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Front cover: Adult female *Vipera berus* and five adult male *Anguis fragilis,* found together under corrugated iron refuges. See article on page 145. (© Jason Steel)

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Effects of road salt, egg predation and alterations to the egg-mass jelly layers on the embryos of spotted salamander *Ambystoma maculatum*

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Road de-icing salt has been used to make winter driving safer. However, its eventual drainage into adjacent water bodies has caused detrimental effects on organisms in those aquatic communities, including amphibians. We used the spotted salamander *Ambystoma maculatum* as a model species and examined whether there would be additive or interactive effects of road salt, egg predation from wood frog *Lithobates sylvaticus* tadpoles, and alteration to egg-mass jelly layers on the hatching rate, incubation period, snout-vent length (SVL) and developmental stage of hatchlings. We found neither main nor interactive effects of salt on the measured traits, which contradicts some of the previous studies. The other body of previous studies, however, showed observational and experimental evidence that amphibian populations became locally adapted to saline water. Given the roadside origin of our study animals, our negative results provide insight into the ubiquity of local adaptation to saline water among amphibian populations. We also found that egg predation and the presence of egg-mass jelly layers negatively affected the incubation period, SVL, or development in the predicted manners based on the O_2 demand-supply balance within egg masses. These responses might be altered by dissolved salt if populations naive to freshwater salinisation were exposed to saline water.

Keywords: aquatic pollution, community ecology, conservation, larval amphibians, *Lithobates sylvaticus*

INTRODUCTION

Despite increasing attention and conservation efforts in recent years, approximately 40 percent of amphibian species have become threatened with extinction due to various direct and indirect human causes, and pollution is one of the major causes along with habitat destruction/fragmentation, invasive species, diseases, light and noise pollution and climate changes (Wake, 1991; Forman & Alexander, 1998; Collins et al., 2009; Lötters et al., 2009; Jones et al., 2017; Dutta, 2018; Lind et al., 2018; Hintz & Relyea, 2019; Fisher & Garner, 2020). Freshwater aquatic habitats adjacent to roads are susceptible to anthropogenic pollution since they trap chemicals from road runoff (Mungur et al., 1995; Dobson et al., 1997; Turtle, 2000; Hayes et al., 2002; Godwin et al., 2003; Collins & Russell, 2009; Denoël et al., 2010; Stoler et al., 2017; Schuler & Relyea, 2018; Schuler et al., 2019). Pollutants entering the water affects the salinity, turbidity and dissolved oxygen levels (Forman & Alexander, 1998; Schuler & Relyea, 2018), which can alter food webs (Jones et al., 2017; Lind et al., 2018; Schuler & Relyea, 2018) and reduce the biodiversity of aquatic communities (Dobson et al., 1997; Forman & Alexander, 1998; Collins & Russell, 2009; Denoël et al., 2010; Stoler et al., 2017; Schuler & Relyea, 2018).

Sodium chloride (NaCl) is one of the pollutants that enter the roadside freshwater ecosystems because it is commonly used as road de-icing salt to make winter travels safer. In the United States, for example, studies reported increases in concentrations of sodium and chloride in roadside aquatic habitats over the past decades (Lagerwerff & Specht, 1970; Godwin et al., 2003; Brady, 2012). Pond-breeding amphibians are the members of aquatic communities facing the detrimental effects of freshwater salinisation (Karraker et al., 2008; Collins & Russell, 2009; Karraker & Gibbs, 2011; Jones et al., 2017; Schuler & Relyea, 2018; Ocampo et al., 2023). Pond-breeding amphibians are vulnerable to aquatic pollutants because they rely on aquatic habitats to complete their life cycle, have semi-permeable skin (Denoël et al., 2010) and tend to be sedentary (Sanzo & Hecnar, 2006). Furthermore, road salt together with other biotic (e.g. predators and competitors) (Karraker & Ruthig, 2009; Jones et al., 2017) and abiotic stressors (e.g. metals, pesticides and fertilisers) (Stoler et al., 2017; Schuler & Relyea, 2018; Lewis et al., 2021) threaten amphibians, often through complex pathways.

The spotted salamander *Ambystoma maculatum* is one of the pond-breeding amphibians commonly found in roadside pools in eastern North America. Acute toxicity

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tests revealed larvae of *A. maculatum* were one of the most sensitive pond-breeding amphibians to chloride in Nova Scotia, Canada (Collins & Russell, 2009). Freshwater salinisation also decreases the survivorship of the embryos (Turtle, 2000; Karraker et al., 2008). Karraker & Gibbs (2011) also found high chloride concentrations dehydrated the thick outer jelly layers surrounding egg capsules of *A. maculatum* and permanently disrupted their osmoregulation, resulting in delayed hatching and reduced hatching success of *A. maculatum* embryos (Karraker & Gibbs, 2011). The jelly layers provide essential protection for developing *A. maculatum* embryos against predators (Ward & Sexton, 1981). However, the wood frog *Lithobates sylvaticus* tadpoles still can significantly lower the survivorship of *A. maculatum* embryos (Petranka et al., 1998; Holbrook & Petranka, 2004). Furthermore, intact jelly layers can reduce the survivorship and developmental rate of the salamander embryos because the thick outer jelly layers interfere with O_2 diffusion throughout the egg mass (Pinder & Friet, 1994; Hale et al., 2017). Therefore, the jelly layers, which protect embryos from predation under normal circumstances, may impose negative impacts on the embryos when the water becomes salinised, and these hypothesised negative effects of salinisation may further be altered by the presence of egg predators that remove the jelly layers by eating them before reaching the embryos inside.

Here, we investigated the effects of road salt, egg predation and manipulation of egg-mass jelly layers on the hatching success, incubation period, developmental stage at hatching and hatchling body size of the spotted salamander *A. maculatum*. In order to investigate the main and interactive effects of these three factors (i.e. salinisation, predation and jelly layers), we conducted a laboratory experiment, in which we created eight treatments by fully crossing the three variables, each of which had two levels: presence/absence of road salt, presence/absence of tadpole predation and presence/absence of jelly layers.

MATERIALS & METHODS

Field procedure

Egg masses of the spotted salamander *A. maculatum* and wood frog *L. sylvaticus* were collected from four vernal pools in Union County, Pennsylvania, USA on 10 April 2018. The vernal pools were monitored for two weeks before the collection day so we knew the age of each egg mass. The first major breeding and oviposition of *L. sylvaticus* occurred between 27 and 29 March 2018, whereas the first major oviposition event of *A. maculatum* occurred on the night of 9 April 2018. *Ambystoma maculatum* deposits two major types of egg masses, clear and white (Ruth et al., 1993), however, only clear egg masses were collected so that the number of embryos inside of each egg mass was visibly assessed before the experiment. Thirty-two salamander egg masses were used for the experiment. Several egg masses of *L. sylvaticus* that were laid on the same day were also collected, from which approximately 500 embryos were isolated for the experiment.

Experimental setup

The experiment consisted of eight treatments based on a factorial design of three main factors: presence/absence of road salt, presence/absence of *L. sylvaticus* tadpoles (predators) and presence/absence of the jelly layers of the salamander egg masses. Each treatment was replicated four times, which resulted in 32 experimental containers. Each contained a single egg mass of *A. maculatum*. In order to calculate a hatching rate, we counted the number of salamander embryos in each egg mass by gently pressing them with a transparent glass sheet and taking photos before placing them into each container (a mean number of embryos per mass ± SE = 124.4 ± 3.6). All containers (container size: 28 cm[L] x 15 cm[W] x 11 cm[H]) were randomly assigned to the eight treatments and arranged in a single incubator under a 14-hour light/10-hour dark cycle with a temperature originally set at 12.7 °C. The National Weather Service Forecast website was used to calculate the historical monthly average temperatures of April and May in this area from 2000 to 2018. Based on the monthly temperature difference between April and May, we increased the incubator temperature by 1.5 °C every Friday during the experiment in order to reflect the general weather patterns of the area.

The treatments with the presence of road salt had a salt (NaCl) concentration of 1000 mg/L (equivalent to 607 mg/L chloride), which is within the range of the ecologically-relevant salt concentration found in vernal pools adjacent to roads (Environment Canada & Santé Canada, 2001; Karraker et al., 2008). Based on the findings of the previous study (Karraker et al., 2008), the salt concentration in our experiment was expected to cause a 40–50% reduction in embryonic survivorship. The treatments that were not assigned with the presence of road salt received aged water. All containers were filled with aged tap water or saltwater up to 7 cm in depth.

Different numbers of *L. sylvaticus* tadpoles were added to the tadpole-treatment containers based on a 10:8 ratio of the number of *A. maculatum* embryos and the number of *L. sylvaticus* tadpoles, which was within the range of the field densities (Takahashi, personal observation). When an egg mass was assigned to the non-jelly layer treatments, the thick, outer, gelatinous layers surrounding the egg capsules were carefully removed, and all of the individual egg capsules were placed in the container filled with aged tap water or salt water. Additionally, we fed rabbit food in all containers with *L. sylvaticus* tadpoles whose hatching occurred between 16 and 24 April 2018. The experiment was started on 25 April 2018 when all of the salamander eggs and tadpoles were placed into their corresponding treatment containers. We fed tadpoles and changed 50% of the water for all containers weekly throughout the experiment.

Data collection

All 32 containers were monitored daily for *A. maculatum* hatchlings between 25 April 2018 and 28 May 2018 when the last hatchling appeared. The number of *A. maculatum* hatchlings from each of the 32 containers was recorded. Collected hatchlings were immediately euthanised with

Table 1. Results of ANOVAs testing the effects of salt, tadpole predation and jelly-layer manipulation on hatching success, incubation period, SVL and developmental stage for *Ambystoma maculatum* embryos. Bold numbers with asterisks indicate statistically significant p-values.

	Hatching success			**Incubation period**			**Snout-vent length (SVL)**			**Developmental stage**		
	df	F-value	p-value	df	F-value	p-value	df	F-value	p-value	df	F-value	p-value
Salt	1,23	1.412	0.249	1,19	12.899	0.329	1,19	5.082	0.970	1,19	2.881	0.305
Tadpole	1,23	2.839	0.108	1,19	0.175	0.68	1,19	24.744	**0.008***	1,19	31.445	**0.0021***
Jelly	1,23	2.034	0.17	1,19	1.006	**0.002***	1,19	0.001	**0.036***	1,19	1.11	0.106
Salt x tadpole	1,23	0.098	0.758	1,19	1.945	0.067	1,19	0.445	0.886	1,19	4.567	0.211
Salt x jelly	1,23	0.018	0.895	1,19	0.075	0.787	1,19	1.053	0.318	1,19	0.041	0.841
Tadpole x jelly	1,23	1.778	0.198	1,19	3.763	0.179	1,19	0.021	0.513	1,19	1.677	**0.046***
Salt x tadpole x jelly	1,23	1.936	0.18	1,19	1.231	0.281	1,19	0.51	0.484	1,19	0.022	0.885

an overdose of MS-222 by immersion and preserved for the determination of developmental stages and body size measurements. The Harrison (1969) standardised salamander staging chart was used in order to identify the developmental stages of the *A. maculatum* embryos. The presence/absence of a leg bud, body colouration, length of balancer, complexity/branching of the gills, and presence/absence of a hind leg bud were used to determine their developmental stages (Harrison, 1969). We used a LEICA EZ4HD dissecting microscope with a built-in digital camera to examine the developmental stages as well as to take pictures of each preserved specimen for body size measurements. We used ImageJ (Schneider et al., 2012) for measuring the total body length, SVL and tail length of each individual hatchling. Over the course of the experiment, the signs of water mould infection were observed in five containers, which were removed from the data analyses. Those containers were two of the no-salt, yes-predators and yes-layers containers; one of the yes-salt, yes-predators and no-layers containers; and two of the yes-salt, yes-predators and yes-layers containers. Therefore, the following data analyses were done using the data from the remaining 27 containers.

Data analysis

The number of hatchlings per container ranged from zero to over 100. If there were under 20 total hatchlings in a container, all of the 20 hatchlings were measured and used for the growth and development analyses. When the number of hatchlings in a container was greater than 20 and less than 100, 20 individuals were randomly selected for the growth and development analyses. Finally, when the number of hatchlings in a container was greater than 100, 20% of the hatchlings (i..e more than 20 individuals) were randomly selected for the growth and development analyses. In total, 2,300 hatchlings were collected, of which 774 individuals were analysed for growth and development.

Three-way analyses of variance (ANOVAs) were run to evaluate the significance of both main and interactive effects of road salt, egg predators and jelly layers on the hatching success, incubation period, developmental stage and SVL of *A. maculatum* hatchlings. When the assumption of normal distribution or heterogeneity was violated, the data was log-transformed before ANOVA tests. All statistical analyses were conducted using the software R (R Core Team, 2021) with the R packages rstatix (Kassambara, 2021) and emmeans (Lenth, 2021).

RESULTS

Hatching rate

There were no significant main effects of salt, predation, jelly layers, nor any interactive effects between the independent variables on the hatching rate of *A. maculatum* embryos (Table 1). However, there was an interesting trend in the absence of predation and jelly layers; these embryos had the highest rate of hatching success compared to all other conditions, as larvae in the absence of these two variables had an average hatching rate of 0.93 ± 0.04 SE compared to 0.56 ± 0.08 SE for the rest of the treatment groups (Table 1; Fig. 1).

Incubation period

The presence of jelly layers significantly increased the incubation period of salamander embryos (P = 0.002), as the average incubation period with jelly layers was 31.9 days ± 0.47 SE, whereas the average incubation period of those without jelly layers was 28.6 days ± 0.81 SE (Table 1; Fig. 2). There were no significant main nor interactive effects of tadpoles and salt pollution on incubation periods (Table 1).

Snout-vent length (SVL)

The presence of tadpoles produced significantly smaller hatchlings (P = 0.008), as the average SVL of hatchlings in the presence of tadpoles was 7.0 mm ± 0.12 SE, whereas those in the absence of tadpoles had an average SVL of 7.2 mm ± 0.10 SE (Table 1; Fig. 3a). The jelly layers also had a significant effect on hatchling SVL (P = 0.036), as hatchlings in the absence of jelly layers had an SVL of 7.2 mm ± 0.10 SE, whereas those in the presence of jelly layers had an average SVL of 7.1 mm ± 0.11 SE (Table 1; Fig. 3b). There was no significant effect of salt, nor were there any significant interactive effects between the independent variables. It is important to note that means and standard errors reported here and used in the figures are observed values while statistical significances were based on linear models, which can create seeming disparities between p-values and reported pairwise means.

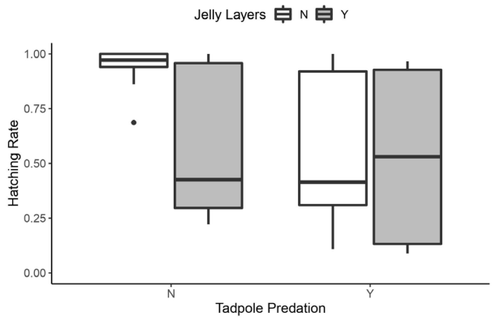

Figure 1. A boxplot showing the hatching rate (i.e. successful hatching) of *Ambystoma maculatum* embryos under four treatments with two independent variables fully crossed: the absence/presence of *Lithobates sylvaticus* tadpole predation and the absence/presence of egg-mass jelly layers.

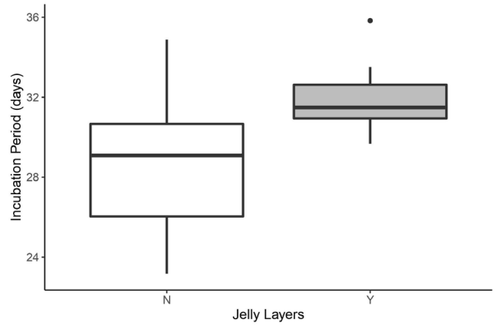

Figure 2. A boxplot showing the incubation period of *Ambystoma maculatum* embryos under two conditions, the absence or presence of egg-mass jelly layers.

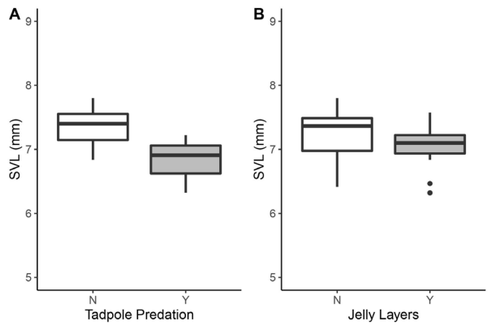

Figure 3. Boxplots showing snout-vent length (SVL) of *Ambystoma maculatum* hatchlings - **A.** under the absence and presence of *Lithobates sylvaticus* tadpole predation and **B.** under the absence and presence of egg-mass jelly layers.

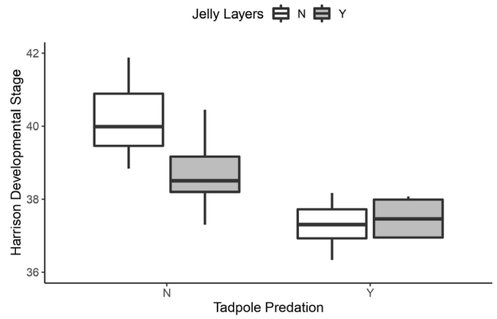

Figure 4. Boxplots Harrison developmental stage of *Ambystoma maculatum* hatchlings under four conditions with two independent variables fully crossed: the absence and presence of *Lithobates sylvaticus* tadpole predation and the absence and presence of egg-mass jelly layers.

Developmental stage

The presence of egg predators alone had a significant effect on the developmental stage of salamander hatchlings (P = 0.0021), as the larvae in the presence of tadpoles had an average Harrison stage of 38.1 ± 0.28 SE, whereas those in the absence of tadpoles had an average stage of 38.9 ± 0.42 SE (Table 1; Fig. 4). The combination of egg predators and jelly layers also had a significant interactive effect on the developmental stage (P = 0.046), as the effect of jelly layers was apparent only in the absence of tadpoles, and those hatched in the absence of both tadpoles and jelly layers were the most developed at hatching with an average Harrison stage of 40.2 ± 0.36 SE (Table 1; Fig. 4).

DISCUSSION

The salt treatments with the intermediate concentration (607 mg/L chloride concentration) in this study did not significantly affect the survivorship or the growth and development of salamander embryos (N = 12 [salt treatments] vs. N = 14 [non-salt treatments]). This result was unexpected when previous studies documented the negative effects of freshwater salinisation on the survivorship of *A. maculatum* embryos (Turtle, 2000; Karraker et al., 2008). Karraker et al. (2008) further showed that (1) embryonic survival was reduced even at relatively low salinity (145 mg/L chloride concentration) though there was notable variation in survivorship from 0% to nearly 100%, and that (2) the average survivorship of embryos at relatively high salinity (945 mg/L chloride concentration) was reduced from 84% to 3%. The notable variation in embryonic survival found in the previous study (Karraker et al., 2008) suggests variation in salt

tolerance among *A. maculatum* egg masses. In fact, there have been many observations reporting amphibians from 144 species in 28 families, including *A. maculatum* (Hardy, 1952), inhabiting saline habitats (Hopkins & Brodie, 2015). Among the 144 species, the evolution of salt tolerance was evident within at least 42 species (Hopkins & Brodie, 2015). Furthermore, Brady (2012) experimentally showed that compared to woodland populations, roadside populations of *A. maculatum* survived better in saline water, by 25% on average, demonstrating local adaptation to saline conditions.

