatively uniform across all days with readings (Mora and Castañeda 2021). Additionally, average daily humidity varied from 60 to 71% in late afternoon and early morning hours to 44% during midday hours. Thus, the bimodal activity pattern exhibited by *K. scorpioides* in PVNP (Figure 4) is a convenient mechanism to reduce the risk of dehydration and avoid extreme temperatures while migrating terrestrially during the dry season. In the forest, turtles remained buried mainly in leaf litter, or hid in rock cavities or among tree buttresses, which further protects estivating turtles from desiccation and predation.

Several behavioral traits of *Kinosternon scorpioides* may account for its ability to withstand desiccation during the estivation period in PVNP (prompted by an extended dry season and the reduction or disappearance of their aquatic habitat): (1) the use of estivating refuges with a lower temperature and higher humidity than the environment; (2) a bimodal daily activity pattern that ensures the turtle´s terrestrial movements occur during hours with optimum conditions of ambient temperature and humidity; (3) juveniles (which are more prone to dehydration than adults) travel shorter distances than adults from the wetland to estivation sites; and (4) the storage of water in the urinary bladder might be a possibility given our observation of fluid voiding during handling.

There are many seasonal wetlands in northwestern Costa Rica that probably support local populations of *K. scorpioides*. The results of this study provide basic information to promote the conservation of these populations that are not in protected areas. Agriculture, development and logging are the top threats to reptiles (Cox *et al.* 2022). These factors negatively impact uplands adjacent to wetlands that are used by turtles as critical components of their overall habitat (Buhlmann and Gibbons 2001). The negative effects of these threats could lead to local extinctions, the prelude to a species global extinction.

Clearly, terrestrial habitats are vitally important for aquatic and semiaquatic turtles (Buhlmann and Gibbons 2001, McKnight and Ligon 2019). The core area needed to protect nesting, estivation and hibernation, and other terrestrial activities, varies by species (Steen *et al.* 2012), but detailed data are essential to conservation. For example, to protect 100% of the *K. scorpioides* nests at PVNP, the core protected area would have to extend 175 m from the wetland, but protection of the first 25 m from the wetland would protect 86.4% of nests (Mora and Castañeda 2021). This observation about the use of terrestrial environments by *K. scorpioides* in PVNP illustrates how studies like the one reported here are necessary to ensure the future of species that are very often associated only with wetland habitats in the public view. We have been able to show that adjacent terrestrial habitats are indispensable for this mud turtle species.

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Macroecology of reproductive modes in the diverse anuran fauna of the Brazilian Atlantic Forest

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Abstract

Macroecology of reproductive modes in the diverse anuran fauna of the Brazilian Atlantic Forest. Anurans exhibit the widest variety of reproductive modes among tetrapod vertebrates. The Atlantic Forest is a hotspot for biological conservation; however, biogeographic documentation of the richness of anuran reproductive modes are underexplored. By overlaying maps of 249 species having reproductive modes with aquatic eggs and 101 species with non-aquatic eggs, we described the geographical patterns of anuran reproductive modes in the Atlantic Forest and identified the main correlates of reproductive diversity of these two major types of anuran reproduction. We found the greatest diversity of reproductive modes in the coastal region of the Atlantic Forest, whereas a smaller number of reproductive modes was found in inland regions of the hotspot. These regions are broadly characterized by deciduous/semideciduous forests and a warmer and more seasonal climate regime in southeastern and southern Brazil. In this climatically harsher region, reproductive modes were more basal and generalized in anurans. Correlative analyses showed that the richness of reproductive modes with aquatic eggs can be described by general combinations of climate, topography, and vegetation types, whereas larger numbers of reproductive modes with non-aquatic eggs are better described by specific variables of temperature seasonality, amount of ombrophilous forests, and rugged topography. The predictors identified here are environmental variables that should be continuously monitored *in situ;* identifying threshold values that could lead a species to a critical conservation status is key to efficiently protecting the rich anuran fauna of the Atlantic Forest.

Keywords: Biogeography, Biological diversity, Neotropical amphibians, Reproductive diversity.

Resumo

Macroecologia dos modos reprodutivos da diversa anurofauna da Mata Atlântica brasileira. Os anuros exibem a maior variedade de modos reprodutivos entre os tetrápodes vertebrados. A Mata Atlântica é um importante *hotspot* para a conservação biológica; no entanto, documentações

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biogeográficas da riqueza de modos reprodutivos de anuros são pouco explorados. Utilizando a sobreposição de mapas de 249 espécies com modos reprodutivos aquáticos e 101 espécies com modos reprodutivos não-aquáticos, nós descrevemos os padrões geográficos dos modos reprodutivos de anuros na Mata Atlântica e identificamos as principais variáveis correlacionadas com a diversidade reprodutiva destes dois maiores tipos de modo reprodutivos. Encontramos uma maior diversidade de modos reprodutivos na região costeira da Mata Atlântica, sendo que um menor número de modos reprodutivos foi encontrado na região interiorana do *hotspot*. Essas regiões são amplamente caracterizadas pela presença de florestas decíduas/semidecíduas em um regime climático mais quente e sazonal na região sudeste e sul do Brasil. Além disso, os poucos modos reprodutivos encontrados nesta região são aqueles considerados mais primitivos e generalizados nos anuros. As análises correlativas mostram que a riqueza de modos reprodutivos com ovos aquáticos está relacionada com combinações gerais de clima, topografia e tipos de vegetação, enquanto que os modos reprodutivos com ovos não-aquáticos são melhores relacionados com variáveis específicas de sazonalidade de temperatura, quantidade de florestas ombrófilas e topografia acidentada. As variáveis identificadas no presente estudo são importantes para serem continuamente monitorados *in situ*, já que a identificação de valores limítrofes de tais preditores que levariam o status de conservação de uma dada espécie para níveis críticos é extremamente importante para a conservação eficiente da rica anurofauna da Mata Atlântica.

Palavras-chave: Anfíbios Neotropicais, Biogeografia, Diversidade biológica, Diversidade reprodutiva.

Introduction

Anurans are the most diverse order of Amphibia with approximately 7,446 species worldwide (Frost 2021). A wealth of adaptations have led anurans to successfully exploit terrestrial and associated freshwater environments; consequently these animals have evolved the greatest reproductive diversity of all tetrapod vertebrates (e.g., Haddad and Prado 2005, Crump 2015, Malagoli *et al.* 2021). Using a combination of traits that includes oviposition sites, ovum and clutch characteristics, rate and duration of development, stage and size of hatchling, and type of parental care, if any (Salthe and Duellman 1973), scientists have identified more than 40 anuran reproductive modes worldwide (Haddad and Prado 2005, Iskandar *et al*. 2014, Kusrini *et al*. 2015, Malagoli *et al*. 2021). Besides elucidating the evolutionary ecology of anurans, the study of reproductive modes assists in biological conservation actions guided by life-history traits related to reproductive requirements (e.g., Loyola *et al.* 2008, Becker *et al.* 2010).

In general, anuran reproductive modes can be split into two major categories (Haddad and Prado 2005): (1) aquatic eggs that are either deposited directly in the water or in aquatic foam nests or bubble nests, or embedded in the dorsum of aquatic females; and (2) terrestrial or arboreal eggs (not in water) that are deposited on the ground, on rocks, or in burrows, as well as on vegetation, associated or not with foam nests, or even carried by adults (on the body of an adult or internally retained in a female oviduct). Additionally, the non-aquatic category includes some species that have direct development of terrestrial eggs, in which the tadpole phase is abbreviated.

The most common reproductive mode is the deposition of eggs and the development of exotrophic tadpoles in lentic water, which is also considered the most basal mode in anurans. Other reproductive modes generally represent a continuum of specializations toward the use of different terrestrial environments (Duellman and Trueb 1994, Haddad and Prado 2005, Crump 2015). Irrespective of whether this trend toward terrestriality exhibits a phylogenetic signal (e.g., Benício *et al.* 2021) or not (e.g., Gomez-Mestre *et al.* 2012, Pereira *et al.* 2015), the evolution of anuran reproductive modes is believed to be a result of repeated independent events of terrestrial breeding strategies from the plesiomorphic fully aquatic breeding (Meegaskumbura *et al.* 2015, Portik and Blackburn 2017). Anurans with non-aquatic eggs still depend on water for reproduction, either for tadpole development or in the form of moist environments for direct development of eggs (Duellman and Trueb 1994). This dependence on water makes these animals highly sensitive to environmental variation from local to regional scales (Werner *et al.* 2007, Rossa-Feres *et al.* 2011, Haddad *et al.* 2013). Various studies have found correlations between species richness (or diversity of reproductive modes) and climatic variables, which are generally attributed to a given trait specialization (e.g., a particular reproductive mode) adapted to a given climatic regime (Vasconcelos *et al.* 2010, Silva *et al.* 2012).

The Atlantic Forest, located along the Brazilian Atlantic coast, is a biodiversity hotspot and the second largest tropical forest in South America (Mittermeier *et al.* 2004). It is home to one of the most diverse anuran faunas in South America (IBGE 2012; Vasconcelos *et al.* 2019), in which anurans exhibit the highest reproductive diversity in the world (Haddad and Prado 2005). Anuran diversity is greatest in the ombrophilous/evergreen forests of the southeastern coastal region (Haddad *et al.* 2013, Vasconcelos *et al.* 2014). Studies focusing on different Atlantic Forest regions (e.g., mostly in southeastern and southern Brazil) found that anuran reproductive diversity is related to taxonomic species richness: larger numbers of reproductive modes occur in more humid forests with rugged topography (Vasconcelos *et al.* 2010, Silva *et al.* 2012, Haddad *et al.* 2013). As described above, anuran reproductive modes can be split into two categories related to their dependence on water: reproductive modes with aquatic eggs and those with non-aquatic eggs. Although most anuran species have an aquatic tadpole phase, species with non-aquatic eggs undergo an embryonic period away from water. Undergoing this critical stage out of water in a dry or humid environment results in differences in survival rates of tadpoles compared to tadpoles of those species that lay eggs in water. We hypothesized that the prevalence of reproductive modes with non-aquatic eggs will be associated with environmental variables such as water or temperature seasonality and that the prevalence of reproductive modes with aquatic eggs will be associated with other variables (e.g., general trends of rainfall or temperature regimes). Our main focus is to elucidate the environmental correlates of these two major categories of reproductive modes in the diverse anuran fauna of the Atlantic Forest.

We mapped reproductive diversity of anurans throughout the Atlantic Forest based on whether a species has an aquatic or non-aquatic reproductive mode. We performed correlative analyses and identified the main correlates of these two major types of reproductive modes. We tested which of the environmental variables (climatic, topographic, and habitat-related variables) were indicative of the type of reproductive mode. We hypothesized that the different types of reproductive modes would have distinct sets of predictors because of their inherent differences in dependence on water. Reproductive modes with aquatic eggs should depend on the accumulated water volume; therefore, we expected that climatic trends such as total annual rainfall and mean annual temperature would be correlates of these modes. Reproductive modes with non-aquatic eggs may not directly depend on accumulated water. A humid environment may be more indicative of the prevalence of these types of reproductive modes. These modes should be correlated with variables associated with climatic or environmental stability such as seasonality in precipitation or temperature and the presence of evergreen forests (e.g., Müller *et al.* 2013).

Material and Methods

Species Data and Study Site

Using the Atlantic Forest species list of anurans compiled by Haddad *et al*. (2013), we downloaded the distribution maps from the IUCN (2017) database and updated the taxonomic nomenclature according to the Amphibian Species of the World database (Frost 2021, updated through January 2020). Anuran species lacking distribution maps in the IUCN (2017) portal (e.g., recently described species) were surveyed using point occurrences to generate their ranges using the minimum convex polygon calculated from the points (e.g., García-Roselló *et al*. 2015, Vasconcelos *et al*. 2019). Species maps with information on reproductive modes were overlapped onto a grid system of the continental Atlantic Forest with resolution of ~50 km (i.e., islands were not considered), allowing generation of a presence/absence matrix of species distributions.

Recently, Nunes-de-Almeida *et al.* (2021) revisited the classification of amphibian reproductive modes and proposed the existence of 71 modes for anurans. Though this classification represents almost twice the number in the classification proposed by Haddad and Prado (2005), we followed the previous classification in this study. Our results are subject to changes under a reanalysis using the classification by Nunes-de-Almeida *et al.* (2021). We believe that such a reanalysis may not result in major changes because some aquatic and non-aquatic reproductive modes proposed by Haddad and Prado (2005) are now divided into new modes, in addition to new modes proposed by Nunes-de-Almeida *et al*. (2021) within both aquatic and non-aquatic categories. Information regarding reproductive modes of each species was taken from Haddad *et al*. (2013), which used the classification by Haddad and Prado (2005). Of the 529 anurans listed by Haddad *et al*. (2013), we compiled distribution maps for 249 species having reproductive modes with aquatic eggs and 101 species with non-aquatic eggs (Appendices I and II). We ignored those species lacking information regarding their reproductive modes, as well as island-endemic species. Many of these species have small ranges and are known only from their type-localities or from a small number of localities (e.g., *Ischnocnema erythromera*, *I. izecksohni*, *Dendrophryniscus proboscideus*, *Frostius erythrophthalmus*, and *Cycloraphus organensis*). Excluding these species is not a critical issue for the present study; according to the niche conservatism hypothesis and the high phylogenetic signal in the reproductive modes of Atlantic Forest anurans (Benício *et al.* 2021), the excluded species are more likely to exhibit the same reproductive modes as other species in the same genus/family, which in turn are already represented by the congeneric species.

Climatic, Topographic, and Habitat-Related Variables

In order to identify the correlates of anuran reproductive diversity, we selected environmental variables that represent potential limits of physiological tolerances for egg development, physical barriers for species distributions, and/or habitat preferences for a given reproductive mode (e.g., Vasconcelos *et al*. 2010, Silva *et al*. 2012, Vasconcelos *et al*. 2014). Mean annual temperature (TEMP), temperature seasonality (TSEASO), annual precipitation (PRECIP), and precipitation seasonality (PRECSEASO) were downloaded from the Chelsa Climate database (Karger *et al.* 2021) at a 1 × 1 km resolution. Annual actual evapotranspiration (AET), which measures the water-energy balance, was downloaded from the Food and Agriculture Organization of the United Nations (FAO: http://www.fao.org/geonetwork/srv/en/metadata.show?id=37233) at a resolution of 10 × 0 km. Topographic heterogeneity (TOPO) was determined using the standard deviation of the mean altitude within each 50 × 50 km grid cell based on the altitude data of 1 × 1 km resolution available at the U. S. Geological Survey portal (https://lta.cr.usgs.gov/GTOPO30). Habitat-related variables were based on how much native forest area is present within a 1 × 1 km resolution. We recognized two forest formations that are predominant in the Atlantic Forest: the deciduous/semideciduous forest (DECIDUOUS) and the ombrophilous forest (also known as EVERGREEN) (Tuanmu and Jetz 2014). For each DECIDUOUS and EVERGREEN map, the grid cell provided information regarding the total area of native forest within this resolution.

Except for the TOPO, which was determined using the standard deviation of the mean altitude of the original layer (1 × 1 km), all explanatory variables were derived from the mean value at each cell of the Atlantic Forest grid system.

Data Analyses

Prior to the analyses, the response (i.e., number of aquatic and non-aquatic reproductive modes) and predictor variables were square root or log transformed to reduce heteroscedasticity and normalize model residuals. The predictor variables were standardized using z-scores to provide comparable regression coefficients (Quinn and Keough 2002).

We performed two approaches to identify the correlates of the reproductive modes: the performance of traditional general linear models (Legendre and Legendre 2012) and spatially explicit simultaneous autoregressive models (SAR; Kissling and Carl 2008). The first approach makes use of ordinary least-squares regression (OLS) that aims to find a straight line that minimizes the sum of squares of the vertical residuals between the observed values and the regression line (Legendre and Legendre 2012). A series of OLS models were performed separately with different sets of predictors for the aquatic and non-aquatic reproductive modes. A model selection was performed based on the Akaike Information Criteria corrected for small samples (AICc) (Burnham and Anderson 2002), of which the model with the lowest AICc was identified as the best model (see similar applications in Boaratti and Silva 2015, Vasconcelos *et al*. 2019). The best models for aquatic and non-aquatic eggs identified by the AICc were also the ones used for the SAR approach.

The second approach, the spatially explicit simultaneous autoregressive models (SAR; Kissling and Carl 2008), was performed because of the presence of spatial autocorrelation in OLS model residuals that violates the assumption of residual independence, which in turn can distort estimates of model parameters. SAR models minimize the effect of spatial autocorrelation and identify the variables that effectively contribute to the richness patterns of the reproductive modes. SAR models are spatially explicit and allow the incorporation of residuals spatially autocorrelated at different classes of errors during the model building. By using spatial correlograms (Moran's *I*), we defined an intermediate value between the first and second class of the spatial correlogram (i.e., alpha = 1.5; neighborhood points separated by 50–100 km) because these were the classes with the highest values of Moran's *I* for aquatic and non-aquatic reproductive modes. OLS, SAR, and the AICc analyses were performed using the software Spatial Analysis in Macroecology (Rangel *et al*. 2010).

Results

Richness Patterns of Reproductive Modes

Reproductive diversity per grid across the Atlantic Forest varied between three and 12 aquatic reproductive modes and between one and 12 non-aquatic reproductive modes (Figure 1). Irrespective of the reproductive mode classification, most reproductive diversity is found along the Atlantic coast, whereas fewer reproductive modes are found in the inland region. This gradient is steeper for the non-aquatic modes (Figure 1).

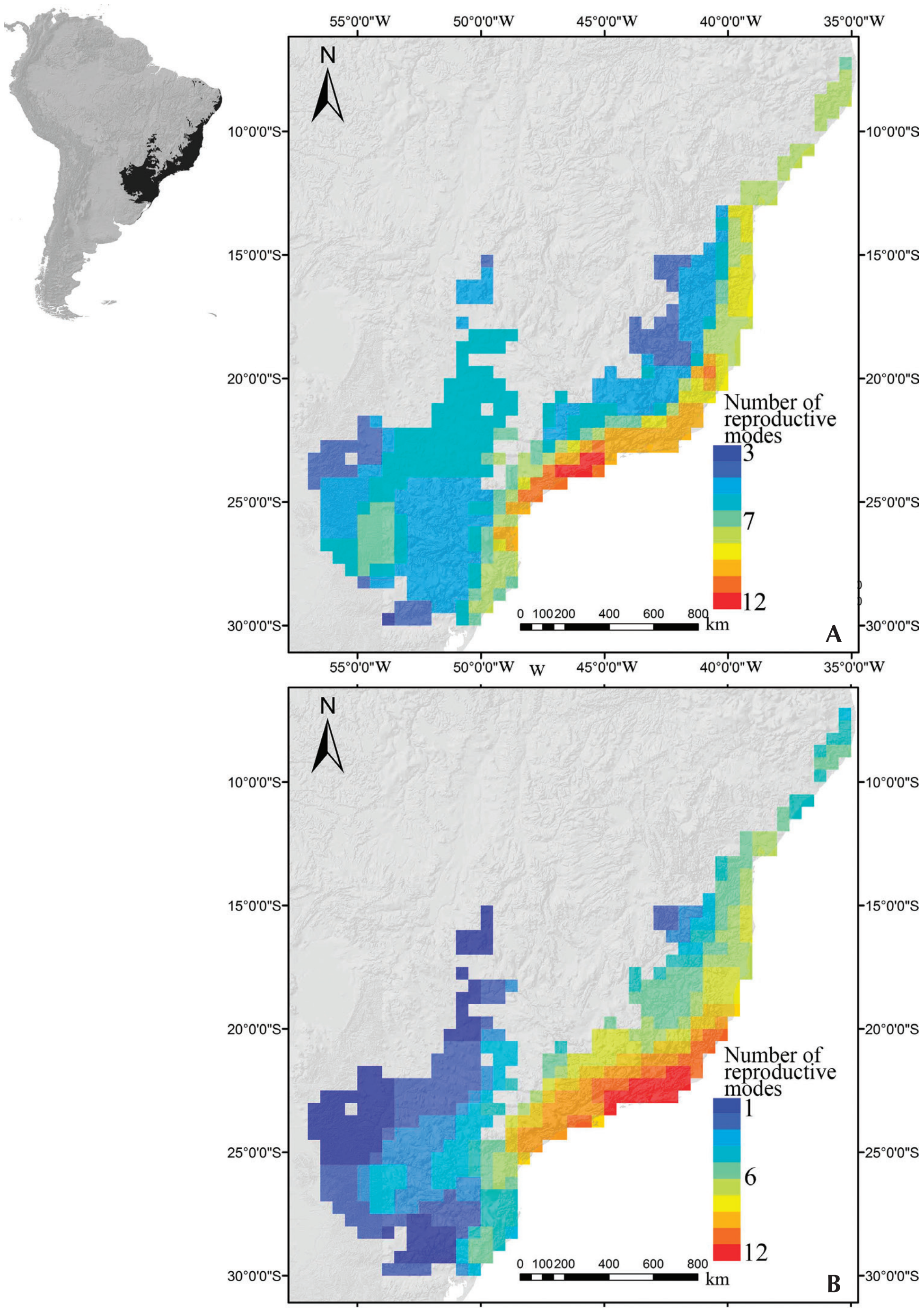

Figure 1. Diversity gradients of the anuran reproductive modes in the Atlantic Forest for the modes having (**A**) aquatic and (**B**) non-aquatic eggs.

Correlates of the Reproductive Modes

OLS models for the aquatic (Table 1) and non-aquatic (Table 2) reproductive modes indicate that the best models have six environmental variables for both categories. The best model of the aquatic reproductive modes (Table 1) includes the climatic variables AET, TSEASO, PRECIP, the topographic variable (TOPO), and the habitat-related variables DECIDUOUS and EVERGREEN. The best model for the non-aquatic reproductive modes (Table 2) includes the climatic variables AET, TEMP, TSEASO, and PSEASO, the topographic variable (TOPO), and the habitat-related variable EVERGREEN.

Regarding the aquatic reproductive modes, the six variables of the OLS (R^2 adj = 0.379, $F = 48.301$, $p < 0.001$) and SAR models (R^2 adj = 0.408, $F = 43.021$, $p < 0.001$) explained relatively similar levels of variance between them. All variables of the OLS and SAR models have significant correlations with the aquatic reproductive modes, except the variable PRECIP for the SAR model (Table 3).

Regarding the non-aquatic reproductive modes, the total variance explained by the OLS and SAR models were relatively higher than those obtained for the aquatic reproductive modes (OLS: R^2 adj = 0.464, $F = 68.211$, $p = 0.00$; SAR: R^2 adj = 0.535, $F = 28.622$, $p < 0.001$). SAR models indicated only three significant correlations with the non-aquatic reproductive modes (TOPO, TSEASO, and EVERGREEN), whereas all variables in the OLS models were significant (Table 3).

In summary, irrespective of the reproductive specialization (aquatic or non-aquatic), we found major reproductive diversity along the Atlantic coast. This region is mostly characterized by the presence of ombrophilous forest, rugged topography, and humid climatic regimes with mild and less seasonality in temperatures. A lower number of reproductive modes was found in inland areas of the Atlantic Forest, mostly in the southeastern and southern Brazilian states. These areas are characterized by having semideciduous and deciduous forests, hot and more seasonal climate regimes, and less rugged topography than the coastal region. Despite the broadly similar diversity patterns of the aquatic and non-aquatic reproductive modes, the main difference between them is the steeper richness gradient in the non-aquatic reproductive modes. Among the selected environmental correlates, we found that the aquatic reproductive modes were better characterized by a general combination of climate, topography, and type of vegetation, whereas the non-aquatic reproductive modes were better described by specific variables related to climate stability (temperature seasonality), prevalence of humid microhabitats (quantity of evergreen forest), and rugged topography.

Discussion

Our initial predictions were supported by the results: (a) the aquatic reproductive modes were correlated with different aspects of climate (either general climatic trends or variables related to climatic seasonality), relief, and vegetation; and (b) the non-aquatic reproductive modes were specifically correlated with predictors related to lower oscillations in temperature on a yearly basis and the presence of evergreen forests in rugged topographic areas, thus emphasizing the necessity of humid forests and stable climate regimes for supporting higher levels of biological diversity (e.g., Müller *et al.* 2013, Vasconcelos *et al.* 2019) and, in the present study, different varieties of anuran reproductive modes with non-aquatic eggs.

Water is necessary for anurans because of their physiological needs (e.g., maintenance of a moist skin for effective cutaneous breathing) and reproductive characteristics (e.g., development of aquatic larvae or presence of humid microhabitats for development of eggs and juveniles with direct development; Duellman and Trueb 1994, Haddad *et al.* 2013). The necessity of water for anurans is reflected here

Table 1. Model selection of the Ordinary Least Square models for the reproductive modes with aquatic eggs. *N* = number of predictor variables in the respective model; AICc = Akaike Information Criterion corrected for small samples (sorted according to the lowest value); AICc WI = evidence support level for the respective model. See Materials and Methods for the abbreviations of variables.

Variables	*N*	AICc	AICc WI
AET+TOPO+TSEASO+PRECIP+DECIDUOUS+ EVERGREEN	6	153.653	0.39
AET+TOPO+TSEASO+PRECIP+PRECSEASO+ DECIDUOUS+EVERGREEN	7	154.679	0.233
AET+TOPO+TEMP+TSEASO+PRECIP+ DECIDUOUS+EVERGREEN	7	155.539	0.152
AET+TOPO+TEMP+TSEASO+PRECIP+ PRECSEASO+DECIDUOUS+EVERGREEN	8	156.119	0.114

Table 2. Model selection of the Ordinary Least Square models for the reproductive modes with non-aquatic eggs. *N* = number of predictor variables in the respective model; AICc = Akaike Information Criterion corrected for small samples (sorted according to the lowest value); AICc WI = evidence support level for the respective model. See Materials and Methods for the abbreviations of variables.

Variables	*N*	AICc	AICc WI
AET+TOPO+TEMP+TSEASO+PSEASO+ EVERGREEN	6	767.956	0.311
AET+TOPO+TEMP+TSEASO+PRECIP+ PRECSEASO+ EVERGREEN	7	769.076	0.178
AET+TOPO+TEMP+TSEASO+PRECSEASO+ DECIDUOUS+EVERGREEN	7	769.286	0.16
AET+TOPO+TEMP+TSEASO+PRECSEASO	5	769.919	0.117
AET+TOPO+TEMP+TSEASO+PRECIP+ PRECSEASO+ DECIDUOUS+EVERGREEN	8	770.289	0.097

Table 3. Results of the non-spatial Ordinary Least Squares (OLS) and spatially explicit Simultaneous Autoregressive models (SAR) (variable importance values ± standard error) to explain the variation in the number of reproductive modes of aquatic and non-aquatic eggs. Significant correlations ($p < 0.05$) are highlighted in bold.

Variables	Aquatic eggs		Non-aquatic eggs	
	OLS	SAR	OLS	SAR
INTERCEPT	**5.735 ± 0.413**	**2.46 ± 0.061**	**2.083 ± 0.025**	**1.602 ± 0.127**
AET	**0.097 ± 0.017**	**0.087 ± 0.016**	**0.114 ± 0.036**	0.045 ± 0.025
TOPO	**0.167 ± 0.016**	**0.111 ± 0.016**	**0.318 ± 0.032**	**0.137 ± 0.025**
TEMP	–	–	**-0.182 ± 0.045**	-0.047 ± 0.038
TSEASO	**-0.964 ± 0.123**	**-0.115 ± 0.026**	**-0.338 ± 0.044**	**-0.223 ± 0.052**
PRECIP	**0.049 ± 0.019**	0.02 ± 0.021	–	–
PSEASON	–	–	**0.172 ± 0.046**	-0.059 ± 0.046
DECIDUOUS	**-0.076 ± 0.019**	**-0.067 ± 0.017**	–	–
EVERGREEN	**0.077 ± 0.018**	**0.079 ± 0.016**	**0.066 ± 0.033**	**0.073 ± 0.024**

by the correlation of aquatic reproductive modes and water-related variables, such as AET and the presence of ombrophilous forests (i.e., evergreen forests). The amount of precipitation per se was only correlated with reproductive modes in the OLS model but not when the spatial autocorrelation was taken into account in the SAR model. This result indicates that the amount of precipitation per se is not always critical for supporting a high variability of aquatic reproductive modes, but an environmental water-energy balance that generates high primary productivity (i.e., places with high AET index) is also important, in addition to the presence of humid microhabitats that are constantly maintained within evergreen forests. Humid conditions may have been ideal for the evolution and establishment of species with aquatic reproductive modes in which the water accumulates in tree holes or in aerial plants, such as the Modes 6, 8, and 14 (sensu Haddad and Prado 2005). For non-aquatic reproductive modes, the SAR model indicated an implicit necessity of water for anurans because of the positive correlation with the presence of ombrophilous forests. The presence of water in the form of humid microhabitats generated by the heterogeneous structure of ombrophilous forests becomes a key opportunity for the development and establishment of reproductive specializations of non-aquatic eggs, such as direct development of terrestrial eggs deposited on the moist forest floor (Mode 23) or in arboreal structures (Mode 27) (Haddad and Prado 2005, Haddad *et al*. 2013).