We collected *A. maculatum* egg masses for this experiment from those pools that had consistently produced a prolific number of pond-breeding amphibian eggs and larvae over the past decade. Interestingly, those vernal pools are located within 50 m of the nearby road where road salt has been used during the winter months. The conductivity measures we took in March 2017 indicated low salinity with conductivity ranging from 35–128 µS/cm (≈ 10.2–37.1 mg/L chloride concentration). Given the proximity to the road, however, it is possible that amphibian populations breeding in those vernal pools have developed local adaptation via repeated exposures to winter and spring salinisation. While our predictions about the effect of salt were rejected, our negative results add to the growing body of literature demonstrating salt tolerance of amphibians (Hopkins et al., 2016; Albecker & McCoy, 2017; 2019; Lai et al., 2019; Albecker et al., 2021).

Previous studies reported negative effects of the firm jelly layers surrounding *A. maculatum* embryos because such thick layers hinder O_2 diffusion throughout the egg mass (Pinder & Friet, 1994; Hale et al., 2017; Takahashi & Ruszala, 2023). We also found that the presence of jelly layers resulted in delayed hatching and smaller-bodied hatchlings. It has been suggested that the evolution of jelly layers has occurred in response to egg predation, and wood frog tadpoles are one of the most common egg predators (Petranka et al., 1998; Holbrook & Petranka, 2004). Therefore, the benefit of jelly layers should be manifested under a certain range of egg predation, which predicts the interactive effect between egg predators and jelly layers (Takahashi & Ruszala, 2023). We found the negative effects of egg predators on growth (i.e. body size; Fig. 3a) and development (i.e. Harrison Stage; Fig. 4) as well as the predicted pattern of the interactive effect between predators and jelly layers on their development, in which the negative effect of jelly layers was apparent only under the absence of predators (Fig. 4). However, we found no interactive effects between egg predators and jelly layers on hatching success, incubation period, and body size (Table 1). Furthermore, the hatching success of embryos without jelly layers under the predation treatment, which was predicted to be very low without the jelly-layer protection against predators, was similar to that with jelly layers under both predator treatments (Fig. 1). These results suggest that the level of egg predation created in our experiment might have been relatively low.

It is important to note that the effect of jelly layers is context-dependent and becomes detrimental when the consumption of O_2 by embryos becomes greater than the available amount of O_2. Diffusion does not provide sufficient O_2 throughout an egg mass, and thus, O_2 provision by symbiotic algae associated with jelly layers critically determines embryonic survival (Pinder & Friet, 1994). Therefore, hypoxia occurs when symbiotic algae do not provide sufficient O_2 (Pinder & Friet, 1994). In nature, these scenarios can occur in deep, cold water or shaded areas where algal photosynthesis becomes limited, or in shallow, warm water where O_2 consumption by developing embryos surpasses O_2 provision via diffusion and algal photosynthesis (Hale et al., 2017). In our experiment, the 14-hour light/10-hour dark cycle likely provided sufficient light for algal photosynthesis, but the constant daily temperature, based on the average regional temperature, without increase during daytime might have reduced photosynthesis capacity during the daytime, creating the O_2 supply-demand balance becoming slightly demand-heavy. This might be the mechanism for the presence of jelly layers resulting in longer incubation period and smaller body size at hatching.

Amphibian embryos are equipped with hatching plasticity and change hatching timing in response to environmental stimuli (Warkentin, 2011). For example, hatching timing tends to shift earlier when embryos are exposed to aquatic egg predators, often resulting in less-developed and smaller-bodied hatchlings. Such environmentally-cued hatching plasticity is adaptive because hatching earlier increases survival from egg predators (Warkentin, 1995; Touchon et al., 2011). In our experiment, the presence of egg predators did not affect the hatching timing of salamander embryos, which did not support the general trend found in Warkentin's review (2011). Furthermore, Takahashi & Ruszala (2023) found that *A. maculatum* embryos hatched earlier in response to *L. sylvaticus* tadpoles only when the embryos were not surrounded by the jelly layers. The lack of hatching plasticity in this study may be because there exists notable intraspecific variation in hatching plasticity in some species. For example, egg clutches of *A. texanum* and *A. barbouri* that hatched earlier in control conditions delayed hatching in response to egg predator flatworms *Phagocotus gracilis* while those that hatched later in control showed no response to the egg predators (Sih & Moore, 1993).

Despite the lack of accelerated hatching in response to egg predators, we found that the presence of the egg predators resulted in less-developed and smaller-bodied hatchlings. The reason for the decreased growth and development in the presence of these predators is likely to be the decreased amounts of algae found within the egg masses as a result of tadpole predation (Petranka et al., 1998). The symbiotic algae convert carbon dioxide (CO_2) produced by the salamander embryos into O_2, which the embryos exploit to survive, grow and develop. Although tadpole predation likely increased O_2 diffusion by thinning the jelly layers, our results suggest that relative to the change in O_2 diffusion capacity, the loss of the symbiotic algae imposed a greater impact on the O_2 supply-demand balance and resulted in O_2 deficient conditions.

Pollution is one of the major contributors to the global amphibian declines, and road salt has been shown to have detrimental effects on embryos and larvae of pond-breeding amphibians (Karraker et al., 2008; Collins & Russell, 2009; Karraker & Gibbs, 2011; Jones et al., 2017; Schuler & Relyea, 2018; Ocampo et al., 2022). The other line of the literature, which has gained recent appreciation by conservation ecologists, also suggests that amphibian populations contain sufficient variation to undergo evolutionary changes in response to freshwater salinisation (Hopkins et al., 2016; Albecker & McCoy, 2017; 2019; Lai et al., 2019; Albecker et al., 2021). Contrary to our predictions, we found no main and interactive effects of salt on the survival, growth and development of *A. maculatum*. In light of local adaptation to saline habitats, negative results such as ours provide valuable insight into the ubiquity of local adaptation to saline water and thus the understanding of how global freshwater salinisation impacts amphibians. Although salt did not impose interactive effects with the two other variables (i.e. egg predators and jelly-layer manipulation), these two variables independently and interactively affected embryonic hatching, growth or development in the predicted manners based on the O_2 supply-demand balance within egg masses. These responses may be altered when embryos from naive populations of *A. maculatum* are exposed to saline water. Future studies should also consider examining the effects of salt pollution on amphibians throughout metamorphosis and sexual maturation. Exposure to saline environments during embryonic and larval periods may also affect resource use, predator avoidance as a metamorph, and phenology as an adult.

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Author Contributions

Conceptualisation: M.K.T.; data collection: J.W., A.W., L.T. & M.K.T; data analysis: J.W., J.R. & M.K.T.; manuscript: J.W., J.R., A.W. & M.K.T.

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Predator morphology affects prey consumption: evidence from an anuran population in subtropical wetlands

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Morphology and diet are key factors in the ecology of organisms, determining aspects of the natural history and evolution of the species. In this work, we evaluated the diet-morphology relationship in an anuran population, measuring the influence of morphological traits on the variation in the diet of individuals of *Leptodactylus luctator*. For this purpose, we collected individuals from a natural grassland habitat in southern Brazil. We analysed the stomach content of individuals and classified the consumed food items up to the classification level of order. We also measured four morphological traits per individual of *L. luctator*: distance between eyes, relative limb length, relative mouth width (gape) and snout-vent length. We applied Linear Mixed Effect Models to evaluate the relationship of anuran morphological traits, number of prey taxa and volume of consumed prey. We tested the hypothesis that the configuration of predator morphological traits determines variations in prey consumption patterns. Our results indicate that the body size of *L. luctator* was not directly related to the diet composition but the individuals' gape is directly and positively related to the number of consumed taxa. This suggests that gape limitation could be a limiting factor in prey selection. The capacity to consume a wide variety of prey taxa could be an advantage in unpredictable environments, especially those with great daily thermal amplitudes such as the subtropical Brazilian grasslands.

Keywords: Leptodactylus luctator, diet, amphibian, ecomorphology, functional ecology,

INTRODUCTION

Amphibians are important predators of small organisms, which represent a considerable portion of the biomass present in ecosystems (Wells, 2007; Rowland et al., 2016). The diet of most amphibian species consists almost exclusively of arthropods, however, although several species have specialised feeding habits, anurans are known for having generalist foraging habits, preying on a wide variety of invertebrate and vertebrate orders (Duellman, 2005; Moser et al., 2019; Oliveira et al., 2022). Among the aspects of the natural history of amphibians, diet is recognised as one of the most important, reflecting their evolutionary history (Duellman & Trueb, 1994; Da Rosa et al., 2002).

Most studies on anuran diet are based on the quantification and description of food items and their relative importance for the species' trophic ecology (Moser et al., 2017; Farina et al., 2018; Moser et al., 2019). However, current ecological studies reinforce the importance of using approaches that integrate the species' ecological-evolutionary aspects (e.g. Queiroz et al., 2015; Leite-Filho et al., 2017; Marques et al., 2019; Dalmolin et al., 2019), which include the relationship within functional traits (such as morphological traits) and ecological variables. Using this approach, the processes that determine the ecological-evolutionary aspects of species become clearer (Marques & Nomura, 2015; Tonkin et al., 2016), and this includes their feeding behaviour (Tozetti & Martins, 2019). Thus, diet stands out as an important factor associated with the ecological and morphological traits of each taxon (Sih & Christensen, 2001).

Foraging strategy is considered a determining factor of the diet in anurans (Toft, 1981; Santos et al., 2004; Piatti & Souza, 2011). Among species that feed on invertebrates, those that are active foragers tend to consume smaller prey that have social habits and slow movements (such as ants and termites) (Toft, 1981). On the other hand, species that have a sit-and-wait type of foraging behaviour generally consume larger and solitary prey such as beetles, orthopterans, and spiders (Strüssmann et al., 1984; Magnusson et al., 1985; Pough & Taigen, 1990). Diet variation is also driven by prey availability (Michelin et al., 2020; Moroti et al., 2021), in particular in generalist species which tend to eat the most abundant prey in their habitat (Maneyro et al., 2004; López et al., 2009; Rebouças & Solé, 2015). However, a substantial

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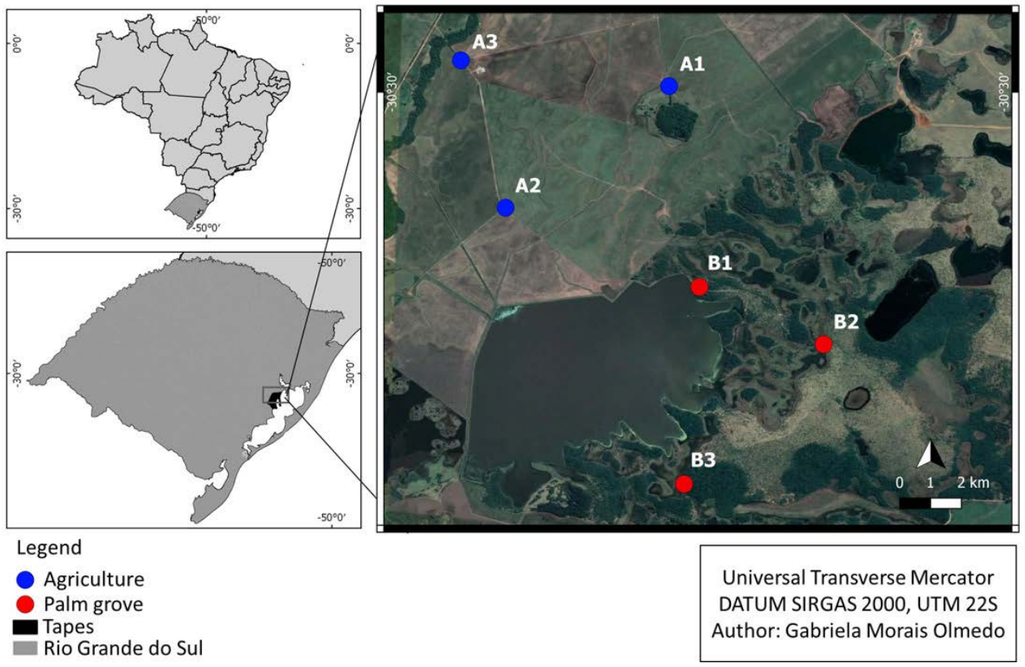

Figure 1. Study area in the municipality of Tapes, Rio Grande do Sul, Brazil. Sampled areas are represented by blue (agriculture matrix) and red (natural palm grove matrix) circles.

part of the variation in diet can emerge in response to inter/intra species functional divergence (Simon & Toft, 1991; Piatti & Souza, 2011). In these cases, the behavioural aspects would be important, but also those characteristics related to the species' physiology and morphology.

In anurans, the association between morphology and diet may become more obvious when individuals of the same species are compared. The morphology of the skull structures, for example, has a great influence on the consumed prey types and have great inter and intra species variation (Emerson & Bramble, 1993; Duellman & Trueb, 1994; Metzger & Herrel, 2005; Cvijanović et al., 2014). Gape size for example, affect the patterns of consumed prey or prey size (Emerson, 1985; Menzies & Parker, 2018). An obvious assumption is that predator body size defines their ability to subdue prey and creates a limit to the maximum size of potential prey. Consequently, the relationship between predator and prey sizes affects the species' trophic niche, as well as on the patterns of divergence in the diet between individuals with different body sizes (Strüssmann et al., 1984; Shine, 1991; Forsman & Lindell, 1993; Araújo et al., 2007). Intrapopulational variation in prey preferences can work as a mechanism to avoid competition, which favours the co-occurrence of close related individuals (Guimarães et al., 2011; Piatti & Souza, 2011). Recently, individual specialisation has gathered attention from ecologists due their contribution to the understanding of trophic specialisations (Bolnick et al., 2003; Xia et al., 2020).

Based on previous studies, many aspects related to the diet of predators (e.g. species composition and biomass) are affected by their morphological traits (such as body size and maximum gape; Araújo et al., 2007; Solé et al., 2017; Tozetti & Martins, 2019). In this work, we evaluated the relationship between the descriptors of feeding behaviour (richness and volume of consumed prey) and the morphological traits of *Leptodactylus luctator* in southern Brazil. Specifically, we tested the hypothesis that a predator's morphological traits (snout-vent length, relative limb length, distance between eyes, and gape) determine the richness and volume of prey consumption. We predicted that the richness and volume of consumed prey have a positive relationship with morphological traits, especially with snout-vent length and gape. As reported by other species, anurans eat the whole prey, the mouth gape being a limiting factor to their ability to ingest (Vitt et al., 2000; Tozetti & Martins, 2019).

MATERIALS & METHODS

Study area

We conducted the study in natural grassland habitats located in the municipality of Tapes, State of Rio Grande do Sul, Brazil (51° 22' 36.8'' S; 30° 25' 58.3'' W; Fig. 1), at 10 m above sea level. The study site covers an area of 1,294.5 ha and includes well-preserved areas of the Pampa Biome (in particular, sandbank formations; Becker et al., 2007). The area stands out for having one of the last

Figure 2. Habitat types present in the study area - **A.** natural palm-grove grasslands, and **B.** agriculture grasslands with irrigated rice and soybean cultivation.

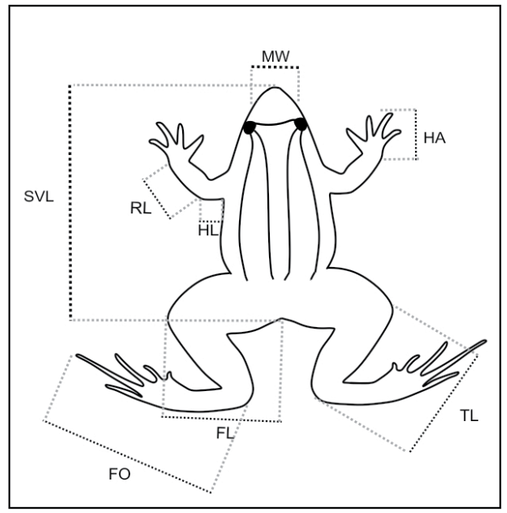

Figure 3. Morphological metrics evaluated in adults of *Leptodactylus luctator*: SVL = snout-vent length; TMP = third metacarpal and phalanx length; RL = radius length; HL = humerus length; FMP = fourth metatarsal and phalanx length; TL = tibia length; FL = femur length; MW = mouth width.

remnant palm grove habitats formed by *Butia odorata* (Barb. Rodr.) Noblick in Brazil. There is an estimated number of 70,000 individuals of *B. odorata* forming a single continuous palm grove spreading over an area of 750 ha (Fig. 2A). The average annual temperature and rainfall in the study region are 18 °C and 1,200 mm, respectively (Maluf, 2000). The area adjacent to the palm groove is used for agricultural activities and is subject to the conventional planting systems. This area has a size of about 800 ha where irrigated rice and soybean are cultivated.

Table 1. Morphological traits measured in adults of *Leptodactylus luctator*

Morphological Trait	Levels	Ecological relevance
Distance between eyes	mm	These traits are associated with the number of individuals and richness of consumed prey orders
Snout-vent length	mm	
Mouth width	Distance between mouth ends (mm)	
Relative limb length	(femur length + tibia length + foot length) / (arm length + forearm length + hand length) - mm	Limb length is associated with the capacity of individuals to search and explore different microhabitats

Data collection

We adopted the nomenclature presented by Magalhães et al. (2020) in which the studied frog population, formerly considered as *L. latrans*, was updated to *L. luctator.* We performed the sampling of individuals of *L. luctator* from September to December 2018. For this purpose, we used visual search (Crump & Scott Jr, 1994) in breeding sites between 20:00 h and 02:00 h. We sampled each area monthly for three consecutive days (N = 12 days). The search was performed by four researchers for one hour in as many breeding sites as possible. Thus, our total sampling effort was 288 search hours. To avoid any effects of ontogeny in our analysis, we restricted collection to adult individuals. The search was conducted in six breeding sites distributed throughout the study area that included a great variety of habitats. Considering this, we selected three breeding sites in natural, preserved grasslands (in the palm groove area) and three breeding sites in grasslands adjacent to agricultural areas (irrigated rice and soybean cultivation; Fig. 2). The choice of the sampled areas occurred so that we could assess the influence of different environmental conditions on the species' diet.

The captured specimens (N = 47), were placed in plastic bags and kept in refrigeration equipment to decrease digestive activity until euthanasia (Moser et al., 2017). The time between capture and euthanasia was less than 2 hours. The collections were carried out under the authorisation of the Federal Agency (SISBIO - authorisation number 66513). Subsequently, individuals were euthanised with xylocaine, fixed with 10% formaldehyde, and preserved in 70% ethanol. In the laboratory, we removed the gastrointestinal contents of each individual, which were also kept in 70% ethanol. We performed the screening of the samples using a stereomicroscope with a magnification range of 10x to 45x. We identified the consumed prey items up to the level of order based on regional literature (Ribeiro-Costa & Rocha, 2002). We then macerated and spread each individual's stomach contents in a Petri dish. Graph paper

Table 2. Results of LMM models showing the relationship between descriptors of feeding behaviour and morphological traits of *Leptodactylus luctator*. DF (degrees of freedom); AICc (Akaike's Information Criterion); R^2m (fixed effects); R^2C (fixed + random effects).

Descriptors	Morphological traits	DF	*F*	*p*	AICc	R^2m	R^2C
Consumed prey diversity	Distance between eyes	47	0.99	0.32	16.18	0.12	0.12
	Mouth width	47	4.02	0.04			
	Relative limb length	47	0.04	0.83			
	Body size (snout-vent length)	47	0.83	0.37			
Total volume	Distance between eyes	47	0.04	0.85	659.25	0.18	0.18
	Mouth width	47	1.50	0.23			
	Relative limb length	47	0.01	0.98			
	Body size (snout-vent length)	47	8.32	< 0.001			

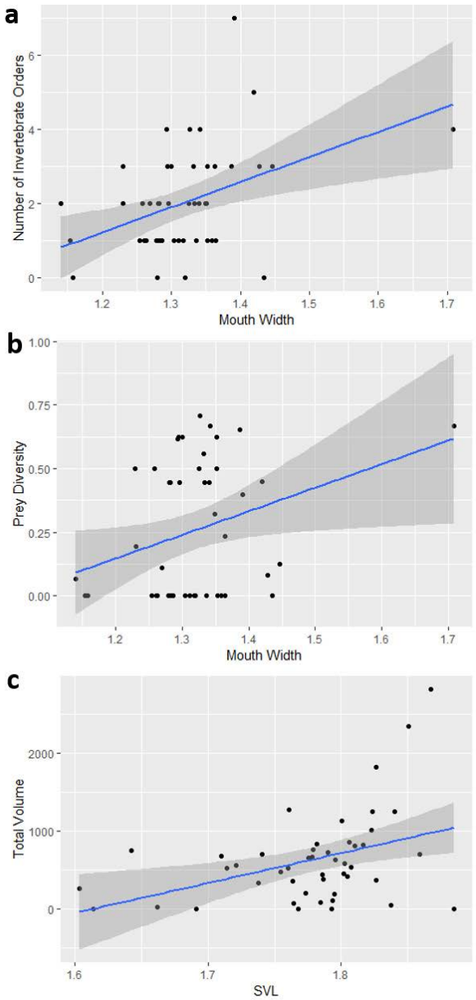

Figure 4. Diet-morphology relationships in *Leptodactylus luctator*. **A.** Relationship between the number of consumed invertebrate orders and gape; **B.** Relationship between the consumed prey diversity and mouth width; **C.** Relationship between total volume of consumed prey and snout-vent length.

(1 mm x 1 mm) was placed under the plate and used to measure the volume (V) and the number of identifiable orders found when categorising the prey (see Moser et al., 2020 for additional details). Prey items were then pressed with a pistil until the entire macerated layer was homogeneous and uniform with a height of 1 mm (Fig. 3). The individuals of *L. luctator* were deposited in the scientific collection of the Laboratório de Ecologia de Vertebrados Terrestres of the Universidade do Vale do Rio dos Sinos. The voucher numbers are available in the supplementary material.

Trait measurement

We evaluated four morphological traits of each individual: distance between eyes, relative limb length, relative mouth width (gape), snout-vent length and anatomical components as RL (radius length), HL (humerus length), FMP (fourth metatarsal and phalanx length), TL (tibia length) and FL (femur length; Fig. 3). We chose these traits based on literature (Marquéz-García et al., 2009) as well on perceived importance for determining the diet composition in several anuran species, including *L. luctator*. The morphological traits were characterised according to the metrics presented in Fig. 3 and Table 1 and based on Marquéz-García et al. (2009).

Statistical analysis

We tested the possible existence of relationships between descriptors of feeding behaviour (consumed prey diversity, total volume and number of consumed invertebrate orders) and morphological traits (distance between eyes, relative limb length, relative mouth width [gape] and snout-vent length) by using Linear Mixed Models (LMM). We used the Rao's quadratic entropy (Q) to assess the diversity of consumed prey. The Rao's quadratic entropy is a measure of diversity which is based on the proportion of the abundance of species present in a sample; in statistical terms, Rao quadratic entropy is equivalent to the Gini-Simpson index, and the dissimilarity range from 0 to 1 (Rao, 1982).

We generated individual models for each descriptor of feeding behaviour. Collection date and habitat were used as random effects. We log-transformed the values of morphological traits before the analysis, and also tested the collinearity between them by using Variance Inflation Factor analysis (VIF; Lin et al., 2011). Results did not indicate a significant correlation between any of the evaluated traits. We used the Quasi-Poisson as link function to model the distribution of the total volume and number of consumed invertebrate orders in relation to the functional traits, in order to avoid or decrease the chances of the overdispersion in our data (Davison & Snell, 1991). For the diversity of consumed prey, we used the gaussian link function, which is specific for data which range from 0 to 1. We tested the significance of each diet-morphology relationship by ANOVA. We then ran these analyses with the packages "MuMin" and "vegan" within R software.

RESULTS

The LMM analysis revealed that the distribution of data related to the total volume of prey consumed was overdispersed (overdispersion > 1). It means that the amount of data that did not adjust to the distribution of the predictor variables (i.e. traits) was preponderant (i.e. excessive amount of residues). On the other hand, data distribution in the model with the number of consumed order showed a relatively low overdispersion (overdispersion = 0.83). In this way, we limited the following analyses to the models in which the number of both prey orders and diversity was considered.

We found a significant and positive relation between gape and the number of consumed prey order and prey diversity (Table 2; Fig. 4a and 4b). Also, the fixed effects (morphological traits) accounted for the total explained variation in both models.

DISCUSSION

Our results showed evidence that variations in some morphological traits are accompanied by differences in the dietary composition of individuals of *L. luctator*. The morphological traits affected each of the evaluated components (prey composition) differently. We found that: (i) body size (snout-vent length) was not directly related to the diet composition and (ii) the larger an individual's gape, the more diverse its dietary composition will be (larger number of prey taxa in the gut). These results are relevant because they highlight a little-explored dimension regarding an anuran diet: intraspecific dietary variation.

In general, species of Leoptodactylidae are considered generalist predators since they consume a great variety of food items (Protázio et al., 2015). This is a common characterisation for many species whose diet is known (Rodrigues et al., 2004; Pazinato et al., 2011; Sugai et al., 2012; Dias et al., 2018). However, we draw attention to the fact that variation in foraging, even subtle ones, can be found when examining the diet at the individual level. In the last decades, the number of studies with focus on anuran diet has been increasing (Piatti & Souza, 2011). More than a description of food items, the idea of testing intrapopulation variation in diet, as well as the relationship between frog size and diet, was well explored by previous studies (Araújo et al., 2007; Borges et al., 2019). On the other hand, our study presents a new approach by adding morphology as a possible driver for dietary differences among individuals. We observed, for example, that body size (snout-vent length) is one of the traits related to dietary variation in *L. luctator*. This relationship is somewhat intuitive since larger animals would theoretically have the greater prey-storage capacity in their digestive tracts (Tozetti & Martins, 2019).

The stomach size of individuals generally increases with body size, so that larger individuals tend to hold a larger volume of prey (Sugai et al., 2012). This hypothesis is reinforced by studies in Uruguayan populations of *L. luctator* (Maneyro et al., 2004), which reported a positive correlation between the body size and the size of consumed prey. In our study, data from prey volume did not fit in our models. In a similar study, Solé et al. (2009) evaluated the diet of the same species in north-eastern Brazil and found no significant correlation between the size of individuals and the volume of consumed prey. These contradictions reinforce the need for more detailed studies between morphological parameters related to pray/predator relationship.

Our data allow us to infer that the dietary patterns of *L. luctator* are determined by gape. While the latter affects the ability to subdue prey, gape acts as a limiting factor in prey selection (Araújo et al., 2007; França et al., 2004; Maneyro et al., 2004). Limits in the gape can restrict the size and, consequently, the variety of prey that can be ingested (Huckembeck et al., 2018). Still, the trend is that the relationship between mouth width and the diversity of consumed prey is linear and positive since animals with a larger mouth opening can consume a wide range of prey categories, which would contain prey items of all sizes (De Carvalho Batista et al., 2011; Sales et al., 2011). These hypotheses are reinforced by the fact that our data indicate gape as the main predictor of prey diversity. The fact that we did not find a relationship between limb length and diet suggests that only specific morphological traits related to foraging affect feeding behavior (Bonte et al., 2012). Relative limb length does not seem to have a major contribution to foraging in anurans (Jeltsch et al., 2013; Bredeweg et al., 2019), although this trait is a conditional factor for access to habitats.

The relationship between a predator's body size and the amount of prey it eats is an ecological consequence of feeding ecology (MacArthur & Pianka, 1966; Charnov, 1976; Duelmann & Trueb, 1994). The possibility of ingesting a larger volume of prey can be an important attribute in unpredictable environments and environments subjected to anthropic modification, especially those with great daily thermal amplitudes (e.g. agricultural areas; Lopez et al., 2015). In this type of environment, sudden temperature drops can decrease the activity of invertebrates, reducing prey availability for anurans (Gibbs & Stanton, 2001; Lopez et al., 2009; Battles et al., 2013). In this case, larger individuals would have accumulated reserves in their stomachs from nights of successful foraging.

Our data reinforce the need to consider aspects of the functional diversity of anurans — especially those

related to their morphology — in future studies that are interested in the diet and trophic ecology of this group. Given the fact that anurans have high degrees of phylogenetic conservation in several traits (including morphological and behavioural traits; Campos et al., 2019), we suggest that these patterns of trait-diet relationship can be extended to other species of the clade Leptodactylidae.

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Patterns of spatial and temporal association between *Zootoca vivipara*, *Anguis fragilis*, *Vipera berus* and *Natrix helvetica* at artificial refuges

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A 15-year data set of reptile observations at corrugated iron refuges was analysed to describe the spatial and temporal associations between species pairs of viviparous lizards *Zootoca vivipara*, slow worms *Anguis fragilis*, northern vipers *Vipera berus* and grass snakes *Natrix helvetica*. Of the two snake species, only the viper is known as a routine lizard predator. We analysed two variables expressing pairwise reptile associations at refuges. The first analysis was of monthly spatial overlap assessed as the proportion of unique refuge positions used in common, but not necessarily used simultaneously. The second analysis was of a more precise spatial and temporal assessment based on counts of reptile pairs cohabitating at the refuges (i.e. using the same refuges simultaneously). Apart from viviparous lizards paired with either of the two snake species, the frequencies for both variables were either as expected or significantly greater than expected by chance; in particular grass snakes cohabited much more frequently than expected with both vipers (+327%) and slow worms (+218%). In contrast, for the viviparous lizard/viper pair both variables were statistically significantly less frequent than expected by chance - monthly overlap (-35%) and cohabitation (-87%). As viviparous lizards are predated by vipers, cohabiting would be much reduced but the significantly lowered monthly spatial overlap may indicate active avoidance. For the viviparous lizard/grass snake pair the spatial overlap was as expected by chance but, as with vipers, the frequency of cohabiting was significantly less than expected by chance (-75%). These observations are discussed in relation to the known kairomonal responses of this lizard to viper and grass snake deposits. Viper deposits are also believed to act as a kairomone for slow worms, but we found no evidence that they avoided vipers, supporting existing literature showing that *V. berus* is unlikely to be an important slow worm predator.

Keywords: species associations, cohabitation, corrugated iron refuges, viper kairomone, chalk grassland

INTRODUCTION

Artificial refuges of galvanized corrugated iron are commonly used for monitoring reptiles that may be found beneath or on them (Dodd, 2016). These refuges give both a suitable fixed reference point and a location where reptiles may be observed singly or in combination. Consequently, observations at refuges are a convenient way to investigate the temporal and spatial overlap between species leading to possible insights into the relationships between species and between conspecifics. For example, the relationship between adult and juvenile smooth snakes *Coronella austriaca* was observed at arrays of refuges where the juveniles appeared to confine themselves to the periphery of the population, which has been interpreted as their avoidance of the cannibalistic adults (Kolanek et al., 2019). Laboratory studies in which potential prey species such as the viviparous lizard *Zootoca vivipara* (Thoen et al., 1986; Van Damme et al., 1995) and slow worm *Angius fragilis* (Cabido et al., 2004) were exposed to chemical deposits from lizard predators such as the smooth snake and northern viper or adder *Vipera berus* have demonstrated clear responses that suggest the chemicals involved are a kairomone used by these lizards to avoid predation. In the case of *Z. vivipara*, the observed responses were changes from normal behaviour, which involved an alternation of activity bouts and basking, to long periods of immobility, interrupted by jerky, hesitant walks, behaviours that would reduce their mobility and hence chances of detection. There appears to have been only a single study outside the laboratory on the behaviours induced by this kairomone. This investigated the associations between viviparous lizards and slow worms with their predator the smooth snake. In this case, records from iron refuges suggested that there was no evidence that either lizard species avoided shelters regularly used by smooth snakes and it was concluded that old chemical cues from smooth snakes apparently did not deter its potential prey from using the same shelters (Ceirans & Nikolajeva, 2017).

A 15-year reptile monitoring project in a chalk grassland reserve in south-eastern England offered an opportunity to document potential spatial and temporal associations between viviparous lizards *Z. vivipara*, slow worms *A.*

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fragilis, northern vipers *V. berus* and grass snakes *Natrix helvetica* at corrugated iron refuges; the two lizard species are potential prey of the viper (Prestt, 1971; Beebee & Griffiths, 2000), while the grass snake is not known as a lizard predator, especially in Britain (Gregory & Isaac, 2004). The associations between the species were examined by comparing differences between the observed and expected overlaps in monthly use of refuge positions and between observed and expected rates of cohabitation at refuges.

MATERIALS & METHODS

The data presented here were collected over 15 years (2008 to 2022) during visits to a chalk grassland reserve (open area of about 10 ha) south-east England (51° 19' N, 000° 11' E), in the reptile active period from March to October (8 months). The number of annual visits varied from 56 to 79, which were mostly undertaken from 09:00 h to 12:00 h. Each visit followed a standard survey path (3 km) and at intervals along the path there were refuge positions where artificial refuges of galvanised corrugated iron (dimensions - 50 cm x 60 cm, 0.5 mm thick, 0.5 g/cm^2) were laid. The refuges were placed on the slopes of this reserve in similar habitat with more or less equal representation of south, south-west and west facing aspects. In 2008, there was a total of 31 refuge positions. The number of refuge positions was gradually increased at the beginning of each year until it reached 50 positions in 2016 and remained constant thereafter. According to anecdotal experience of surveying this site, the two snake species were frequently found at refuges when digesting food or sloughing, the viviparous lizards were typically basking on refuges or beneath them sloughing or just sheltering, and slow worms were more permanent residents. Occasionally, viper cadavers were encountered and the stomach contents of these was examined to give some information on the diet of this species.

Records were made of the reptiles at refuges, overwhelmingly under the refuges but occasionally on them. This investigation was part of a much more detailed study where reptiles were also recorded along the survey path and at refuges made from roofing felt, we have only reported reptile observations from the corrugated iron refuges. This is because we wished to make comparisons between all four reptile species and, apart from slow worms, corrugated iron refuges provided considerably more data.

Statistical analyses

Three main analyses were conducted using the collected data. The first analysis explored the relationship between the number of each reptile species at refuges and the number of available refuges. To give a measure of the spatial and temporal distribution of reptile species, their use of refuges was then subject to two further different analyses. The first analysis considered monthly overlap of species pairs in their use of refuge positions (use of same refuge positions irrespective of whether animals were found together or not). The second analysis concerned the degree to which individuals of pairwise species combinations were cohabiting (species present together at refuges at the same time).

Correlation of reptile encounters and refuge numbers

Variation in the monthly rate at which the four reptile species were found at corrugated iron refuges was compared using a plot of $\log_{10}$ transformed monthly means with their standard deviations. The relationship between this encounter rate at refuges and the number of different refuges occupied monthly by each species was then examined by preparing a log/log plot of these two variables and the strength of this relationship tested using the non-parametric Spearman's rank correlation on the transformed data set. The Spearman's rank test was used as data, even when log-transformed, did not meet the assumption of Normal distribution required for parametric analysis.

Monthly spatial overlap of refuge occupancy by species pairs

The first analysis was designed to answer the question - 'To what extent do the species pairs overlap in their choice of refuge positions?'. This was assessed by comparing the observed frequency of monthly overlap for each species pair with the expected frequency of overlap. The analysis was initiated by making a count of the number of unique refuge positions that each species had occupied in each of the 120 months of the study. Then a count was made of the number of unique refuge positions that had been occupied at some time during the month by each of the six pairwise species combinations (Table 1). As an illustration, if we were operating 50 refuge positions then the maximum 'overlap' would be 50 and the minimum would be 0, i.e. there was no attempt to quantify the number of overlaps at the same refuge position. The total count of unique refuge positions (number refuge positions x number months, summed over 15 years) was 5264 or 658 for each of the eight study-months (March to October) across the 15 years of study.

The probability of each species alone occupying a refuge was determined by dividing the occupancy frequency (number of unique refuge positions occupied each month) by the total number of unique refuges inspected each month for the 15-year study (658). Then the probability of any two species pair using the same refuge position during a given month ($P_{overlap}$) was estimated as the product of the two single species probabilities. Data were then analysed using simulation under the null hypothesis that species distributions are independent of one another. This approach, which does not require assumptions of independence to be met (Dugard et al., 2012), has been taken as in our data repeated measures from the same population violates the assumption of independence of data points made by more conventional statistical methods. The analysis was undertaken using base R (version 4.2.0) in RStudio (version 2022.2.02) (R Core Team, 2022). For each species pair, for each observation (i.e. for each month for each year, or 120 observations), the expected number of overlaps was drawn from a binomial distribution with

the number of observations set to the number of unique refuges present that month and the probability of overlap set to $P_{overlap}$. The mean number of simulated overlaps was then calculated. This process was repeated 9,999 times to generate a frequency distribution of simulated observed counts under the null hypothesis, against which the observed mean overlap (representing the 10,000th repeat) was compared. The proportion of this distribution more or less than (whichever was less) the observed mean was then used to calculate two-tailed p-values by doubling the 1-tailed p-value. As calculations were made individually for each observation (i.e. each month for each year), variation driven by year and month is accounted for and the analysis considers overall trends across the dataset. Two-tailed tests were used as there was no *a priori* prediction of direction of any effect. Probability values were treated as statistically significant if $p_{2\text{-tailed}} < 0.05$.

Cohabitation at refuges by species pairs

The second analysis was designed to answer the question, 'Do the reptiles tolerate the presence of each other under the same refuges?'. We counted instances where pairs of the four reptile species were observed actually cohabiting. There was a total of 44,536 refuge observations (number refuge positions x number site visits, summed over 15 years) during the study. To assess the statistical significance of the extent to which different species pairs were observed cohabiting, the observed total monthly counts for species pairs were compared with expected counts. We determined the frequency at which each species alone was found at refuges by dividing the observed numbers of refuges occupied by each species over 15 years (slow worm: 3802 refuges, viviparous lizard: 1661 refuges, viper: 1478 refuges, and grass snake: 330 refuges) by the total number of refuge inspections (44,536). The probability of any two species cohabiting ($P_{cohabit}$) was the product of the two refuge use rates. These data were analysed in the same manner as overlap data, using simulation, but with the binomial distribution probability parameter set at $P_{cohabit}$.