Temperature oscillation throughout the year was a predictor for both aquatic and non-aquatic anuran reproductive modes. Regions having less temperature variation throughout the year supported more anuran reproductive modes. Greater variations in temperature values (especially for higher temperatures) may lead to lower values of humidity or to faster evaporation of water bodies. More stable temperatures may lead to breeding sites with enough humidity or volume of water throughout the year, which in turn should allow for the presence of a variety of reproductive modes in such regions. This result reinforces the idea that climatically stable regions support higher levels of biological diversity (e.g. Carnaval *et al*. 2009), as seen in the southeastern ombrophilous forests of the Atlantic Forest that support the highest diversity of reproductive modes. Warmer and more seasonally variable regions in inland areas of the Atlantic Forest support fewer types of reproductive modes but include the most common anuran reproductive mode (Mode 1: eggs and exotrophic tadpoles in lentic water; sensu Haddad and Prado 2005) and reproductive modes of wide-ranging species with aquatic (e.g., *Physalaemus cuvieri,* Mode 11) or non-aquatic (e.g., *Leptodactylus fuscus,* Mode 30) eggs associated with foam nests that typically avoid desiccation in these climatically harsher environments (Haddad and Prado 2005, Santos *et al*. 2009).

Topography was also identified as a correlate of anuran reproductive modes. Mountain uplifts favored diversification of different taxa and anuran reproductive modes (Haddad and Prado 2005, Rangel *et al*. 2018, Benício *et al*. 2021). This process was not the only source of selective pressures leading to the evolution of diverse reproductive modes (Haddad and Prado 2005). A region with high reproductive diversity depends on the presence of different aquatic/humid environments found within forests (Müller *et al*. 2013). When such a region is intersected by mountain chains, the major breeding habitats may be inaccessible to some populations, which in turn may have become more isolated. Evolution will favor those organisms that exploit the many small, humid microhabitats that become available to them. Prior to mountain uplifts, organisms of an ancestral species maintained gene flow among themselves, leading to common reproductive behaviors. The splitting of populations because of mountain uplifts, coupled with the constant presence of humid/aquatic microhabitats within evergreen forests, was an ideal scenario that favored new reproductive strategies, promoted genetic differentiation and speciation. In areas lacking ombrophilous forests or constant environmental humidity, higher rates of allopatric speciation in mountainous areas might still occur, but most species would probably exhibit generic reproductive modes with eggs and exotropic tadpoles in lentic (Mode 1) or lotic (Mode 2) environments or egg deposition in subterraneous foam nests (Modes 30 and 32 sensu Haddad and Prado 2005). The influence of topography on the evolution of anuran reproductive modes is corroborated by the high phylogenetic signal found for Atlantic Forest anurans. Benício *et al.* (2021) found that a higher speciation rate occurred when the coastal Atlantic Forest mountains emerged. Many of these coastal species, some of them from a particular genera or family (e.g., *Brachycephalus, Dendrophryniscus, Fritziana, Crossodactylodes,* Cycloramphidae) have unique reproductive modes associated with bromeliads, arboreal structures, or the forest floor, or have direct development, modes that are absent in inland regions where the climate is drier and the topography is not rugged.

The greater reproductive diversity found in coastal regions of the Atlantic Forest is broadly congruent with the taxonomic richness of anurans (Vasconcelos *et al*. 2010, 2014, 2019). Silva *et al*. (2012) found a positive correlation between the taxonomic richness and the number of reproductive modes in 27 anuran communities in the southeastern Atlantic Forest. These authors found that reproductive modes of these communities have a nested pattern of distribution and are correlated with high levels of environmental humidity. Localities with a low number of reproductive modes, mostly located in inland and drier regions, present the most generalized and widespread anuran reproductive modes. Silva *et al*. (2012) also found higher variability and unique types of reproduction in humid areas along the Atlantic Forest coast. Besides this nested pattern of distribution of reproductive modes in the Atlantic Forest, the absence of some anuran lineages with diversified reproductive modes in inland regions suggests that physiological or ecological tolerances have prevented coastal species and clades from occupying the inland region (i.e., the niche conservatism hypothesis; Wiens *et al.* 2010, Benício *et al.* 2021). Ecological traits that species and clades retain over their evolutionary history (i.e., a given set of combination of reproductive characteristics) play a strong role in shaping anuran communities and different reproductive modes across regions with different climatic regimes in the Atlantic Forest (Benício *et al.* 2021).

Our study identified predictors of reproductive modes of the diverse anuran fauna in the Atlantic Forest. Our results may be of interest to herpetologists and ecologists who monitor climate variation and the fragmentation of natural areas. Our data may help to identify threshold values for predictors that would be invaluable in establishing the conservation status of a given species (e.g., Rueda *et al.* 2015). In light of the current biodiversity crisis, dozens of Atlantic Forest anurans with diverse reproductive modes have been predicted to have no climatically suitable breeding areas by 2050 under various climate change scenarios (e.g., Vasconcelos *et al*. 2018). Future studies integrating the conservation biogeography under different climate change scenarios (e.g., Vasconcelos and Prado 2019) and the environmental integrity that supports high levels of anuran reproductive diversity might be promising approaches leading to an effective anuran conservation plan in the highly diverse Atlantic Forest.

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Appendix I. *Species list having aquatic reproductive modes and the respective reproductive mode number (sensu Haddad* et al. *2013). Mode 1: Eggs and exotrophic tadpoles in lentic water. Mode 2: Eggs and exotrophic tadpoles in lotic water. Mode 3: Eggs and early larval stages in constructed subaquatic chambers; exotrophic tadpoles in streams. Mode 4: Eggs and early larval stages in natural or constructed basins; subsequent to flooding, exotrophic tadpoles in ponds or streams. Mode 5: Eggs and early larval stages in subterranean constructed nests; subsequent to flooding, exotrophic tadpoles in ponds or streams. Mode 6: Eggs and exotrophic tadpoles in water in tree holes or aerial plants. Mode 8: Eggs and endotrophic tadpoles in water in tree holes or aerial plants. Mode 10: Bubble nest floating on pond; exotrophic tadpoles in ponds. Mode 11: Foam nest floating on pond; exotrophic tadpoles in ponds. Mode 13: Foam nest floating on water accumulated in constructed basins; exotrophic tadpoles in ponds. Mode 14: Foam nest floating on water accumulated on the axils of terrestrial bromeliads; exotrophic tadpoles in ponds. Mode 15: Eggs hatch into exotrophic tadpoles.*

Aparasphenodon arapapa (6), *A. brunoi (1), Aplastodiscus albofrenatus* (5), *A. albosignatus* (5), *A. arildae* (5), *A. cavicola* (5), *A. cochranae* (5), *A. ehrhardti* (5), *A. eugenioi* (5), *A. flumineus* (5), *A. ibirapitanga* (5), *A. leucopygius* (5), *A. perviridis* (5), *A. sibilatus* (5), *A. weygoldti* (5), *Arcovomer passarellii* (1), *Bokermannohyla astartea* (6), *B. capra* (2), *B. caramaschii* (2), *B. carvalhoi* (2), *B. circumdata* (1 and 2), *B. gouveai* (1 and 2), *B. hylax* (4), *B. luctuosa* (2 and 4), *B. martinsi* (2), *Ceratophrys aurita* (1), *Chiasmocleis alagoana* (1), *C. atlântica* (1), *C. capixaba* (1), *C. carvalhoi* (1), *C. leucostica* (10), *C. schubarti* (1), *Crossodactylodes bokermanni* (6), *Crossodactylus aeneus* (3), *C. caramaschii* (3), *C. dispar* (3), *C. gaudichaudii* (3), *C. grandis* (3), *C. schmidti* (3), *Dasypops schirchi* (1), *Dendrophryniscus berthalutzae* (8), *D. brevipollicatus* (8), *D. carvalhoi* (8), *D. krausae* (8), *Dendropsophus anceps* (1), *D. bipunctatus* (1), *D. branneri* (1), *D. elegans* (1), *D. giesleri* (1), *D. meridianus* (1), *D. microps* (1), *D. minutus* (1), *D. nahdereri* (1), *D. nanus* (1), *D. novaisi* (1), *D. oliveirai* (1), *D. pseudomeridianus* (1), *D. sanborni* (1), *D. seniculus* (1), *D. soaresi* (1), *D. studerae* (1), *Elachistocleis cesarii* (1), *E. erythrogaster* (1), *Frostius pernambucensis* (8), *Hylodes asper* (3), *H. babax* (3), *H. cardosoi* (3), *H. charadranaetes* (3), *H. lateristrigatus* (3), *H. nasus* (1), *H. ornatus* (3), *H. otavioi* (3), *H. perere* (3), *H. perplicatus* (3), *H. phyllodes* (3), *H. regius* (3), *H. sazimai* (3), *Hyophryne histrio* (1), *Hypsiboas albomarginata* (1), *H. albopunctata* (1), *H. atlanticus* (1 and 2), *H. bischoffi* (1), *H. caingua* (1), *H. caipora* (2), *H. crepitans* (4), *H. curupi* (2), *H. exastis* (4), *H. faber* (1 and 4), *H. guentheri* (1), *H. joaquini* (2), *H. latistriatus* (2), *H. leptolineatus* (1 and 2), *H. marginatus* (2), *H. pardalis* (4), *H. polytaenius* (1), *H. pombali* (1 and 2), *H. prasinus* (1 and 2), *H. pulchellus* (1), *H. punctatus* (1 and 2), *H. raniceps* (1), *H. semiguttatus* (1 and 2), *H. semilineatus* (1 and 2), *H. stellae* (1 and 2), *Itapotihyla langsdorffii* (1), *Leptodactylus flavopictus* (13), *L. labyrinthicus* (11), *L. latrans* (11), *L. natalensis* (13), *L. podicipinus* (13), *L. vastus* (11), *L. viridis* (11), *Limnomedusa macroglossa* (1), *Lithobates palmipes* (1), *Macrogenioglottus alipioi* (1), *Melanophryniscus vilavelhensis* (6), *M. admirabilis* (1), *M. cambaraensis* (1), *M. dorsalis* (1), *M. moreirae* (1), *M. simplex* (2), *M. tumifrons* (1), *Odontophrynus americanus* (1), *O. carvalhoi* (2), *O. maisuma* (1), *Paratelmatobius cardosoi* (1), *P. gaigeae* (1), *Phyllodytes acuminatus* (6), *P. edelmoi* (6), *P. gyrinaethes* (6), *P. kautskyi* (6), *P. luteolus* (6), *P. melanomystax* (6), *P. tuberculosus* (6), *P. wuchereri* (6), *Physalaemus aguirrei* (11), *P. atlanticus* (11), *P. crombiei* (11), *P. cuvieri* (11), *P. erikae* (11), *P. feioi* (11), *P. jordanensis* (11), *P. kroyeri* (11), *P. lateristriga* (11), *P. lisei* (11), *P. maculiventris* (11), *P. maximus* (11), *P. moreirae* (11), *P. nanus* (11), *P. obtecus* (11), *P. olfersii* (11), *P. signifer* (11), *P. spiniger* (11 and 14), *Pipa carvalhoi* (15), *Proceratophrys appendiculata* (1 and 2), *P. avelinoi* (2), *P. bigibbosa* (2), *P. boiei* (1 and 2), *P. brauni* (2), *P. cristiceps* (2), *P. laticeps* (1 and 2), *P. melanopogon* (1 and 2), *P. renalis* (2), *P. sanctaritae* (2), *P. schirchi* (2), *P. subguttata* (2), *Pseudis bolbodactyla* (1), *P. cardosoi* (1), *P. fusca* (1), *P. minuta* (1), *Pseudopaludicola falcipes* (1), *Rhinella abei* (1 and 2), *R. achavali* (1), *R. crucifer* (1 and 2), *R. dorbignyi* (1), *R. fernandezae* (1), *R. granulosa* (1), *R. henseli* (1 and 2), *R. hoogmoedi* (1), *R. icterica* (1 and 2), *R. jimi* (1), *R. ornata* (1 and 2), *R. pygmaea* (1), *R. schneideri* (1 and 2), *Scinax agilis* (1), *S. albicans* (2), *S. alter* (2), *S. angrensis* (1), *S. argyreornata* (1, *S. ariadne* (2), *S. aromothyella* (1), *S. auratus* (1), *S. belloni* (2), *S. brieni* (2), *S. caldarum* (1), *S. carnevallii* (1), *S. catharinae* (2), *S. crospedospilus* (1), *S. cuspidatus* (1), *S. duartei* (1), *S. eurydice* (1), *S. flavoguttatus* (2), *S. fuscomarginatus* (2), *S. fuscovarius* (1), *S. granulatus* (1), *S. hayii* (1), *S. heyeri* (2), *S. hiemalis* (1), *S. humilis* (1), *S. imbegue* (1), *S. insperatus* (6), *S. juncae* (1), *S. littoralis* (1), *S. littoreus* (6), *S. longilineus* (2), *S. luizotavioi* (1 and 2), *S. machadoi* (2), *S. obtriangulatus* (2), *S. pachycrus* (1), *S. perereca* (1), *S. perpusillus* (6), *S. ranki* (1), *S. rizibilis* (1), *S. similis* (1), *S. squalirostris* (1), *S. strigilatus* (1 and 2), *S. trapicheiroi* (1), *S. tymbamirim* (1), *S. uruguayus* (1), *S. v-signatus* (1), *S. x-signatus* (1), *Scythrophrys sawayae* (1), *Sphaenorhynchus botocudo* (1), *S. caramaschii* (1), *S. palustris* (1), *S. planicola* (1), *S. prasinus* (1), *S. surdus* (1), *Stereocyclops incrassatus* (1), *S. parkeri* (1), *Trachycephalus typhonius* (1), *T. atlas* (1), *T. dibernardoi* (1), *T. imitatrix* (1), *T. lepidus* (1), *T. mesophaeus* (1), *T. nigromaculatus* (1 and 2), *Xenohyla truncate* (1).

Appendix II. *Species list having non-aquatic reproductive modes and the respective reproductive mode number (sensu Haddad* et al. *2013). Mode 18: Eggs on ground or rock above water; upon hatching, exotrophic tadpoles move to water. Mode 19: Eggs on humid rocks, in rock crevices, or on tree roots above water; exotrophic semiterrestrial tadpoles living on rocks and rock crevices in a water film or in the water–land interface. Mode 20: Eggs hatching into exotrophic tadpoles that are carried to water by adult. Mode 21: Eggs hatching into endotrophic tadpoles that complete their development in the nest. Mode 23: Direct development of terrestrial eggs. Mode 24: Eggs hatching into exotrophic tadpoles that drop in lentic water. Mode 25: Eggs hatching into exotrophic tadpoles that drop in lotic water. Mode 27: Eggs hatching into froglets. Mode 30: Foam nest with eggs and early larval stages in subterranean constructed nests; subsequent to flooding, exotrophic tadpoles in ponds. Mode 32: Foam nest in subterranean constructed chambers; endotrophic tadpoles complete development in nest. Mode 36: Eggs carried on dorsum or in dorsal pouch of female; endotrophic tadpoles in bromeliads or bamboo. Mode 37: Eggs carried on dorsum or in dorsal pouch of female; direct development into froglets.*

Adelophryne pachydactyla (23), *Adenomera ajurauna* (32), *A. araucaria* (32), *A. bokermanni,* (32), *A. marmorata* (32), *A. nana* (32), *A. thomei* (32), *Agalychnis aspera* (18), *A. granulosa* (25), *Allobates olfersioides* (20), *Brachycephalus alipioi* (23), *B. didactylus* (23), *B. ephippium* (23), *B. ferruginus* (23), *B. guarani* (23), *B. hermogenesi* (23), *B. margaritatus* (23), *B. nodoterga* (23), *B. pulex* (23), *B. toby* (23), *B. tridactylus* (23), *B. vertebralis* (23), *Cycloramphus lutzorum* (19), *C. acangatan* (21), *C. boraceiensis* (19), *C. brasiliensis* (19), *C. dubius* (19), *C. eleutherodactylus* (21), *C. izecksohni* (19), *C. juimirim* (19), *C. rhyokonastes* (19), *C. valae* (19), *Dendropsophus berthalutzae* (24), *D. decipiens* (24), *D. haddadi* (24), *Euparkerella brasiliensis* (23), *E. cochranae* (23), *E. robusta* (23), *Fritziana fissilis* (36), *F. goeldii* (36), *F. ohausi* (36), *Gastrotheca albolineata* (37), *G. ernestoi* (37), *G. fissipes* (37), *G. fulvorufa* (37), *G. megacephala* (37), *G. microdiscus* (37), *G. prasina* (37), *G. pulchra* (37), *G. recava* (37), *Haddadus binotatus* (23), *Holoaden bradei* (23), *H. luederwaldti* (23), *Ischnocnema bolbodactyla* (23), *I. concolor* (23), *I. guentheri* (23), *I. henselii* (23), *I. hoehnei* (23), *I. juipoca* (23), *I. manezinho* (23), *I. nasuta* (27), *I. parva* (23), *I. sambaqui* (23), *I. verrucosa* (23), *I. vizottoi* (23), *Leptodactylus cupreus* (30), *L. furnarius* (30), *L. fuscus* (30), *L. gracilis* (30), *L. jolyi* (30), *L. mystaceus* (30), *L. mystacinus* (30), *L. notoaktites* (30), *L. plaumanni* (30), *L. spixi* (30), *L. troglodytes* (30), *Myersiella microps* (23), *Paratelmatobius poecilogaster* (18), *Phasmahyla spectabilis* (25), *P. cochranae* (25), *P. exilis* (25), *P. guttata* (25), *P. jandaia* (25), *Phrynomedusa marginata* (18), *Phyllomedusa bahiana* (24), *P. burmeisteri* (24), *P. distincta* (24), *P. iheringii* (24), *P. nordestina* (24), *P. rohdei* (24), *P. tetraploidea* (24), *Pristimantis paulodutrai* (23), *P. ramagii* (23), *P. vinhai* (23), *Sphaenorhynchus pauloalvini* (24), *Thoropa lutzi* (19), *T. miliaris* (19), *T. saxatilis* (19), *Vitreorana eurygnatha* (25), *V. uranoscopa* (25), *Zachaenus parvalus* (21).

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Morphological variability and age structure in a population of *Bufo verrucosissimus* (Anura: Bufonidae) from Artvin, Turkey

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Abstract

Morphological variability and age structure in a population of *Bufo verrucosissimus* (Anura: Bufonidae) from Artvin, Turkey. We investigated morphological diversity (external body traits, coloration patterns and skin structure) and age structure in a population of Caucasian toad (*Bufo verrucosissimus*) from Lake Borçka Karagöl, situated in the vicinity of Artvin on the northeastern border of Turkey and Georgia. Age was determined using phalangeal skeletochronology. The external morphological variability was assessed using the linear measurement of 27 body-related characters and the photographs of each specimen. According to the results, the means of head length and head width were found significantly lower in Karagöl population as opposed to Caucasian populations, but average body size did not differ among all populations. Coloration and pattern features of the specimens from Karagöl were identical to those of Caucasia. Most of the females have brown tones and males are usually olive green and brown. The indiscrete elongated dark bands on parotoids did not extend behind the gland. PCA analysis confirmed a clear separation between the sexes and a high degree of female-biased sexual size dimorphism was determined based on the body size (SDI index: +0.41). The constructed Von Bertalanffy growth curve models yielded similar profiles in both sexes. Body size and age were significantly correlated in both models, but growth coefficient value was higher in males. Accordingly, the mean age of the Karagöl population is greater in both sexes, but the lifespan and maximum age are greater in Caucasus populations.

Keywords: Body measurements, Caucasian toad, Life history, Skeletochronology.

Resumo

Variabilidade morfológica e estrutura etária em uma população de *Bufo verrucosissimus* (Anura: Bufonidae) de Artvin, Turquia. Investigamos a diversidade morfológica (características externas do corpo, padrões de coloração e estrutura da pele) e estrutura etária em uma população do sapo caucasiano *Bufo verrucosissimus* do Lago Borçka Karagöl, situado nas proximidades de Artvin, no nordeste da fronteira da Turquia com a Geórgia. A idade foi determinada utilizando

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esqueletocronologia falageal. A variabilidade morfológica externa foi avaliada utilizando a medida linear de 27 caracteres relacionados ao corpo e fotografias de cada espécime. De acordo com os resultados, as médias do comprimento e largura da cabeça foram significativamente menores na população de Karagöl do que nas populações caucasianas, mas o tamanho médio do corpo não foi diferente entre as populações. As características de coloração e padrão dos espécimes de Karagöl foram idênticas às do Cáucaso. A maioria das fêmeas apresenta tons castanhos, e os machos são geralmente verde oliva e castanhos. As faixas escuras alongadas não se estendem por alémde das glândulas paratóides. A análise PCA confirmou uma separação clara entre os sexos, e um elevado grau de dimorfismo sexual nas fêmeas foi determinado com base no tamanho do corpo (índice SDI: +0,41). Os modelos construídos de curva de crescimento de Von Bertalanffy produziram perfis semelhantes em ambos os sexos. O tamanho do corpo e a idade foram significativamente correlacionados em ambos os modelos, mas o valor do coeficiente de crescimento foi mais elevado nos machos. Consequentemente, a idade média da população de Karagöl é mais elevada em ambos os sexos, mas a expectativa de vida e a idade máxima são maiores nas populações do Cáucaso.

Palavras-chave: Esqueletocronologia, História de vida, Medidas corporais, Sapo-caucasiano.

Introduction

The Caucasian toad, *Bufo verrucosissimus* (Pallas, 1814), occurs throughout the Caucasian diversity hotspot classified as Transcaucasia covering the center of Caucasia including Artvin (on the northeastern of Turkey) and Ciscaucasia covering the southernwest of Russian Federation representing the northern part of the Caucasus (Orlova and Tuniyev 1989, Kidov 2009, Recuero *et al.* 2012, Tuniyev *et al.* 2014). In addition, the presence of the toad was also reported in the south of Turkey (Antalya, Mersin, Osmaniye, Hatay) (Özdemir *et al.* 2020) and from Lebanon (Moukhtara) (Jablonski and Sadek 2019). The species was previously delimited under four morphology based subspecies, namely *B. verrucosissimus verrucosissimus* (Pallas, 1814) spreading from the Black Sea coast of Turkey to the inner part of Georgia and extending to Great Caucasus mountains, *B. verrucosissimus turowi* (Krasovsky, 1933) from in a narrow zone at the north of Greater Caucasus, *B. verrucosissimus circassicus* (Orlova and Tuniyev, 1989) from the northern coastal side of Greater Caucasus Mountain Range, and *B. verrucosissimus tertyschnikovi* (Kidov, 2009) inhabiting Stavropol Krai of the Russian Federation. However, the latest genetic studies have indicated the presence of three different lineages (Pisanets *et al.* 2009, Garcia-Porta *et al.* 2012, Özdemir *et al.* 2020) rather than morphological units and the subspecies are no longer recognized (Frost 2021).

Bufo verrucosissimus is known from Lake Karagöl (41°23' N, 41°51' E; 1,450–1,480 m a.s.l.,) is situated in the district of Borçka (Artvin city) near the northeastern border of Turkey and Georgia (Figure 1). It is a 5-ha freshwater lake with a maximum depth of 25 m. Streams and rainfall are the sources feeding the lake (Kopar and Sever 2008). The lake and its surrounding have a rich fauna and flora with a mean air temperature of 2.0–6.1°C. Lake Karagöl and its surroundings are part of the Eastern Black Sea Mountains (Eastern Pontides) shaped by Alpine orogeny and volcanism events. The lithology in and around the lake consists of volcano-sedimentary sequences. The site has a rich potential in terms of streams and resources fed by rain and spring waters in addition to the continuously flowing rivers (Kopar and Sever 2008, Nergiz and Alar 2019).

The lake has an ice cover (10–14 cm) in February and the active period for anurans was from March to October. A total of five species of anurans—*Rana macrocnemis* Boulenger, 1885,

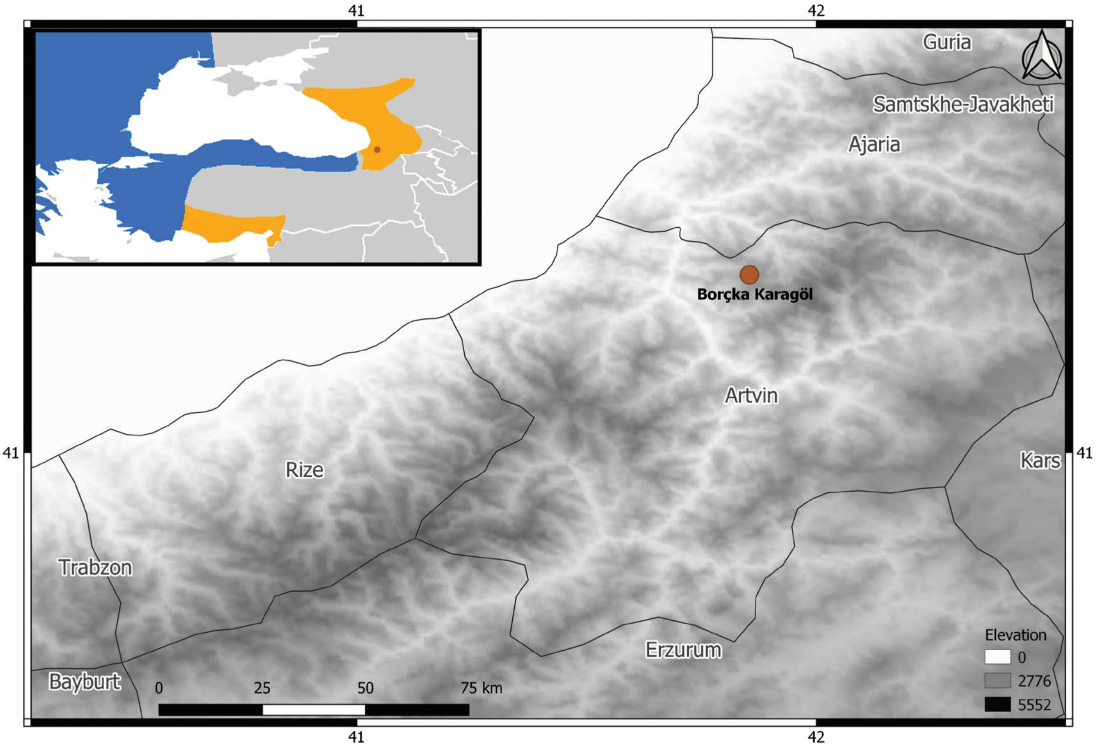

Figure 1. The location map of Lake Karagöl, Artvin, Turkey. The inset map was modified from Özdemir *et al.* (2020) and represents the distribution of *Bufo verrucosissimus* (yellow) and *B. bufo* in surrounding regions (blue).

Pelophylax ridibundus (Pallas, 1771), *Hyla orientalis* Bedriaga, 1890, *Pelodytes caucasicus* Boulenger, 1896, and *Bufo verrucosissimus*—and two newts—*Mertensiella caucasica* (Waga, 1876) and *Ommatotriton ophryticus* (Berthold, 1846)—were found in the Lake Karagöl and its vicinity (Afsar *et al.* 2018).