The data for the monthly spatial overlap and cohabitation analyses can be found in Supplementary Material on the BHS website and and the R code at https://github.com/CJMichaels/Hodges-et-al-2024.-Spatial-and-temporal-association.

RESULTS

Variation in encounter rates at refuges

All four reptile species were encountered at corrugated iron refuges during the reptile active season of March to October (Fig. 1). The mean encounter rate of slow worms, however, was generally greater than the other three species, while grass snakes were always encountered at much lower rates than the other three taxa.

Monthly spatial overlap in refuge occupancy

The total 15-year monthly counts of different refuge positions used individually by the four reptile species show that the greatest number of different positions

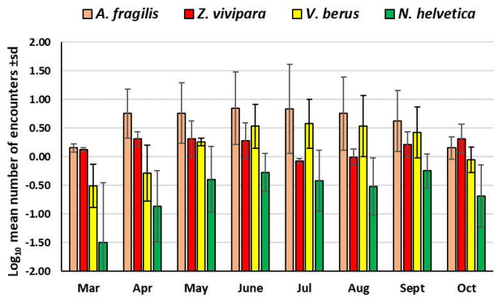

Figure 1. Mean monthly encounter rate (log scale) ± standard deviation for four reptile species on a chalk grassland reserve recorded from corrugated iron refuges, 2008 to 2022

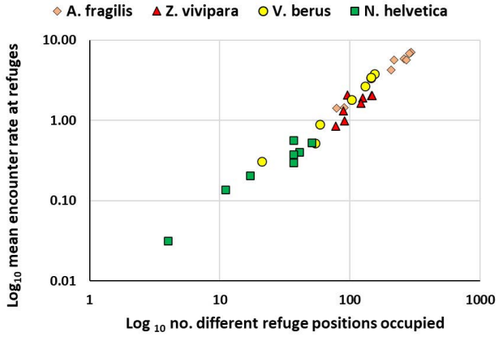

Figure 2. A log/log plot of the mean monthly encounter rate of four reptile species against the monthly total number of different refuge positions occupied during the eight-month reptile active season from 2008 to 2022

was occupied by slow worms (1708, 32% of total). While viviparous lizards (896, 17% of total) and vipers (812, 15% of total) were similar and grass snakes much lower (235, 4% of total). When the monthly encounter rates for each species (Fig. 1) are plotted against the monthly totals for the number of different refuge positions occupied (Fig. 2) the two are strongly correlated ($r_s = 0.98$, $n = 32$, $p < 0.001$). This suggests that for all four species, the number of different refuges at which the species were found is a function of their encounter rates (potentially a proxy for abundance).

When the observed and expected numbers of spatial overlaps of species pairs at different refuge positions were compared, those for the viviparous lizard/viper pair were significantly lower than would be expected by chance (*Zv/Vb*, Table 1), i.e. there was less overlap in the use of the same refuge positions than would be expected if refuge use by each species was independent of the other. The counts of refuge positions used by viviparous lizards with either grass snakes or slow worms were no different from those expected by chance (*Zv/Nh*, *Zv/Af*, Table 1). In the case of the three other pairwise species combinations, observed counts of shared refuge positions were all greater than expected by chance (*Af/Vb*, *Af/Nh* & *Vb/Nh*, Table 1).

Table 1. Pairwise comparisons of the observed and expected degree of spatial overlap between four reptiles species (*Vb = Vipera berus, Zv = Zootoca vivipara, Nh = Natrix helvetica, Af = Anguis fragilis*) expressed as counts of the same refuge positions occupied by both species at some time during any given month (over 15 years), and the statistical probability of the difference between observed and expected values occurring by chance, significant p values are in bold.

Species Pair	Observed	Expected	$p_{2\text{-tailed}}$	Direction
ZV/VB	0.733	1.132	**< 0.0001**	Lower by -35%
ZV/NH	0.35	0.337	0.857	-
ZV/AF	2.65	2.460	0.178	-
AF/NH	1.192	0.733	**< 0.0001**	Higher by +63%
AF/VB	3.2	2.505	**< 0.0001**	Higher by +28%
VB/NH	0.85	0.367	**< 0.0001**	Higher by +132%

Table 2. Pairwise comparisons of the monthly (for 15 years) observed and expected count of cohabitation by four reptile species (*Vb = Vipera berus*, *Zv = Zootoca vivipara*, *Nh = Natrix helvetica*, *Af = Anguis fragilis*), at corrugated iron refuges and the probability of the difference between observed and expected values occurring by chance, significant p values are in bold.

Species Pair	Observed	Expected	$p_{2\text{-tailed}}$	Direction
ZV/VB	0.058	0.46	**<0.0001**	Lower by -87%
ZV/NH	0.025	0.103	**0.001**	Lower by -75%
ZV/AF	1.05	1.183	0.165	-
AF/NH	0.742	0.236	**<0.0001**	Higher by +214%
AF/VB	1.317	1.052	**0.008**	Higher by +25%
VB/NH	0.392	0.091	**<0.0001**	Higher by +331%

Cohabitation at refuges by species pairs

Species pairs cohabiting at the corrugated iron refuges (Fig. 3) were not uncommon. The total 15-year counts of cohabiting species pairs were as follows *Zv/Vb* - 7, *Zv/Nh* - 3 , *Zv/Af* - 126, *Af/Nh* - 89, *Af/Vb* - 158 and *Vb/Nh* - 47. The number of viviparous lizards paired with either vipers (*Zv/Vb*, Table 2) or grass snakes (*Zv/Nh*, Table 2) were statistically significantly lower than expected by chance. In contrast, vipers paired with grass snakes or with slow worm (*Vb/Nh*, *Af/Vb,* Table 2) or slow worms with grass snakes (*Af/Nh*, Table 2) and were all cohabiting significantly more than expected by chance. For the viviparous lizard/slow worm pair (*Zv/Af*, Table 2) the observed counts of cohabiting pairs were no different than expected by chance.

The only cases where species pairs were observed cohabiting less frequently than expected by chance were between the viviparous lizard and the two snake species. There were only seven occasions when a viviparous lizard and a viper were found cohabiting. These observations were made in the months of May to September in three years (2010 x 2, 2015 x 3, 2020 x 2), and the vipers involved were 1 adult female, 4 sub adults and 2 juveniles while the viviparous lizards were 3 adult females, 1 adult male, 1 sub adult and 2 juveniles. Likewise, there were only 3 occasions when a viviparous lizard and a grass

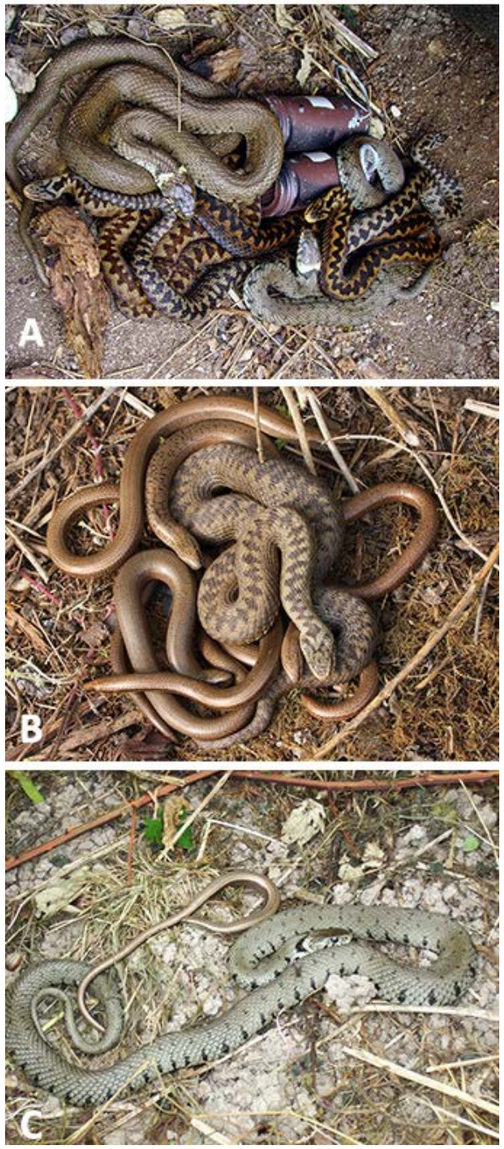

Figure 3. Reptile species pairs found together under corrugated iron refuges - **A.** Adult *Vipera berus* and *Natrix helvetica* (the canisters hold data loggers for a different study), cloudy eyes indicate that both grass snakes are soon to shed their skins, **B.** Adult female *Vipera berus* and five adult male *Anguis fragilis*, a particularly good example from a different study site nearby, and **C.** Sub adult *Anguis fragilis* with an adult female *Natrix helvetica.*

Table 3. Summary of the average observed/expected differences between species pairs for monthly spatial overlap in the use of the same refuge positions and cohabitation at corrugated iron refuges (*Vb = Vipera berus*, *Zv = Zootoca vivipara*, *Nh = Natrix helvetica*, *Af = Anguis fragilis*)

Reptile pair	*Zv/Vb*	*Zv/Nh*	*Zv/Af*	*Af/Nh*	*Af/Vb*	*Vb/Nh*
Spatial overlap	35% less than expected	As expected	As expected	63% more than expected	28% more than expected	132% more than expected
Cohabitation	87% less than expected	75% less than expected	As expected	214% more than expected	25% more than expected	331% more than expected

snake were found cohabiting. These observations were confined to the months of June to September in three years (2008 x 1, 2014 x 1, 2019 x 1) and the grass snakes involved were two sub adults and one adult male, while the lizards were one adult male, one adult female and one sub adult.

Observation on adder prey species

Fifteen viper cadavers were found over the course of the study. Their stomach contents included viviparous lizards, voles *Microtus* sp, wood mice *Apodemus sylvaticus* and shrews *Sorex* sp, with no slow worms despite these being a very commonly encountered species. Furthermore, there were no observations of slow worms being eaten by vipers and the two species were observed cohabiting beneath refuges more frequently than any other species combination.

DISCUSSION

All four reptile species inhabiting the chalk grassland reserve used the corrugated iron refuges throughout the reptile active period, however encounter rates varied by month across the year probably related to weather conditions and physiological and reproductive status of the individuals concerned. It was of interest to note that at the two extremes of the reptile active season, March/April and October, the two lizard species were encountered at greater rates than the two snake species suggesting a greater use of refuges in cooler weather and possibly, at least in the case of the viviparous lizard, earlier emergence from, and later entry into, hibernation (an observation made by Viitanen, 1967). For all species, the observed encounter rates, a proxy for abundance, were positively correlated with the number of different refuge positions occupied. This indicates that as the encounter rates rose more refuge positions were occupied instead of simply more reptiles appearing at the same refuges, which would instead have resulted in a sharp increase in the degree of aggregation. It also implies a relatively high degree of uniformity in the underlying physical characteristics of refuges. The degree of monthly spatial overlap between the four reptile species in their use of the same refuge positions and the extent to which these species were observed cohabiting at refuges is summarised in Table 3.

Deviations from expected values for spatial overlap and cohabitation are likely to arise from either attraction or avoidance between species pairs and/or from a convergence between species in their preference for particular microhabitats. Predator-prey relationships would also potentially have a significant bearing on this. The northern viper is known to be a frequent predator of the viviparous lizard (Prestt, 1971; Beebee & Griffiths, 2000). In the case of slow worms, Prestt (1971) reported that *A. fragilis* amounted to only 2% of the stomach contents of *V. berus* and our own observations showed no evidence of the slow worm as a prey species. Slow worms are well protected with osteoderms in their skin (Beebee & Griffiths, 2000) and would be relatively difficult to grasp and swallow. It seems probable that they are rarely eaten by *V. berus*. Grass snakes are not known to be lizard predators but feed on fish, amphibians and small mammals (Gregory & Isaac, 2004). Our interpretations of the observed associations between the four reptile species are as follows.

Association between viviparous lizards and vipers

The association between viviparous lizards and the vipers gave significantly lower than expected values for both overlap in use of the same refuges, less by 35%, and in cohabitation less by 87% (Table 3). Viviparous lizards are a common food item of vipers so any lizards cohabiting with vipers at refuges are likely to be eaten thus reducing the chances of finding them together. Over 15 years the two species, on a monthly basis, occupied a similar number of different refuge position, viviparous lizards 17% of the total available and vipers 12%. Nevertheless, on the same monthly basis and in effectively the absence of the other species, their degree of overlap in the use of these refuge positions was significantly and substantially lower than expected by chance. This raises the possibility that some other explanation than simply predation may account for the difference. It seems reasonable to dismiss the possibility that viviparous lizards and vipers simply have different refuge preferences since there was a strong similarity between all refuge positions and the overlap of both vipers and viviparous lizards with the other two species was as expected or greater than expected suggesting that viviparous lizards and vipers would likely have similar preferences for the refuges on offer. It is known from laboratory studies that there is a kairomonal response of viviparous lizards to hexane-soluble chemical deposits from the skins of vipers (Thoen et al., 1986; Van Moorleghem et al., 2020) that has been observed to result in a suite of behaviours that may

reduce mobility and hence the chances of detection followed by predation. However, the reduction in overlap in refuge use could be taken as evidence that viviparous lizards are avoiding those refuges that have been used by vipers, where chemical deposits from vipers may act as a kairomone. If that is the case, then the kairomone not only produces a local response that reduces mobility but leads these lizards to actively avoid those places frequented by vipers. Laboratory studies of the responses of viviparous lizards to vipers have involved confined environments where escape or avoidance have not been options. Our observations suggest that in a more natural context, where attenuated chemical signals indicate that a viper is not in the immediate proximity, it may be 'safe' to show a locomotor response that results in avoidance/escape.

Association between viviparous lizards with grass snakes

The association between viviparous lizards and grass snakes differs from that with vipers in that the overlap was as expected by chance but, as with vipers, cohabitation was less than expected (Table 3). It is known that viviparous lizards respond to grass snake chemical deposits in the laboratory, but that the response is weaker than that to vipers and the lizard is probably able to differentiate between the two species (Thoen et al., 1986). It would appear that, although they do avoid cohabiting at refuges with grass snakes, viviparous lizards do not avoid refuges that have been used by them. Whether or not they avoid grass snakes themselves is uncertain because grass snakes and vipers show a greater than expected overlap in both choice of refuge position and in cohabiting (Table 3) so that any impacts the presence of grass snakes may have had would likely be confounded with that to vipers at shared refuges. Nevertheless, the result suggests that even if grass snake deposits had some effect this would have been weaker than that of the viper kairomone. To confirm this would require a study of associations between viviparous lizards and grass snakes in a habitat without vipers.

Association between viviparous lizards and slow worms

Both the overlap in refuge use and cohabiting between viviparous lizards and slow worms were no more or less than expected by chance. It might have been expected that there would be some convergence in their choice of refuges, or at least the microhabitats represented by the refuges, so that the associations would have been greater than expected. However, as vipers were using many of the same refuges as slow worms and there is evidence that viviparous lizards avoid vipers, it is likely that this has diminished the association with slow worms. Whether or not viviparous lizards' choice of refuges converges with that of slow worms would need to be investigated in a habitat without vipers.

Association between slow worms and vipers or grass snakes

The monthly overlap of slow worms with vipers was greater than expected (Table 3). This suggests that slow worms do not avoid vipers and that there is a convergence in the species preferences for the microhabitats of refuge positions. Also, more vipers and slow worms were observed cohabiting than expected by chance, this is perplexing as laboratory studies have revealed that slow worms do respond to the swabs taken from the skin of either *V. berus* or *Vipera ammodytes* (Telenchev et al., 2021); in the case of *V. ammodytes* slow worms do form a significant part of the diet (Anđelković et al., 2021). It seems that although slow worms may be aware of northern vipers they do not avoid them. We did not examine the relationship between a slow worm's size and its likelihood of sharing a refuge with *V. berus*, this would be an informative area of further research. The difference between observed and expected overlap in refuge use and cohabitation of grass snakes and slow worms was even greater than for vipers and slow worms. Slow worms feed on invertebrates such as slugs and worms that favour moist habitats while a large portion of grass snake diet comprises amphibians that are also found in more moist habitats. This preference for more moist habitats may account for active selection of the same refuges in a situation where slow worms do not actively avoid grass snakes.

Association between vipers and grass snakes

The two snakes species both occupied the same refuge positions and cohabited more frequently than expected by chance. In the absence of predator-prey relationships between the two snake species, it seems likely that the cryptic behaviours of both species when shedding their skins (Fig. 3) or digesting food may lead both species to select the same refuges; ones that offer more conducive environmental conditions and greater crypsis, at roughly the same times of the year. In Poland, *V. berus* and the eastern grass snake *Natrix natrix* have been documented sharing basking sites without any appearance of competition or other interference while in England during a time when male and female *V. berus* were in courtship the arrival of three *N. helvetica* at a shared site resulted in aggressive behaviour from the male vipers, this was interpreted as a behaviour specific to their reproductive condition (Dawson & Baker, 2020).

Besides cohabiting at refuges, reptiles may also cohabit at hibernacula. Viitanen (1967) lists cases from Scotland and Denmark of viviparous lizard hibernating with vipers *V. berus* and in the case of Finland mentions viviparous lizards, grass snakes and slow worms as well as the amphibians, the common frog *Rana temporaria* and common toad *Bufo bufo*, hibernating together with vipers. He comments that "joint hibernation is fairly common but the number of individuals remains low compared to the number of vipers." He also observes that "the arousal of the viviparous lizard from hibernation occurs about a week before vipers." The seeming regularity of viviparous lizards hibernating with vipers is in contrast to their apparent avoidance at refuges. If response to the viper kairomone leads to avoidance behaviour then it seem reasonable to speculate that at lower temperatures the threshold of response is raised to such an extent that that avoidance may be suppressed. This may pose no threat to the lizards as at lower temperatures the snakes are not feeding, furthermore the lizards are expected to leave hibernacula before the vipers become active.

Our results may be interpreted as viper kairomone stimulating active avoidance by viviparous lizards rather than just a reduction in mobility as observed in laboratory studies. The kairomone would also appear to be persistent enough to remain active after a viper has moved away. To confirm this tentative avoidance response requires further study using an experimental approach to detail the behaviours involved and to quantify the relationship between response and the kairomone. Additionally, our pairwise analyses cannot account for more complex multi-species interactions in refuge choice and further research with more data from sites with different combinations of reptile species may extend these methods to further explore species interactions in a landscape.

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Aspects of the demography of two *Podarcis muralis* populations in anthropogenic modified habitats in western France, based on a non-invasive sampling method

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Urbanisation impacts on both abiotic and biotic characteristics of the environment and is likely to bring new selective pressures on animal species living within these areas. The common European wall lizard *Podarcis muralis* adapts well to urbanisation and indeed may be described as the archetypical urban lizard. In this paper we investigated some aspects of the demography of two populations of *P. muralis* from western France, one living in a hedgerow system situated in an agricultural area on the edge of a village and a second in an urban garden. The active year in both populations was from February–March until October–December, the latter in the urban garden where temperatures were higher. Numbers decreased from around June then increased again during September but this varied annually and between populations. Diversity and equality indexes for both populations were high, especially in females, indicating a range of individuals and their frequency of presence. Both populations were therefore stable despite only limited numbers of lizards being present for more than one year. Male lizards with high presence were more frequently seen in the company of females than males that were seen less frequently. Hatchling lizards were seen from June after the spring mating period with a second period of hatchlings appearing during autumn. This supports the notion that females annually deposit two clutches of eggs in the area.

Keywords: lizards, *Podarcis muralis*, demography, western France, non-invasive sampling

INTRODUCTION

Whereas many reptile populations have been monitored in order to analyse their demography (e.g. Guiller et al., 2022; Kusrini et al., 2022), quantitative data on reptiles living in anthropised landscapes, especially at the level of the individual, are still limited (French et al., 2018; Doherty et al., 2020). For example, since reptiles have a limited ability for dispersal and relatively small home ranges, they are more likely to be exposed to increased risk of population decline and collapse due to habitat modifications (Doherty et al., 2020; Guiller et al., 2022). One species that thrives in urban areas is the European wall lizard *Podarcis muralis* (e.g. Capula et al., 1993; Williams, 2019). This species has successfully colonised many new areas, including urban environments, where they are non-natural due to pet trade escapes or deliberate introductions (e.g. Gherghel et al., 2009; Allan et al., 2011; Kolbe et al., 2013; MacGregor et al., 2017; Williams, 2019; Williams et al., 2021) and can be regarded as a model species to understand how urban lizards are able to colonise and persist in anthropogenic environments.

Several studies have examined *P. muralis* population ecology within its natural range (e.g. Barbault & Mou, 1988) including in urban environments (e.g. Edsman, 1990; Meek, 2014a; 2014b; 2020; Heym et al., 2013; Lazic et al., 2017; Williams, 2019). Edsman (1990; unpublished PhD thesis) described a five-year study of behaviour and territoriality of individual lizards living on an ancient stone wall in Fiesole, central Italy. The study was primarily focused on the causes and consequences of male territoriality in *P. muralis* based on individually marked lizards. The results showed that males defended long term territories encompassing the home ranges of resident reproductive females. Females were evenly distributed on the wall and hence larger male territories contained more females. Edsman concluded that females remained in male territory because it offered access to optimal basking places, which is important for reproduction.

In this paper, we describe the results of a three-year study in western France on two populations of *P. muralis* situated less than 1 km from one another, a hedgerow in an agricultural area and an urban garden. This means that both populations experienced a similar climate but occupied structurally different habitats,

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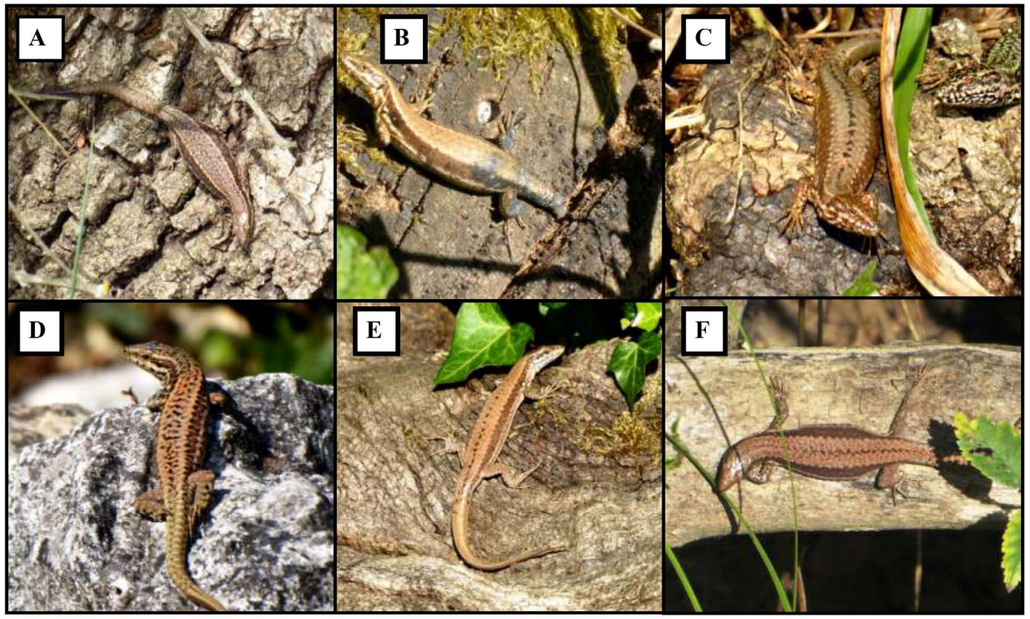

Figure 1. Examples of patterns and colours of females from the two populations. Lizards **A, B, C** and **F** recorded in the hedgerow, **D** and **E** in the garden. Lizards A-E have all experienced autotomy with B also experienced some injury.

for example floral composition and different thermal regimes (e.g. Battles & Kolbe, 2019; Campbell-Staton et al., 2020). Most demographic studies rely on the capture-mark-release-recapture (CMRR) method that provides robust information, for example capture enables direct measurement on growth rates and age structure. However, it is also time-consuming and invasive necessitating disturbance to the subject animals, which potentially risks dispersal and distortion of population characteristics including behaviour and survival rates of individuals (Wilson & McMahon, 2006). So, when possible, it would be useful and important to employ alternative methods than CMRR, which are those that do not require the capture and handling of individuals of the target species. The densities and unique individual dorsal pattern markings of *P. muralis* enabled the tracking of individuals over time (Figs. 1 & 2). Thus, we employed for data collection a photographic-mark-recapture method that minimises disturbance to the lizards and once they become adapted to the researchers presence (Dustin et al., 2020) facilitates data collection. Although errors in identification have occurred using photographic identification they have usually concerned animals with poorly defined markings (e.g. Choo et al., 2020). However, individual *P. muralis* often vary greatly (Figs. 1 & 2) and when numbers are relatively low identification errors are minimised. Non-invasive methods, including photographic-mark-recapture, have in recent years gained importance as a research tool to minimise these effects (Choo et al., 2020). Here we employ the method to ask the following questions:-

1. When collecting data on the duration of presence of individuals in the two populations, we noticed that some individuals remained within the population over the entire three-year study period but that others did not. We also noticed that there was a correlation between the number of times that an alpha male was sighted and the likelihood that it would be seen basking in the presence of females. These two aspects of the demography of the two populations are analysed further here. Is high male presence (= high sighting frequencies) associated with male-female presence? We answer this question by comparing counts of individual males and their corresponding frequencies in the company of females.
2. In a previous study, Meek & Luiselli (2022a) showed that *P. muralis* were generally social lizards with high frequencies of communal basking. This might suggest that tail breaks and their frequencies are due to predation pressure and not primarily a result of intra-specific conflicts. We then asked the question, what are the tail breakage levels and are there differences between populations and between males and females?
3. What was the long-term presence of individual lizards in the study areas?

MATERIALS & METHODS

Study areas

The study was carried out during 2020, 2021 and 2022 on two populations of *P. muralis* in a hedgerow (PH) and urban garden (PG). The study area lies on the edge of the village of Chasnais (46° 27 N, 1° 53 E), western France and was selected because of the numbers of lizards present that also quickly habituated to observer presence minimising observer effect (e.g. Diego-Rasilla, 2003b). The garden area was rectangular shaped with an area of 1197 m^2 and

hedgerow 190 m in length (Meek & Luiselli, 2022a). Both habitats were structurally relatively simple. The hedgerow consisted of mostly low growing bush *Rubus fruticosus* and *Hedera helix*, ash tree *Fraxinus excelsior* and oak *Quercus robur*, with open sunlit patches and shaded areas that facilitated thermoregulatory opportunities (Meek & Luiselli, 2022b) and detection. The urban garden had more open areas with approximate 40% cover compared to around 90% cover in the hedgerow but many plant species were non-natural. Both areas had water present, a drainage ditch running parallel to the hedge and several small man-made ponds in the garden. By June the drainage ditch alongside the hedgerow was usually devoid of water but was present in the garden ponds all year. Both study areas are open systems with no barriers preventing lizards from entering or moving outside the study areas.

Lizard sampling

Allowing for inclement weather, sampling was approximately even across seasons and carried out 5–6 days each week for around 45–60 minutes daily, usually from around 08:30 h but up to around 13:00 h. In the hedgerow habitat, sampling was mostly confined to the morning (first lizards seen around 09:00 h) until midday due to the habitat being in sun most of the day. Sampling was between February to December in the garden and February to October in the hedgerow. Data were collected by a single observer walking along the hedgerow covering 1140 m (= 6 x 190 m) and around six times around the garden area and photographing any lizards detected. All sighted lizards were photographed using a Lumix DMC-TZ70 camera set on Intelligent-Auto for rapid use. However, a number were discarded due to low quality identification (e.g. out of focus), which was expected using this setting. Identification was by colour, markings, tail loss and the position of loss, and abnormalities of re-grown tails if present (See examples in Figs. 1 & 2). When possible several photographs of each lizard were taken, but only the best quality used for each daily sighting. Using one photograph per lizard per daily sampling session was adequate to register that the lizard was present on that day (Welbourne et al., 2020).

Statistical analysis

The Shannon-Weiner index (Spellerberg & Fedor, 2003) was repurposed to calculate lizard diversity based on individual presence. The method is normally used to quantify diversity in species assemblages and is based

Figure 2. Examples of male lizards. Lizard **A** was a male recorded in high frequencies in the hedgerow during the three-year study period. Lizard **B** a male recorded in high frequency in the garden from 2020 into early 2021 and known from 2018 indicating a minimum five years in age. Lizard **C** is an example of a male from the garden population with a likely viral papiloma on the flank (Baxter & Meek, 1988). Lizards in **D** were two of the four males that entered the garden after the disappearance of lizard B, photographed here in a territory dispute.

on the probability of sighting a single lizard two or more times in succession if the lizards in the population were encountered at random. It evaluates the numbers of individual lizards that were present in each study area and their sighting frequencies. In theory the method has no limit with high scores indicating high diversity (many lizards and high frequency of presence) and a zero score when only a single lizard is present. The index is calculated from

$$H = -\sum[(p_i) \times \log_e(p_i)]$$

where the Shannon-Weiner index index score *H* is derived from p_i the numbers of individual lizards and their sighting frequencies. The *H*-values were then employed to calculate the degree of equability *E* of individual presence derived from

$$E = H / \log_e(K)$$

where *H* are the Shannon index values and *K* the number of individuals in the sample. Equability values range from 0 to 1, where a theoretical value of 1 indicates all individuals were sighted an equal number of times. Low *E*-values indicate individual lizard presence was unequal. Values of *H* and *E* were found for males and females separately and for pooled samples of males, females and juveniles.

We then compared the distributions of female sighting frequencies between years for both populations using a Kruskal-Wallis non-parametric test. This pools and ranks the number of lizards and sighting frequencies in each population and then returns the ranked data to their original columns and compares the rankings using a χ^2 test. The null hypothesis is that female numbers and individual frequency of presence is the same for each year. Post hoc, when appropriate, is by Dunn`s test.

Regression analysis was used to compare count frequencies of solitary males with numbers of males in association with females. To improve the fit, males in association with females were treated as the dependent variable y after transformation to logarithms ($\log_e$) and male counts as the independent variable x. If a zero count was present in the y-array, $\log_e y(+1)$ was applied. This gives an equation of the form

$$\text{Log}_e y = m \pm \varepsilon x + b$$

where $\log_e y$ (or $\log_e y+1$) is the number of males with females and x the number of corresponding solitary male counts in linear form with ε white noise error (Gotelli & Ellison, 2004). The null hypothesis is $m = 0$, which would indicate no relationship between solitary male numbers and males with females; significant positive departures from m indicative of males with high presence counts are more likely to be seen with females. Departures from $m = 0$ were evaluated using a *t*-test at n-2 degrees of freedom (Bailey, 1995).

If it is assumed that males have a greater chance of having access to females they should be present over most of the active time period. If so, are all lizard sighting frequencies a measure of the length of time they were present in the study area? This notion was tested by regressing the number of days (N_{days}) each lizard was observed between first and last annual sightings against the numbers of times they were counted (N_{mc}). In the regression, days between first and last sighting were arbitrarily treated as the independent variable x (N_{days}) and sighting frequencies as the dependent variable y (N_{mc}). No relationship between the two would be indicated if the regression coefficient did not differ significantly from 0. In all tests alpha was set at 5%, with Minitab V17, and various internet statistical sites used for data analysis (e.g. Rain, 2023).

Table 1. Summary statistics for two *P. muralis* populations during the three-year study period based on identified lizards. For juveniles only counts of sighting are given due to difficulties of individual identification

	# of lizards	# of sightings (photo-graphs)	Ratio of individual females to males	Ratio of female to male sighting frequency counts	Ratio of adult to juveniles sightings (m+f)/j
Garden					
2020 Males	2	83			
2020 Females	15	122	7.5	1.46	7.32
2020 Juveniles	-	28			
2021 Males	5	62			
2021 Females	13	95	2.6	1.37	11.21
2021 Juveniles	-	14			
2022 Males	3	59			
2022 Females	13	88	4.33	1.49	14.7
2022 Juveniles	-	10			
Hedgerow					
2020 Males	4	37			
2020 Females	12	66	3.0	1.78	12.87
2020 Juveniles	-	8			
2021 Males	5	84			
2021 Females	13	113	2.6	1.34	49.25
2021 Juveniles	-	4			
2022 Males	4	92			
2022 Females	17	135	4.25	1.46	28.37
2022 Juveniles	-	8			

RESULTS

General overview

Number of identified lizards and their sighting frequencies for both populations are shown in Table 1 and graphically in Figures 3A and 3B. During the three-year period a total of 54 adults were identified in the hedgerow, which were sighted 541 times, with 51 adults sighted 577 times in the garden. Most sightings were from late February to March, with usually the alpha male appearing first (see below), but sightings declined from June to August then increased during September. Alpha male sightings (black cells in Figures 3A and 3B) were greater than other males (cross hatched cells) but were fewer late in the year, especially compared to females.

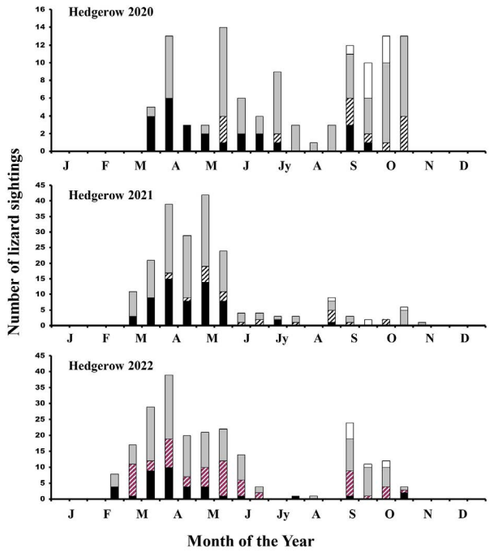

Figure 3A. Annual sightings (15-day intervals) in the hedgerow. Black cells represent male lizards with high sightings, crosshatched other males, females grey cells and open cells hatchling/juveniles. See text for further details

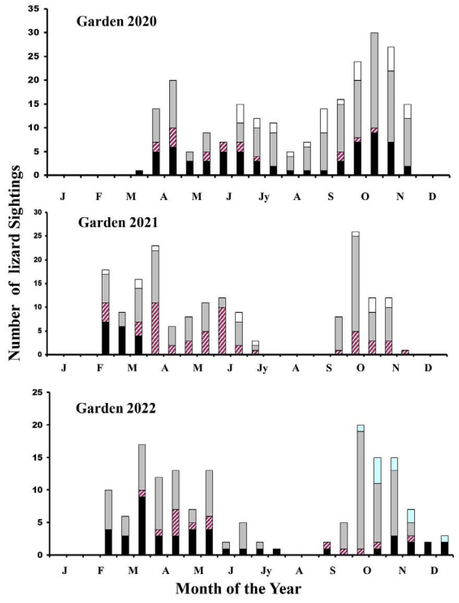

Figure 3B. Annual sightings (15-day intervals) in the garden. Cell differentiation is given in Fig. 3A. See text for further details

Table 2. Results from the Shannon-Weiner analysis (*H*) of numbers of individuals and their relative abundance derived from sighting frequencies (*Σ*n). *E* values indicate equality of presence is in part calculated from the Shannon-Weiner results (see text). The eveness score ranges from 0 to 1 with 1 indicating equal individual presence. Pooled results indicates males, females and juveniles. The low *E* values for males reflect alpha male presence. See text for further details.

	Males	*Σ*n	Females	*Σ*n	Juveniles	*E* (pooled)	*H* (pooled)	*E* (males)	*H* (males)	*E* (females)	*H* (females)
Hedge											
2020	4	37	12	66	8	0.92	2.62	0.69	0.96	0.97	2.41
2021	5	84	13	113	4	0.85	2.46	0.59	0.82	0.93	2.38
2022	4	92	17	135	8	0.90	2.77	0.91	1.27	0.91	2.57
Garden											
2020	2	83	15	122	28	0.86	2.49	0.73	0.51	0.95	2.57
2021	5	62	13	95	14	0.92	2.65	0.99	1.59	0.86	2.15
2022	3	59	13	88	10	0.85	2.40	0.65	0.71	0.88	2.27

Females (grey cells in Figs. 3A and 3B) were present in greater numbers than males with female/male ratios (in terms of individuals) in the hedge from 2.6 to 4.25/1 and in the garden from 2.6 to 7.5/1. These ratios changed when adjusted for female/male sighting frequencies with the hedgerow population female/male ratios from 1.34 to 1.78/1 and the garden 1.37 to 1.49/1. The full results are shown in Table 1.

Sightings of hatchlings/juveniles - the latter are defined as offspring born late the previous year, were greater in the garden area (52 versus 20), possibly due to ease of detection. First sightings were usually from June in the garden although most were detected during September/October with September the main month of sightings in the hedgerow.