Age and body size are two important indicator of amphibian life history (Üzüm *et al.* 2020) and their relationship is critical when assessing evolutional-ecological processes. Although age structure of *B. verrucosissimus* was studied in a population at the border of Caucasia (Gokhelashvili and Tarkhnishvili 1994, Tarkhnishvili 1994, Zazanashvili and Mallon 2009), no data exist from Turkey. In this study, we describe comprehensive morphology and age structure of a local population of *B. verrucosissimus* from Lake Karagöl.

Materials and Methods

A total of 29 adult specimens (14 ♀♀, 15 ♂♂) were collected during the breeding season in 2019. Initially, the toads were anesthetized with 250 mg/L MS222. Thereafter, 27 external morphological characters (Özdemir *et al.* 2020) were measured for each of the toads: snout–vent length (SVL), length of the head (LHEAD), width of the head (WHEAD), minimum distance between the nostrils (INTNOS), distance between the nostril and the tip of snout (NOSTIP), minimum distance from the nostril to the anterior corner of the eye (NOSEYE), eye–

tympanum distance (EYETYM), horizontal diameter of the eye (DEYE), diameter of the tympanum (DTYM), length of the parotoid (LPAR), distance between the elbows with humerus kept perpendicular to the body axis (WGRASP), radio ulna length (RADUL), length of the hand (LHAND), length of the first finger (L1FING), length of the femur (LFEM), length of the tibia (LTIB), length of the tarsus (LTARS), length of the foot (LFOOT), minimum distance from the distal extremity of the inner metatarsal tubercle and the web between the third and fourth digit (WEB), length of the metatarsal tubercle (LMET), interorbital distance (IOD), anterior parotoid distance (PDA), posterior parotoid distance (PDP), left parotoid width (LPW), right parotoid width (RPW), length of the inner metatarsal tubercle (LIMT) and width of the inner metatarsal tubercle (WIMT).

All measurements were taken by the same researcher with using a digital calliper to the nearest 0.01 mm. The sex of adult specimens was determined in the field using secondary sexual characters. To examine qualitative traits, each individual was photographed. After sampling process, the individuals were released to the area where captured. This study was done with the permission of the local ethic committee (Republic of Turkey Recep Tayyip Erdogan University Local Ethics Committee for Animal Experiments, approval reference number: 2019/9).

Morphological Analyses

Captured specimens were analysed using photographs of their external body traits, coloration patterns and skin structure with reference to the former studies (Orlova and Tuniyev 1989, Sinsch *et al.* 2009). To make numerically explicit the morphological features of the Karagöl population, measurements were computerized using SPSS v22 (IBM 2013) and then transferred in R environment using *foreign* package (R Core Team 2021). Descriptive statistics of each character were calculated with the *psych* package (Revelle 2019). Normality assumptions were confirmed using the Shapiro-Wilks test. We used a One Sample t-test to compare our data with the average measurements of previous studies. To compare the means of characters between sexes, the Student t-test was carried out using the *stats* package (R Core Team 2021). The sexual dimorphism index (SDI) was calculated according to the formula introduced by Lovich and Gibbons (1992): the mean SVL of the bigger sex was divided by the mean SVL of the smaller sex and subtracted from one (SVLbig/SVLsmall-1) to achieve an approximate relationship of sexual size dimorphism (SDI > 0 when females are larger than males, SDI < 0 when males are larger than females). To visualize the separation between sexes, log10-transformed data were subjected to PCA analysis using *FactoMineR* (Le *et al.* 2008) and *factoextra* (Kassambara and Mundt 2020) packages. Data visualization was performed using *ggplot2* (Wickham 2016), *grid* and *gridExtra* (Auguie 2017) packages. All the analyses were run using R Programming Language (R Core Team 2021).

Skeletochronology

The 4th toe of the hind limb was clipped for each toad and preserved in 95% ethanol for the skeletochronology. We applied standard skeletochronology procedure following Castanet and Smirina (1990) to elicit the age structure of the Lake Borçka Karagöl population. Phalanges were manually cleaned from soft tissues and stored in 70% ethanol. Samples were washed in the tap water approximately one hour, decalcified in 5% nitric acid around 1.5 hours. Cross sections of 18 µm were obtained from second phalanges using a freezing microtome (Thermo Shandon Cryostat, Germany). The cross sections were stained with Ehrlich’s haematoxylin around 15 min. Photos were taken using an Olympus BX51 microscope at 200× and 400× magnifications for each individual. Age estimation was done counting the lines of arrested growth (LAGs) that develop during hibernation, the only period of arrested activity in the year. The calculation was done by two independent researchers (N. Özdemir and C. Dursun) using an Olympus BX51 microscope (Figure 2). To avoid probable errors of the age estimation due to medullary resorption, we used diaphysis sections in which the periosteal bone size reaches its maximum and that of the medullar cavity is at its minimum (Oromi *et al.* 2016).

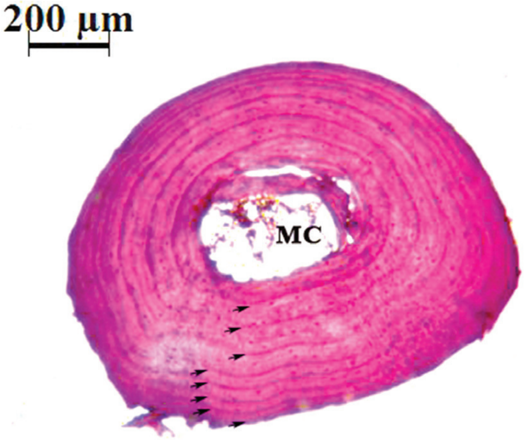

Figure 2. Cross-section image through phalanges of eight years old female specimen.

Since the age classes are discrete variables, we compared age differences between sexes using the Mann-Whitney U test by comparing median values in the *stats* package. We predicted growth curve models under the typical Von Bertalanffy's equation modified by Beverton and Holt (1957): $L_t = L_\infty\{1-\exp[-k(t-t_0)]\}$ where Lt is the expected or average length at the time (or age) t, L_∞ is the asymptotic average length, k is the so-called Brody growth rate coefficient and t_0 is a modelling artifact that is said to represent the time or age when the average length was zero. To obtain parameter values and execute the analyses *FSA* (Ogle *et al.* 2021), *FSAdata* (Ogle 2019), *FSAsim* (Ogle 2020) and *nlstools* (Baty *et al.* 2015) packages were used following the guide *fishR Vignette* (Ogle 2013). To visualize growth curve, we also added a hypothetical individual to the dataset using the reference of Afrin *et al.* (2019) under the given parameters: SVL at metamorphosis is fixed to mean 12.00 mm and age at metamorphosis is 0.3 year. Since we lacked the individuals between 0–4 years in females, the growth curve did not fit the data. Therefore, we estimated the body length of a female at age 2 years to a shape consistent with the curve of the male dataset and we applied the missing value analysis based on regression which is advisable method to fill the gaps in data for the constructed models (Raghunathan 2004, Zhang 2016, Johnson *et al.* 2021).

Results

Morphology

The head is not proportionately larger compared to the body size. Snout is wide and short, and rostrum is extensively rounded. The parallel positioned parotoid glands are apparent but narrow. Dorsal surface is covered with large, round, and smooth warts. Ventral side has small smooth knobs. The warts at the corners of mouth and flanks of the head bear thorny keratinized spines. Females are wartier and spinier. Males have relatively smooth skin with fewer spine intensity. Males mostly have a green dorsum, rarely brown. Females are generally brown but some have a dorsally brick-red and brown-greenish mixture. A clear majority of specimens had irregular dark spots locating on dorsal warts. Ventral side is off-white, cream colored and covered with generally visible brown patterns. The bands located on parotoids are indiscrete and never extending beyond parotoids. The laterally extended light bands are more observable in females but absent in a few individuals (Figure 3).

Descriptive statistics of the measurements are summarized in Table 1. The data indicated that females were larger than males in all cases. Also, significant differences were found in the

Figure 3. The specimens representing both sexes from the population of Lake Karagöl: (**A**) A male specimen; (**B**) A female specimen.

investigated characters between sexes ($p < 0.05$). The first principal component explained 83% of the variance; 91.09 % of the total variance was explained by four principal components. All variables showed positive loadings for PC1 which is representing the variation relevant to the size. The highest loads were observed for WHEAD, LHEAD and SVL, respectively. In the second principal component RADUL and PDP had the highest positive loads whereas NOSTIP and LIMT showed the lowest negative loads (Table 2). Males and females are clearly distinguished in the morphospace based on size, but they represented similar shape characteristic corresponding to the explained variance by PC2 (Figure 4). Sexual dimorphism index was calculated + 0.41 and this rate verified female-biased sexual size dimorphism, as well.

Skeletochronology and Intraspecific Phenotypic Variation

The median values of age were found 5 and 7 years for males and females, respectively. Mann-Whitney U test also pointed the significant difference between sexes in terms of age structure (W = 34.5; $p < 0.01$). The average age was found 5.13 and 6.64 years for males and females, respectively. Males aged between 2–7 years and females 5–8 years (Table 3).

The Von Bertalanffy's growth curves adequately fitted the relationship between age and SVL in the constructed models. The models represented identical growth trajectories in terms of curve shapes (Figure 5). The estimated asymptotic SVL of females was 101.72 ± 1.55 mm with 95% confidence interval between 98.36

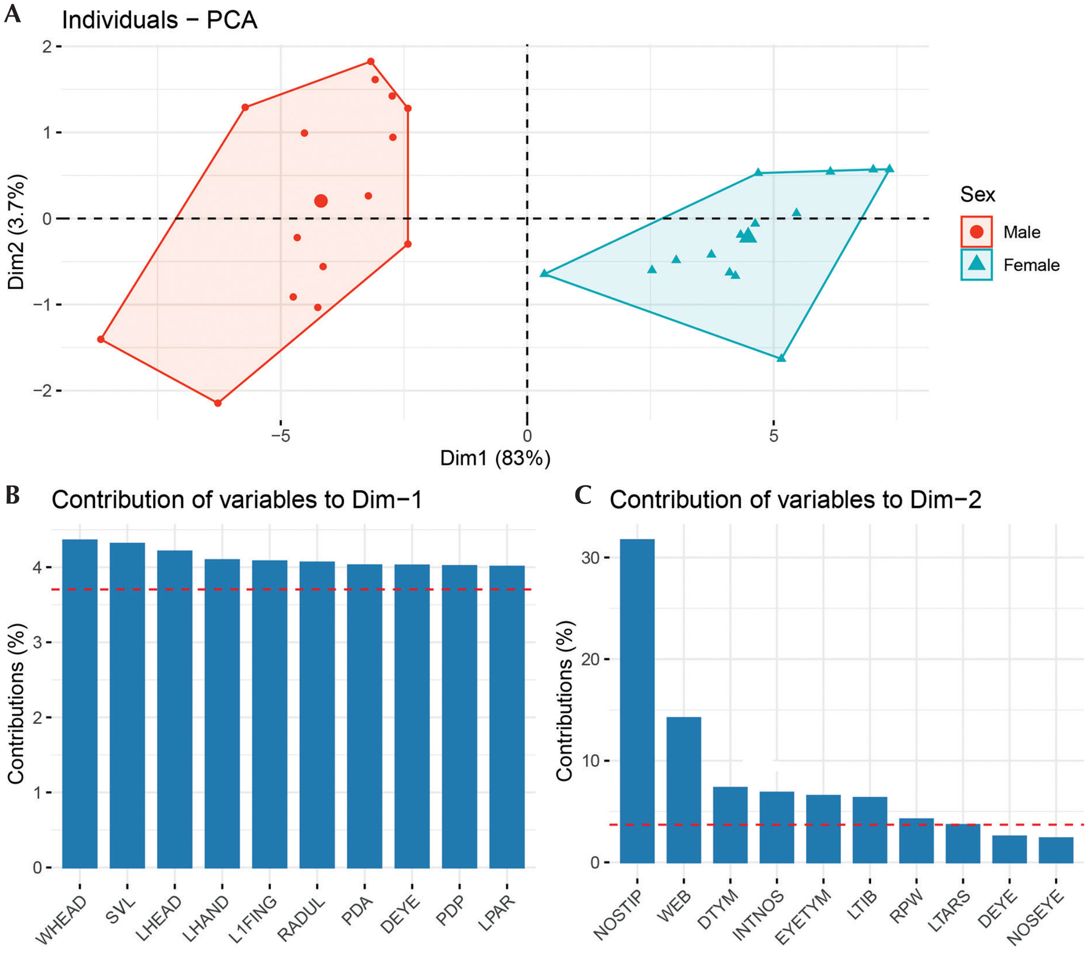

Figure 4. The scatter plot of PCA analysis based on the morphometric measurements (**A**), and the top ten variables contributing to PC1 (**B**) and PC2 (**C**).

and 105.07 mm and it is lower than the maximum SVL (112.59 mm). The growth coefficient (K) is 0.88 ± 0.16 (confidence interval: 0.51–1.24). On the other hand, the estimated asymptotic SVL of males (75.55 ± 0.91 mm) was lower than females with 95% confidence interval between 73.57–77.53 mm. However, K was calculated as 0.99 ± 0.14 (confidence interval: 0.67–1.31) which is higher than females. The estimated asymptotic SVL was not higher than maximum recorded SVL value (79.86 mm). The final models were found statistically significant for all parameters ($p < 0.05$). The correlation of age and SVL was higher in males (r = -0.60) than females (r = -0.44). The negative direction of the correlation values indicated that the growth is decreasing in higher ages.

The intraspecific comparison yielded statistically significant results between Karagöl and Caucasus populations. Caucasian populations

Table 1. Descriptive statistics of morphometric characters (SE: Standard error of mean; Min: Minimum value; Max: Maximum value). Test results and significance values of each character were given in the same line. Significance levels were flagged with asterisk (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Positive test values indicate female biased differences.

Characters	Mean ± SE (mm)	Min	Max	Mean ± SE (mm)	Min	Max	Test Value	Sig.
	Males			Females				
SVL	73.91 ± 1.05	65.74	79.86	102.67 ± 1.49	90.86	112.59	$t = 15.946$	***
LHEAD	17.15 ± 0.32	14.98	19.52	24.13 ± 0.47	21.12	27.89	$t = 12.511$	***
WHEAD	23.67 ± 0.34	20.23	25.29	33.12 ± 0.56	28.73	36.56	$t = 14.728$	***
INTNOS	4.83 ± 0.12	3.88	5.66	6.04 ± 0.10	5.34	6.99	$t = 7.637$	***
NOSTIP	3.01 ± 0.14	2.22	4.15	3.60 ± 0.11	2.92	4.32	$t = 3.154$	**
NOSEYE	3.68 ± 0.12	3.01	4.47	5.14 ± 0.14	4.27	5.83	$t = 7.942$	***
EYETYM	2.68 ± 0.11	2.00	3.27	4.25 ± 0.11	3.79	4.97	$t = 10.350$	***
DEYE	7.08 ± 0.14	5.84	7.90	8.94 ± 0.20	7.53	10.33	$t = 7.747$	***
DYTM	2.54 ± 0.13	1.91	3.49	3.17 ± 0.17	2.07	4.37	$t = 3.031$	***
LPAR	15.57 ± 0.36	12.38	17.72	22.33 ± 0.43	19.85	24.56	$t = 11.978$	***
WGRASP	61.57 ± 1.11	52.39	70.48	74.72 ± 1.67	64.70	88.33	$t = 6.655$	***
RADUL	24.57 ± 0.35	22.08	27.11	30.52 ± 0.56	26.07	33.51	$t = 9.102$	***
LHAND	19.40 ± 0.42	14.85	21.63	26.60 ± 0.60	22.67	30.10	$t = 9.918$	***
L1FING	7.74 ± 0.24	5.50	9.39	12.04 ± 0.35	9.36	13.90	$t = 10.376$	***
LFEM	31.27 ± 0.58	27.28	35.68	41.55 ± 0.77	37.15	46.68	$t = 10.231$	***
LTIB	19.79 ± 0.29	17.55	21.78	27.13 ± 0.52	23.48	30.28	$t = 12.558$	***
LTARS	18.08 ± 0.28	16.67	20.17	24.14 ± 0.50	21.49	27.30	$t = 10.773$	***
LFOOT	34.20 ± 0.62	29.44	38.16	40.28 ± 0.73	35.42	44.26	$t = 6.374$	***
WEB	20.40 ± 0.47	16.89	23.76	23.78 ± 0.59	19.86	27.07	$t = 4.504$	***
LMET	2.71 ± 0.11	1.94	3.41	3.90 ± 0.10	3.34	4.40	$t = 7.961$	***
IOD	8.67 ± 0.24	7.43	10.41	12.34 ± 0.33	9.79	14.62	$t = 9.111$	***
PDA	17.06 ± 0.31	14.77	19.20	23.13 ± 0.58	19.39	28.22	$t = 9.433$	***
PDP	24.95 ± 0.50	20.59	28.27	35.70 ± 0.92	30.85	42.03	$t = 10.483$	***
RPW	5.27 ± 0.07	4.61	5.66	8.17 ± 0.20	7.10	10.08	$t = 13.686$	***
LPW	5.17 ± 0.08	4.37	5.66	7.93 ± 0.30	5.08	10.08	$t = 9.117$	***
LIMT	4.42 ± 0.10	3.56	4.91	6.32 ± 0.14	5.08	7.47	$t = 10.854$	***
WIMT	2.52 ± 0.06	1.95	2.83	3.41 ± 0.10	2.72	4.13	$t = 7.791$	***

Table 2. Principal component loadings of PC1–PC4 extracted from correlation matrix.

Characters	PC1	PC2	PC3	PC4
SVL	0.2076	-0.0897	0.0043	-0.0408
LHEAD	0.2051	-0.1096	0.0769	0.0834
WHEAD	0.2087	-0.0697	0.0345	0.1113
INTNOS	0.1932	0.2619	-0.0683	-0.0502
NOSTIP	0.1325	0.5630	-0.4834	0.0614
NOSEYE	0.1858	0.1541	0.0841	0.5773
EYETYM	0.1812	-0.2557	-0.0478	0.3107
DEYE	0.2005	0.1596	-0.0902	-0.0046
DTYM	0.1295	0.2707	0.7908	-0.0076
LPAR	0.2001	0.0413	-0.0501	-0.0732
WGRASP	0.1972	0.1181	0.0885	0.0348
RADUL	0.2015	0.0034	0.0489	-0.0579
LHAND	0.2023	0.0209	-0.0445	-0.1145
L1FING	0.2019	-0.0315	-0.0245	0.0877
LFEM	0.1991	0.0124	-0.1035	0.0422
LTIB	0.1929	-0.2518	0.0007	-0.2471
LTARS	0.1930	-0.1917	0.1133	-0.2867
LFOOT	0.1931	0.1495	0.1188	-0.1504
WEB	0.1767	0.3768	0.0337	-0.3127
LMET	0.1831	-0.1027	0.0033	0.3826
IOD	0.1993	0.0259	0.0503	0.1225
PDA	0.2006	-0.1089	-0.0619	0.0732
PDP	0.2003	-0.0387	-0.0807	-0.1574
RPW	0.1991	-0.2057	-0.1379	-0.1016
LPW	0.1984	-0.1186	0.0437	0.1702
LIMT	0.2000	-0.1274	-0.0343	0.0001
WIMT	0.1875	-0.1498	-0.1492	-0.2082
Eigenvalue	22.40	1.00	0.72	0.40
Variance (%)	82.99	3.71	2.90	1.48
Cumulative variance (%)	82.99	86.70	89.61	91.09

Table 3. Descriptive statistics of age groups in both sexes of *B. verrucosissimus* from Lake Karagöl (SVL: Snout–vent length, SE: Standard error of mean).

Male Age Groups

Age	*N*	Mean SVL ± SE (mm)	Range (mm)
2	1	66.58 ± 0.00	66.58
4	4	71.82 ± 1.15	69.45–73.89
5	3	73.48 ± 1.46	70.61–75.41
6	5	76.62 ± 0.98	74.81–79.86
7	2	77.60 ± 0.89	76.7–78.49

Female Age Groups

Age	*N*	Mean SVL ± SE (mm)	Range (mm)
5	1	102.83 ± 0.00	102.83
6	4	99.02 ± 2.88	90.86–104.23
7	8	101.34 ± 1.85	93.3–107.7
8	1	108.45 ± 0.00	108.45

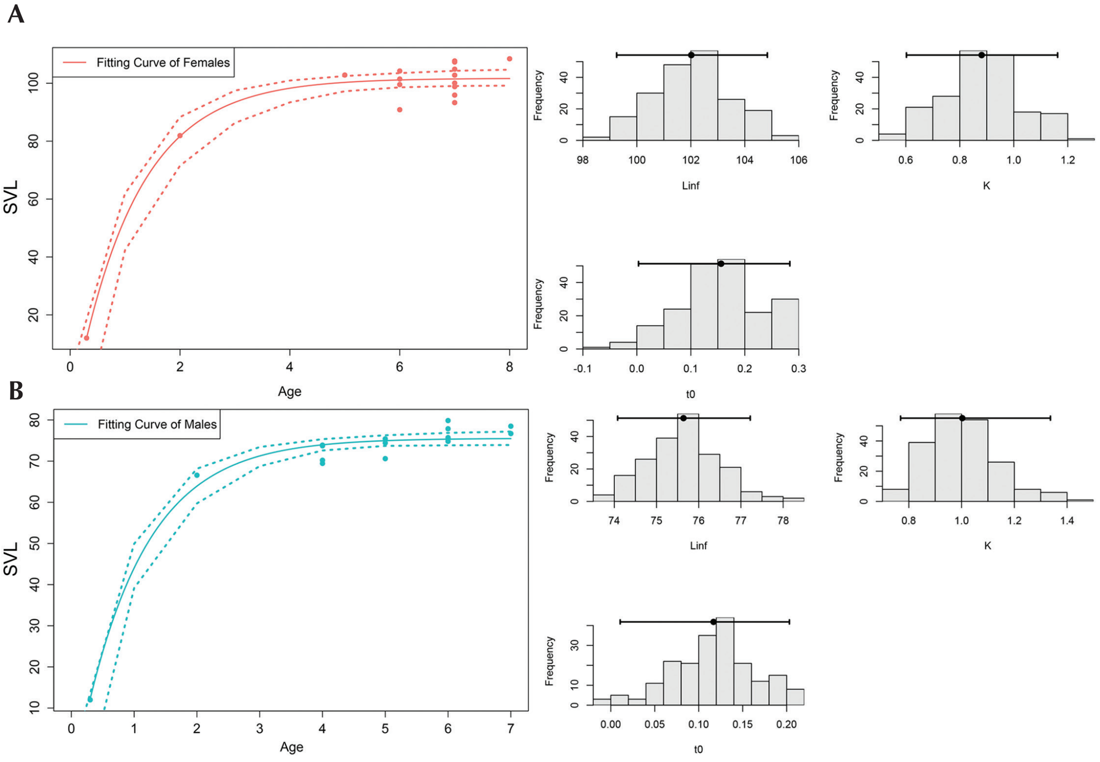

Figure 5. The Von Bertalanffy growth models and bar graphics of the relevant parameters for females (**A**) and males (**B**).

have larger body proportion in comparison with Karagöl population. Even though the size of body segments is larger in the other populations, the body length was not significantly different between Lake Karagöl and the population which was previously described as *B. verrucosissimus turowi* subspecies (Table 4).

Discussion

In this study, we described the morphological characteristics of Lake Karagöl populations of *Bufo verrucosissimus.* At first, we tested the body size difference between sexes and the results confirmed a high degree of female-biased sexual size dimorphism which is common phenomenon in anurans (Kupfer 2007) regarded as an outcome of sex-specific patterns of sexual and natural selection. Numerous studies have reported female-biased sexual dimorphism in the genus *Bufo* (Gittins *et al.* 1980, Reading 1991, Tarkhnishvili 1994, Kutrup 2001, Cvetković *et al.* 2005, 2007, Litvinchuk *et al.* 2008, Mozaffari and Moghari 2012, Cadjenović *et al.* 2013, Arntzen *et al.* 2013, Ergül-Kalayci *et al.* 2019, Dursun *et al.* 2022). Female biased sexual size dimorphism is a common phenomenon in 90% of anuran species (Shine 1979). The larger body of females is a fecundity advantage in explosive breeder species due to faster growth rate and delayed reproduction to allocate energy (Hoglund and Robertson 1987, Kuhn 1994, Tomašević *et al.* 2008) besides different age structure of breeding population between sexes (Kusano *et al.* 2010).

We examined the population with regard to smooth and slightly spined warts. Females have more keratinized spines on the warts than males. The well-developed (thorny) spines were found on the chest and legs, and the lateral side of the head. Cadjenović *et al.* (2013) implied that *Bufo bufo* (Linnaeus, 1758) males were commonly characterized with poorly expressed warts with thorn-like ends, whereas most females have visible warts with very developed thorn-like ends on the head region. Arntzen *et al.* (2013) described similar observations between the sexes of *B. bufo* and *Bufo spinosus* Daudin, 1803 species. Therefore, the density of the keratinized spines may be a sexual dimorphic feature as noted in Lake Karagöl population. Dry warty skin is presented as main characteristic of bufonid species (Pough 2015) with the spines originating from epidermis (Regueira *et al.* 2016). The density of spines on the head was used to discriminate *Bufo* taxa (De Lange 1973, Lüscher *et al.* 2001, Arntzen *et al.* 2013). A high density of keratinized spines on the head was observed in *B. spinosus* and some *B. bufo* populations. Therefore, we assume that the lower density of spines is specific of *B. verrucosissimus.*

In this study, we revealed the age structure of the Lake Karagöl population for the first time. The range of ages was between 2–8 years in the population, but median differences indicated that females are significantly older than males. Growth models showed similar curves that described the correlation between age and SVL. The models indicated that slower growth was observed after 2–3 years for males and 3–4 years for females and was supported by the higher growth coefficient of males. In amphibians, males generally reach sexual maturity one year earlier than females (Miaud *et al.* 1999, Leclair *et al.* 2005, Liao *et al.* 2010). Reading (1991) implied that the minority of *B. bufo* males and females reached sexual maturity at ages of two and four years in the United Kingdom. Ergül-Kalayci *et al.* (2019) reported a significant decrease in the growth at the age of maturity ranging from 2–3 years in three different *B. bufo* populations in Turkey. Matushkina *et al.* (2015) mentioned that *Bufo eichwaldi* Litvinchuk, Borkin, Skorinov, and Rosanov, 2008 males and females reach sexual maturity after 2 and 3 winters, respectively. Therefore, our results are consistent with the previous studies in terms of growth patterns. The main reason behind delayed sexual maturity of females is related to allocation of energy to reproduction like in majority of amphibian species (Gibbons 1984, Pizzatto and Marques 2006, Yu *et al.* 2022).

Table 4. The results of One Sample t test among Karagöl population and Caucasian populations. Analyses were run with the mean values of previous studies against to our data. Positive t values are representing Karagöl biased differentiation, whereas that of negative for the other populations. Significance levels were flagged with asterisk (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Non-significant differences were presented as "n.s".