Diversity of individual presence

Results from the Shannon-Weiner analysis (*H*) of males, females and the pooled sample are shown in Table 2 along with the *E* values indicative of population equality. The lower *E* and *H*-values for males reflect fewer male numbers and their skewed presence but females showed high scores in both indexes and thus higher equality of presence compared to males (Fig. 4). The χ^2 Goodness of Fit tests comparing the rankings of individual lizard sightings indicated the annual counts of females and their individual

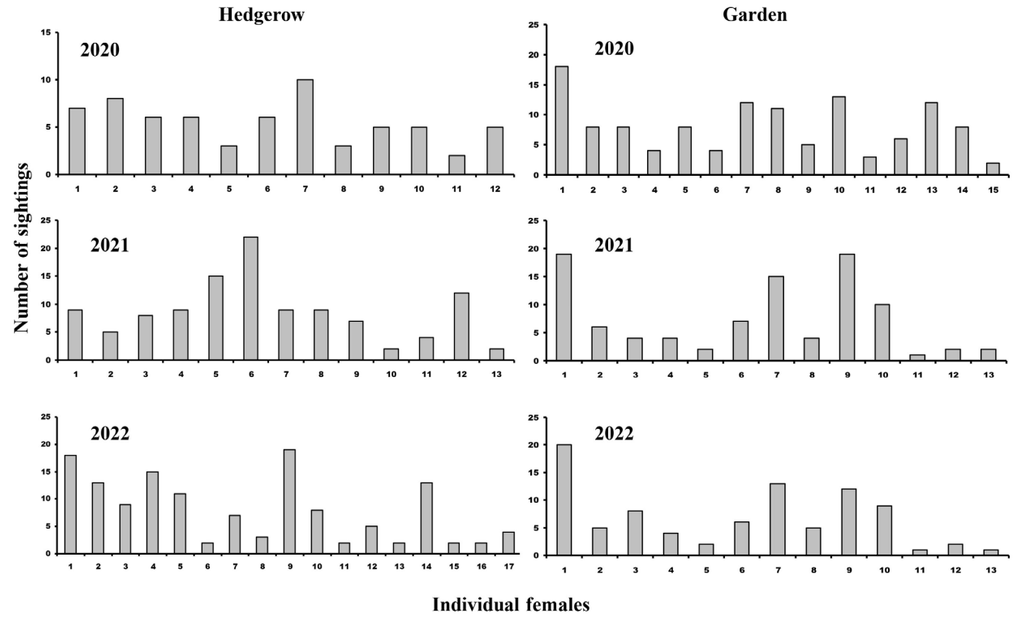

Figure 4. Number of individual females and their frequencies. Details are given in Tables 1 & 2.

frequencies were in good agreement; Garden χ^2 =1.3, P = 0.52, Hedgerow χ^2 = 1.93, P = 0.38. When year counts for individual females in each study locality were pooled and compared, sighting frequencies between populations were consistent; χ^2 = 3.41, P = 0.64, d.f. = 5 supporting the *E*-score results. This suggests healthy populations in both study populations' in all years.

The Kruskal Wallis χ^2 Goodness of Fit tests indicated that differences in annual male counts were significant; Garden (2020) χ^2 = 27.7, (2022) χ^2 = 49.2 both P < 0.0001; Hedge (2020) χ^2 = 36.6, (2021) χ^2 = 106.1, 2022, χ^2 = 21.6 all P < 0.0001. The exception was during 2021 in the garden population when each male was sighted in approximately similar frequencies that ranged from 13–21 times (χ^2 = 2.77, P = 0.6, 4 d.f.). This latter result was likely due to the early disappearance in 2021 of the 2020 alpha male, which was not seen after 28 March (Fig. 2B). Four males subsequently appeared in the study area and although sighted in approximate equal frequency this was generally during different time periods (see Fig. 2D).

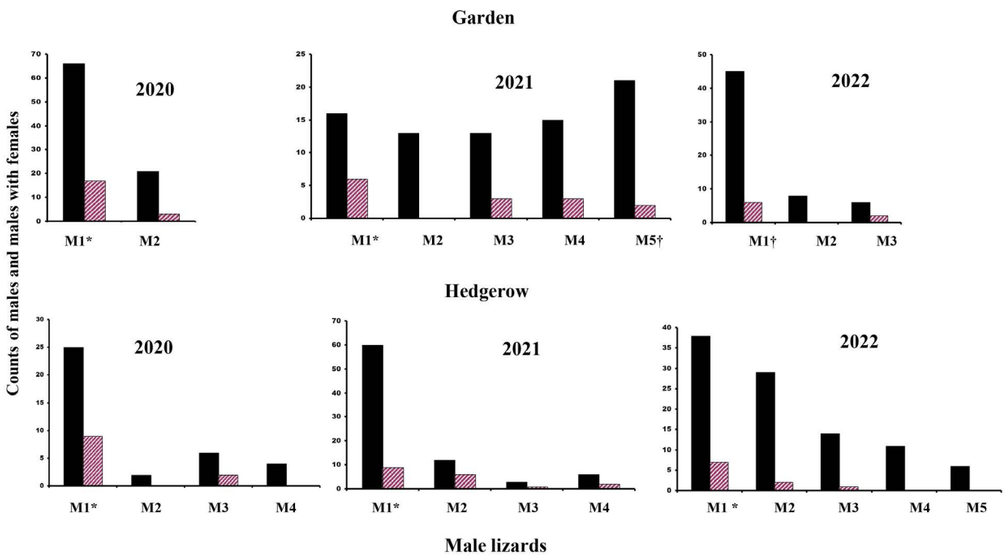

Figure 5. Male sightings (black cells) and their frequencies with females (cross hatched). Asterisk indicates same individual males in each population, with crosses a male that entered the garden in 2021 and was present in 2022.

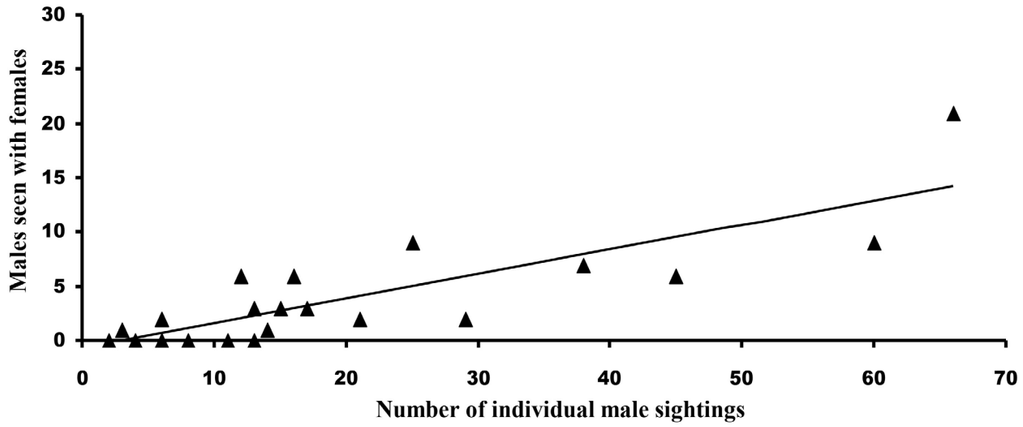

Figure 6. Graph of the general relationship (data from years and study sites data pooled) of male count frequencies and their presence with females. The line running through the data represents the regression equation given in the text. Data points situated on the x-axis indicate individuals not seen in female presence.

Male occurrence with females

Frequency counts of each male and the number of times they were present with females (N_{mf}) as the dependent variable was strongly associated with male frequency counts (N_{mc}). The regression equation was

$$N_{mf} = 0.04 \pm 0.007N_{mc} + 0.4$$

with 0.007 the white noise error ε. The regression coefficient of 0.04 was significantly different from a theoretical 0 (no-effect) coefficient (t = 5.73, P < 0.001). This suggests that males with high frequency counts were more likely to be alpha males with greater access to females (question 1). This indirectly supports the findings of Edsman (1990) that females mate mainly with male territory holders. Figure 5 shows histograms of individual males and their frequencies of associations with females. Figure 6 shows the general trends derived from the regression analysis with the line drawn through the data points calculated from the equation.

Sighting frequencies and time intervals

Regression of the number of male lizard sightings (N_{mc}) against time intervals (in days (N_{days})) between first and final sighting produced a positive regression coefficient

$$N_{mc} = 0.096 \pm 0.04\text{time } (N_{days}) + 3.73$$

with the coefficient significantly different from a no-effect (m = 0) of N_{days} on N_{mc} (t = 2.44, P = 0.02). The data for females gave similar positive results with the regression coefficients significantly different from 0: Garden m = 0.025 ± 0.01, t = 2.53, P = 0.02; Hedgerow m = 0.33 ± 0.01, t = 3.36, P = 0.002. This result indicates that frequencies of lizard sightings and length of time between sightings are strongly linked. The data are shown in Figure 7 with regression lines calculated from the equations.

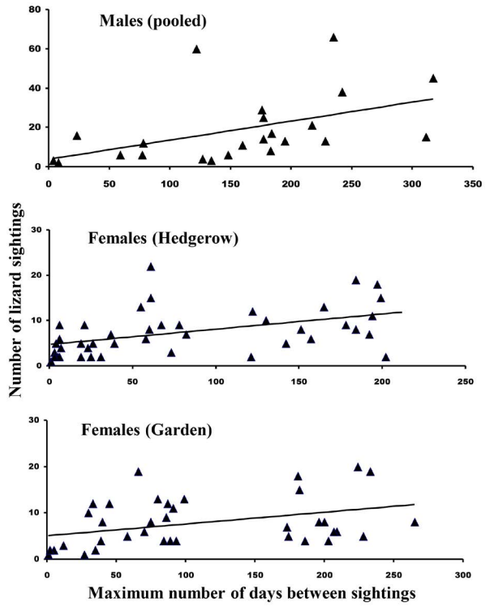

Figure 7. Graphs showing the relationship between lizard sightings frequencies and number of days between first and last sightings

Tail loss

Pooling the year data for male tail loss in both populations gave male tail loss at 66.7% in the hedgerow (12 of 18 males) and 55.5% (5 of 9 of males) in the garden population. These figures are corrected for lizards that were present for 2 or more years with assumed no further instance of autotomy and hence they were only counted as tail loss once. Female tail loss was in good agreement in both populations with 47.7% (21 of 44) in the hedge and 48.6% (18 of 37) in the garden population. However

tail loss varied within years. For example during 2021 all 5 males in the hedgerow had experienced tail loss at some time with 76% (13 of 17) of females showing high numbers in the garden during 2020 (question 2).

Temporal presence

Year to year presence (question 3) indicated that only two males (Fig. 2) in the hedgerow population were present for the three-year study period. Five individuals (2 in the hedgerow and 3 in the garden) were present for two years with four (2 in each study area) detected for just one year. This gives, for both areas 18.2% of males present for the three-year period and 45.5% for two-years. Females were present in greater number than males but in the garden population only three of 15 (20%) of 2020 females were seen continuously for the three-year study period. In the hedgerow four of the 2020 females were present in 2021. It should be noted however that absence of sightings does not necessarily indicate mortalities since both study areas were open habitats with no physical barriers to prevent the lizards going in or out of the study areas.

DISCUSSION

The diversity and equality results showed strong similarities in the numbers of individual lizards and their frequencies, either when comparing habitats or years. This dynamic is likely a key factor in enabling populations to remain stable over time despite general fluctuations in numbers over the wider area (Meek, 2020) and low year-on-year presence. A general trend in both populations was high spring activity followed by declines from around June in both populations (Table 2) with increased sightings from September through to October or December. This is indicative of a bimodal activity pattern due to a midsummer gap in activity as found in the sympatric green lizard *Lacerta bilineata* and other Mediterranean lizards (Luiselli et al., 2022; Meek & Luiselli, 2024) with the hotter dryer midsummer period likely driving the decline in sightings. These observations are similar to those observed in previous studies, including areas where they are non-natural (e.g. Allan et al., 2011; Rugiero et al., 2021; Luiselli et al., 2022). Water availability has been cited as a factor constraining activity during hot and dry periods (e.g. Carneiro et al., 2017; Kearney et al., 2018), with mass-related differences in metabolic rate and fasting endurance contributing to explaining the patterns (Luiselli et al., 2022).

One notable difference between the two populations was times of winter den entrance. This occurred during October in the hedgerow but November and occasionally December (2022) in the garden population and may be examples of climatic and habitat structure influencing activity levels. Measurements of temperatures during spring and autumn showed an approximate 2 °C mean higher ambient air temperatures in the garden, which has a more closed aspect due to a surrounding fence/hedgerow. This supports the urban heat island effect (Alberti, 2015; Campbell-Staton et al., 2020).

Individual female lizards were sighted at wide frequencies from 1 to 20 in the garden and 2 to 22 in the hedgerow (Fig. 4). However, some lizards that were sighted only a few times also had long intervals between sightings suggesting these were possibly transients moving through the study areas (Fig. 7). Therefore in these lizards, although not present for a second year or third year, such low or absence of counts does not necessarily imply mortality. Recent data on sympatric *L. bilineata* in the hedgerow found low frequency of presence of individual lizards with new individuals appearing after the midsummer gap and an absence of those present earlier in the year. This suggests extensive moment across the hedgerow system (Meek & Luiselli, 2024).

The higher frequencies of females in the company of high prominence males (question 2; Fig. 3) agrees well with Edsman`s (1990) study where males held territories and females moved along a wall through these territories resulting in males with larger territories having greater access to females. However Edsman also found that males with smaller territories also had access to females. This is supported here; males with lower annual sighting counts were also observed with females, albeit in lower frequencies (Fig. 3). Edsman (1990) indicated that females only copulated with territorial males and also remained in a male's territory because it offered access to optimal basking sites, important for female reproduction. *Podarcis muralis* is a communal basker with females regularly sharing optimum basking sites with males, other females and occasionally subadults (Meek & Luiselli, 2022a). In the present study, male conflicts were observed only in the garden during 2021, when the resident alpha male that had been present outside the study period since at least 2018 disappeared during late March. Four new males entered the territory and a series of male-to-male conflicts were seen (example in Fig. 2C). Serious conflicts between females were not seen. However it is likely that conflicts, especially between males, were more frequent than observed.

The frequencies of autotomy, defined here as lizards with absent, shortened or regrown tails (examples in Fig. 1), were relatively high in both populations. However given the extent of communal basking (Meek & Luselli, 2022a) and observed limited incidences of intra-specific aggression, suggests that autotomy is primarily due to predation attempts since there is little evidence that *P. muralis* suffers tail loss during intra-specific conflict (Brown et al., 1995). Males, both in the hedgerow and garden were seen more frequently active and seen to travel over more extensive distances, up to 30–40 m in single bouts of activity. This may partly explain tail loss differences since movement is known to increase predation risk in lizards (Cooper, 2003). Potential predators regularly seen in both study areas were adults and juveniles of the saurophagous snake *Heirophis viridiflavus*, aspic vipers *Vipera aspic* (e.g. Meek, 2014a; Rugerio et al., 2021) and green lizards *Lacerta bilineata*, (Meek & Luiselli, 2022a; 2022b; 2024). Birds and mammals, including mustelids and domestic cats, were occasional sightings. Other studies have shown differences in frequencies of autotomy between lizard populations, for example, between *P. muralis* living in high and low predation sites (Diego-Rasilla, 2003a). Mortalities

from disease are also known in lacertid lizards and several were seen in the garden population with skin disorders (examples in Fig. 1B and Fig. 2C). The latter resembled the papillomas of the skin often observed in lacertid lizards, that are often fatal (e.g. Lopez & Bons, 1981; Baxter & Meek, 1988).

Previous studies of wall lizard populations indicate differences in demography including mortalities and age structures. For example, mortality rates in a Slovenian population were 17% but male-female sex ratio was 1:1 (Vogrin, 1998), this differs from the higher female numbers in this study. Longevity also differs. Two populations in Turkey were reported with life spans from 14–16 years (Eroglu et al., 2018) considerably longer than the estimated age of the two alpha males in Figure 2, which allowing for three years to reach full size was estimated at six or seven years.

Relatively low year-to-year lizard permanence of around 18–20% might suggest high mortaility levels or lizards moving out of the study areas to other areas. If this is due to mortalities, long term population stability (Meek, 2020) would be maintained by the capacity of *P. muralis* to recover from very low numbers due to fast generation times (Barbault & Mou, 1988; Bauwens & Díaz-Uriarte, 1997) including involving a dynamic of increased juvenile survivorship that is proportional to declines in adult numbers (e.g Žagar & Carretero, 2012; Ineich et al., 2022). Moreover, the twice-per-year reproductive effort found here and in other French populations (Barbault & Mou, 1988) increases the capacity of *P. muralis* for population recovery. Unfortunately, hatchlings and juveniles were more difficult to detect and identify, especially in the hedgerow, which presented greater plant cover and hence limited accurate measurements of numbers present. The general trends indicated most hatchling/juvenile sightings occurred during the latter part of the year, which suggests the hatchlings sighted at this time were from the second clutch and slightly larger individuals were from the first clutches from spring matings. Mortality is likely high in these smaller classes not only from predators that include the sympatric *L. bilineata* but also adult wall lizards (Simović & Markov, 2013; Žagar & Carretero 2012; Ineich et al., 2022).

One of the key findings of our study is the importance of urban gardens and hedgerow systems for lizards, especially *P. muralis*, in anthropogenic modified environments. However, expansion of agriculture and urban areas are now a feature of large areas of Europe, and can potentially negatively impact on reptile population persistence. Hedgerows, for example, are not just corridors for many species to access prime habitat, they represent year-round habitat for many species that include *P. muralis*. In addition, snakes *Hierophis viridiflavus*, *Natrix helvetica* and *Vipera aspis* are frequent visitors along with various mammals and bird species. Hedgerows therefore play a crucial part in population persistence, whether to serve as permanent habitat or pathways between prime habitat. However, hedgerows have experienced declines during the last 100 years due to changes to intensive crop farming (Robinson & Sutherland, 2002; Guiller et al., 2022) and are disappearing rapidly (Guiller et al., 2022). Their loss could impact on metapopulation persistence not just for reptile populations but other animal species, emphasising that legislation and conservation measures are needed to conserve these systems (e.g. Simberloff & Cox, 1987). Habitat protection is difficult to achieve, but along with raising the awareness of local authorities and public awareness of their importance, other possible avenues are possible. For example herpetological or other environmental organisations should be encouraged to purchase natural areas not just pristine habitats but also hedgerow systems, which if securely preserved maintain a future for habitat connectivity. An example of land purchase policy is that operated by the British Herpetological Society in the UK who in partnership with other organisations, for example the Royal Society for the Protection of Birds (RSPB), regularly purchase prime habitat. This ensures habitat continuity and thus increases the community of conservationists and ecologists stake in the future of habitat for reptiles and other life forms.

In summary, our study showed that both populations were active from February–March until October–December. We observed a mid-summer gap of very low or zero numbers during approximately from June to September including in the garden population despite the presence of ponds as a water source. Two batches of hatchling lizards were seen, the first around June and a further batch during autumn indicating females deposit two clutches annually. Regression analysis indicated male lizards with high presence were more likely to be seen with females than males that were seen less frequently. Diversity and equality analysis indicated stable populations in both study localities with a wide range of individuals and their frequency of presence.

Author Contributions

Conceptualisation: R.M. and L.L.; methodology, R.M. and L.L.; formal analysis, R.M.; field work/data collection, R.M.; writing original draft preparation, R.M., L.L. and RAA; writing, review and editing, R.M. and L.L. All authors have read and agreed to the published version of the manuscript.