Study-Subspecies		Males							Females						
	Characters	***N***	**Mean**	**Min**	**Max**	**SD**	**Test Value**	**Sig.**	***N***	**Mean**	**Min**	**Max**	**SD**	**Test Value**	**Sig.**
Tarknishvili and Gokhelashvili (1999)	SVL	27	77.08	69.60	85.60	4.29	t = -3.018	**	8	115.1	104.00	124.20	6.37	t = -8.339	***
	LHEAD	27	19.41	18.00	21.80	0.86	t = -7.164	***	8	26.49	23.30	28.80	1.80	t = -5.025	***
	WHEAD	27	25.03	22.80	30.00	1.51	t = -3.993	**	8	39.90	37.00	44.70	2.85	t = -12.219	***
	INTNOS	27	8.37	7.20	10.10	0.72	t = -28.79	***	8	12.11	11.20	13.70	0.74	t = -62.395	***
	LPAR	27	15.30	13.5	18.00	2.09	t = 0.732	n.s	8	23.50	20.30	26.80	2.12	t = -2.6951	*
	LFEM	27	26.29	24.00	30.90	2.23	t = 9.003	***	8	36.44	33.70	39.30	1.68	t = 6.611	***
	LTIB	27	28.52	25.10	31.80	2.22	t = -29.785	***	8	38.45	36.00	41.50	1.94	t = -21.834	***
	Characters	***N***	**Mean**	**Min**	**Max**	**SD**	**Test Value**	**Sig.**	***N***	**Mean**	**Min**	**Max**	**SD**	**Test Value**	**Sig.**
Orlova and Tuniyev (1989)	SVL	13	85.88	78.00	97.00	4.65	t = -11.396	***	10	119.98	113.00	124.00	4.00	t = -11.613	***
	LHEAD	13	22.46	20.00	25.00	1.41	t = -16.840	***	10	29.55	27.00	32.50	1.64	t = -11.550	***
	WHEAD	13	27.96	25.00	30.00	1.30	t = -12.594	***	10	41.17	38.50	43.00	1.45	t = -14.507	***
	INTNOS	13	4.28	3.70	4.80	0.29	t = 4.479	***	10	6.08	5.00	6.70	0.51	t = -0.418	n.s
	LPAR	13	19.04	16.00	23.00	2.34	t = -9.512	***	10	24.94	23.00	27.00	1.20	t = -6.010	***
	LFEM	13	37.38	32.00	43.00	3.21	t = -9.685	***	10	48.00	43.00	53.00	3.13	t = -8.340	***
	LTIB	13	34.42	30.00	40.00	2.52	t = -49.908	***	10	43.30	41.00	47.00	1.71	t = -31.193	***
	Characters	***N***	**Mean**	**Min**	**Max**	**SD**	**Test Value**	**Sig.**	***N***	**Mean**	**Min**	**Max**	**SD**	**Test Value**	**Sig.**
Orlova and Tuniyev (1989)	SVL	1	73.50	-	-	-	t = 0.389	n.s	2	102.75	91.50	114.00	-	t = -0.053	n.s
	LHEAD	1	19.50	-	-	-	t = -7.450	***	2	25.00	23.60	26.40	-	t = -1.847	n.s
	WHEAD	1	25.40	-	-	-	t = -5.079	***	2	38.15	36.30	40.00	-	t = -9.006	***
	INTNOS	1	7.00	-	-	-	t = -17.646	***	2	6.15	5.30	7.00	-	t = -1.137	n.s
	LPAR	1	17.20	-	-	-	t = -4.473	***	2	23.10	22.40	23.80	-	t = -1.774	n.s
	LFEM	1	35.50	-	-	-	t = -6.466	***	2	46.15	45.00	47.30	-	t = -5.947	***
	LTIB	1	32.00	-	-	-	t = -41.654	***	2	41.50	41.10	41.90	-	t = -27.719	***

Table 4. *Continued.*

Study-Subspecies	Characters	N	Mean	Min	Max	SD	Test Value	Sig.	N	Mean	Min	Max	SD	Test Value	Sig.
		Males							**Females**						
Orlova and Tuniyev (1989)	SVL	10	81.01	75.60	86.90	3.51	*t* = -6.759	***	1	123.20	-	-	-	*t* = -13.774	***
	LHEAD	10	20.20	17.40	26.20	2.31	*t* = -9.670	***	1	28.40	-	-	-	*t* = -9.098	***
	WHEAD	10	26.11	23.40	28.00	1.52	*t* = -7.163	***	1	42.40	-	-	-	*t* = -16.723	***
	INTNOS	10	4.60	4.00	5.30	0.44	*t* = 1.863	n.s	1	6.00	-	-	-	*t* = 0.403	n.s
	LPAR	10	16.80	14.70	19.80	1.64	*t* = -3.375	**	1	26.40	-	-	-	*t* = -9.371	***
	LFEM	10	30.32	24.00	36.70	4.24	*t* = 2.401	*	1	47.00	-	-	-	*t* = -7.047	***
	LTIB	10	30.41	22.40	33.40	3.35	*t* = -36.231	***	1	42.00	-	-	-	*t* = -28.684	***
	Characters	**N**	**Mean**	**Min**	**Max**	**SD**	**Test Value**	**Sig.**	**N**	**Mean**	**Min**	**Max**	**SD**	**Test Value**	**Sig.**
Kidov (2009)	SVL	6	69.75	64.00	75.50	4.41	*t* = 3.957	***	2	101.6	99.70	103.50	-	*t* = 0.717	n.s
	WHEAD	6	24.32	23.00	25.50	0.92	*t* = -1.909	n.s	2	38.60	37.00	40.20	-	*t* = -9.877	***
	INTNOS	6	3.93	3.40	4.20	0.30	*t* = 7.362	***	2	6.35	6.30	6.40	-	*t* = -3.193	***
	LPAR	6	15.67	14.10	17.40	1.29	*t* = - 0.281	n.s	2	22.2	22.10	22.30	-	*t* = 0.297	n.s
	LFEM	6	31.85	30.20	33.60	1.31	*t* = -0.218	n.s	2	46.05	45.20	46.90	-	*t* = -5.818	***
	LTIB	6	28.17	27.20	28.80	0.61	*t* = -28.591	***	2	38.30	38.20	38.40	-	*t* = -21.545	***

Although we presented the first report on the Turkish populations, the age structure of *B. verrrucosissimus* was studied in Caucasia. Gokhelashvili and Tarkhnishvili (1994) extensively investigated life-history traits of six anuran species during two consecutive years at the border of central Georgia, which has similar ecological conditions with the area of Lake Karagöl. The vegetation is mainly composed of mixed forest [*Abies nordmanniana* (Steven) Spach, *Picea orientalis* (L.) Peterm., *Carpinus caucasica* Grossh., *Fagus orientalis* Lipsky], the elevation is between 900–1200 m and the annual precipitation is 1000 mm (Tarkhnishvili 1993). In this context, they aged 105 adult *B. verrucosissimus* specimens (49 males, 56 females), and the age varied between 2–9 years (mean: 4.04 years) in males and 3–10 years in females (mean: 5.96). Tarkhnishvili (1994) indicated that the maximum age is reached around 9–10 years for both sexes of the species. Accordingly, the lifespan and maximum age are higher in Georgian populations. Iskanderov (2009) noted that the sampled Caucasian toads from Azerbaijan were aged 5–8 years and 75–80% of the population ranged between 8–12 cm in body size. Recently, Afrin *et al.* (2022) showed that the age varied between 3–7 years in males, and 5–12 years in females on the Stavropol uplands in Russia. A shorter lifespan is generally explained by early maturity and high reproductive investment while delayed maturity is observed in long-lived amphibian species (Guarino *et al.* 2010, Oromi *et al.* 2012, Stănescu *et al.* 2016; Székely *et al.* 2018). However, these patterns can also be observed between different populations of the species under severe environmental pressures or stressors. For instance, Zamora-Camacho and Comas (2017) reported a lower mean age of *Epidalea calamita* (Laurenti, 1768) populations from agrosystems than natural habitats, and they implied that agrosystem toads compensate for lesser reproductive event with more breeding attempt. Marangoni *et al.* (2018) observed life history traits in *Chacophrys pierottii* (Vellard, 1948) due to aridity effect. Moreover, forest destruction, drying of wetlands, intentionally killing and road mortality are destructive effects on hygrophilous forest species. Zazanashvili and Mallon (2009) noted that degradation of natural sites and the effect of tourism are serious threats to *B. verrucosissimus* since the species can not hide from potential enemies. According to the Afsar *et al.* (2018), the main threats to amphibians in Lake Karagöl and surroundings are hotel wastes, camping and picnicking activities, and noise pollution. Moreover, the introduction of carp fish by resident people may cause the decline of the Lake Karagöl population due to the invasion of breeding sites, egg and tadpole mortality, and avoidance of breeding habitats. Therefore, we assume that the difference between the longevity of Georgia and Karagöl populations may be related to these factors.

The Lake Karagöl population differs from the toad populations inhabiting Caucasia in terms of body size, with Caucasus populations being significantly larger (see Tables 2 and 4). The maximum length of the species measured in Caucasia is 154 mm (Gumilevsky 1939). Moreover, the findings obtained in previous studies also validated the size difference between Lake Karagöl and Caucasian populations (Tarkhnishvili 1993, Litvinchuk *et al.* 2008, Iskanderov 2009, Sinsch *et al.* 2009). In amphibians, the geographic distributions of morphological traits can be influenced by different environmental factors such as precipitation, temperature, water balance and altitude (Bidau *et al.* 2011, Green 2015, Boaratti and Silva 2015, Guo *et al.* 2019) as well as life-history traits (Miaud *et al.* 1999, Morrison and Hero 2003, Womack and Bell 2020). The ranges of the populations compared in this study are characterized by different floristic districts of the Caucasus (Oganesian 1995) with different altitudes. Ergül-Kalayci *et al.* (2019) studied body size variation of three *B. bufo* populations from different altitudes (Trabzon, 1090 m a.s.l.; Kastamonu, 925 m a.s.l.; Yalova, 65 m a.s.l.) and they reported that Trabzon population was smaller than the others. We also revealed similar patterns in terms of the body size variation between *B. verrucosissimus* populations and the Lake Karagöl population. Those at the highest altitude had smaller body sizes and proportions. However, the previous studies reported no difference (Cvetković *et al.* 2009) or smaller body size in the lowlands (Hemelaar 1988) for *B. bufo*. Ergül-Kalayci *et al.* (2019) suggested that body condition can show negative correlation with the amount of snowfall, and it can reduce the body size due to short annual activity periods due to shortening the feeding period between the metamorphosis and first hibernation. Moreover, Womack and Bell (2020) investigated evolution of anuran body size using 2434 species and they indicated that the effect of altitude and microhabitats caused size differences. Therefore, we propose that the intraspecific body size variation of *B. verrucosissimus* populations is caused from the environmental characteristics affecting phenotypic plasticity. Lastly, the presence of carp fish in Lake Karagöl can be taken into consideration, because Lardner (2000) noted that *B. bufo* tadpoles tend to respond to predator presence by reducing their growth rate to tolerate weak swimming ability. This behavioral mechanism may be a defensive behavior in *B. verrucosissimus*.

The means of head length and head width were significantly lower in Karagöl specimens as opposed to Caucasian populations. The members of the Karagöl population have narrower and shorter heads. The highest head values were recorded in the populations from Agurchik and Krepostnaya (Orlova and Tuniyev 1989). In addition, we revealed similar differences between populations in the following characters: femur, tibia, and parotoid length (see Table 2). Therefore, the population inhabiting Lake Karagöl is smaller than Caucasian representatives in terms of head proportion and body segments. Most of the variation in head size is generally driven by body size representing allometry for amphibians. However, these differences might be due to the diet preferences and local environmental conditions. Crnobrnja-Isailović *et al.* (2012) described the head size and width differences between the sexes of *B. bufo* based on the prey size, and they suggested that the prey items of females have higher proportions opposed to male diets. Hudson *et al.* (2018) studied the geographical divergence in head width of *Rhinella marina* (Linnaeus, 1758) populations. They noted that the head width of samples from native ranges was higher than the translocated populations. Moreover, the head width was correlated with maximal ingestible prey size and prey-handling ability of the toads. Lastly, the authors proposed that the geographical divergence in relative head widths is a result of rapid evolution and the adaptation to the local conditions causing plasticity. These character differences were also reported for different species such as *Boana albopunctata* (Spix, 1824) (as *Hypsiboas albopunctatus;* Guimarães *et al.* 2011), *Bufotes boulengeri* (Lataste, 1879) (as *Bufo boulengeri;* Nabil and Sarra 2011), and *Pelophylax saharicus* (Boulenger, 1913) (as *Rana saharica;* Amor *et al.* 2009). Concerning the head measurements, the observed differences between *B. verrucosissimus* populations yield similar patterns with the other amphibian species, a result of plasticity as found in body size variation. Even though INTNOS was evaluated as a distinguishing character between populations, it was not totally supported by our measurements except for the round rostrum shape in the Karagöl population (Orlova and Tuniyev 1989, Kidov 2009).

Most females demonstrated brown shades whereas males were usually olive green-brown, and dark brown ventral sides occurred in both sexes. Color and pattern features of the specimens from Lake Karagöl are identical to Murgul-Artvin (Kutrup 2001) and Georgia populations from The Black Sea coast (Orlova and Tuniyev 1989, Tarkhnishvili and Gokhelashvili 1999, Sinsch *et al.* 2009). Although reddish brown males were rarely reported from Georgia, they were not observed in Karagöl. The remaining *B. verrucosissimus* populations were characterized by various shades of brown (Orlova and Tuniyev 1989). The elongated dark bands extending posteriorly along parotoid glands were previously noted as a specific character in the populations which were formerly assessed as *B. verrucosissimus verrucosissimus* (Sinsch *et al.* 2009). However, the bands were not discrete in the Karagöl samples. Zhelev *et al.* (2020) studied color polymorphism in two distinct *Bufotes viridis* (Laurenti, 1768) populations in Bulgaria and they reported that one of the populations mostly had morphs with dark backgrounds, whereas the other population was dominated by light colored individuals. They explained the observed variation by environmental differences due to the habitat characteristics relevant to vegetation type which can play a role in thermoregulation as well as defensive adaptations as camouflage seen in numerous anuran species (Hoffman and Blouin 2000, Zakharova *et al.* 2012, Zhelev *et al.* 2014, Wassef *et al.* 2019). Regarding our results, we also suggest that eco-geographical conditions and habitat characteristics may explain the observed polymorphism in *B. verrucosissimus.*

In sum, our study described general morphological traits and the lifespan and age distribution of the Karagöl population for the first time. Further studies are needed on the life-history traits of this population using ecological parameters.

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Predatory influence of dragonfly larvae and water scorpions on eggs and tadpoles of *Indosylvirana temporalis* (Anura: Ranidae)

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Abstract

Predatory influence of dragonfly larvae and water scorpions on eggs and tadpoles of *Indosylvirana temporalis* (Anura: Ranidae). We assessed in the laboratory the vulnerability of Bronzed frog (*Indosylvirana temporalis*) eggs and tadpoles to two potential sit-and-wait insect predators, larvae of a dragonfly (*Pantala flavescens;* Odonata: Libellulidae) and adult water scorpions (*Laccotrephes* sp.; Hemiptera: Nepidae). We exposed a series of different developmental stages of *I. temporalis* (from eggs to metamorphic climax stage) to these two predators. The results of this study showed that larvae of *P. flavescens* preyed on eggs and tadpoles of *I. temporalis* but only to stage 36. *Laccotrephes* sp. did not prey on eggs of *I. temporalis* but on tadpoles of all stages (22 to 42). This difference in predation rate was likely due to the gape size of the predators. The larvae of *P. flavescens* are gape-limited and cannot prey on larger tadpoles (above stage 36). Adults *Laccotrephes* sp. are non-gape-limited predators, using a segmented beak to pierce *I. temporalis* and suck the body fluids. They captured small to large tadpoles by quickly grabbing and immobilizing them using the front pair of raptorial legs. The present study shows that both predatory insects are a threat to *I. temporalis* at early and later stages of larval development.

Keywords: Developmental stages, Eggs, Hemiptera, *Laccotrephes* sp., Odonata, *Pantala flavescens,* Predator-prey interactions.

Resumo

Influência da predação por larvas de libélulas e escorpiões d'água sobre ovos e girinos de *Indosylvirana temporalis* (Anura: Ranidae). Avaliamos em laboratório a vulnerabilidade de ovos e girinos de rã-de-bronze (*Indosylvirana temporalis*) a dois potenciais insetos predadores do tipo senta-e-espera, larvas de uma libélula (*Pantala flavescens*; Odonata: Libellulidae) e escorpiões-d'água adultos (*Laccotrephes* sp.; Hemiptera: Nepidae). Expusemos uma série de diferentes estágios de desenvolvimento de *I. temporalis* (do estágio de ovos ao estágio de clímax metamórfico) a esses dois predadores. Os resultados deste estudo mostraram que as larvas de *P. flavescens* preda ovos e girinos de *I. temporalis*, mas apenas até o estágio 36. *Laccotrephes* sp. não predou ovos de *I.*

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temporalis, mas girinos de todas os estágios (22 a 42). Essa diferença na taxa de predação foi provavelmente devida ao tamanho da boca dos predadores. As larvas de *P. flavescens* são limitadas pelo tamanho da boca e não podem apresar girinos maiores (acima do estágio 36). Os adultos de *Laccotrephes* sp. não são limitados pelo tamanho da boca, utilizando um bico segmentado para perfurar *I. temporalis* e sugar os fluidos corporais. Capturaram girinos pequenos a grandes, agarrando-os rapidamente e imobilizando-os com o auxílio do par frontal de apêndices raptoriais. O presente estudo mostra que ambos os insetos predadores são uma ameaça para a *I. temporalis* nas fases iniciais e posteriores do desenvolvimento larval.

Palavras-chave: Estágios de desenvolvimento, Hemiptera, Interações predador-presa, *Laccotrephes* sp., Odonata, Ovos, *Pantala flavescens*.

Introduction

Amphibians, especially anurans (frogs), have complex life history strategies consisting of an aquatic and a terrestrial stage (Wilbur 1980). Eggs and larvae of most anurans are vulnerable to a wide range of predators including both invertebrates and vertebrates (Eterovick and Fernandes 2002, Johnson *et al.* 2003, Portheault *et al.* 2007, Burbano-Yandi *et al.* 2018, Mogali 2018, Mogali *et al.* 2020a, b). Anurans usually deposit their eggs either in temporary or permanent ponds or streams with continuous or intermittent flowing water (Hiragond and Saidapur 1999, Eterovick and Barata 2006, Mogali *et al.* 2016, 2017), resulting in early developmental stages being highly vulnerable to predation (Alford, 1999). As a consequence of this predation stress, larval anurans have developed various kinds of defensive mechanisms such as secretion of toxic substances that make eggs or larvae unpalatable or toxic to predators (Gunzburger and Travis 2005), a drastic reduction in activity levels to avoid being detected (Chovanec 1992, Mogali *et al.* 2011, 2012, Gómez 2019), increased use of refuges (Kopp *et al.* 2006, Hossie and Murray 2010, Sanders *et al.* 2015, Mogali *et al.* 2019, 2022), increased activity in order escape predators (Mogali *et al.* 2021), or aggregation and formation of schools (Waldman and Adler 1979, Watt *et al.* 1997, Spieler and Linsenmair 1999), depending upon the species or perceived predation threat.

In Southern India (Western Ghats), Bronze frogs, *Indosylvirana temporalis* (Günther, 1864), breed from September to January (post-monsoon season). They typically breed along the edges of gently flowing streams and/or in pockets of still water along streams; larvae are present in the aquatic habitats until March or April (Saidapur 2001). The larvae are mostly bottom dwellers that thrive on detritus and algal matter (Hiragond and Saidapur 2001). Visibility is low in these habitats because of shadows from vegetation and because the benthic area is naturally covered by leaf litter and detritus (Mogali *et al.* 2019). These habitats harbor both vertebrate and invertebrate predators, although the number of vertebrate predators (e.g., fishes, snakes, and birds) is lower based on personal observations. The streams are home to several types of predatory invertebrates, including beetles (*Dineutus* sp.), crabs (*Barytelphusa* sp.), dragonfly larvae (*Pantala flavescens, Bradinopyga geminata*), damselfly larvae (*Ceriagrion cerinorubellum*) mayfly larvae (*Baetis* sp.), water striders (*Cylindrostethus* sp.), backswimmers (*Notonecta* sp.), water scorpions (*Laccotrephes* sp., *Ranatra* sp.), caddis fly larvae (*Leptocerus* sp.), and leeches. Among these many predatory invertebrates, water scorpions (*Laccotrephes* sp.; Hemiptera: Nepidae) and dragonfly larvae (*Pantala flavescens;* Odonata: Libellulidae) are the most voracious predators that actively feed on anuran tadpoles including *I. temporalis*. Although we observed that other predatory invertebrates cause damage to anuran eggs or tadpoles, their impact was minimal.

Larvae of dragonflies are considered ambush/sit-and-wait, gape-limited predators. Adult water scorpions are also ambush/sit-and-wait predators, but they are not gape-limited. Both predators have excellent vision. In nature, they are well camouflaged in vegetation or detritus, where they ambush unsuspecting prey including tadpoles of *I. temporalis* (Mogali *et al.* 2020a, b). Although their life histories are remarkably similar to diving beetles that are known to prey on and severely threaten anuran larval populations (Kruse 1983, Cabrera-Guzmán *et al.* 2012, Gould *et al.* 2019, Valdez 2019), the cryptic nature of dragonfly larvae and water scorpions has discouraged the study of predation on tadpoles. Although arthropods are not typically regarded as major vertebrate predators, they can have a large impact on vertebrate populations and communities (McCormick and Polis 1982, Valdez 2020, Nyffeler and Altig 2020). Our study investigates whether one or if both of these predators negatively affects larval populations of *I. temporalis*.

In this experiment, we exposed a series of developmental stages of *I. temporalis* (i.e., from egg to metamorphic climax) to freely hunting predators, *P. flavescens* (gape-limited) and *Laccotrephes* sp. (not gape-limited). Hence, we hypothesized that there should be a difference in the predation rate between the two predatory insects.

Materials and Methods

Developmental stages are according to Gosner (1960). Eggs (stages < 10, *N* ~ 8000) from eight clutches and tadpoles (stages 25–28, *N* ~ 500) of *Indosylvirana temporalis* were collected from a stream in the Western Ghats near Anmod village (15.43088° N, 74.37360° E), Karnataka State, India, in November 2015 and immediately brought to the laboratory. Eggs were placed in a glass aquarium (90 × 30 × 15 cm) containing 25 L of aged (dechlorinated) tap water and used as stock for the experiment. Hatching occurred five days later at stage 19. The tadpoles were reared in another glass aquarium (90 × 30 × 15 cm) containing 25 L of aged tap water and mainly used as food for predators. Upon reaching the feeding stage (stage 25), the tadpoles were fed boiled spinach to sustain growth and development.

Larvae of *P. flavescens* (*N* = 60), and adult *Laccotrephes* sp. (*N* = 60) were collected from the same location as the eggs and tadpoles. Because both insects are highly cannibalistic in nature (Mogali *et al.* 2020a, 2021), they were reared individually in plastic tubs (14 cm diameter and 7 cm depth) with 500 mL of aged tap water to avoid cannibalism. All predators were fed each day with 5–8 tadpoles of *I. temporalis*.

Predatory Experiments with Dragonfly Larvae and Water Scorpions

In these experiments, a series of trials was conducted. In each trial, 20 individuals in the same developmental stage (egg stages 10–12 and tadpole stages 17–42) of *I. temporalis* and of comparable body sizes were released into a tub (32 cm diameter and 14 cm depth) containing 3 L of aged tap water and allowed to familiarize themselves to the tub for 15 min. One *P. flavescens* (late instar, 35 mm long) or one adult *Laccotrephes* sp. (35 mm long excluding the siphon length) that had been starved for 48 hours was introduced gently into a tub and left there for a period of 24 hours. After the trial period, the number of prey that survived or had been predated was noted. Five trials were conducted for each developmental stage using each predator. For each trial, a new set of eggs or tadpoles (*N* = 20) was used. The test tub was washed thoroughly before each trial.

Including both experiments, we carried out 270 experimental trials over a 90-day period. Each trial started at 07:00 h and ended at 07:00 h the next day. A minimum of five trials (of a particular developmental stage of prey) was conducted each day. An average of 2–3 days between experiments allowed the eggs or tadpoles to attain the next developmental stage. All experimental prey were healthy, and tadpoles were well fed with boiled spinach before trials, but they were not provided food during the trials. For each trial, a new set of eggs or tadpoles were used. Predators (*P. flavescens* or *Laccotrephes* sp.) used in the experiments were healthy, and all individuals were fed daily with a sufficient number of tadpoles, except that prior to use in experimental trials predators were starved for 48 hours. Individual predators (either *P. flavescens* or *Laccotrephes* sp.) were used in up to three trials each and were re-used only after a gap of at least five days. Over the course of our experiments, both predator species grew in size. For all experimental trials, we used only last instars of *P. flavescens* of comparable body size; similarly, we used only adult *Laccotrephes* sp. of comparable body size. During the course of our experiments, only the larvae of 10 *P. flavescens* metamorphosed into adult dragonflies. All experimental trials were carried out at room temperature (25°C). The data on the number of eggs/tadpoles consumed by the two predators at each developmental stage were analyzed by the Mann-Whitney U test.

Results

The number of eggs (stages 10–12) or tadpoles (stages 17–42) of *I. temporalis* consumed by the predators is given as mean ± SE (Table 1). Larvae of *P. flavescens* consumed both eggs and tadpoles of *I. temporalis* but consumed tadpoles only up to stage 36. They did not kill or consume any tadpoles in stages 37–42. Adults of *Laccotrephes* sp. did not consume eggs of *I. temporalis*, but they consumed tadpoles from stage 22 until metamorphic climax.

The results also show that *P. flavescens* consumed a significantly greater number of eggs/tadpoles of early developmental stages (stages 10–12 and 17–23) compared to those that of *Laccotrephes* sp. (Table 1). However, there was no significant difference in the number of tadpoles consumed by both predators at stage 24 (Table 1). From stage 25 to 42, *Laccotrephes* sp. consumed significantly greater number of tadpoles compared to those that of *P. flavescens* (Table 1).

Discussion

In aquatic environments, amphibian eggs and larvae are vulnerable to various types of predators (McCormick and Polis 1982, Kruse 1983, Gould *et al.* 2019, Valdez 2019) but differences exist in the risk and intensity of predation among predator species (Portheault *et al.* 2007, Cabrera-Guzmán *et al.* 2012). In the present study, we observed differences in the number of tadpoles or eggs of *I. temporalis* consumed by two species of predatory insects (*P. flavescens* and *Laccotrephes* sp.). Larvae of *P. flavescens* preyed upon eggs and tadpoles to stage 36 of *I. temporalis*. They were apparently not able to kill or consume tadpoles from stages 37–42. *Laccotrephes* sp. did not prey on eggs of *I. temporalis* but consumed tadpoles of all stages, i.e., from 22–42.

We believe these differences in patterns of predation risk are related to gape size of the predators. The larvae of *P. flavescens* are gape-limited predators (Mogali *et al.* 2016), and cannot prey on larger tadpoles (above stage 36). Other mechanisms may counteract this limitation. For example, like adult diving beetles, they may time their offspring to hatch at the same time as tadpoles (Valdez 2019, Gould *et al.* 2019). Adult *Laccotrephes* sp. are non-gape-limited and use a segmented beak to pierce *I. temporalis* and suck their body fluids. They are able to capture small to large tadpoles by quickly grabbing and immobilizing the tadpoles by using the front pair of raptorial legs (Mogali *et al.* 2020b). Even though starved for 48 h, the water scorpions did not eat eggs of *I. temporalis*. Perhaps the small, non-mobile eggs of *I. temporalis* may not catch the attention of adult water scorpions or they may not feed on small prey. An earlier study revealed that water scorpions mostly feed on moving prey (Ohba and Swart 2009).

Table 1. Number of eggs/tadpoles of different developmental stages of *Indosylvirana temporalis* (prey) consumed by the predators, *Pantala flavescens* (sit-and-wait, gape-limited; total length 35.05 ± 0.62 mm) and *Laccotrephes* sp. (sit-and-wait, non-gape-limited; 35.30 ± 0.85 mm; excluding the siphon length) in 24-hour trial periods. Data represent mean ± SE and analyzed by Mann-Whitney U test. *N* = 5 trials for each stage.