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Spatial ecology of the Endangered and endemic Sagalla caecilian *Boulengerula niedeni* in the Eastern Arc Mountains of Kenya

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Caecilians (Order Gymnophiona) are generalist predators of soil invertebrates, and may therefore play an important role in tropical soil ecosystems. However, their fossorial lifestyle and the associated difficulties in surveying them have caused a deficit in data for the majority of species. We applied a systematic approach and an intensive sampling strategy to an Endangered and evolutionarily distinct caecilian from the Eastern Arc Mountains, the Sagalla caecilian *Boulengerula niedeni*. We investigated the association between habitat type and caecilian occupancy across its entire range, the Sagalla Hill, Kenya, and explored the relationship between several variables (land use type, surface soil temperature, soil compactness and landowner prediction of caecilian presence) and its presence in different habitats. We found no significant effects of any of the investigated variables in predicting caecilian presence across the Sagalla landscape. Instead, our findings suggest that the species survives at least as well in agricultural landscapes as it does in areas with indigenous vegetation, with an estimated density of around 900 caecilians per hectare. A bimodal distribution of sizes and weights of captured specimens suggests ongoing successful breeding and recruitment. This suggests that there is a case for cautious optimism with regard to the status of *B. niedeni*. Our work could act as a useful pilot for further, improved caecilian surveys in the Eastern Arc Mountains and beyond, to improve our understanding and conservation of these overlooked fossorial amphibians.

Keywords: amphibian, Gymnophiona, Herpelidae, soil ecology, amphibian conservation, Taita Hills

INTRODUCTION

Amphibian populations are undergoing global declines with as many as 42% of assessed species now threatened with extinction (Leudtke et al., 2023). Habitat deterioration, climate change and disease are thought to be some of the leading causes of population declines (e.g. Pounds et al., 2006; Wake & Vredenburg, 2008, Scheele et al., 2019; Leudtke et al., 2023), however, the vast majority of amphibian research has been biased towards anuran and caudate amphibians, with the lesser known third order, the legless, primarily soil dwelling Gymnophiona (commonly referred to as caecilians), receiving relatively little attention (e.g. Gower & Wilkinson, 2005; de Oliveira Ferronato, 2019).

The subterranean lifestyle of many caecilians has precluded them from various biodiversity surveys, perpetuating the impression that they occur in low densities and have little impact on ecosystem function (Gower & Wilkinson, 2005; Decaëns et al., 2006). However, some studies have found certain species of caecilian to be abundant in particular localities (e.g. Bhatta, 1997; Oommen et al., 2000; Measey, 2004; Kupfer et al., 2005), suggesting their impact on ecosystem function may be underestimated. Moreover, as generalist predators of soil ecosystem engineers (SEE), such as ants, termites and earthworms (e.g. Measey et al., 2003; Gaborieau & Measey, 2004; Kupfer et al., 2005; Jones et al., 2006; Kouete & Blackburn, 2020), locally abundant caecilians may exert some degree of regulation over SEE populations, further underscoring the need for greater research into these interactions (e.g. Measey et al., 2003).

Caecilians could also prove to be useful indicator species for soil ecosystems (Measey, 2006), particularly with the unpredictable effects that anthropogenic climate change is likely to have on soil ecosystems worldwide (Copley, 2000; Decaëns et al., 2006). With reports of several caecilian species being commonly encountered in low-intensity agricultural landscapes (Hebrard et al., 1992; Measey, 2004; Jared et al., 2015; Malonza, 2016), these species could be indicators of soil degradation or pesticide toxicity, though this requires more investigation (Oommen et al., 2000; Gower & Wilkinson, 2005).

A contributing factor to the paucity of caecilian research is the challenge of surveying them. Soil is an opaque, dense and heterogeneous medium that requires considerable energy and destructive force to sample comprehensively (Decaëns et al,. 2006; Measey, 2006). A basic component of caecilian surveys is manually

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excavating the soil, a labour-intensive process with a relatively low return in terms of the number of caecilians sighted per unit of effort expended, partly due to the often patchy yet poorly understood distribution of many caecilian species (Largen et al., 1972; Measey et al., 2003; Gower & Wilkinson, 2005). This challenge has inhibited the development of effective tools for measuring ecological parameters for most caecilian species, though efforts by Measey (2006) to develop a standardised methodology have been a step in the right direction. This challenge has resulted in over half (97/193) of caecilian species being assessed as Data Deficient by the IUCN (IUCN, 2023), with few quantitative historical datasets against which to compare current populations (Measey et al., 2009). For this reason, statements on declines in caecilian species are often founded on recent surveys having returned no individuals from sites of known historical presence (Gower & Wilkinson, 2005 and references therein), or inferences made from broader soil herpetofaunal declines (Pooley et al., 1973; Measey et al., 2009).

The focal species for this study was the Sagalla caecilian *Boulengerula niedeni*, an Endangered herpelid caecilian that is endemic to Sagalla Hill in south-eastern Kenya. Sagalla is located approximately 30 km east of the comparatively well-studied Taita Hills, and together they form the northernmost reach of the Eastern Arc Mountains, a global biodiversity hotspot (Myers et al., 2000; Malonza et al., 2010). Described in 2005 (Müller et al., 2005), this species was previously seldom distinguished from earthworms (phylum Annelida) by the inhabitants of Sagalla, who generally consider it of little use or value, a known issue for East African caecilians (Loader et al., 2003). A naming contest held in 2006 was successful in raising awareness for the species, with the name Kilima-mrota eventually chosen, meaning "thin burrowing animal" in the KiSagalla language (Wojnowski & Malonza, 2009). The species is assessed as Endangered by the IUCN Red List (IUCN SSC, 2013) and recognised as a global priority for conservation on the basis of its evolutionary distinctiveness (Isaac et al., 2012; Gumbs et al., 2018). It has a species action plan (Nature Kenya, National Museums of Kenya, Kenya Wildlife Service & Kenya Forest Service, 2015) and may have benefited from targeted attempts to restore native habitat by planting indigenous trees and replacing exotic eucalyptus with indigenous trees (Malonza, 2016; IUCN SSC, 2013).

The objective of this study was to quantitatively assess the distribution and occupancy of *B. niedeni* across its distributional range, to better inform future conservation work aimed at protecting the biodiversity of Sagalla Hill. We sought to test if land use type was correlated with species' occurrence across this heavily altered landscape. An ancillary objective was to collect data on environmental and soil variables, to explore the species' preferred habitat conditions. To the best of our knowledge it is one of the most intensive sampling efforts of an African caecilian amphibian, and the first to sample at randomly selected sites across the entire range of a caecilian species.

MATERIALS & METHODS

Ethics and Biosecurity statement

This project was approved by the ethics committee of Imperial College London's Department of Life Sciences. All methods used in this study were non-invasive and did not require a UK Home Office Licence and were compliant with the BHS Ethics Policy (British Herpetological Society, 2017). Research was carried out under the Taita Taveta Wildlife Forum's research permits issued by the Kenya Wildlife Service and Kenya Forest Service. To reduce the risk of pathogen transmission between sites and individuals we disinfected all equipment between sites and all animals were handled with powder-free nitrile gloves which were changed between individuals. A euthanasia protocol was established in case of accidental injury of a caecilian during the digging process: individuals whose survival was judged to be unlikely (3 individuals) were immediately euthanised by the ventral application of 20% benzocaine cream (OrajelTM) and preserved before being deposited at the National Museum of Kenya.

Data collection

Fieldwork was carried out on Sagalla Hill in south-eastern Kenya (3.499° S, 38.580° E, 580–1450 metres above sea level) in May and June of 2016, in the transition season between the long rains (March−May) and the cooler, dry winter (June−September). Caecilians were sought at pre-determined sites on a systematic grid covering the whole of Sagalla Hill, with the intent to produce data for occupancy modelling. Given that the semi-arid lowland habitat surrounding Sagalla represents a barrier to amphibian dispersal and that the nearby Taita Hills have been well sampled and only ever produced the closely related yet distinct Taita caecilian *Boulengerula taitanus* (Malonza & Measey, 2005), we assumed that the entire range of *B. niedeni* was covered by this study. A three-person team visited 76 sites over 36 days, between the times of 07:00 h and 11:00 h to avoid the heat of mid-day. A total of 204.8 person hours were spent digging for caecilians. We estimated the total number of sites that could feasibly be visited within the planned research period and created a grid with this number of sites spaced evenly across the entire study area, resulting in a constant spacing of 400 m between sites (Fig. 1). This even spacing of sites ensured that all land use types were sampled roughly in proportion to their occurrence on the landscape. As it was highly unlikely that individual caecilians moved between sites within the duration of data collection, we assumed that the 400 m spacing between sites meant that each site could be considered as an independent sample. We allocated each site to one of three categories of land use: 1) agricultural land, which includes active and fallow fields as well as areas dominated by fruiting trees, 2) heavily anthropogenically disturbed land, including urban centres, habitations, school compounds and non-native pine and Eucalyptus plantations, and 3) natural, relatively undisturbed areas, including unmanaged shrubby or forested areas and the last remaining patch of Sagalla's indigenous forest. Given

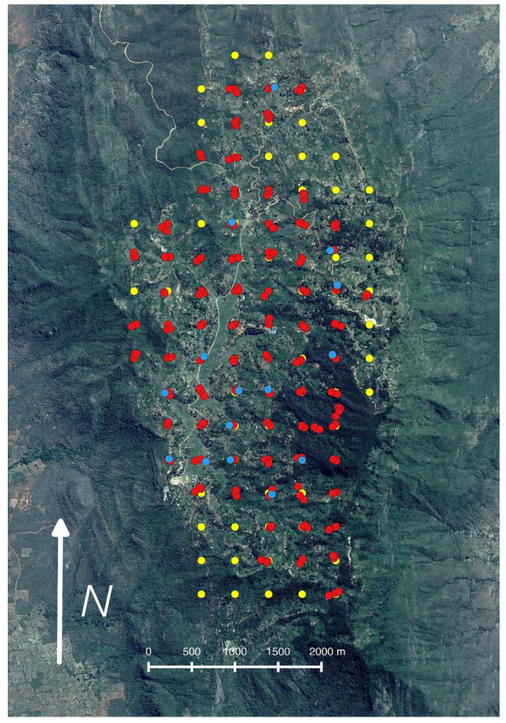

Figure 1. Sagalla caecilian survey sites, including unsampled locations in yellow, and surveyed sites in blue (caecilian present) and red (no caecilians found) on Sagalla Hill, Kenya. Note supplementary sampling in the indigenous forest habitat (darker green).

the small remaining area of the indigenous forest habitat, it was over-sampled, with two sites added equidistantly between existing indigenous forest sites (i.e. 200 m apart) to increase the coverage of this habitat.

To find caecilians, a standardised search protocol was developed by the author (BT) with the input of David Gower and Mark Wilkinson (Natural History Museum, London). At each of the 76 sites, three 1.5 m x 1.5 m plots were dug, giving a total of 228 plots. Digging used a systematic method, with two workers standing side by side and digging forward, to a targeted depth of 30 cm. The depth of the excavated plot followed previous studies (Measey et al., 2003; Measey, 2004). The digging tools used were local implements known as jembes, which consist of a metal blade (approx. 15 x 15 cm) fixed roughly perpendicularly to a 1 m long wooden handle, which is raised overhead and swung downwards. These tools have been widely used to excavate caecilians, including congeneric species (e.g. Malonza & Measey, 2005; Measey et al., 2006; 2012).

Plots were dug as near as possible to the exact GPS marker at each site, with the intent to retain its randomness. However, it was not always possible to dig on the exact GPS location, either due to the terrain (boulders, dangerously steep gradient) or due to landowners withholding their consent for digging on their property, as was often the case with cultivated fields. In such cases, the first plot was dug as near as possible and never more than 10 m away from the GPS marker.

The second and third plot dug at each site were situated in perceived favourable caecilian microhabitat within 100 m of the GPS marker, with the exact locations selected according to the following criteria, in decreasing order of priority: 1) maximal apparent soil moisture, 2) proximity to banana plants, 3) proximity to fruiting trees, 4) maximal apparent soil fertility/organic matter content (leaf piles, cow dung). These selection criteria were based on a review of previous caecilian surveys where favourable caecilian habitat had been described (e.g. Hebrard et al., 1992; Bhatta, 1997, Measey, 2004; Gower & Wilkinson, 2005; Kupfer et al., 2005; Kouete & Blackburn, 2020). In rare cases where none of these criteria could be met, the second and third plots were dug in effectively random microhabitat exactly 30 m east and west of the GPS marker.

After each plot was dug, a suite of environmental variables was recorded. Soil moisture, soil pH, and ambient light were measured with a Mudder 3-in-1 Moisture/pH/Light meter. Soil temperature was measured using a ThermaPen (Electronic Temperature Instruments Ltd) inserted to a depth of 5 cm while the soil compaction was measured using a Humboldt H-4200 Soil Penetrometer on bare soil, with 3 replicates of each variable taken at the edge of each plot following the manufacturers guidelines and only after the plot had been excavated, to prevent disturbing caecilians within the plot prior to excavation. (Table S1, see supplementary material). Excavated material was carefully checked for the presence of caecilians. Captured caecilians were weighed with a Pesola Light Line spring scale, their total length measured to the nearest mm by placing the caecilian in a clear, Ziploc bag and manipulating the caecilian so that it lies straight along the inside edge of the bag where its total length was then measured with a haberdashery tape. Caecilians were released at the point of capture.

In addition to the 288 systematically distributed plots, a further 63 exploratory plots were dug using the same protocol as above, but selected opportunistically, either at sites with known historical presence (from Malonza et al., 2010), or at sites in habitat expected to be favourable to caecilians based on prior information and experience during the survey. These plots yielded additional specimens whose length and weight data added to the analysis of morphology.

Landowner survey

In cases where the site fell on private land, a short standardised social survey was incorporated into conversation while seeking landowner permission to dig a plot on their property. All landowners spoken to were over 18 years of age, gave consent for data to be collected and used, and were informed of their right to withdraw their consent at any time. These conversations were carried out in the local KiSagalla language and

three questions were asked: 1) Have you heard of the Sagalla caecilian? (identified by its KiSagalla name, kilima-mrota), 2) Do you recognise it from these images? and 3) Have you ever found any Sagalla caecilians on this property? For the second question, eight images were carefully selected for clarity and unambiguity and presented to the respondent, including two images of *B. niedeni* and three species with similar morphology (two images of each). *Boulengerula niedeni* is commonly misidentified as either a worm or a snake, therefore we included images of both a common earthworm (phylum Annelida) and a regionally abundant, similarly coloured snake (genus Amblyodipsas). Lastly, pictures of a south-east Asian caecilian (genus Ichthyophis) which have a striking yellow stripe along the length of their body and are therefore impossible to confuse with *B. niedeni* were included as a control (Fig. S1, supplementary material). The purpose of this was to verify the landowner's ability to recognise the caecilian from multiple options, prior to collecting data on its predicted presence at the site.

Statistical analysis

While occupancy modelling was originally planned using the three replicate plots at each site as repeat observations, it was not possible due to a lack of any repeat observations in the dataset, a well-recognised problem with occupancy models running on sparse data (Welsh et al., 2013). We therefore analysed apparent occupancy (effectively the product of true occupancy and detection probabilities) without correcting for variation in detectability.

Generalised Linear Mixed Effects Models (GLMM) with binomial errors were run in the software R (R Core Team, 2015) using the presence/absence of *B. niedeni* as a response variable and a set of environmental data as explanatory variables (full set of variables collected in Table S1, supplementary material) Explanatory variables were first grouped according to collinearity, and those considered most likely to have an effect on caecilian distribution, based on a review of the caecilian literature and consultation with experts, were retained as fixed effects. These were land use type, surface soil temperature, soil compactness and landowner prediction of caecilian presence.

Land use type was included in the model because it is hypothesised to play an important role in determining caecilian habitat use and therefore distribution, as evidenced by several recent studies reporting higher densities of caecilians from agricultural land than adjacent indigenous habitat (Hebrard et al., 1992; Oommen et al., 2000; Gower & Wilkinson, 2005; Malonza, 2016; Jared et al., 2015).

Three environmental variables, soil moisture, soil pH and ambient light were disregarded prior to data analysis, due to suspected inaccuracy of the equipment used in the field, as the readings reported by the meter were the same when soil was visibly moist and visibly dry. Soil surface temperature and soil compactness were selected both to test their own effects on caecilian distribution and to serve as proxies for weather conditions and soil type, respectively. Several caecilian species have been shown to exhibit preferences for less compacted soil (Ducey et al., 1993) and soil compaction is considered a potential threat to *B. niedeni* (Malonza, 2016) but one that has not yet been quantified. Finally, the landowner prediction variable was included to test the hypothesis that local ecological knowledge may be a more efficient method to gather data on the presence of a particular amphibian species (Harpalini et al., 2015; Pan et al., 2016; Turvey et al., 2018; Kanagavel et al., 2020).

A random effect of site identity was used to account for spatial dependency between multiple plots dug at each sample site and thus avoid risk of pseudoreplication. All possible combinations of the four fixed effects were fitted (16 models in total), and Akaike weights were used to evaluate strengths of evidence for particular models and variable effects (Burnham & Anderson, 2002). The maximal importance scores of each variable were assessed by summing the Akaike weights of all models with that variable, with a score close to 1 denoting a strong effect and a score < 0.8 indicating little to no effect. We limited our models for consideration to those within 6 AIC units of the top model (Richards, 2008). We also applied the 'nesting rule' within this model set, wherein models that are more complex versions of those that have a lower AIC are disregarded (Richards, 2008).

To test support for size-based clusters among captured caecilians, partitioning around medoids was used, with optimum number of clusters evaluated by average silhouette width (Hennig, 2023).

Minimum caecilian densities, allowing for the possibility that not all individuals within plots would be found, were estimated by dividing numbers of caecilians found by the area of plot sampled, with standard errors and log normal confidence intervals derived from the empirical between-site variance in density (Borchers et al., 2002). Total population size was estimated by multiplying estimated density by total area of the site within which the survey was planned (1216 ha).

RESULTS

Sample characteristics

Of the 228 plots dug at 76 systematically sampled sites, 112 (49.1%) were in agricultural areas, 71 (31.1%) in areas of high human disturbance, such as school yards, households, or plantations of eucalyptus or pine, and 45 (19.7%) in natural areas minimally affected by the human population, such as unmanaged, shrubby areas and the last remaining patch of the indigenous forest on Sagalla Hill.

In total, 31 caecilians were collected in 15 of the 228 plots, representing an encounter rate of 6.6% and a catch per unit effort of 0.15 caecilians per hour. Disaggregating into land use types, plots dug in agricultural fields saw an encounter rate of 7.1% (8/112), while those dug in areas of high disturbance had 7.0% (5/71) and the natural areas were lowest with 4.4% (2/45). See Figure 1 for a site map identifying plots where caecilians were encountered at systematically selected sites. An additional 25 caecilians

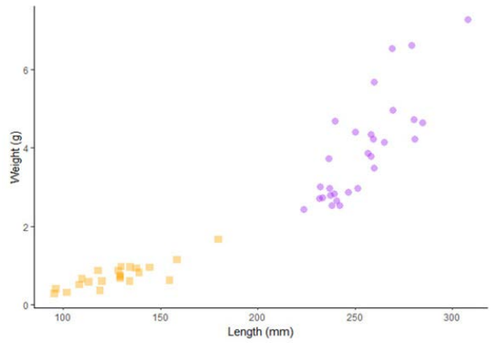

Figure 2. Length and weight distribution of specimens of Sagalla caecilian *Boulengerula niedeni* sampled in this study. Contrasting symbols and colours represent membership of the two groups identified by cluster analysis, likely representing age classes.

were captured in ten of the 63 opportunistically surveyed plots (15.8% encounter rate). Three caecilians (5.6%) were severely injured during excavation and were euthanised.