Stages	**Prey total length (mm)**	**Number of eggs/tadpoles consumed by the predator**		
		Pantala flavescens	*Laccotrephes* sp.	U and *p* values
10–12 (eggs)	1.84 ± 0.06 (diameter)	0.60 ± 0.24	0.00 ± 0.00	U = 5.000, *p* = 0.050
17	2.56 ± 0.02	0.80 ± 0.20	0.00 ± 0.00	U = 2.500, *p* = 0.014
18	2.84 ± 0.03	1.20 ± 0.20	0.00 ± 0.00	U = 0.000, *p* = 0.004
19	3.02 ± 0.07	1.40 ± 0.24	0.00 ± 0.00	U = 0.000, *p* = 0.005
20	3.92 ± 0.04	1.60 ± 0.24	0.00 ± 0.00	U = 0.000, *p* = 0.005
21	4.80 ± 0.05	2.20 ± 0.20	0.00 ± 0.00	U = 0.000, *p* = 0.003
22	5.58 ± 0.07	2.60 ± 0.24	1.00 ± 0.44	U = 2.000, *p* = 0.021
23	6.78 ± 0.04	3.20 ± 0.37	2.00 ± 0.00	U = 2.500, *p* = 0.018
24	7.64 ± 0.06	4.00 ± 0.31	3.40 ± 0.40	U = 8.000, *p* = 0.288
25	9.42 ± 0.04	7.80 ± 0.66	13.60 ± 0.67	U = 0.000, *p* = 0.009
26	17.16 ± 0.05	5.80 ± 0.58	11.40 ± 0.50	U = 0.000, *p* = 0.009
27	20.13 ± 0.01	5.20 ± 0.37	8.40 ± 0.25	U = 0.000, *p* = 0.008
28	24.32 ± 0.01	4.20 ± 0.20	6.20 ± 0.20	U = 0.000, *p* = 0.005
29	24.72 ± 0.02	3.40 ± 0.25	5.80 ± 0.20	U = 0.000, *p* = 0.006
30	28.35 ± 0.02	2.60 ± 0.25	5.60 ± 0.25	U = 0.000, *p* = 0.007
31	28.90 ± 0.03	2.20 ± 0.20	5.40 ± 0.25	U = 0.000, *p* = 0.006
32	29.02 ± 0.05	2.00 ± 0.00	4.80 ± 0.20	U = 0.000, *p* = 0.004
33	29.33 ± 0.01	1.60 ± 0.25	4.60 ± 0.25	U = 0.000, *p* = 0.007
34	30.41 ± 0.03	1.00 ± 0.00	4.40 ± 0.25	U = 0.000, *p* = 0.005
35	30.65 ± 0.02	0.60 ± 0.25	4.20 ± 0.20	U = 0.000, *p* = 0.006
36	31.70 ± 0.02	0.20 ± 0.20	4.00 ± 0.00	U = 0.000, *p* = 0.004
37	32.29 ± 0.03	0.00 ± 0.00	3.60 ± 0.25	U = 0.000, *p* = 0.005
38	32.46 ± 0.02	0.00 ± 0.00	3.20 ± 0.32	U = 0.000, *p* = 0.005
39	32.65 ± 0.02	0.00 ± 0.00	2.80 ± 0.37	U = 0.000, *p* = 0.005
40	33.24 ± 0.02	0.00 ± 0.00	2.60 ± 0.25	U = 0.000, *p* = 0.005
41	33.43 ± 0.01	0.00 ± 0.00	2.40 ± 0.25	U = 0.000, *p* = 0.005
42	33.80 ± 0.02	0.00 ± 0.00	2.60 ± 0.25	U = 0.000, *p* = 0.005

Pantala flavescens are relatively ineffective egg predators but they actively feed on small tadpoles. The immobile eggs or less mobile early stages of tadpoles of *I. temporalis* (stages < 23) may not readily catch the attention of sit-and-wait predators. In nature, other aquatic predators, such as caddis fly larvae, leeches, water beetles, water boatman, and other small insects or their larvae mainly feed on eggs of *I. temporalis* (e.g., Henrikson 1990, Cabrera-Guzmán *et al.* 2012). To counteract such predator pressure, tadpoles of *I. temporalis* have been shown to have higher survivorship by reducing their activity levels (Mogali *et al.* 2012). They also seek refuge sites in the presence of predators (Mogali *et al.* 2019, 2021) and complete their larval period early with a larger body size (Mogali *et al.* 2016). It is unknown what effect these predators have on natural populations of tadpoles of *I. temporalis*. The present study shows that the two predatory insects studied can be threats to *I. temporalis* at most stages of larval development.

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Sensory basis of food detection in tadpoles of *Polypedates maculatus* (Anura: Rhacophoridae): an experimental approach

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Abstract

Sensory basis of food detection in tadpoles of *Polypedates maculatus* (Anura: Rhacophoridae): an experimental approach. The mechanism of food detection in tadpoles of *Polypedates maculatus* was experimentally tested. We used a rectangular glass test tank with stimulus zones in opposite ends to provide visual and/or chemical food. For visual cues, boiled spinach was placed inside a glass container, and for chemical cues boiled spinach was placed in a mesh cage. Each tadpole of *P. maculatus* (either at an early or medium developmental stage) was held at the center of the test tank for acclimation. The tadpole was released, and we recorded whether it approached or did not approach the caged food. Tadpoles of all stages failed to detect food using visual cues. Tadpoles of all stages detected food using chemical cues. In tests using chemical cues, they spent the majority of their time (69.3% by early stage tadpoles and 87.3% by medium-stage tadpoles) near the container with food than in the end with no containers or with only visual food cues. Tadpoles in medium stages spent more time near food (18.1% of total time) than tadpoles in early stages. These findings indicate that tadpoles of *P. maculatus* detect food by chemical sensory mechanisms rather than visual ones. Tadpoles in medium stages spent more time near food than tadpoles in early stages indicating that time spent foraging increases as tadpoles grow.

Keywords: Anuran larvae, Chemical cues, Developmental stage, Food detection behavior, Visual cues.

Resumo

Base sensorial da detecção de alimento em girinos de *Polypedates maculatus* (Anura: Rhacophoridae): uma abordagem experimental. O mecanismo de detecção de alimento em girinos de *Polypedates maculatus* foi testado experimentalmente. Utilizamos um aquário retangular de teste com zonas de estímulo em seus extremos opostos, fornecendo exclusivamente alimento visual e/ou químico. Para o estímulos visual, espinafre cozido foi colocado dentro de um recipiente de vidro, e para os estímulo químico, espinafre cozido foi colocado em uma gaiola de tela nas zonas opostas do aquários de testes. Cada girino de *P. maculatus* (na fase inicial ou média de

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desenvolvimento) era mantido no centro do aquário para a aclimatação, sendo depois liberado e deixado que se aproximasse ou se afastasse do alimento das gaiolas. Os girinos de ambas as fases não conseguiram detectar o alimento por meio de sinais visuais. Passaram quase o mesmo tempo na zona que abrigava o alimento no recipiente de vidro e na zona oposta, mantida vazia. Os girinos de ambas as fases detectaram o alimento unicamente por meio de estímulos químicos. Nos testes com estímulos químicos, os girinos passaram a maior parte do tempo (69,3% para girinos na fase inicial e 87,3% para girinos na fase média) perto do recipiente que liberava sinais químicos, em comparação com os recipientes vazios ou apenas com estímulos visuais na zona oposta do aquário. Curiosamente, o estudo também mostrou que os girinos na fase média estão mais associados ao alimento (18,1% do tempo total) do que os girinos de fase inicial. Essas descobertas evidenciaram que os girinos de *P. maculatus* detectam o alimento por mecanismos sensoriais químicos em vez de visuais. Além disso, os girinos de fase média permanecem mais associados ao alimento do que os girinos de fase inicial, o que indica claramente que a taxa de forrageio aumenta nos girinos de fase avançada.

Palavras-chave: Comportamento, Estágio de desenvolvimento, Estímulos químicos, Estímulos visuais, Larvas de anuros.

Introduction

Amphibians and especially anurans exhibit a great diversity of reproductive modes and reproduce in a great variety of habitats, including phytotelmata, forest litter, and burrows (Duellman and Trueb 1986, Haddad and Prado 2005). Tadpoles of the majority of anurans complete their development in different types of lotic and lentic water bodies (Saidapur *et al.* 2009, Mogali *et al.* 2011, 2012, 2020, 2021). A number of studies have shown that anuran tadpoles respond to a wide variety of stimuli that include tactile, chemical, and visual cues and exhibit appropriate behavioral responses (Stauffer and Semlitsch 1993, Hoff *et al.* 1999, Mogali *et al.* 2015). Most anuran tadpoles are found in murky or turbid water with poor visibility. Anuran tadpoles in general are near-sighted and it is unlikely that they use vision to detect distant objects (Hoff *et al.* 1999). The perception of chemical cues seems to play a vital role in behavioral and physiological responses in tadpoles.

Tadpoles of most tropical anurans live in temporary water bodies that are rich in resources. They are consequently exposed to a wide range of larvae of invertebrate predators and to some vertebrate predators, mainly fishes (Saidapur 2001). Tadpoles feed on microorganisms, algae, and detritus depending upon their oral apparatus and prey availability (Sekar 1992, Hoff *et al.* 1999, Saidapur 2001, Santos *et al.* 2016, Protázio *et al.* 2020).

The Indian tree frog, *Polypedates maculatus* (Gray, 1830), is widely distributed in India. In Southern India, populations breed only during the rainy season. Females deposit eggs in foam nests that are attached to vegetation such as bushes over water, under rocks, or adhered to walls of cement cisterns filled with water (Mohanty-Hejmadi and Dutta 1988, Girish and Saidapur 1999). Early embryonic development (up to stage 23 of Gosner 1960) occurs inside the foam nests, after which tadpoles drop into the water where they undergo further development and metamorphosis. Foam nests, including those of *P. maculatus,* supply adequate oxygen (Seymour and Loveridge 1994) and can maintain adequate temperatures for development (Dobkin and Gettinger 1985). Foam nests also protect eggs and tadpoles from predation in early developmental stages and prevent the eggs from desiccating (Heyer 1969, 1975, Downie 1990, Mogali 2018). Tadpoles of *P. maculatus* are bottom dwellers (in temporary and permanent water bodies) and forage on detritus and algal matter (Hiragond and Saidapur 2001). Visibility is generally low in these habitats because of turbid water and detritus. Little is known about tadpoles dwelling in environments with limited visibility (Fouilloux *et al.* 2022). In this context, we studied how tadpoles of *P. maculatus* detect their food using laboratory experiments.

Materials and Methods

Developmental stages are according to Gosner (1960). Four foam nests of the Indian tree frog, *Polypedates maculatus,* were located in temporary ponds during the early monsoon season (June 2017) on the Karnatak University Campus (15.440407° N, 74.985246° E), Dharwad, Karnataka State, India. The foam nests were attached to vegetation about 20 cm above the water. They were collected and placed separately in plastic tubs (32 cm diameter and 14 cm deep) with 1 L of dechlorinated, aged tap water and substratum collected from the same pond. The tadpoles emerged at stage 23 after 5–6 days. Tadpoles from all four foam nests were mixed to normalize genetic differences among the groups and were reared in a glass aquarium (75 × 45 × 15 cm) containing 15 L of aged tap water. When tadpoles reached the feeding stage (stage 25) they were provided with boiled spinach *ad libitum*. The mechanism of food detection in *P. maculatus* was studied at different developmental stages: early (stages 27–28) and medium (stages 35–36). In all experimental trials, tadpoles were of comparable body sizes and developmental stages (early stage tadpoles: total length 26.50 ± 0.65 mm ($\bar{x}$ ± SE); *N* = 25; medium stage tadpoles: total length 49.20 ± 1.20 mm ($\bar{x}$ ± SE); *N* = 25).

A rectangular glass tank (90 × 30 × 15 cm) was used for the food detection experiments (Figure 1). A central line perpendicular to the long axis of the test tank was drawn on the outside of the bottom, dividing it into two equal compartments referred to as stimulus zones A and B. The food (boiled spinach, 1 g) was placed either in a transparent glass container (9 cm diameter × 14 cm height) or within a mesh cage (10 cm diameter × 15 cm height) wrapped in cheesecloth. We assumed that food placed in the transparent glass container would block chemical cues but provide visual information, while food placed in the mesh cage wrapped with cheesecloth would block visual cues but allow diffusion of chemical cues in the water. Prior to each trial, the tank was filled with aged tap water to a height of 3 cm. The stimulus zones were reversed between the trials.

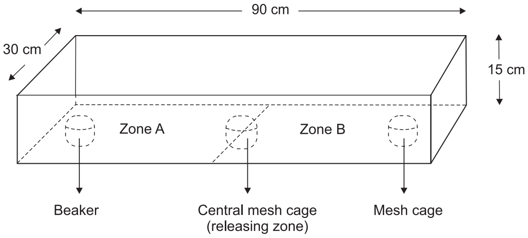

Figure 1. The design of the test tank for investigating the mechanism of food detection by the tadpoles (at early and medium stages of development) of *Polypedates maculatus*. The dotted central line visually divides the test tank into two zones (A and B). The central area was used to release test tadpoles. Containers in the opposite zones indicate areas where food was presented either in a transparent glass container or a mesh cage wrapped with cheesecloth.

In each trial, a single tadpole (*P. maculatus*) was tested. A tadpole starved for 24 hours (either at an early or medium stage of development) was placed in an open-ended mesh cage (10 cm diameter × 15 cm height) wrapped with cheesecloth. The mesh cage was placed in the center of the tank for 5 min to allow the tadpole to acclimate as well as to perceive visual and/or chemical food cues. The tadpole was then released by gently lifting the mesh cage without disturbance, allowing it to freely move in the tank. The time spent by the tadpole in each stimulus zone during the trial period was recorded for 10 min. Our assumption was that when tadpoles detected food, they would spend more time in the zone with food than in the opposite zone without food. Failure to detect food would result in random movements of tadpoles in the tank. After each trial, the tank was washed and the water was changed. Each tadpole and container of food was used only once. All trials were conducted under natural photoperiod and temperature. The daily temperature of the testing room in the laboratory varied between 25–26°C.

End-bias tests were conducted to check whether the tadpoles showed bias toward either end of the test tank or for either type of container used for placing the food (a glass container and an open-ended cylindrical mesh cage wrapped with cheesecloth) (Sugur *et al.* 2008). These tests involved four sets of trials: (1) with no containers at either end of the test tank; (2) with a glass container containing water level with that in the test tank at one end and the other end with nothing; (3) with a mesh cage wrapped with cheesecloth in one end and nothing in the other end; and (4) with a glass container and a mesh cage wrapped with cheesecloth placed at the opposite ends of the tank. The time spent by a tadpole in each zone in a given trial was recorded. We carried out the end-bias tests separately using early and medium developmental stages of tadpoles. In each set, 25 trials were conducted (4 types of end-bias tests × 2 types of test tadpoles, early and medium stages of development × 25 trials for each set = total of 200 end-bias tests).

Tests involving detection of food based on visual or chemical cues by the tadpoles were conducted as follows. In tests for food detection based on visual cues, a glass container containing 1 g of boiled spinach was placed at one end of the test tank and the opposite end was provided with a glass container containing water level with that in the tank (*N* = 25 trials). In tests for food detection based on chemical cues, an open-ended mesh cage wrapped with cheesecloth containing 1 g of boiled spinach was placed at one end of the tank and a similar cage but devoid of food was placed at the opposite end (*N* = 25 trials). In tests for food detection based on both visual and chemical cues, a glass container containing 1 g of boiled spinach (visual food cues) was place in one end of the tank, and a mesh cage wrapped with cheesecloth containing 1 g of boiled spinach (chemical food cues) was placed in the other end (*N* = 25 trials). The amount of time spent in each stimulus zone by a tadpole was recorded and analyzed using the Wilcoxon matched-pairs signed-ranks test (SPSS software ver. 16.0).

Results

Mechanism of Food Detection at Early Stages of Development

In the end-bias tests, tadpoles of early stages moved freely throughout the tank, showing no bias toward any particular zone of the test tank (Z = -0.331; *p* = 0.741; Table 1). The placement of the glass container or mesh cage made no difference to space use by the tadpoles.

In tests involving food providing only visual cues, there was no significant difference in the time spent by tadpoles between the stimulus zones with glass containers with or without food, even though the food was visible through the glass container at one end of the tank (Z = -0.414; *p* = 0.679; Table 1). In trials involving food providing only chemical cues, tadpoles spent significantly more time in the zone with food inside the mesh cage wrapped with cheesecloth compared to the mesh cage without food (Z = -4.347; *p* = 0.000; Table 1). In tests involving food providing both visual and chemical cues, tadpoles spent a significantly greater amount of time in the zone with food in the mesh cage wrapped with cheesecloth compared to the zone providing visual cues of food through the glass container (Z = -4.346; *p* = 0.000; Table 1).

In the 25 trials with only visual cues of food in glass containers, no tadpoles approached the container or touched it. In contrast, in 16 of 25

Table 1. The amount of time spent by tadpoles of *Polypedates maculatus* (at early stages of development; stages 27–28) in response to visual/chemical cues of food, boiled spinach. [#]Wilcoxon matched-pairs signed-ranks test. [*]Significantly different.

Tests	**Mean time spent ± SE (s)**		***Z* and *p* values[#]**
	Zone A	Zone B	
End-bias	302.16 ± 8.07	297.84 ± 8.07	*Z* = -0.331; *p* = 0.741
Blank (A) vs. Visual (B)	294.92 ± 16.22	305.08 ± 16.22	*Z* = -0.414; *p* = 0.679
Blank (A) vs. Chemical (B)	184.44 ± 9.39	415.56 ± 9.39	*Z* = -4.347; *p* = 0.000[*]
Visual (A) vs. Chemical (B)	176.40 ± 8.69	423.60 ± 8.69	*Z* = -4.346; *p* = 0.000[*]

trials involving only chemical cues of food, tadpoles touched the mesh cage with their snouts, presumably sensing chemical cues from food that could not be seen but was sensed through chemical cues. In 17 of 25 trials involving both visual and chemical cues of food, tadpoles touched the mesh cage with their snouts, presumably trying to reach the food, but no tadpoles approached or touched the glass containers.

Mechanism of Food Detection at Medium Stages of Development

In the end-bias tests, tadpoles at medium stages moved freely throughout the test tank (Z = -0.978; *p* = 0.328; Table 2). They showed no bias toward any particular zone of the tank or toward the containers (glass container or mesh cage).

In tests involving food providing only visual cues, there was no significant difference in the time spent by the tadpoles between the stimulus zones with glass containers with or without food (Z = -0.243; *p* = 0.808; Table 2). In trials involving food providing only chemical cues, the tadpoles spent significantly more time in the zone with food inside the mesh cage compared to the zone with the mesh cage without food (Z = -4.373; *p* = 0.000; Table 2). In tests involving food providing both visual and chemical cues, tadpoles spent a significantly greater amount of time in the zone with food in the mesh cage compared to the zone providing visual cues of food (Z = -4.372; *p* = 0.000; Table 2).

In the 25 trials with only visual cues of food, no tadpoles approached the glass container or touched it. In contrast, in 22 of 25 trials involving only chemical cues of food, tadpoles touched the mesh cage with their snouts, presumably trying to reach the food that could not be seen but was sensed through chemical cues. In 22 of 25 trials involving both visual and chemical cues of food, tadpoles touched the mesh cage with their snouts, presumably trying to reach the food, but no the tadpoles approached or touched the glass containers.

Discussion

In Southern India, tadpoles of *P. maculatus* occur in temporary and permanent water bodies that are generally turbid or murky with low visibility (Hiragond and Saidapur 2001, Saidapur *et al.* 2009, Mogali 2018). The ability of these tadpoles to detect food based on visual cues may be limited. It is generally believed that anuran tadpoles are near-sighted (Hoff *et al.* 1999, Mogali 2018). If so, visual detection of food or prey would be limited. In our laboratory experiments, tadpoles of *P. maculatus* at early and medium developmental stages were tested to understand the mechanism of food detection in

Table 2. The amount of time spent by tadpoles of *Polypedates maculatus* (at medium stages of development; stages 35–36) in response to visual/chemical cues of food, boiled spinach. #Wilcoxon matched-pairs signed-ranks test. *Significantly different.

Tests	Mean time spent ± SE (s)		Z and p values#
	Zone A	Zone B	
End-bias	296.66 ± 8.64	303.34 ± 8.64	$Z = -0.978$; $p = 0.328$
Blank (A) vs. Visual (B)	300.60 ± 14.80	299.40 ± 14.80	$Z = -0.243$; $p = 0.808$
Blank (A) vs. Chemical (B)	76.04 ± 5.83	523.96 ± 5.83	$Z = -4.373$; $p = 0.000$*
Visual (A) vs. Chemical (B)	71.88 ± 6.11	528.12 ± 6.11	$Z = -4.372$; $p = 0.000$*

clear water. Tadpoles in both early and medium stages did not detect the food (boiled spinach) placed in transparent glass containers, even though they were starved for 24 hours, indicating the ineffectiveness of visual cues. Blocking visual food cues did not limit detection of food that is solely based on water-borne chemical cues (see Sugur *et al.* 2008). Tadpoles in both early and medium stages moved toward chemical cues from food hidden in a mesh cage wrapped in cheesecloth. A large proportion of tadpoles of both stages (68% by early and 88% by medium stages) touched the mesh cage with their snouts, presumably trying to reach the food that could not be seen. No tadpoles touched the glass containers with visible food, indicating that tadpoles of *P. maculatus* detect food using chemical cues.

Our results conform to an earlier study on tadpoles of *Indosylvirana temporalis* (Veeranagoudar *et al.* 2004), *Sphaerotheca breviceps* (Sugur *et al.* 2008), and *Clinotarsus curtipes* (unpubl. data). The present study shows that tadpoles in medium stages spend more time (18.1%) near food than tadpoles in early stages. More tadpoles in medium stages (88%) touched the mesh cages with food than tadpoles in early stages (68%). Time spent foraging increases as tadpoles grow.

The ability to detect food based on chemical cues may have evolved under poor or low visibility conditions, such as in murky or turbid water or benthic areas covered by leaf litter and detritus. This ability may allow tadpoles to forage at night. All stages of herbivorous tadpoles like *P. maculatus* forage during the night hours (SMM personal observations). The present study shows that tadpoles of *P. maculatus* of different developmental stages detect food through chemical, not visual, senses. Failure to detect food based on visual cues by the tadpoles of *P. maculatus* supports the general view that anuran tadpoles, especially those dwelling in turbid water, have poor vision.

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SHORT COMMUNICATION

Field observations of *Ambystoma altamirani* at near-freezing conditions in the Sierra de las Cruces, Mexico

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Keywords: Ice, Montane, Salamander, Stream.

Palabras claves: Arroyo, Hielo, Montaña, Salamandra.

Palavras-chave: Gelo, Montanha, Riacho, Salamandra.

Amphibian populations living at high elevations or latitudes often face harsh environmental conditions, including freezing winter temperatures. At the more northern extent of their distribution and at high elevations, salamanders in the genus *Ambystoma* have been observed at or near freezing temperatures. For example, Radi and Beachy (2013) observed larval *Ambystoma mavortium* Baird, 1850 active under ice in North Dakota, and *Ambystoma* often migrate during snow events at near or below freezing temperatures (McClure 1943, Anderson 1967). Freezing of ponds and freezing temperatures can cause significant mortality for adult and larval *Ambystoma* [e.g., adult *A. maculatum* (Shaw, 1802) in North Carolina (Harris 1980) and larval *A. opacum* (Gravenhorst, 1807) in Connecticut (Herstoff and Urban 2014)]. In addition, at least some *Ambystoma* cannot tolerate freezing (Storey and Storey 1986).

The response of more southern *Ambystoma* to freezing conditions, such as populations in central Mexico, is unknown. Indeed, to our knowledge, no published observations of Mexican *Ambystoma* under freezing or near freezing conditions exist. As part of an on-going

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study of populations of the Mountain Stream Siredon, *Ambystoma altamirani* Dugès, 1895 in the mountains near Mexico City (Lemos-Espinal *et al.* 2016, Villanueva-Camacho *et al.* 2020, Villarreal-Hernández *et al.* 2020, Gómez-Franco *et al.* 2022), we have regularly visited and surveyed streams throughout the year. Here we report the first observation of *A. altamirani* during a freezing event.

On 27 December 2021, while surveying a population of *A. altamirani* at the Llano Las Navajas-Arroyo Los Axolotes (19°3212.2 N, 99°2952.7 W; 3479 m a.s.l.), Sierra de las Cruces, state of México, Mexico, we observed a 4- to 7-cm thick ice layer on the surface of the stream with only occasional small stretches free of ice. Large portions of the stream channel lacked water entirely. Water was concentrated in the deep and narrow parts of the stream and absent from shallow and wide sections.

At 10:10 h we observed a juvenile *A. altamirani* (~ 60 mm SVL) that looked to be in good condition (i.e., apparently healthy and alert). This individual was in a small section of the stream that had ice along the sides. Water temperature in the middle of the stream was 6.4°C, and the air temperature was 12.8°C. The stream at this point was 60 cm wide and 30 cm deep. The salamander was on the bottom of the stream in a small cavity of sand and dark brown mud away from the crevices and overhangs where inactive salamanders are typically found. The icy environmental conditions along with the thickness of the mud bottom of the stream prevented us from capturing the individual. We continuously surveyed a 1-km section of the stream from 10:00 to 14:00 h, taking water temperatures of around 3.4°C near the ice layer. The juvenile *A. altamirani* was the only amphibian that we observed during this survey.

We have been visiting this stream for seven years and have observed that the activity of this population decreases from November to February, sometimes with no observations in the months of December and January, probably due to freezing conditions (see Lemos-Espinal *et al.* 2016, Villanueva-Camacho *et al.* 2020, Villarreal-Hernández *et al.* 2020). According to the climate data from a nearby meteorological station 15231 (Iturbide Dam; National Water Commission, CONAGUA, accessed 06 January 2022) for the period 1977–2015, the lowest monthly average temperatures for the study area occur in November (8.0°C), December (6.9°C), January (6.8°C), and February (7.7°C). This is the first time that we have observed an active individual (i.e., not found in a crevice or under cover) in this stream with a thick layer of ice.

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Short Communication

Predation on *Xenophrys zhangi* (Anura: Megophridae) by *Heteropoda* sp. (Araneae: Sparassidae) in Nepal

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Keywords: Amphibians, Huntsman Spider, Natural history, Predator-prey interaction, Zhang's Horned Frog.

Palavras-chave: Anfíbios, Aranha-caçadora, Interação predador-presa, História natural, Rã-de-chifres-de-zhang.

Anurans in post-metamorphic stages are highly vulnerable to predation, and many are preyed upon by invertebrates such as spiders and aquatic insects (Menin *et al.* 2005, Toledo 2005, Luria-Manzano *et al.* 2020). Zhang's Horned Frog, *Xenophrys zhangi* (Ye and Fei, 1992), is the smallest frog among three species in the genus *Xenophrys* found in Nepal and can be identified by a W- or V-shaped mark on its dorsum (Bhattarai *et al.* 2020). It occurs near small streams in moderately disturbed secondary forests and is widely distributed in central and eastern Nepal between 500 and 1,000 m a.s.l. Juveniles are mostly found near rocks close to the water channel (Schleich and Kästle 2002, Bhattarai *et al.* 2020, Khatiwada *et al.* 2021).

Spiders are among the most common and abundant predators in terrestrial ecosystems (Turnbull 1973, Coddington and Levi 1991, Nyffeler and Sunderland 2003). A global review of spiders that feed on frogs by Nyffeler and Altig (2020) reported a total 106 species of spiders that prey on frogs. Huntsman Spiders in the genus *Heteropoda* Latreille, 1804 are mostly distributed in tropical Asia and Australia (Sethi and Tikader 1988, Airamé and Sierwald 2000, Jäger 2008). Feeding behavior of *Heteropoda* sp. in captivity by Airamé and Sierwald (2000) reported that three events of frog larvae were preyed upon belonging to Megophryidae family. In Nepal, both frogs and spiders are understudied (Siliwal and Molur 2007, Bhattarai *et al.* 2018). We present here the first record of predation on *Xenophrys zhangi,* by *Heteropoda* sp., an addition to our knowledge of the interactions between frogs and spiders in Nepal.

On 14 April 2022, the first author was on a nocturnal herpetological survey along Phewa stream, Mangsebung, Ilam. At 19:40 h, he observed that a Huntsman Spider had grasped

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both hind limbs of a juvenile *Xenophrys zhangi* on the bank of the stream. (Figure 1A). Both frog and spider remained stationary when the flashlight was focused on them for about two minutes. The observer dimmed the flashlight to observe the behavior. The frog tried to escape by stretching its fingers, but the spider had seized the frog's hind limbs and remained motionless. After approximately 1.5 min the prey appeared immobile with fatigued hind limbs. The sluggish frog did not open its mouth or produce any distress call. The spider slowly pulled the frog toward a safe place under a rock. When the observer again focused the flashlight on them, the spider stopped pulling the prey and remained motionless between rocks until the observer dimmed the light again. When the observer focused the light away from spider, it escaped into rock crevices with its prey (Figure 1B).

Generally, vertebrates (mostly snakes) are considered major predators of frogs. However, studies suggest that spiders are common predators of frogs (Menin *et al.* 2005, Toledo 2005). Spiders of the genus *Heteropoda* inhabit rock crevices and tree bark and are known to hunt on the surface of shallow water (Airamé and Sierwald 2000). In general, predation tactics correlate with predator-prey size relationships (Toledo *et al.* 2007, Wells 2007). Frogs and spiders are competitors and have cross predation (Polis *et al.* 1989, Gaiarsa *et al.* 2012). Spiders frequently overpower frogs that are larger than themselves, whereas frogs exclusively kill spiders of smaller size than themselves (Labanick 1976, Parmelee 1999, Hirai and Matsui 2002, Arroyo *et al.* 2008). This behavior happens because spiders have extra-intestinal digestion whereas frogs swallow their prey whole (Nyffeler and Altig 2020). To the best of our knowledge, the interactions between frogs and spiders are not documented in Nepal. This study is the first observation of predation on *Xenophrys zhangi* by a species of spider, suggesting that detailed studies on their cross predation are needed.

Acknowledgments.—We would like to thank Kiran Thapa Magar, arachnologist from Nepal, for identification of the spider. This observation was made during herpetological work supported by the Society for the Study of Amphibians and

Figure 1. (**A**) A Huntsman Spider (*Heteropoda* sp.) preying upon a Zhang's Horned Frog (*Xenophrys zhangi*) and (**B**) dragging its prey into rock crevices.

Reptiles (SSAR), Josh's Frog, the Roger Williams Park Zoo, the Amphibian Survival Alliance (ASA), and Idea Wild to Bivek Gautam for a Salamander Conservation Project in Nepal. We also would like to thank Jeevan Gurung, Jash Hang Rai, and Netra Koirala for field assistance.

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SHORT COMMUNICATION

Communal nesting and clutch characterization of *Lygodactylus klugei* (Squamata: Gekkonidae) from Brazilian semiarid region

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Palavras-chave: Lagartos, Neonatos, Ovipostura, Ovos, Nordeste do Brasil, Reprodução.

Communal nesting is a reproductive tactic used by many lizards (Graves and Duval 1995, Carvajal-Ocampo *et al.* 2019). It refers to egg laying by different conspecifics or by different species in a single shared nest (Espinoza and Lobo 1996). Of the different hypotheses explaining this behavior, avoidance of predation (Somaweera 2009) and the shortage of suitable nesting places (Rand 1967, Oda 2004) are the most accepted. This behavior, in addition to favoring offspring, can also be advantageous to females because depositing eggs in a place already chosen by other females requires less energy and results in lower predation risk during the search for nesting sites (Graves and Duvall 1995, Montgomery *et al*. 2011).

Recorded observations of communal nesting in lizards of different families have increased in neotropical regions, including Teiidae (Magnusson and Lima 1984, Filadelfo *et al*. 2013), Dactyloidae

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(Montgomery *et al.* 2011), Sphaerodactylidae (Oda 2004, Oliveira *et al.* 2014, Carvajal-Ocampo *et al.* 2019, Sales *et al.* 2020), Phyllodactylidae (Vitt 1986, Rigui *et al.* 2004, Ávila and Cunha-Avellar 2005, Cassimiro and Rodrigues 2010, Lima *et al.* 2011, Domingos *et al.* 2017), and Gekkonidae (Sousa and Freire 2010, Bezerra *et al.* 2011). Communal nests have been reported for the gekkonid genus *Lygodactylus* on the African continent (Greer 1967, Simbotwe 1983, Rodríguez-Prieto *et al.* 2010), but currently no records of this behavior have been reported for South American species.

The genus *Lygodactylus* Gray, 1864 is composed by 64 species: 62 occur in Africa and Madagascar, and two occur in South America (Uetz *et al.* 2022). In Brazil, in addition to these two species, *Lygodactylus wetzeli* (Smith, Martin, and Swain, 1977) and *Lygodactylus klugei* (Smith, Martin, and Swain, 1977), three putative cryptic species have been detected (Lanna *et al.* 2018). *Lygodactylus klugei* is one of the smallest Brazilian gekkonids and has diurnal and arboreal habits (Vitt and Ballinger 1982, Galdino *et al.* 2011, Teixeira *et al.* 2013). The species has a wide distribution in the Caatinga, extending from the state of Piauí to the state of Bahia. It also occurs in Dry Tropical Forest enclaves within the Cerrado (Vanzolini 1974, Rodrigues 2003, Mesquita *et al.* 2017, Lanna *et al.* 2018, Costa *et al.* 2021).

Lygodactylus klugei is a relatively well-known species, with studies detailing its diet (Vitt 1995, Galdino *et al.* 2011, Teixeira *et al.* 2013, Aximoff and Felix 2017, Silva *et al.* 2021), morphology (Vitt and Ballinger 1982), behavior (Teles *et al.* 2018), annual activity (Passos *et al.* 2016), parasitology (Anjos *et al.* 2011), and phylogeography (Lanna *et al.* 2018, Lanna *et al.* 2019). In terms of sexual dimorphism, females have larger body sizes and males have larger heads (Vitt 1986, Galdino *et al.* 2011). Reproduction is continuous throughout the year; egg mass is not related to female size, and each female deposits only two eggs per clutch (Vitt 1986). Herein we present the first records of communal nesting for *L. klugei* and provide additional information on egg and neonate morphometry.

Occasional records of nests of *L. klugei* were obtained from five localities between March 2018 and July 2019 (Figure 1). Three localities were in the state of Rio Grande do Norte (Campus Central da Universidade Federal Rural do Semi-Árido (UFERSA) and Estação Experimental - municipality of Mossoró, and Floresta Nacional de Açu - municipality of Assu), one in the state of Pernambuco (Sítio Russa Mansa - municipality of Itapetim) and one in the state of Ceará (countryside of the municipality of Mauriti). All measurements were taken to 0.01 mm. We measured egg length, diameter, and mass at the Rio Grande do Norte and Ceará localities. At the Pernambuco and Ceará localities, we assessed incubation time in the laboratory and measured egg mass and neonate snout–vent length (SVL). We recorded the color (white, yellow, light yellow, and grey) of the eggs when they were found in nature.

We used data from 25 eggs (from five localities) and five neonates (two from Itapetim and three from Mauriti) from both single and communal nests. We provide the median and the 1st and 3rd quartile for the morphometric egg measurements (length, diameter, and mass) and the mean and standard deviation for the morphometric neonate measurements (SVL and mass) (Table 1).

Material collected in the states of Pernambuco and Ceará was deposited in the Laboratório de Herpetologia da Universidade Federal de Campina Grande (LHUFCG), Patos municipality, state of Paraíba (LHUFCG 2632–2637). Samples from Rio Grande do Norte state were deposited in the Coleção Herpetológica do Semiárido (CHSAR), in Mossoró municipality, state of Rio Grande do Norte (CHSAR 028–031, 1058–1059, 1319–1320).

Of the eight sampled nests (six in Rio Grande do Norte state, one in Pernambuco state, and one in Ceará state), some contained one egg (*N* = 3), while others contained two (*N* = 1), three

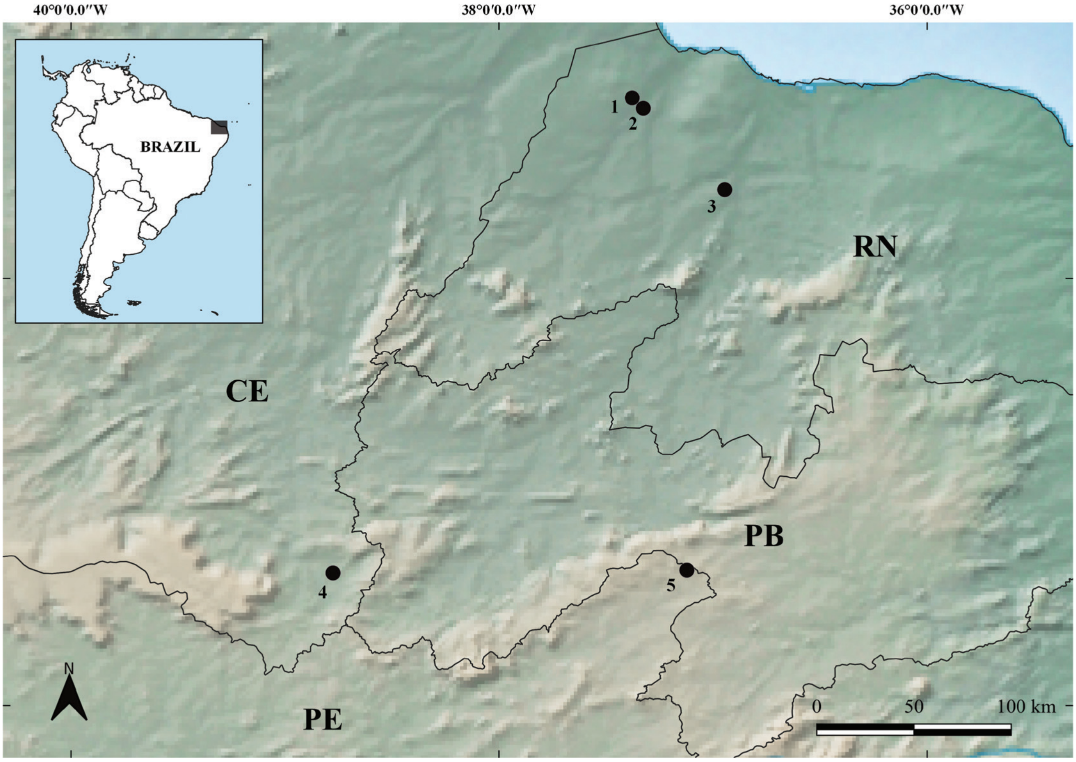

Figure 1. Localities of northeastern Brazil with *Lygodactylus klugei* communal nesting records: 1. Estação Experimental and 2. Campus Central of the Universidade Federal Rural do Semi-Árido, both in Mossoró municipality, Rio Grande do Norte state; 3. Floresta Nacional de Açu, in Assu municipality, Rio Grande do Norte state; 4. Locality in Mauriti municipality, Ceará state; and 5. Sítio Russa Mansa, in Itapetim municipality, Pernambuco state. Legend for Brazilian states: RN, Rio Grande do Norte; PB, Paraíba; PE, Pernambuco; CE, Ceará.

(*N* = 1), four (*N* = 1), six (*N* = 1), and eight eggs (*N* = 1) (Figure 2). Although this species is known for having a fixed clutch size of two eggs (Vitt 1986), we recorded three nests of *L. klugei* (37.5%) with a single egg and another nest with three eggs (12.5%). If these nests were created by young and/or small adult females, it is also possible that one of the eggs was preyed upon. However, given that the eggs of communal nests with three or more eggs (62.5%) were joined by the shells in different positions to each other (Figure 2), it seems unlikely that the nests observed with only one egg were the result of predation events on an individual egg. Other species in the genus *Lygodactlylus* produce clutches with two eggs that are often attached to each other (Glaw and Vences 1994, Bruse *et al.* 2005, Rodríguez-Prieto *et al.* 2010). The size of communal nests of *L. klugei* suggests the participation of two to four females per nest, which is similar to observations recorded for *Lygodactylus thomensis wermuthi* (Peters, 1881) in São Tomé and Príncipe in Central Africa. At least three females participated in egg deposition in those nests (Rodríguez-Prieto *et al.* 2010).

We observed nests during a rainy month (March), as well as during months with low or no precipitation (July and August) (Table 2),

Figure 2. *Lygodactylus klugei* communal nests in northeastern Brazil. Communal nesting with (**A**) eight eggs found in Mauriti, Ceará state, (**B**) three eggs in a cactus trunk in Itapetim, Pernambuco state, (**C**) four eggs in a tree trunk at the Estação Experimental, in Mossoró, Rio Grande do Norte state, and (**D**) six eggs found at the Floresta Nacional de Açu, in Açu, Rio Grande do Norte state.

reinforcing the notion that *L. klugei* has continuous breeding behavior, as observed by Vitt (1986). Egg features, such as hard eggshells and small clutch sizes (1–2 eggs), may be related to the continuous breeding behavior of this species throughout the year, which occurs in many species of gekkonids (Vitt 1986, Serrano-Cardozo *et al.* 2007, Anjos and Rocha 2008, Díaz-Pérez *et al.* 2017). Microenvironments selected for oviposition can provide suitable conditions for incubation in terms of moisture and temperature even in arid and semiarid environments like the Caatinga (Vences *et al.* 2004).

Most of the communal nests recorded in this

Table 1. Comparison of *Lygodactylus klugei* egg and neonate measurements from the present study, from Vitt (1986), and from other species in the genus. SVL = snout–vent length, NA = not available. Legend for localities: [a]Exu, Brazil, [b]Kafue Flats, Zambia, [c]Island of São Tomé, São Tomé and Príncipe, [d]Mount Namuli, Mozambique, [e]Lake Ihotry, Madagascar.

Species	Egg length (mm)	*N*	Egg diameter (mm)	*N*	Egg mass (g)	*N*	Neonate SVL (mm)	*N*	Neonate body mass (g)	*N*	Source
L. klugei	6.67 (Q1 6.49; Q3 6.89)	22	5.35 (Q1 5.17; Q3 5.51)	22	0.10 (Q1 0.08; Q3 0.14)	25	12.47 ± 0.31	5	0.04 ± 0.03	5	Present study
L. klugei	6.57 ± 0.06	94	4.48 ± 0.05	94	0.07 ± 0.002	94	14.00	94	0.04 ± 0.03	5	Vitt 1986[a]
L. capensis	6.40 ± 0.76	~24	4.70 ± 0.57	~24	NA			NA	NA	NA	Simbotwe 1983[b]
L. chobiensis	6.50 ± 1.22	~90	4.70 ± 0.73	~90	NA		15.17 ± 0.34	6	NA	NA	Simbotwe 1983[b]
L. thomensis wermuthi	6.00	16	NA	NA	NA		13.00	2	NA	NA	Rodrígez-Prieto *et al.* 2010[c]
L. regulus	7.7–8.00	NA	NA	NA	NA		16.80	NA	NA	NA	Portik *et al.* 2013[d]
L. verticillatus	4.50–6.70	13	3.00–5.25	13	NA		8–12	NA	NA	NA	Vences *et al.* 2004[e]

Table 2. Communal nesting features of *Lygodactylus klugei* found in four of the five study localities. Legend for Brazilian states: CE, Ceará, PE, Pernambuco, RN, Rio Grande do Norte. EE = Estação Experimental, FLONA-AÇU = Floresta Nacional de Açu.

Localities and dates	Number of eggs	Egg color	Substrate	Incubation time (days)
Mauriti, CE (20 July 2019)	8 (Figure 2A)	White (*N* = 6) and yellow (*N* = 2)	Tree trunk	32
Itapetim, PE (21 March 2018)	3	Yellow (*N* = 1), light yellow (*N* = 1) and grey (*N* = 1)	Cactus trunk (Figure 2B)	Not available
EE, RN (15 August 2018)	4 (Figure 2C)	White	Tree trunk (Figure 2C)	37 ± 14
FLONA-AÇU, RN (18 August 2018)	6 (Figure 2D)	White	Tree trunk	37 ± 14

study were found in tree trunks (Table 2), which is similar to nest microhabitats recorded by Vitt (1986). Individuals of this species are often active on tree trunks and branches of shrubs of different species in the Caatinga (Vitt 1986, Teixeira *et al.* 2013, Aximoff and Felix 2017, Silva *et al.* 2021) and less commonly observed in cacti, as found in this study.

Of the eight eggs observed in the Mauriti communal nest, two that were yellow did not hatch. Three of the neonates escaped from the terrarium. Therefore, the morphometric data was recorded for three remaining individuals in this locality. None of the eggs (with yellow, light yellow, and grey coloration) in the Itapetim communal nest hatched (Table 2). Thus, we used data of two neonates from one of the four eggs at the Estação Experimental communal nest was broken during handling; thus, we presented the measurements of 25 eggs.

Non-white eggs produced by this species did not hatch successfully in two different localities (Mauriti-CE and Itapetim-PE) (Table 2). No information about nonviable egg color was found in the literature for avian or non-avian reptiles. Nevertheless, this attribute could be explored in experimental studies about development/ incubation of oviparous species under different environmental conditions or in conservation projects that breed species in captivity with the aim of future re-introductions. Although the incubation period varied between 32 and 37 days in the laboratory (Table 2), the exact date of oviposition was not known. We cannot determine an accurate estimate of the incubation period for this species.

The median egg length, diameter and mass recorded here were similar to the measurements found by Vitt (1986) for a population in Exu-PE (Table 1). Additionally, the length and diameter of the eggs of *L. klugei* did not seem to differ greatly from other congeneric species found on the African continent (Table 1). Eggs with a length of approximately 6.5 mm may constitute an ancestral characteristic of the *Lygodactylus* genus. The mean neonate body size and mass recorded here were lower in relation to the population studied by Vitt (1986). Mean body size was also smaller than that of the three African species (*Lygodactylus chobiensis, L. thomensis,* and *L. regulus*) (Table 1).

Although Vitt (1986) did not record communal nesting in *L. klugei* in his long-term study, we recorded at least one communal nest in four relatively distant localities (between 62 and 290 km). This behavior could be more common than previously assumed for the species. Our findings about communal nesting and neonate morphometry of *L. klugei* expand the knowledge of reproductive ecology and behavior of *Lygodactylus,* contributing to the understanding of reproductive strategies of gekkonid lizards from highly seasonal dry environments, as the Brazilian semiarid region.

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SHORT COMMUNICATION

Seasonal pattern of basking activity in *Nerodia sipedon* (Serpentes: Colubridae) along a western Michigan lakeshore, USA

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Keywords: Basking, Diel activity, Environmental conditions, Northern Water Snake.

Palavras-chave: Aquecimento ao sol, Atividade diária, Condições ambientais, Serpente-aquática-do-norte.

The Common Watersnake, *Nerodia sipedon* (Linnaeus, 1758), typically basks close to the water's edge (Laurent and Kingsbury 2003, Burger *et al*. 2004, Stanford *et al*. 2010). In addition, *Nerodia* spp. often bask on structures overhanging the water (Hebrard and Mushinsky 1978), and *N. sipedon* readily use anthropogenic structures in urban area (Pattishall and Cundall 2009).

Environmental conditions can influence basking and activity in *N. sipedon*. *Nerodia sipedon* basked more frequently when there was little cloud cover (i.e., when it was sunnier) (Burger *et al*. 2004). Burger *et al*. (2004) found that *N. sipedon* basked when air temperatures were between 12 and 30°C and when water temperature was > 15°C. Observed diel variation in activity by *N. sipedon,* with basking occurring primarily early in the morning and late in the afternoon (Robertson and Weatherhead 1992, Ernst *et al*. 2012) with aquatic activity in the middle of the day (Tiebout and Cary 1987), may reflect the diel pattern of environmental conditions. For example, *Nerodia fasciata* (Linnaeus, 1766) and *N. taxispilota* (Holbrook, 1838) enter the water when the water temperature is greater than air temperature and aerially bask or leave the water when air temperature is higher than water temperature (Osgood 1970, Robertson and Weatherhead 1992).

Previous studies examining basking and activity of *N. sipedon* have been conducted in natural, relatively undisturbed marsh habitat (Robertson and Weatherhead 1992), in a national wildlife refuge (Ernst *et al.* 2012), and a canal in an urbanized area (Burger *et al*. 2004). Thus, these studies do not allow for a direct comparison in basking activity between habitats with varying levels of human influence in a single lake. Pattishall and Cundall (2008, 2009) found that *N. sipedon* make use of urban areas of a stream, and make extensive use of anthropogenic structures to bask but their movement and site fidelity differs between areas of the stream that vary in human influence.

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We examined the numbers of *N. sipedon* observed along the shore of a lake in suburban Kalamazoo County, Michigan, USA that has habitats ranging from basically natural to heavily human influenced. In particular, we evaluated the potential role of date, time of day, and environmental conditions (air temperature, wind speed, and sky conditions), as well as extent of human influence, on the number of *N. sipedon* observed. Our study therefore provides information on how human influence within a single lake may influence the basking activity of *N. sipedon*.

As part of a study of turtle basking behavior (Hoinville and Smith, unpubl. data), we established 26 survey stations every 100 m along the perimeter of Lake Hill'n Brook and Howard Lake (Figure 1) in Kalamazoo County, Michigan, USA (42.246226, -85.632203). Stations were used to facilitate the checking of the entire shoreline by a single observer in a concise time frame. Each survey station was an approximately 6 m stretch of shoreline. When observing each survey station, the observer was usually at least 10 m and often > 15 m from shore. Making observations at these distances from the shore appeared to minimize any disturbance of the snakes. These survey stations represented the range of available habitats and included sites with a variety of human influence (Figure 2). The majority of stations (16 of 26; 61.5%) were along sections of the shore where the shoreline

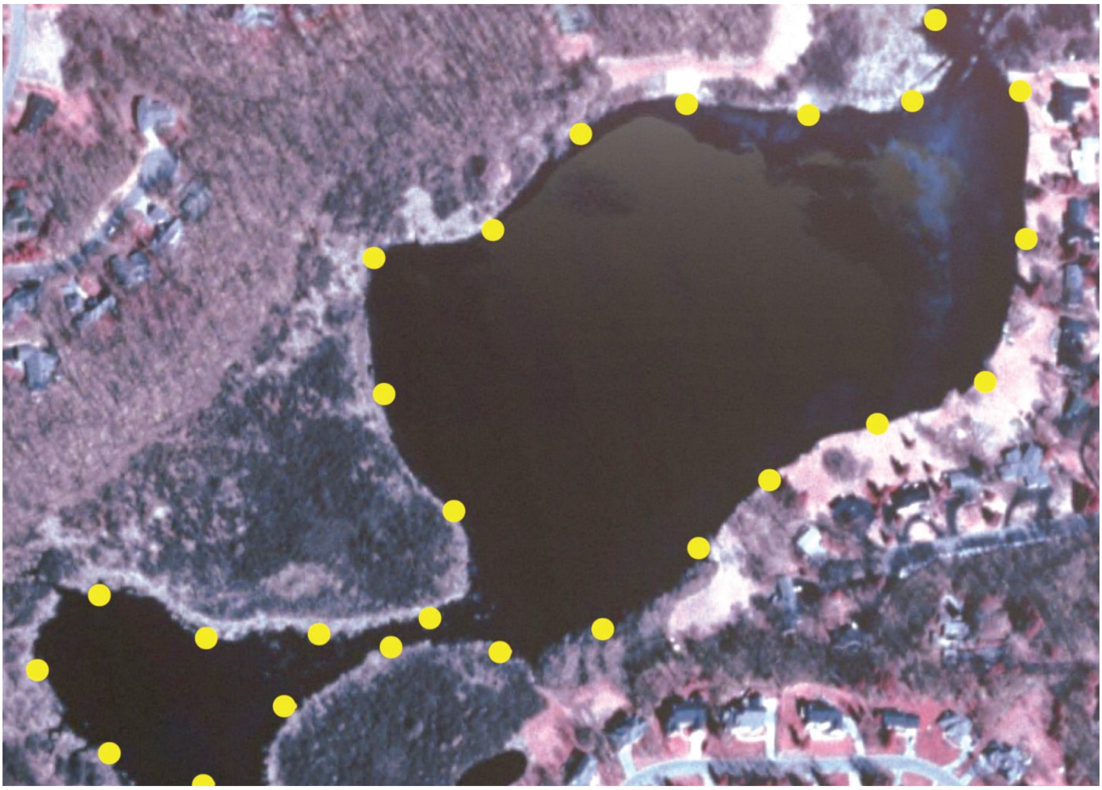

Figure 1. Map of survey stations (yellow dots) along the shoreline of Lake Hill'n Brook and Howard Lake in Kalamazoo County, Michigan, USA. Base map credit: United States Department of Agriculture, Natural Resources Conservation Service, published 20 May 2002.

Figure 2. Photographs of representative survey stations with differing levels of human influences (**A**: Low, **B**: Intermediate, **C**: High) along the shores of Lake Hill'n Brook and Howard Lake in Kalamazoo County, Michigan, USA. Photos: EJH.

was relatively natural, with at least 15 m of marsh, swamp, or forest separating the shore from any human influence (Figure 2A). Five of the 26 survey stations (19.2%) had intermediate levels of human influence, where the shoreline was natural but the station was adjacent to or near artificial habitats (e.g., lawn, artificial beach) (Figure 2B). The six remaining stations (19.2%) were along shoreline that had high level of human influence, where the shoreline was artificially built up with wooden railroad tie seawalls, artificial rock substrate, or metal seawalls and with lawns extending right up to the water (Figure 2C).

From 18 May to 17 July 2020, one of us (EJH) conducted surveys on 40 d (i.e., most weekdays unless limited by weather) and counted the number of *N. sipedon* basking at each station from a canoe. We assumed *N. sipedon* were basking since they were in locations and postures suggesting basking (see Hebrard and Mushinsky 1978, Laurent and Kingsbury 2003, Burger *et al.* 2004, Stanford *et al.* 2010). We began surveys four times a day: 09:00 h (N = 20), 12:00 h

(N = 33), 15:00 h (N = 37), and 19:00 h (N = 32). Due to safety considerations, we did not conduct surveys during high winds or storms. At the start of each survey, we recorded air temperature, wind speed, and sky conditions [sunny (sunny and partly sunny) or cloudy (cloudy or mostly cloudy)]. We did not record snakes observed outside the survey stations.

We used generalized linear models (binomial distribution, logit link) to examine the effects of date, date^2, time of day (as a categorical variable), air temperature, wind speed, and sky conditions on the total number of *N. sipedon* observed basking during a survey (i.e., number of snakes observed pooled across all survey stations) for each survey. We compared candidate models (see Appendix I for all candidate models examined) using AICc to determine the best models. In addition, we used a chi-square test to compare the observed number of snake observations made among the three levels of human influence relative to the number expected if snakes were using the stations at random with respect to the level of human influence. We used JMP Pro 15.2 (SAS Institute, Cary, NC) for the statistical analyses.

Over the course of the study we made a total of 38 observations of individual *N. sipedon,* with a range of 0–5 in any given survey, with snakes observed on 14 of the 40 d (35%) surveys were conducted. We provide the five best overall models in Table 1. The best overall model included only date and time of day. Indeed, date and time of day appeared in all five of the best models. Date^2 and air temperature appeared in two of the five best models. Wind speed appeared in one of the best models. In general, the greatest numbers of *N. sipedon* observed were found early in the study period from 17 May to 8 June, followed by a period of several weeks when no snakes were observed from 18 June to 17 July (Figure 3A). During the day, the greatest number of *N. sipedon* observed was in the morning at 09:00 h, with very few individuals observed basking at 19:00 h (Figure 3B).

We observed *N. sipedon* at the relatively natural survey stations much more than they would be expected by chance, and at stations with intermediate or high levels of human influence much less than by chance (Natural: 35 observed, 23.4 expected; Intermediate: 3 observed, 7.3 expected; High: 0 observed, 7.3 expected; χ^2_2 = 15.58, p = 0.0004).

The most dramatic result of our observations is the strong degree to which *N. sipedon* used the most natural shoreline habitat stations and the apparent avoidance of shoreline with even intermediate levels of human influence. In contrast, Pattishall and Cundall (2009) found *N. sipedon* used a wide variety of anthropogenic structures, even using urban areas of a stream more than natural areas. Burger *et al*. (2004) found that *N. sipedon* in an urban canal quickly entered the water when approached by humans.

Our observations on the daily pattern of *N. sipedon* activity are similar to those from other

Table 1. The five best models from the candidate generalized linear models examining the number of *Nerodia sipedon* observed basking during individual surveys along the shores of Lake Hill'n Brook and Howard Lake in Kalamazoo County, Michigan, USA.

Model	AICc	DAICc
Date + Time	46.375	-
Date + Time + Air	47.266	0.891
Date + Date^2 + Time	47.585	1.211
Date + Date^2 + Time + Air	47.757	1.382
Date + Time + WS	48.188	1.813

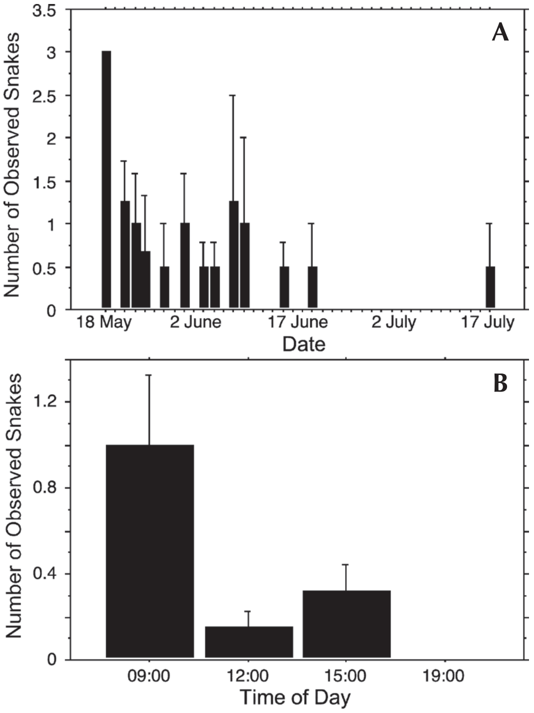

Figure 3. Mean ± SE number of *Nerodia sipedon* observed along the shores of Lake Hill'n Brook and Howard Lake in Kalamazoo County, Michigan, USA (**A**) each day during the study period, and (**B**) at each time of the day.

locations, with basking being most frequently observed early in the morning (e.g., Robertson and Weatherhead 1992, Ernst *et al*. 2012). Our results are also consistent with the observation that *N. sipedon* tend to shift to aquatic activity in the middle of the day (Tiebout and Cary 1987). This pattern is likely related to thermoregulation in *N. sipedon,* whereby body temperatures increased in the morning followed by constant temperatures for most of the day and declined in the evening (Brown and Weatherhead 2000).

We observed a peak in basking from late May to early June. This is slightly earlier than the observed seasonal activity of *N. sipedon* in Pennsylvania and Iowa that was highest in June and lasted from May to September (Klimstra 1958, Hughes *et al*. 2018). Meshaka *et al*. (2008) found *N. sipedon* under cover boards in northeastern Ohio most often in May and August. The observed seasonal pattern of basking activity by *N. sipedon* in our study may again reflect thermoregulatory pressures or opportunities. As the summer progresses, water temperatures likely increase, thereby potentially increasing the use of aquatic habitats relative to basking habitats (see Osgood 1970, Robertson and Weatherhead 1992, Burger *et al*. 2004). In addition, the higher numbers of observations in May and early June may be related to greater activity of *N. sipedon* during the breeding season, which typically occurs in May and June over much of its range (Bauman and Metter 1977, Mushinsky 1979, Aldridge 1982, Weatherhead *et al*. 1995).

Our results are consistent with Stevenson (1985) identifying diel and seasonal activity patterns as the most important behavioral mechanisms of body temperature regulation in terrestrial ectotherms. However, the presence of air temperature and wind speed in at least one of the five best models indicates environmental parameters may also be playing a role and may be indirectly included in both the time of day and day of the year variables. The model results emphasize the likely importance of thermoregulation driving the patterns of basking in *Nerodia sipedon* that we observed.

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Appendix I. *Candidate General Linear Models (Binomial distribution, Logit link) for the number of* Nerodia sipedon *observed along the shores of Lake Hill 'n Brook and Howard Lake in Kalamazoo County, Michigan, USA. Models are given in order of AICc. Date = day of year, Time = time of day, Air = air temperature, Sky = sky condition, WS = wind speed.*

Model	AICc	DAICc
Date + Time	46.375	-
Date + Time + Air	47.266	0.891
Date + Date^2 + Time	47.586	1.211
Date + Date^2 + Time + Air	47.757	1.382
Date + Time + WS	48.188	1.813
Date + Time + Sky	48.518	2.143
Date + Time + Air + WS	49.155	2.780
Date + Date^2 + Time + WS	49.284	2.909
Date + Time + Air + Sky	49.289	2.914
Date + Date^2 + Time + Air + WS	49.411	3.036
Date + Date^2 + Time + Air + Sky	49.569	3.194
Date + Date^2 + Time + Sky	49.792	3.417
Date + Time + Air + WS + Sky	51.226	4.851
Date + Date^2 + Time + Air + WS + Sky	51.288	4.913
Date + Date^2 + Air	52.313	5.928
Date + Air	54.934	8.559
Date	55.704	9.329
Date + Date^2	55.770	9.395
Date + Air + Sky	56.119	9.744
Date + Air + WS	56.718	10.343
Time + Air + WS	56.888	10.513
Date + Date^2 + WS	57.109	10.734
Date + WS	57.206	10.831
Air	57.314	10.939
Time + Air	57.482	11.107
Date + Sky	57.637	11.262
Date + Date^2 + Sky	57.867	11.492
Date + Air + WS + Sky	57.896	11.521
Air + WS	57.945	11.570
Time + Air + WS + Sky	58.931	12.556
Air + Sky	59.084	12.709
Date + WS + Sky	59.111	12.736

Appendix I. *Continued.*

Model	AICc	DAICc
Air + WS + Sky	59.458	13.083
Time + Air + Sky	59.514	13.139
Time + WS	59.628	13.253
WS	62.104	15.729
Time	62.944	16.569
WS + Sky	64.138	17.763
Time + Sky	65.118	18.743
Sky	66.570	20.195

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SHORT COMMUNICATION

Plastic ingestion by the Indian snakes *Ptyas mucosa* and *Coelognathus helena helena* (Serpentes: Colubridae)

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Keywords: Common Trinket Snake, Conservation, Indian Rat Snake, Plastic pollution.

Palavras-chave: Conservação, Poluição por plásticos, Serpente-rato-indiana, Serpente-*trinket*-comum.

Plastic pollution has become a critical environmental issue (Elena-Diana *et al.* 2016, Santos *et al.* 2021). Plastic is so harmful that some countries, including India, have imposed a ban on the use of plastic. Ingestion of plastics occurs in many taxa ranging from terrestrial animals, such as cows, to marine animals, such as turtles. In the present study, we report the consumption of plastic along with prey by the snakes *Ptyas mucosa* (Linnaeus, 1758) and *Coelognathus helena helena* (Daudin, 1803) from India, a novel observation of anthropogenic debris affecting wildlife.

Individuals were obtained by visual encounter while answering a rescue call. We measured snout–vent length in mm using string stretched from the snout to the posterior margin of the anal plate. The abdomen of *P. mucosa* was examined by X-rays, and *C. h. helena* was examined by feeding. No snakes were sacrificed or dissected.

Ptyas mucosa, the Indian Rat Snake, is widely distributed in Asia, is semi-arboreal, diurnal, and one of the fastest snakes (Wogan *et al.* 2021, Uetz *et al.* 2022). Although *Ptyas mucosa* is common and widely distributed according to the International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species, populations of *Pytas mucosa* are decreasing (Wogan *et al.* 2021). It inhabits both mesic and xeric habitats in montane, forest, coastal, open fields, and agricultural areas (Parmar and Tank 2019). The diet of *Ptyas mucosa* depends on age and habitat; juveniles feed on insects, reptiles, and frogs and shift to mammals, birds, fishes, amphibians, snakes, and other reptiles as they grow (Saha and Chaudhuri 2017, Chaudhuri *et al.* 2018).

Snakes usually do not feed on inanimate objects. Sharma *et al.* (2016) reported *Ptyas mucosa* feeding on an onion, a male contraceptive, a piece of cloth, discarded socks, and a polythene roll, indicating that this behavior could have been the result of some attractive chemical

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stimulus such as fish, rodent, human, or other mammalian odor. According to Sharma *et al.* (2016), human odor acts as a stimulus to attract snakes, but observations recorded in the present study conclude that the odor of rodents or other prey rather than human odor acts as a stimulus to snakes.

On 08 December 2014, we discovered a *P. mucosa* in the garden of an urban residence. The snake was captured near Big Bazar, Piplod-Magdalla (21°09'32" N, 72°46'16" E). Its total body length was 2072 mm, snout–vent length was 1472 mm, and mid-body circumference was 119 mm. The captured individual regurgitated five bottles containing pills along with five dead rat pups (Figure 1). Bottles were made of propylene plastic (70 × 20 mm in length and width), and the volume of each bottle was 8 ml. The snake was taken to a hospital where a radiologist performed an abdominal X-rays that determined no bottles remained in the snake's stomach.

The Common Trinket Snake, *Coelognathus helena helena,* known as Roop Sundari in the Gujarati language, is a medium-sized snake widely distributed in India (Uetz *et al.* 2022). It inhabits plains, hills with forest and plantations, leafy trees, and plants and bushes (Whitaker and Captain 2008). The diet of *C. h. helena* consists of small mammals, lizards, snakes, and frogs (Whitaker and Captain 2008). Deshmukh *et al.* (2018) reported consumption of shed skin and scavenging behavior (feeding on a discarded piece of chicken) by *C. h. helena*.

At 09:40 h on 28 September 2016, we rescued an individual of *C. h. helena* from ONGC Phase 1 [a residential colony for Oil and Natural Gas Corporation Limited (ONGC) staff], Magadalla, Surat, India (21°08'31" N, 72°45'02" E). This individual had a 152-mm long cassette tape extending from its anus (Figure 2). The total length of the individual was 1372 mm, the snout–vent length was 1166, and the mid-body circumference was 61 mm. The individual was collected and fed two wild mice in an attempt to cause it to expel the tape. After two days, on 01 October 2016, the snake egested a 7-m length of coiled tape. The individual was kept under observation for 24 hours and then released into its natural habitat.

Figure 1. *Ptyas mucosa* showing five plastic bottles and five newborn rats that were regurgitated by the snake. Photo by DSP.

Based on our observations, we hypothesized that a rat birthed a litter among discarded medicine bottles and that *Ptyas mucosa* ingested the bottles in addition to the young rats, likely because the bottles carried the odor of the juvenile rats. Deshmukh *et al.* (2017) reported the consumption of plastic bags by the Common Krait, *Bungarus caeruleus* (Schneider, 1801). They hypothesized that the bag was eaten because it carried the smell of meat. In this study, we also found a 7-m cassette tape ingested by *C. h. helena*. We attribute this ingestion as accidental by the individual. *Coelognathus h. helena* is a constrictor snake and may have ingested the tape simultaneously with prey. Another possibility could be that the snake ingested prey that had previously ingested the tape. We believe that these ingestions were not triggered by human odor because they were ingested along with prey. Our findings suggest that the regurgitation method (used for *P. mucosa*) and feeding method (used for *C. h. helena*) are the most efficient methods to expel anthropogenic debris. Our findings also show that banning

Figure 2. (**A**) Dorsal view of *Coelognathus helena helena* with a cassette tape emerging from the vent. (**B**) Ventral view of *Coelognathus helena helena* showing the tape emerging from the vent. Photo by DSP.

plastic, especially single-use plastic, is necessary for the protection and conservation of wildlife and the environment.

Acknowledgments.—We thank Mr. Dipak Patel for giving us access to his property for rescuing *Ptyas mucosa,* Mr. Vasudev Limbachiya for obtaining mice and feeding *Coelognathus helena helena,* and the Forest Department of Surat, Gujarat, for their logistic support.

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SHORT COMMUNICATION

New prey items of *Crotalus campbelli* (Serpentes: Viperidae) from Mexico

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Keywords: Campbell´s Dusky Rattlesnake, Coleoptera, Diet, *Sceloporus bulleri, Sceloporus unicanthalis, Stenopelmatus.*

Palabras claves: Cascabel oscura de Campbell, Coleoptera, Dieta, *Sceloporus bulleri*, *Sceloporus unicanthalis*, *Stenopelmatus*.

Palavras-chave: Cascavel-escura-de-campbell, Coleoptera, Dieta, *Sceloporus bulleri*, *Sceloporus unicanthalis*, *Stenopelmatus*.

Campbell´s Dusky Rattlesnake (*Crotalus campbelli* Bryson Jr., Linkem, Dorcas, Lathrop, Jones, Alvarado-Díaz, Grünwald, and Murphy, 2014) is a small montane rattlesnake that is part of the *Crotalus triseriatus* group (Bryson *et al.* 2014). *Crotalus campbelli* is endemic to western Mexico and occurs in the states of Colima and Jalisco, inhabiting rocky open areas within pine-oak and cloud forests (Bryson *et al.* 2014, Heimes 2016). The species is not included in the NOM-059-SEMARNAT-2010; thus, it does not receive protection by the Mexican government (SEMARNAT 2010) and is not considered by the IUCN Red List of Threatened Species, it is Highly Vulnerable according to its Environmental Vulnerability Score of 17 (see Johnson *et al.* 2017). Information about its natural history is scarce, and only one unidentified rodent has been reported as part of its diet under the synonym of *Crotalus triseriatus* (Wagler, 1830) (Mociño-Deloya *et al.* 2014). Here we report new prey items from specimens collected during field surveys.

We conducted field surveys in 2019–2020 in thigh elevations of Sierra de Manantlán, Colima, Mexico (19.395085° N, 103.896438° W; WGS84; 2200 m a.s.l.) in order to locate individuals of this species. The habitat is characterized by the presence of pine-oak forest with outcrops of karst rock (Padilla-Velarde *et al.* 2006). We found four *C. campbelli.* One adult male was found during a predation event while consuming a lizard and was not disturbed or captured. Two specimens, a juvenile female and a newborn male, were captured alive, handled with tongs and herpetological tubes and gently palpated in

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search of stomach contents or scats. The juvenile female was released in the same place of capture, and the newborn male was euthanized by injecting sodium pentobarbital, 60–100 mg/kg intracelomically. One newborn female was found dead, and a mid-ventral incision was made to determine the presence of food remains in the gut. We measured the snout–vent length (SVL; ± 1 mm) and tail length (TL; ± 1 mm) with a measuring tape, determined sex by cloacal probing or by everting the hemipenes, and recorded date, locality, number, and identity of prey items. We calculated the weight ratio (WR) by dividing the mass of the prey by the mass of the snake. The collected snakes were fixed with 10% formalin, and then housed, along with their prey remains, in individual containers with 70% ethanol. Scats were deposited in 70% ethanol and both specimens and scats were deposited in the herpetological collection of the Universidad Autónoma de Aguascalientes (UAA-REP). Dorsal scales of lizard prey were compared with scales of lizards from UAA-REP that were from the same locality where the rattlesnakes were found. Arthropods were identified based on the characters of the remains found; for Jerusalem crickets, we followed Weissman *et al.* (2021). The known distribution of prey species was also used as a criterion for species-level identification. The data reported for both the snakes and their prey follow Maritz *et al.* (2021).

The four specimens of *Crotalus campbelli* from field surveys were examined. An adult male *C. campbelli* ca. 400 mm in total length was found on 31 July 2004 at 10:00 h while consuming an adult female *Sceloporus bulleri* Boulenger, 1895 (Buller's Spiny Lizard) headfirst, with the tail apparently freshly autotomized (Figure 1). No additional data were collected in order to avoid interrupting the predation event. The sex of the rattlesnake was determined based on the relatively lighter coloration and longer tail; the sex of the lizard was determined by the absence of enlarged post-anal scales and the absence of blue coloration on the abdomen and thighs (vs. present in males). We identified the lizard as *S. bulleri* because only this species of the *Sceloporus torquatus* group occurs in the area (Reyes-Velasco *et al.* 2020). We estimated the weight of the male *C. campbelli* to be approximately 45 g based on a specimen of similar size of the closely related *Crotalus pusillus* Klauber, 1952 (UAA-REP 875). The female *S. bulleri* was at least 95 mm in SVL and 70 in TL, with an estimated weight of 35 g based on a female *S. bulleri* of similar size (UAA-REP 835). These weight estimates yielded a WR of at least 0.78.

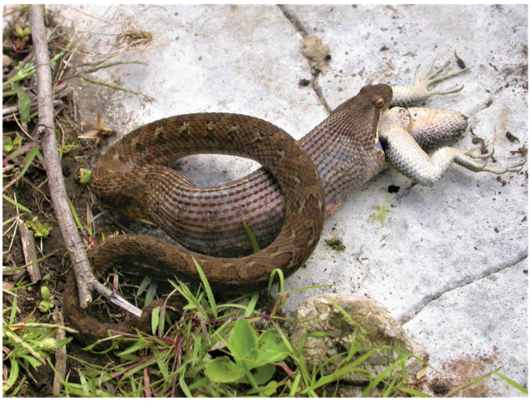

Figure 1. A female lizard *Sceloporus bulleri* being preyed upon by an adult male *Crotalus campbelli,* found in an open area with karst outcrops in pine-oak forest in the Sierra de Manantlán, Colima, Mexico. Photo: JMJ.

Another juvenile female *C. campbelli* was found on 07 July 2019 (UAA-REP 831). The snake measured 353 mm in SVL and 34 mm in TL. The scat contained scales belonging to *Sceloporus unicanthalis* Smith, 1937 (Southwestern Bunchgrass Lizard), the only species of the *Sceloporus scalaris* group that occurs in the area (Reyes-Velasco *et al.* 2020). The snake was released at point of capture and only the scat was collected. On 26 September 2020 two additional specimens of *C. campbelli* were found at the same locality: a newborn female (UAA-REP 778; 171 mm SVL, 15 mm TL) was found dead at 14:20 h, probably killed by local people. After dissection, we found a *Stenopelmatus faulkneri* Weissman, 2021 (Faulkner's Jerusalem cricket) partially digested in the intestines and ingested headfirst. The second specimen was a newborn male (UAA-REP 830; 171 mm SVL, 16 mm TL) found under a log at 13:10 h. Following palpation, a scat was obtained that contained the partially digested remains of an unidentified coleopteran larva.

These are the first reported records of consumption of *Sceloporus bulleri, Sceloporus unicanthalis, Stenopelmatus faulkneri,* and coleopteran larvae by *Crotalus campbelli.* In addition, *S. faulkneri* represents a new state record for Colima (see Weissman *et al.* 2021). The new prey of *C. campbelli* reported here, along with the unidentified rodent reported by Mociño-Deloya *et al.* (2014), are the only known prey. These results indicate that adults of *C. campbelli* feed on lizards and small mammals, while juveniles may also consume arthropods. Other closely related species [*Crotalus aquilus* Klauber, 1952, *Crotalus lepidus* (Kennicott, 1861), *Crotalus ravus* Cope, 1865, and *Crotalus triseriatus*] are known to consume vertebrates such as lizards (mainly *Sceloporus* spp.) and small mammals as adults, and arthropods as juveniles (Klauber 1972, Holycross *et al.* 2002, Mendoza-Hernández *et al.* 2004, Güizado-Rodríguez *et al.* 2016, Mociño-Deloya 2016).

The ingestion of crickets (Orthoptera) by rattlesnakes has been documented in *Crotalus aquilus, Crotalus pusillus* (Armstrong and Murphy 1979), *Crotalus ravus* (Sánchez-Herrera 1980), and *Sistrurus miliarius* (Linnaeus, 1766) (Mitchell 1903); some may have been *Stenopelmatus.* Specific reports of rattlesnakes consuming *Stenopelmatus* spp. include *C. ravus* (Campbell and Armstrong 1979), and *C. triseriatus* (Klauber 1972). The presence of arthropods in the gut of rattlesnakes has typically been regarded as secondary ingestion because the gut contents also contained vertebrate remains (Holycross *et al.* 2002, Mociño-Deloya 2016). However, because the arthropods were consumed by newborn *C. campbelli* in this study, it is confirmed that they were prey items, not secondary ingestion.

The WR estimated for the adult male *C. campbelli* and its *S. bulleri* prey is fairly high, which may induce significant costs such as long handling and processing time, vulnerability to predators, or even mortality (Avila-Villegas *et al.* 2005, Loughran *et al.* 2013). However, the energetic benefits of a large prey may outweigh the cost of subduing and ingesting it. The high WR found in this study is consistent with an emerging pattern for vipers. It is well known that vipers occasionally eat large prey with a high WR, even higher than WR = 1.0 (Greene 1983, 1992, Loughran *et al.* 2013).

We recommend continuing to document the diet of this and other poorly studied pitvipers (prey preference, ontogenetic change, sexual differences, and seasonal changes in diet) to obtain a better understanding of their natural history. Additionally, these data may help generate better conservation strategies for species of pitvipers.

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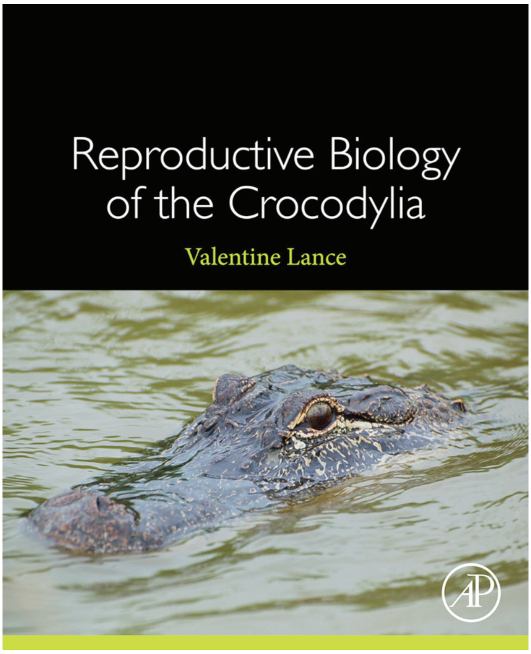
Editor: Ross D. MacCulloch

Lance, V. 2022. **Reproductive Biology of the Crocodylia.** Academic Press, Elsevier. 157 pp.

Val Lance's new book, *Reproductive Biology of the Crocodylia,* is more than a treatise on crocodilian reproduction *per se*. The 12 chapters, all well-written and easily read, track the development of knowledge about crocodilian reproduction, and the research subjects in which Val Lance has actively participated. Some research directions proved fruitful, and others not, but all have added to the steadily building knowledge base on crocodilian reproduction, and the identification of knowledge gaps where future research is likely to be rewarding. There is a strong element of research detective investigation throughout the book, which is an integral part of the motivation of dedicated researchers.

For context, Chapter 1 deals with the phylogeny and fossil history of crocodilians, and Chapter 2, with the history of reproductive research (Chapter 2), which gives true credit to pioneering researchers, going back to the times of Herodotus (484-425 BC). Fascinating and insightful, in these times, when historical research is often ignored. All aspects of crocodilian reproduction are dealt with comprehensively (Chapters 3 to 9), after which the focus shifts to how knowledge of crocodilian reproduction is linked to real life problems and opportunities, such as crocodilian farming (Chapter 10), environmental contamination (Chapter 11) and conservation (Chapter 12). For the people around the world whose lives are intertwined with crocodilians and research into them, it is a "must read" book.

Val Lance started researching alligator reproduction in Louisiana (1979), at a time when crocodilian research was starting to build globally. Virtually all wild populations had been depleted from unregulated and unsustainable historical harvests, yet knowledge of population dynamics, which is needed to improve conservation, was rudimentary. The 1970s and 1980s saw basic knowledge about crocodilians increase exponentially, with key researchers sharing their information, results and advice freely. The IUCN-SSC Crocodile Specialist Group (CSG) created an umbrella for information exchange, as it does today. But in those early days many aspects of crocodilian reproductive biology were poorly understood. Val Lance's pioneering research at that time, filled many of the gaps with which researchers were struggling. The extensive knowledge he and others have gained on crocodilian reproduction, and the enthusiasm and commitment of researchers, is addressed well in *Reproductive Biology of the Crocodylia.*



Life history parameters and ecology (Chapter 3), is more than the title suggests. It discusses issues such as biased sex ratios, multiple paternity and nest site fidelity, along with crocodilian anatomical and biochemical traits that are unique and in need of targeted research. The role played by fat bodies and peritoneal canals is still known superficially. Likewise, the endocrine system and history of research into it, identifies major findings and gaps in current knowledge. In addressing growth, sexual maturity and senescence (Chapter 4) it is clear that as in turtles, the size and age at which maturity is reached vary geographically, and appear to be related to climate, food availability, growth rates and social triggers. The mechanism that control adaptability about when maturity is reached, in different contexts, remains unclear.

The male reproductive system is described (Chapter 4), with the single phallus, long recognised as different to the dual hemipenes of snakes and lizards. For most crocodilians (Chapter 5) there is relatively little information on testes size and its seasonal cycling outside of American alligators. The timing of their courtship is triggered by changes in ambient temperature, rather than photoperiod, which are more extreme for alligators than most crocodilian species. The scattered information available suggests other environmental cues may be used by other species in different climatic regimes.

The female reproductive system is similar to birds, but with both left and right ovaries present and functioning (Chapter 7). The vast literature on ovulation and egg production in commercially valuable poultry, such as chickens, stands in stark contrast to our limited understanding of ovulation and egg formation in crocodilians. But what we do know is well described here. Unlike birds, that lay eggs one at a time, the whole clutch of crocodilian eggs is held within the oviducts until laying. In alligators, calcium is withdrawn from bones for shell secretion, and replaced after laying. Courtship behavior may be what stimulates ovulation. Sperm storage occurs, but it is not known how long viable sperm can be held in the oviducts. Secretions from paracloacal and gular glands are associated with courtship, but their role is not understood. Nesting is described, along with the evolutionary significance of the amniotic egg, the relationships between clutch size and female size, and the history of research into most aspects of nesting.

The discussion of temperature-dependent sex determination (TSD) in crocodilians (Chapter 9), tracks its discovery in the late 1970s and explains a series of experiments to try and better understand how it operates in crocodilians. Although with alligators TSD is clearly capable of producing strongly biased sex ratios in nests, and does so, it does not as yet explain the strongly female-biased sex ratios in juveniles and subadult alligators in the wild.

The treatment of crocodilian farming (Chapter 10) describes the history of keeping crocodilians in captivity, which has been done for thousands of years. The history of alligator farming in the United States of America is comprehensive, and the problems encountered with captive breeding remain difficult to understand. Relative to many crocodilian species, captive breeding of alligators, on a commercial scale, has been difficult, and considerable research aimed at understanding why has been carried out. The problems seem to be more associated with malfunctioning of the female reproductive processes malfunctioning than with the males, although effects of ambient temperatures on spermatozoa survival cannot be discounted. Regardless, alligator farm production has switched largely to ranching (collecting wild eggs), which has proved totally sustainable. Captive breeding is one of a number of areas where alligators seem to differ from many crocodile species, although the reasons why are unclear.

That disruption of reproduction in alligators and other crocodilians can be caused by environmental pollution (Chapter 11) has been well established with alligators, in some locations, and may be implicated with die-offs of both Indian gharials and Nile crocodiles. The discussion of this issue, along with insights into heavy metal concentration in alligators is both valuable and informative.

Much of Val Lances work has been done in association with programs in which crocodilians are being managed and used sustainably for economic benefit. Hence his insights into conservation (Chapter 12) are both pragmatic and realistic. The commercial benefits derived from sustainably using crocodilians — at least the species that have commercial value — can be used to generate incentives to conserve. Trade in skins is now mostly legal and regulated nationally and internationally, and is not the significant threat it was in the 1960s. Val Lance is correct in identifying habitat loss and spreading urbanisation as a far more insidious threats to many wild crocodilian populations than is trade.

In overview, *Reproductive Biology of the Crocodylia* is a rather unique book, directed at both researchers and crocodilian enthusiasts. It tracks Val Lance's personal interest and research into alligators, his extensive travels and research into other crocodilian species, his obvious commitment to historical research and to the reality that scientific research proceeds from corrected error to corrected error — only occasionally making a giant step forward. Of equal importance are the many areas identified where there remain significant research opportunities. The book is both a good read and an important contribution to our knowledge on crocodilian reproduction.

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OBITUARY

William E. Duellman (1930–2022)

His endless study and legacy on the Ecuadorian amphibians

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William Edward Duellman (Bill) passed away on February 25, 2022, at age 91. Two years earlier, in December 2019, Bill described a hylid frog—hylids and hemiphractids were his most loved amphibians—in a publication in the Brazilian journal *Phyllomedusa* (Duellman 2019). This new yellow-eyed Colombian spiny-backed treefrog was named as *Osteocephalus omega,* and its specific epithet—the last letter in the Greek alphabet—was intended to call attention to his realization that this was going to be the last description he would write. This meticulous publication (having him as the sole author) was the epilogue of his species descriptions during a long, multifaceted life dedicated to the systematics, evolution, ecology, morphology, and natural history of amphibians, and the building of one of the largest herp collections and herpetological academic programs in the world at The University of Kansas (KU).

Bill´s biography was written in part and with many details in his book *Herpetology at Kansas: A Centennial History* (Duellman 2015). However, the efforts to write his complete biography, including his strengths and weaknesses, and to recover the essence and legacy of Bill, one of the most influential and prolific herpetologists of this era, is a pending issue. But given that Bill´s life was closely tied to the Neotropics, and specially to Ecuador, Peru, and Central America, we write this essay to briefly summarize and highlight a few aspects of his monumental contribution, especially as related to Ecuadorian amphibian research which we (as his students) partially witnessed and in which we participated.

The numbers are unreal! Bill described or co-described 252 (currently recognized as valid) species of frogs from the Neotropics (93 from Ecuador), a monumental task for a scientist! He started publishing at a young age; Bill was 17 years old when he co-authored his first paper, a range extension of Kirtland's snake (Wood and Duellman, 1947).

The first species that he described was the Peters' Shiny Peeping Frog (*Tomodactylus petersi;* now in the genus *Eleutherodactylus*) from Mexico (Duellman 1954). Sixty-five years later, in 2019, Bill completed, on his own terms, his contributions on new species, with the description of *Osteocepahlus omega*. By no means, however, did he intend to finish his writing, despite his advanced age and acute pain caused by some twisted sacral vertebrae. On the contrary, he was in a fresh dawning of a herpetologist and fully committed to the *Encyclopedia of Ecuadorian Amphibians,* an opus on which his extraordinary intellect, passion, and heart were placed. Two weeks before passing away, Bill met online with Patricia A. Burrowes, Joseph R. Mendelson, and Ignacio de la Riva (former graduate students and postdoc) at an intensive care unit at the hospital, and he told them that he would soon be going home because he had to finish the work of the amphibians of Ecuador. Unfortunately, his wish did not materialize and he could not continue with the encyclopedia. Nonetheless, most of his contributions were already in place and the opus will be published soon.

Bill´s activity in Ecuador could be divided in two major periods, the first in which he was directly working in Ecuador from 1966–1990, when he and his KU students carried out field work, and another one from 1988–2022 when he and Linda Trueb (his wife and scientific partner) mentored Ecuadorian students, specialized in systematics, evolutionary biology, and ecology at KU, and continued publishing on Ecuadorian frogs.

Bill´s first period began in 1966, when his taxonomic research and resulting publications already included Ecuadorian frogs. For this first paper, on the Neotropical genus *Smilisca,* he examined Ecuadorian specimens of *S. phaeota* deposited at several museums in the U.S., and it was published by the Museum of Natural History of the University of Kansas (Duellman and Trueb, 1966). Next would be a paper that appeared in 1968 in the U.S. journal *Herpetologica,* in which Bill reviewed the taxonomic status of some Americas hylid frogs, among them the Ecuadorian *Trachycephalus coriaceus,* based on specimens from Limoncocha (Duellman 1968). Then, in 1969, three consecutive papers dealt with the Ecuadorian frogs: *Agalychnis buckleyi* (Duellman 1969a), *Atelopus ignescens*, *A.* sp. (*spumarius* at that time) (Duellman and Lynch, 1969), and *Dendropsophus carnifex* (Duellman 1969b). The latter was the first new species described by Bill from Ecuador. These initial studies were followed by an explosion of research and publications about Ecuadorian taxa. During his life (between 1968 and 2020), Duellman published 73 titles that included Ecuadorian frogs of a total of 386 herp publications; 68 of the 73 were journal publications, whereas the others were components in five of his major books: *The Hylid Frogs of Middle America* (1970), *The Biology of an Equatorial Herpetofauna in Amazonian Ecuador* (1978), *The South American Herpetofauna* (1979), *Patterns of Distribution of Amphibians: A Global Perspective* (1999), *Marsupial Frogs: Gastrotheca & Allied Genera* (2015). Also, in seven of the journal publications, he authored (1) or coauthored (6) with Ecuadorian researchers.

Bill´s field work in Ecuador started in November 1966, when he was 36 years old. With this trip, Bill began his lifelong journey related to Ecuador, toward which he focused a large portion of his professional life. He flew from Miami to Quito and then traveled, for the first time, to the Amazonian rainforest, specifically to Santa Cecilia, on the banks of Río Aguarico, in Sucumbíos Province. The forest was virgin or only slightly disturbed; it was located at 340 m above sea level and right on the Equator. In a prolog of a photography coffee table-book of Ecuadorian frogs, he described his experience at Santa Cecilia as follows:

> *"As darkness fell, I donned my boots and a headlamp and walked along a trail in the rainforest. Everywhere I looked I saw frogs of all sizes, shapes and colors—big green frogs that made a soft cluck call, large brown frogs with acute snouts that had a laugh-like call, and many kinds of small yellow frogs, some of which had red feet. These were only the tree frogs. By day on the ground there were various kinds of toads and poison-dart frogs. And then there was the frog with a long, fleshy nose and what seemed like horns on its head. Had I died and gone to heaven inhabited by the world's greatest diversity of frogs? No, I was in the Oriente* (Amazonia) *of Ecuador."* (Duellman 2009).

In this first trip of 11 days, he collected 191 specimens of amphibians and reptiles at Santa Cecilia and Limoncocha adding to his 30,933 field series of amphibians and reptiles he had collected before in U.S., Mexico, Guatemala, El Salvador, Honduras, Costa Rica, Nicaragua, Panama, and Venezuela. Bill would return to conduct field work in the Lago Agrio region (Santa Cecilia, Limoncocha, Puerto Libre) in 1967, 1968, 1969, 1971, and 1972. At that time the oil industry in the Ecuadorian northern Amazonian had just begun its activities, and deforestation along with some of the worst oil spills in world history would shortly follow (Kimerling 1993). Nonetheless, the virgin equatorial rain forest at Lago Agrio and surroundings in Ecuador offered Bill a unique opportunity to lead and conduct inventories and ecological studies, and to document natural histories, sometimes walking behind or stopping bulldozers that were clearing the forests. At Santa Cecilia, one of his graduate students, Martha L. Crump, undertook an analysis of the ecology and reproductive modes in a tropical anuran community with the assistance of John E. Simmons (the renowned collection manager at KU). Bill commented on his student Marty *"What a significant addition (to the field team) that turned out to be!"* Marty is now one the main authorities on amphibian awareness and conservation, having written books such as *In Search of the Golden Toad* (2000) and *Extinction in Our Times: Global Amphibian Declines* (Collins and Crump 2009).

Numerous papers were written by Bill, John D. Lynch, and others, describing new species from this region, but the completion was reached with the publication, in 1978, of his opus entitled *The Biology of an Equatorial Herpetofauna in Amazonian Ecuador*. In this study, he revealed a record of the greatest diversity of herps for a single site in the world and provided novel information on the ecology and natural history of numerous species. He also challenged what was the rule (mostly from the avian works by Robert MacArthur) at the time in community ecology. Duellman emphasized: *"Herpetological communities in aseasonal tropical forests are not structured in the same way as bird communities. The models generated from bird communities do not generally apply to herpetological communities."* This landmark monograph was well received and soon began to be an obligatory lecture for anyone interested in herps, natural history, and tropical communities. This monograph and his impressive book *Biology of Amphibians* (Duellman and Trueb 1986) inspired several Ecuadorian herpetologists, among whom was Coloma, who later undertook graduate studies in Systematics and Ecology at KU, with Bill as his advisor.

Bill carried out a total of 11 fieldwork trips to Ecuador (1966, 1967, 1968, 1969, 1970, 1971, 1972, 1974, 1975, 1984). They varied in length from a short one in 30–31 January 1971 to the longest one from 6 March–18 May 1975. This field work provided Bill with the opportunity to travel throughout much of Ecuador, including the lowlands of the Pacific coast, the cloud forest of the Andes (western and eastern slopes), the highlands, and the Amazonian lowlands, and collect 6981 specimens of amphibians and reptiles. Needless to say, he collected for the first-time hundreds of species new to science. For example, in just 3 days on 20, 21, and 23 October 1971, after walking at the margins of the Azuela River in Napo Province, in the cloud forest of the Amazonian slopes of the Andes, Bill (with Simmons, Collins, and MacBryde) found 12 new species of frogs (4 centrolenids, 1 bufonid, 2 dendrobatids, 5 *Pristimantis*).

During his field work, Bill was accompanied mostly by his graduate students and some colleagues: Henry S. Fitch, William G. Saul, Linda Trueb, Martha L. Crump, Stephen Edwards, James W. Waddik, Werner C. A. Bokermann, Ildefonso Muñoz B., Thomas H. Fritts, Charles F. Walker, Sandy Echternacht, Bruce MacBryde, Joseph T. Collins, John E. Simmons, Dana T. Duellman (daughter), Alan Savitzky, Patricia A. Burrowes, and David M. Hillis. As part of his and KU's focus on Ecuador, Bill mentored additional KU students who participated in his fieldwork in Ecuador until 1990. Among them were John D. Lynch, David C. Cannatella, Richard Montanucci, John J. Wiens, and David A. Kizirian, who did collecting trips by themselves.

The KU graduate students mentioned above currently are widely recognized in the herpetological world and the contributions of most of them to the knowledge of Ecuadorian herps have been immense. Their studies were summarized by Bill (Duellman 2015), and herein we briefly highlight the taxonomic contributions of Lynch, Edwards, Cannatella, and Hillis. Lynch's field work in Ecuador added 8410 specimens to the KU collection and described or co-described the amazing number of 116 new amphibian species from Ecuador. Bill and Lynch's combined efforts resulted in seven titles, including three of the most important and classic studies, one on glassfrogs (Centrolenidae) (Lynch and Duellman 1973) and two on cutín frogs *Eleutherodactylus* (most of them currently under *Pristimantis*) (Lynch and Duellman 1980, Lynch and Duellman 1997), in which they jointly described numerous new species to science. Bill also supervised Cannatella's Master's thesis on the systematics of the *Phyllomedusa* (currently *Agalychnis*) *buckleyi* group work in Ecuador. Cannatella has published descriptions of 13 species of Ecuadorian frogs, and has continued a strong research and academic cooperation program on dendrobatids and *Engystomops* with Ecuadorian partners during the 2010s, extending Bill´s legacy to his contemporary graduate students to this day. Another one of Bill´s students who did fieldwork in Ecuador was Edwards, who undertook a phenetic taxonomic revision of the Neotropical genus *Colostethus* (currently under *Allobates, Aromobates, Colostethus, Ectopoglossus, Hyloxalus, Epipedobates, Leucostethus, Mannophryne, Paruwrobates, Rheobates, Silverstoneia*), in which he recognized 19 species as new, some of them from Ecuador. He published two papers about Ecuadorian frogs, and most of his dissertation research (Edwards 1974) remained unpublished before Bill, Lynch, Enrique La Marca, Juan A. Rivero, and Coloma began to undertake such difficult tasks. In 1988, Bill suggested that Coloma review the Ecuadorian *Colostethus* for his Master's and enthusiastically supported additional field trips. In the same year Bill described (with Lynch) an Ecuadorian species from Cordillera de Kutukú, in which they expressed their frustration in the etymology paragraph as follows:

> *"The specific epithet is Latin meaning irritating or rasping. We use the name in reference to the exasperation endured for nigh onto a decade and a half by students of neotropical frogs awaiting the publication of a revision of* Colostethus *by Stephen R. Edwards."* (Duellman and Lynch 1988).

The completion of Bill´s travels to Ecuador and the beginnings of a new stage in Peru was marked by his 3-month field work on marsupial frogs in the Ecuadorian Andes in 1984, in which he was accompanied by David Hillis (among others). Hillis spent a memorable time with Bill, collecting and studying frogs in the Ecuadorian Andes. Hillis would remark:

> *"He lived and breathed herpetology. In my mind, I see him with a headlight on, catching frogs."*

Bill and Hillis would publish two seminal systematic papers, one on frogs of the *Hyloscirtus larinopygion* Group (Duellman and Hillis 1990) and other paper describing three new species of marsupial frogs, and resolving taxonomic problems, and their phylogenetic relationships (Duellman and Hillis 1987). When Bill began his journey of discovery of *Gastrotheca* in the Andes in 1967, only two species of biphasic marsupial frogs (*G. riobambae* and *G. lojana*) were known from the Ecuadorian Andes, and four monophasic species (*G. guentheri, G. plumbea, G. testudinea,* and G. *weinlandii*) were known from Ecuador. Currently, 20 species are known and such a task took him about half of a century. After his paper with Hillis, in 2015, he published his major book about the marsupial frogs on which the Ecuadorian species were included (Duellman 2015). He also contributed, along with Ecuadorian researchers, studies on tree frogs (Duellman and Coloma 1993, Ron *et al.* 2018), endangered harlequin frogs (Ron *et al.* 2003, Coloma *et al.* 2007, 2010, Guayasamin *et al.* 2010), and the description of additional

Andean marsupial frogs (Carvajal-Endara *et al.* 2019).

By the end of his *Gastrotheca* and Andean Ecuadorian trip, he gave a somewhat emotional lecture in an event that filled an auditorium in Quito at Pontificia Universidad Católica del Ecuador (PUCE) on April 3, 1984. In his lecture, entitled "Los Batracios Andinos: 20 años de estudios y los prospectos para el futuro," he referred to the unparalleled wealth of frogs of Ecuador. He went further and to complain about the difficulties and bureaucratic frustrations of obtaining collection permits for research—paradoxically, a similar lament still is shared by Ecuadorian scientists. As an epilogue of his talk, he said with remarked emphasis, the following:

> *"Interesting and necessary research could be done here in the parks of Quito. And I have a question. Where are the Ecuadorian, Peruvian, and Bolivian herpetologists? The richest region in the world herpetologically, and there are no herpetologists here."* Translated from Spanish (hear the original audio at: http://www.anfibiosecuador.ec/recursos/audio/DuellmanTalk-PUCE_3April1984.wav. Courtesy of Guillermo Paz-y-Miño).

In the Ecuadorian nationalistic mood of that time, Bill´s reclaim was not necessarily well received, especially by the local bureaucracy and some of the biologists. Anyway, that day, he would not know that in the auditorium was the seed of the first Ecuadorian herpetologists: Ana Almendáriz C. and Luis A. Coloma. The former was in charge of the Escuela Politécnica Nacional herp collections since 1983 and the latter has been making collections for Ecuadorian museums since 1982. Bill also could not imagine that 4 years later, Coloma would be his and Linda´s first Ecuadorian graduate student at the University of Kansas. In fact, Coloma arrived in KU in January 1988 and completed his MA in 1991 and PhD in 1997, after conducting systematics studies on *Colostethus* and *Atelopus*, and then he was followed by other Ecuadorians: Santiago R. Ron (1996–1998, MA), Omar Torres-Carvajal (1999–2005, MA and PhD), and Juan M. Guayasamin (2001–2007, MA and PhD).

When Coloma arrived in Lawrence, Kansas, in January 1988 he stayed a few days at Bill and Linda´s house and he was received with welcoming and open arms. He recounts this experience as follows:

> *"After visiting the amazing herp division facilities and museum and being presented to the herp people therein I went to his house. As darkness fell, after dinner I walked downstairs to his and Linda´s large office. Everywhere I looked I saw hundreds of reprints and books of all sizes, shapes and colors—the big opus of Biology of Amphibians, the two volumes of Hylid frogs of Middle America, the huge volume of the Amphibian Species of the World (promoted by him and edited by his student Darrel R. Frost). There were also large original paintings and illustrations hanging on the wall such as the colorful plates of the frogs and reptiles from Santa Cecilia and the skull drawings of casque-headed frogs (done by Linda Trueb). And then, there were brand new computers, and all ordered in perfection. Had I died and gone to heaven? No, I was at Bill´s and Linda´s empire of knowledge."*

Two and a half decades after his last visit to Ecuador, he had these generous words when talking about Ecuadorians.

> *"The people of Ecuador are fortunate to have such talented scientists who can convey their knowledge so concisely. The people of Ecuador are also fortunate to live in a land of such great frog diversity, but they also are obligated to maintain that diversity for future generations of both frogs and humans."* (Duellman 2009).

In 1997, Bill's planned completion of work with Ecuadorian frogs was clearly stated in the influential monograph about the *Eleutherodactylus* (now most are in the genus *Pristimantis*) in western Ecuador:

> *"Little did we image [sic] three decades ago that the 20th Century would be waning before our endeavors on the* Eleutherodactylus *in western Ecuador would come to fruition. In the intervening years we have learned much about these frogs and have become increasingly aware that there is so much yet to be known. We leave this challenge to our successors, to whom we bid: !Que les vaya bien!"* (Lynch and Duellman 1997).

Bill retired in 1997 to become, most likely, the most prolific Curator Emeritus in the field of

Herpetology. Some of his most important publications—a revised edition of *Hylid Frogs of Middle America* (2001), *Cusco Amazónico: the Lives of Amphibians and Reptiles in an Amazonian Rainforest* (2005), *Terrestrial Breeding Frogs (Strabomantidae) in Peru* (2009), *Marsupial Frogs: Gastrotheca & Allied Genera* (2015), *Herpetology at Kansas, A Centennial History* (2015), and the yet unfinished *Encyclopedia of Ecuadorian Amphibians* (Coloma and Duellman, in prep.)—were part of his "retirement" hobbies.

Bill's influence on Ecuadorian herpetologists also extends to Omar Torres-Carvajal and Santiago R. Ron, both currently working at PUCE on reptiles and amphibians, respectively. Torres-Carvajal is the leading researcher on Ecuadorian Squamata, having made numerous contributions that follow the KU tradition (e.g., Torres-Carvajal 2000, 2003). Ron has also played a role in amphibian studies, with a focus on systematics of frogs, especially of the genus *Engystomops* (e.g., Ron *et al.* 2006) and *Osteocephalus* (e.g., Ron and Pramuk 1999).

When Guayasamin started his graduate studies at KU (2001), under the mentorship of Linda Trueb, Bill always was in his office next to the entrance of the Herpetology Division. His door always open, his mind always working. Every printed publication was perfectly organized, as were his field notes, slides, and recordings. Although the idea of "integrative taxonomy" may have a catchy new air, it was clear that for Bill that was just day-to-day taxonomy. The collections that Bill and his students obtained in the 1970s were so complete that they even included tissues for allozyme studies. These very tissues were used 40 years later to generate the first molecular phylogeny of Ecuadorian harlequin frogs, co-authored by Bill (Guayasamin et al. 2010). Also, Bill's work on direct-developing frogs (Lynch and Duellman 1997) and glassfrogs (Lynch and Duellman 1973) inspired Guayasamin to pursue these taxa for his MA and PhD theses, transitioning from the classic Truebean osteology (Guayasamin 2004, Guayasamin and Trueb 2007) to the novelty of molecular systematics in centrolenid frogs (e.g., Guayasamin et al. 2008, 2009). Many productive Ecuadorian herpetologists have been inspired and enchanted by Bill's work, as well. For example, Ana Almendáriz C., Alejandro Arteaga-Navarro, Sofía Carvajal-Endara, Diego Cisneros-Heredia, Mauricio Ortega-Andrade, Mónica Páez-Vacas, Carolina Reyes-Puig, Juan Carlos Santos, Verónica Urgiles, and Mario Yánez-Muñoz all visited him at KU or interchanged correspondence for many years.

It is difficult to measure the impact of Bill in Herpetology, but we can certainly say that the status of Ecuador as one of the most diverse countries on Earth in terms of amphibian species, is Bill's legacy. We can also say that we, the authors of this note, had in Bill and Linda true mentors, friends, and colleagues. The University of Kansas Natural History Museum was a second home where we listened to endless frog stories, worked in one of the largest collections of Neotropical amphibians, and learned how to study these beautiful animals in the heaven of amphibian academy. Everything in this very special, blue (democrat) town, called Lawrence, surrounded by prairies, and on the shoulders of the herpetology giants.

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(**A**) From left to right: Linda Trueb, Charles F. Walker, Ildefonso Muñoz B. William E. Duellman, and Thomas H. Fritts at Santa Cecilia, Provincia Sucumbíos, Ecuador, April 1969. (**B**) Bill recording a treefrog near Tandayapa, Provincia Pichincha, Ecuador. April 1975. (**C**): Bill at the Second Latin American Congress of Herpetology in Mérida, Venezuela in 1990 (photographer unknown). (**D**): Bill with three of his Ecuadorian students in Lawrence, Kansas, in September 2005. From Left to right: Omar Torres Carvajal, Luis A. Coloma, Juan M. Guayasamin. Photos by Blanca Muñoz (A), Linda Trueb. (B, D).

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- **Labeling figures:** Labels must be consistent on a figure and among all figures included in the article. **Use a sans serif font** that is common to Windows and Macintosh platforms (e.g., Arial). Subunits of multipart figures must be labeled with capital letters (A, B, C) placed in the **upper, left-hand area** of each unit. The letters **should be about 10 points large** (not to exceed 12 pt); they must be identical in size and typeface on each figure included in the manuscript.

INSTRUCTIONS TO AUTHORS

Labeling within figures (e.g., anatomical parts, legends on axes of graphs, etc.) **should be in the range of 8–9 pt and in a sans serif font, such as** Arial. Scale bars should be labeled with their values on the face of the figure (e.g., 5 mm); **the minimal size of lettering that may be used is 7 points** in a sans serif font for scale bars, longitude and latitude on maps, etc.

- **Vector graphics:** *Maps, graphs, and line drawings* should be prepared with an illustration program such as Adobe Illustrator, CorelDRAW, or Deneba Canvas. Graphs and maps generated in other programs (e.g., Sigma Plot, Excel) can be imported into these illustration programs and manipulated (or used as a template to produce a new drawing) to produce an acceptable figure at the size intended for publication. Similarly, drawings executed by hand, should be scanned (300–600 dpi) and imported into an illustration program in which they can be sized and labeled for publication. Follow the instructions for labeling provided above, along with the following guidelines for illustrations at column and page widths.
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  - ✓ Tick marks on graphs should be on the outside of the axis line. Sized for publication, they are between 3 and 5 points in length and 0.25 pt in weight. Longitude and latitude marks should be on the inside of the map border.
  - ✓ All maps must have an appropriate scale in kilometers.
  - ✓ Overlapping symbols and lines must be counter shadowed with white.
  - ✓ Export completed image as a TIF document for submission.
- **Raster graphics:** *Photographs (color and gray-scale [black & white]) and tone (gray-scale) renderings* should be submitted as a RGB document in TIF format sized for publication (described above) at a resolution between 300 and 600 dpi (after reduction/sizing). To label raster images, import them into a vector graphic program, follow the directions above, and export the completed image as a TIF document for submission.

Editorial conventions.

- **Taxonomy.** All generic and specific names must appear in italics. At the first mention of a species in any paragraph, provide its complete binomial name; in subsequent references to the same species, the generic name may be abbreviated. The first citation of a species must include the authority and date, but the authority does not have to be cited in the References. Hierarchical taxa are separated with colons (e.g., Anura: Leptodactylidae). New taxonomic names should not appear in the Abstract or Keywords.
- **Dashes.** There are three kinds of dashes. Short dashes (-) are used as hyphens. En-dashes (–) are used to denote ranges (e.g., 5–10, May–September) and the minus sign in mathematics. Em-dashes (—) are used in Tertiary Headings, and frequently as a substitute for parentheses and colons. There should be no space on either side of any of these dashes.
- **Numbers and units.** All measurements are noted in Arabic, unless the number starts a sentence.
  - ✓ Measurements include distances, areas, dimensions, volumes, weights, time (e.g., hours, days, seconds, minutes), temperatures, etc. **Standard SI units are used**—e.g., time: 08:16 h; distances and areas: 7 km, 12.5 mm, 17,840 ha; geographic coordinates: 04°43'23" S; temperature: 24°C. To indicate degrees, use a degree sign (°), not a superscript oh (^{o}). Note that degrees and minutes are straight quotation marks or prime signs; do not use curly quotes.
  - ✓ Use the **double-digit rule** for numbers other than measurements. Numbers less than 10 are spelled out—e.g., "… nine animals were sampled"; numbers of 10 and more are denoted in Arabic—e.g., "… but 10 larvae were collected."
- **Citations.** Authorities are cited in text as follows. Single: (Caballero 1944); double: (Bursey and Goldberg 2006); three or more (Goldberg *et al.* 2002). Note use of "and" and italics for "et al." Multiple text citations should be listed in chronological order and separated by commas—thus: (Crump 1974, Duellman 1978a–c, 1980, Duellman and Trueb 1986). Two or more publications by the same author should be cited in the following pattern: (Vanzolini 1991, 1992) or Cadle (1984a, b, 1985).
- **References.** All publications cited in the text (except taxonomic authorities) must be included in the References in alphabetical order. "Gray literature" (e.g., technical reports, theses, dissertations that have limited distribution or are difficult to identify and acquire) should be avoided. Follow the formats shown below.

✓ **Normal journal articles:**

Vanzolini, P. E. 1993. A new species of turtle, genus *Trachemys,* from the state of Maranhão, Brazil (Testudines, Emydidae). *Revista Brasileira de Biologia 55:* 111–125.

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

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

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

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

✓ **Chapter in an edited volume:**

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

✓ **Unpublished thesis or dissertation:**

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

✓ **Book:**

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

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

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

✓ **Software:**

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

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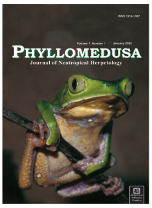
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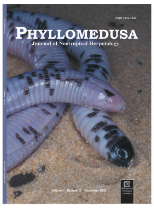
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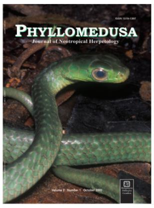
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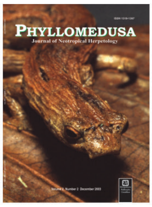
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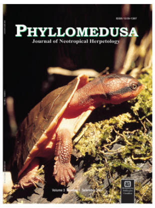
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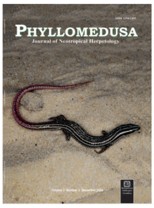
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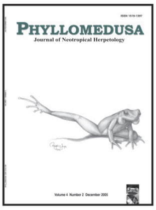
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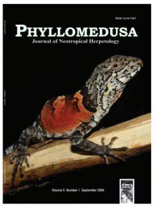
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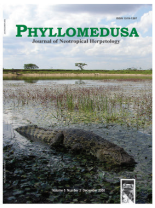
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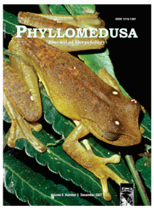
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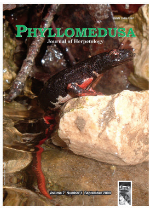
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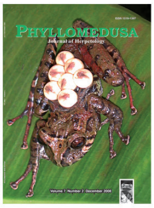
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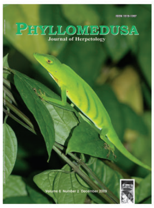
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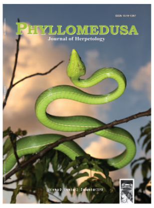
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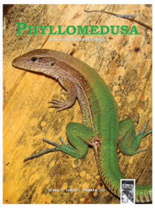
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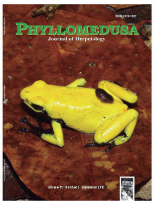
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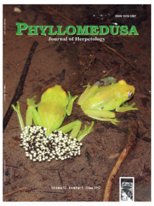
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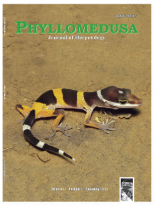
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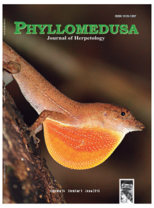
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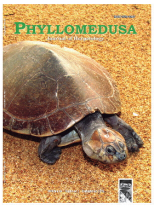
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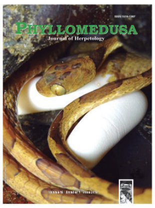
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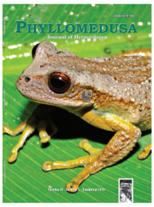
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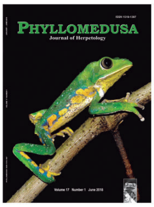
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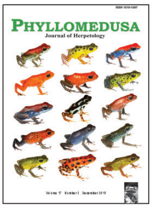
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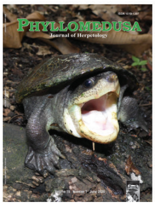
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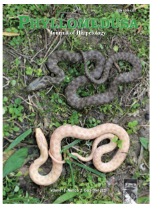
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