The first plots dug at the pre-determined GPS marker for each sites contained fewer caecilians than the second and third plots that were dug in favourable caecilian microhabitat within 100 m of the GPS markers. Five out of the 31 caecilians were found at the systematically selected sites, whereas the favourable caecilian microhabitat held 26/31 of the specimens encountered.

Overall, captured individuals averaged 2.6 g in weight (range 0.4–7.2 g) and 200 mm in length (range 95–310 mm), but cluster analysis strongly supported the existence of two size groups (Fig. 2). Smaller individuals (presumed juveniles, n = 22) averaged 0.8 g (0.4−1.8 g) and 128 mm (95−180 mm) while larger individuals (presumed adults, n = 29) averaged 3.9 g (2.8−6.4 g) and 254 mm (220− 310 mm).

Landowner prediction survey

Of the 228 plots dug, 61.0% (139/228) were on private land, requiring that a landowner be approached for permission, while the remaining sites were located on public land. Of the 139 landowners approached, 87.8% (122/139) correctly identified *B. niedeni* from the images presented to them. Of these 122 landowners, 79.5% (97/122) confirmed that they had found the caecilians on their property before. This suggests that landowners potentially hold good information on caecilian presence. Furthermore, the encounter rate at these 97 plots where presence of caecilians were reported was 8.3% (8/97), compared with 0% (0/25) for plots on land where the owner did not report caecilians.

Mixed Effects Modelling

Of the remaining four predictors of the probability of encountering caecilians at a site, none showed any clear evidence of an effect. All models within the top set were nested versions of the top model (the null model), suggesting that they should be disregarded from consideration, and maximal importance scores were low for all variables (0.46 for soil surface temperate in the highest case; Table 1).

Table 1. Comparison of all permutations of the binomial GLMM of caecilian encounter probability, ranked according to AICc, with the following covariates (variable importance, i.e. summed AIC weights of containing models, in brackets): 1 = land use type (0.11), 2 = landowner prediction (0.28), 3 = soil compaction (0.3) and 4 = surface temperature (0.46).

Covariates	df	AICc	ΔAICc	Weight
[. . . .]	2	97.5	0.00	0.230
[. . . 4]	3	97.8	0.24	0.204
[. . 3 .]	3	98.8	1.29	0.102
[. 2 . .]	3	99.5	1.95	0.087
[. 2 . 4]	4	99.5	1.97	0.086
[. . 3 4]	4	99.6	2.05	0.083
[. 2 3 .]	4	100.8	3.25	0.045
[. 2 3 4]	5	101.3	3.79	0.035
[1 . . .]	4	101.7	4.16	0.029
[1 . . 4]	5	101.9	4.39	0.026
[1 . 3 .]	5	103.0	5.51	0.015
[1 2 . .]	5	103.6	6.06	0.011
[1 . 3 4]	6	103.6	6.10	0.011
[1 2 . 4]	6	103.8	6.23	0.010
[1 2 3 .]	6	105.1	7.55	0.005
[1 2 3 4]	7	105.6	8.08	0.004

Density

Caecilian density in plots selectively located in habitat expected to be favourable for caecilians at systematically selected sites was estimated at 721 ha^{-1} (SE 261, log normal 95% confidence interval 282–1287) or 0.07 m^2. While a return of only five individuals at a single randomly selected plot prevents precise estimation, we can tentatively calculate a lower site wide average density of 347 ha^{-1} (SE 347, log normal 95% confidence interval 68–1775) or 0.03 m^2. Extrapolating the average density per ha to the entire study area suggests a total population of 422,222 (CI 82,576–2,158,867).

DISCUSSION

Taken together, we suggest that our results indicate a degree of cautious optimism regarding the status of *B. niedeni*. Although we found the species in only a small proportion of sample plots (6.6%), we believe that this low encounter rate largely reflects the low efficiency of our survey method. Caecilians are inhabitants of moist forest, and their localised distribution is usually strongly correlated with soil moisture (Gundappa et al., 1981; Gower & Wilkinson, 2005; Kupfer et al., 2005; Jared et al., 2015; Malonza, 2016). The absence of soil moisture and light intensity data is therefore a significant shortfall of

this study. However, for the variables we did explore, we found no strong habitat associations, suggesting that the species is adaptable and able to exploit a range of habitats, including heavily disturbed land uses. This suggests that much of the species' range remains habitable, despite widespread and intense disturbance. Consistent with this, a large majority (80%) of farmers reported the presence of the species on their land. Furthermore, even without correcting for imperfect detectability, abundance in the targeted plots was substantial, with an estimated density of around 350 caecilians per hectare overall, and twice that in favourable habitat. Furthermore, this density estimate could be an underestimate if caecilians were able to react to digging by escaping from the plot before being detected, or if caecilians typically burrow below 30 cm depth in the soil. While our overall density estimate from randomly located plots is tentative due to the small number of caecilians discovered, taken together, our results suggest that the total *B. niedeni* population could well number in the hundreds of thousands. Density estimates for *B. niedeni* in favourable habitat are similar to density estimates reported for two congeneric species (*B. boulengeri* and *B. taitana*) from forest habitat at a similar time of year (Measey, 2004; Measey & Barot, 2006). Finally, caecilians encountered were clearly grouped in two distinct size and weight classes which we interpret as juveniles and adults, suggesting ongoing breeding and potential recruitment.

No effects of land use type, soil temperature or compaction, and landowner knowledge had a detectable association with the presence of caecilians. This lack of any effect of habitat types on caecilian presence may be due to the species' adaptability to anthropogenically disturbed habitats (Gower & Wilkinson, 2005). Combined with the relatively small area of indigenous vegetation remaining on Sagalla Hill, these caecilians may have little choice but to occupy modified landscapes. Moreover, this is consistent with the findings of a number of caecilian studies (Hebrard et al., 1992; Oommen et al., 2000; Jared et al., 2015), which hypothesised that the benefits of irrigation in agricultural fields may outweigh the drawbacks of living in a disturbed habitat. Indeed, many agricultural fields in Sagalla are irrigated, particularly those near the Sagalla river. While the encounter rates found in this study were lower than in previous surveys of other caecilian species at a similar time of year (Oommen et al., 2000; Measey et al., 2003; Malonza & Measey, 2005), this may not be representative of a decline in abundance as the site selection process in our study used a different sampling design (evenly spaced and random rather than grouped in areas with high probability of caecilian occurrence).

The reproductive mode of *B. niedeni* is not known for certain. The closely related species *B. taitanus* is oviparous and has altricial young that are nursed via the hypertrophic skin secretions of the female (Malonza & Measey, 2005; Kupfer et al., 2006). The altricial young of *B. taitanus* have a total length of approximately 28 mm when they hatch (Kupfer et al., 2006), are unpigmented and undergo an ontogenetic shift in pigmentation becoming increasingly pigmented, with pigmentation developing from a darker mid-dorsal band which gradually broadens over time (Nussbaum & Hinkel, 1994). The young become independent of their mothers when they reach approximately 86 mm in length (Kupfer et al., 2006). Females of this species have an annual reproductive cycle (Raquet et al., 2015) and they are known to nest in January after the short rainy season (Kupfer et al., 2006). It is likely that this reproductive mode is also exhibited by *B. niedeni* and that our surveys therefore took place after the breeding and nesting season. The few juvenile *B. niedeni* that are known were unpigmented on their ventral and lateral surfaces (Müller et al., 2005), indicating that a similar ontogenetic shift in colouration also occurs in this species. The smallest individual *B. niedeni* we encountered had a total length of 95 mm and all were well pigmented indicating that animals in the smaller size class were juveniles which had already completed the ontogenetic shift in colouration. No nests were excavated which could reflect seasonality of breeding but nests might have been missed. The presence of two clear size categories of specimens which might indicate ongoing breeding as well as the presence of an annual breeding cycle like other congeneric species.

Our sampling strategy encountered a number of operational difficulties on the ground due to the inherently destructive nature of the sampling protocol. Because of the local dependence on subsistence agriculture, farmers would understandably not allow any part of their field to be dug up while their crops were sown. For this reason, many of our plots were forced away from their systematically placed GPS marker, introducing bias to the random sampling portion of the survey. This also may cause slightly sub-optimal habitat to be sampled in our first plots, as fields are often fringed by hedgerows, streams or roads. One solution to this issue could be to synchronise future caecilian surveys with the annual harvest that occurs in February in Sagalla, so that the soil is only disturbed once. Additionally, any additional caecilian mortality caused by the digging process would be minimised by sampling during a regular digging event that would occur either way, while also allowing for a greater volume of soil to be sampled. However, this might risk disturbing nests if caecilians breed at this time of year. Such a strategy should not be undertaken in habitat types that would otherwise be undisturbed until more information on the time of breeding and parental care is known. This information might be gathered by the further questioning of local land users.

Another important limitation to our study was the failure to account for imperfect detection of caecilians while sampling the survey sites, for a number of reasons. First, due to the high amount of effort needed to manually excavate the soil, only a relatively small volume of soil was sampled in comparison to the total volume available to the species. Second, caecilians would have likely been disturbed at the onset of digging and could have moved out of the plot before they were excavated. It should, however, be mentioned that an escape response was not clearly evident in our field experience, as in many cases

the caecilians captured from a plot were discovered after digging had been ongoing for a few minutes. Third, it is possible, but unlikely, that caecilians may have been excavated but not spotted by either of the diggers, thus allowing the individuals to escape back into the soil.

The primary limitation inhibiting statistical analysis of the data was the low encounter rate of caecilians (15/228 plots, 6.9%). There are several ways in which future caecilian surveys could increase their encounter rate. Some avenues for future exploration include the potential use of eDNA (Thomsen & Willerslev, 2015) in the detection of caecilian presence within the soil using easily extracted soil cores. If viable, this would allow sampling without crop disruption while also shedding light on depths of caecilian activity. Secondly, a caecilian survey with mechanical digging could decrease the effort involved while increasing the speed at which the soil is removed, thus minimising the likelihood of missing individuals due to an escape response. However, mechanical excavators are expensive and cumbersome and, in many cases, including Sagalla Hill, would be unable to reach sites targeted during a caecilian survey, thus only under very specific circumstances could their superior digging power be effectively used.

The landowner prediction survey showed that most landowners were able to correctly identify the target species and that of these, 79.5% of respondents had encountered *B. niedeni* on their property before. The encounter rate of *B. niedeni* at plots where presence of caecilians was reported was relatively high and no caecilians were detected in plots where land owners did not report the presence of the species. This is consistent with landowners providing reliable reports of caecilian presence, although the small sample of absences here precludes strong conclusions on this. A limitation with our survey strategy was that we did not ask when *B. niedeni* was last seen. The climate and land use may have changed over time and once-suitable habitat may have become unsuitable. Whilst our work shows that information from local respondents may be of use in providing information on this particular species, future studies should include estimates of last sighting date. Surveys on fossorial vertebrates have a unique set of challenges, setting them quite apart from the average ecological survey of cryptic species (e.g. Gower & Wilkinson, 2005; Measey, 2006). Our ecological survey of *B. niedeni* highlighted these challenges but nonetheless produced some interesting findings to advance our knowledge of the species. We provide the first population estimate for the species which provides an important baseline for the future study of population trends overtime. Furthermore, this work supports the growing evidence for apparent tolerance for agricultural and human-disturbed habitat as well as evidence of ongoing breeding. While their apparent survival in human-disturbed landscapes may warrant cautious optimism, the species remains a narrow-range endemic, necessarily restricted to a very small area. *B. niedeni* was first assessed as Critically Endangered in 2006 on the basis of its extremely limited extent of occupancy, although it was later downlisted to Endangered (EN) in 2012 (IUCN, 2020). The current assessment is very close to becoming out-of-date. Our study indicates that *B. niedeni* probably still qualifies for being assessed as EN in accordance with the IUCN Red List of Threatened Species categories and criteria B1ab(iii) (see IUCN, 2012).

More generally, there remains an urgent need to enhance our understanding and protection of soil ecosystems worldwide. It is hoped that our study can inform similar work for caecilians and other fossorial vertebrates.

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SHORT NOTE

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Defensive plasticity in South American rattlesnakes *Crotalus durissus*

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Reptiles exhibit defensive responses in diverse ways, influenced by situational factors. Snakes, such as the South American rattlesnake *Crotalus durissus*, display complex defensive responses, adapting tactics based on circumstances when encountering potential predators. Environmental and intrinsic parameters shape snake behaviour during predator encounters. This study employs distinct stimuli to investigate behavioural changes and whether snakes adapt defensive strategies. We compare the behaviours of rattlesnakes before and after an aversive stimulus involving physical contact. Following encounters with higher-risk stimuli, snakes become more defensive. Findings reveal that snakes alter strategies based on threat intensity. This phenomenon might be influenced by the potential cost of defense and prior interactions with predators. The study underscores the intricate nature of snake defensive behaviour.

Keywords: behavioural plasticity, antipredator behaviour, aversive stimulus, behavioural cost, active behaviours

Many animals possess the ability to assess and behaviourally influence the risk of predation (Ibáñez et al., 2014; Lima & Dill, 1990), consequently generating defensive responses in different ways, depending on circumstantial factors (Sazima, 1988). Therefore, it is possible to hypothesise the existence of a component of choice in the mechanisms used by the individual when confronted with a dangerous situation (Sih et al., 2012).

Snakes can exhibit a complex repertoire of defensive responses, switching from one tactic to another depending on the conditions at the moment of encountering a potential predator (Llewelyn et al., 2010; Stankowich & Blumstein, 2005). Currently, it is known that many environmental parameters determine how snakes modulate their defensive behaviour in various situations of predator encounter, such as temperature (Citadini & Navas, 2013; Webb et al., 2001), distance from refuge (Stankowich & Blumstein, 2005) and intrinsic parameters such as sex (King, 2002), ontogeny (Vitt, 2000) and body condition (Andrén, 1982). Venomous snakes are ideal for investigating changes in defensive strategy, as they incur high costs associated with striking (e.g. venom expenditure, potential prey damage, increased vulnerability to predators due to extended head and neck, muscle fatigue (Hayes et al., 2002), and they possess a wide variety of warning behaviours (Greene, 1988).

The South American rattlesnake *Crotalus durissus* is a snake with a highly extensive distribution, spanning from the northern to the southern regions of the South American continent. Due to its high adaptability, it easily occupies urban areas and is susceptible to attacks from predators belonging to various animal groups, including birds, mammals and even other squamates (Flores-Villela et al., 1990; Daltry et al., 1996). Similar to other members of the Crotalinae subfamily, rattlesnakes possess an infrared sensory system in their facial organs (Gracheva et al., 2010; Noble & Schmidt, 1937) enabling them to detect electromagnetic radiation invisible to most other animals (Goris, 2011).

The ability to distinguish the degree of danger in a predatory attack and how this ability changes with experience remains relatively unexplored by science. Given this, we enquire whether snakes belonging to the species *C. durissus* have the ability to alter their defensive repertoire according to the level of danger posed by a predatory attack, and whether a change in behaviour patterns is possible after undergoing experiences of greater peril. Our hypothesis is based on the notion that *C. durissus* will modify its defensive behaviours when confronted with different predatory stimuli. Furthermore, following exposure to a more dangerous stimulus, we posit that the snakes would become more aggressive, exhibiting increased active behaviours in order to evade their predator.

For this purpose, four snakes of the *C. durissus* species (2 males and 2 females) were used, approved by the ethics committee for the use of animals (CEUA-8444011020) originating from natural environments in the state of São Paulo, Brazil. All animals were adults with snout-vent lengths corresponding to the adult phase (> 83 cm SVL)(Barros et al., 2012). The experiments were conducted between 20 and 24 July 2020, with two days post-collection, from 14:00 h to 16:00 h, in a room at 25 °C. The experimental test involved placing the animal in the centre of an arena measuring 2.025 m^2 (1.5 m x 1.35 m x 1 m), constructed with an aluminum base plate and foam walls according to Alves-Nunes et al. (2023). Each individual snake was allowed to acclimate in the centre of the arena for 15 minutes individually. The experiments were carried out once for each animal, involving a total of three stimuli.

Subsequently, the first stimulus (Stimulus 1) was administered, involving a plastic bottle containing 37 °C water, mimicking the temperature of its natural predators. The stimulus object was approached toward the snake's head without making physical contact, at a constant speed with 1-second intervals. The duration of the experiment ranged from one to four minutes. Following stimulus 1, a one-minute interval was given. After this interval, the animals were exposed to a higher-stress stimulus involving physical contact (Physical stimulus). This was achieved using a snake catcher to apply pressure to three regions of the snake (tail, mid-body and behind the head), each stimulus lasting five seconds with a 3-second interval between each stimulus. The duration of the stimulus ranged between one and four minutes. Following the conclusion of this phase, another 1-minute interval was provided, allowing the snake to rest.

Immediately after this, the animals were subjected to a third stimulus (Stimulus 2). This stimulus was similar to the first, where a warm bottle approached the snake without contact. These stimuli represent relevant cues for predation events carried out by opossums, which are homeothermic predators that engage in compression of all three regions of the snake's body (Almeida-Santos et al., 2000). This investigation aimed to determine whether the snake's behaviour changed in response to the same stimulus before and after a more perilous experience.

The experiments were recorded and subjected to behavioural analysis, involving documentation of the duration of all behaviours and the duration of predatory stimuli in seconds. The behaviours were categorised into two behavioural groups: passive and active (Citadini & Navas, 2013; Silva, 2016). Passive behaviours encompassed all immobility-related behaviours or actions that increased the distance between the snake and the predatory stimulus (immobility, body retraction, coiling, hiding the head, retracting the head, moving away and fleeing). On the other hand, active behaviours comprised actions involving alert signals and a reduction in the distance between the snake and the aggressor (thrashing, body rotation, striking, biting, strike pose and sigmoid position) (Figures S1 & S2, see supplementary material).

The data were subjected to analysis using a Generalised Linear Mixed Model (GLMM), employing a binomial distribution and a logit link function. This analysis was conducted using the "lme4" package in R version 4.2.2. This approach was chosen because the snake has only two possible behavioural decision options (active or passive). The fixed variable was the type of experiment (with three levels of stimulus: Stimulus 1, physical stimulus and Stimulus 2. The random effect was the individual snake itself. The variance explained by the random variable was lower than the predictor variable (type of stimulus), with a proportion of explained variance of 0.176 and 0.824, respectively. Therefore, the type of stimulus explains more variance in the data than the individual behavioural differences of *C. durissus*.

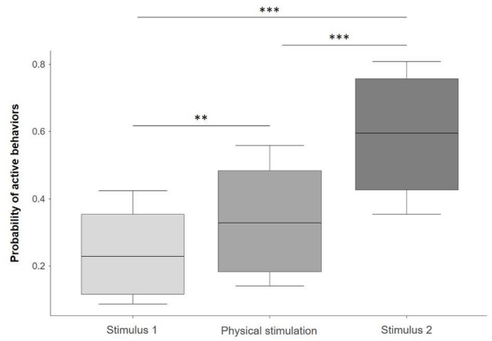

Figure 1. Probabilities of active behaviours of *Crotalus durissus*. Active behaviour probabilities depending on the stimulus. Stimulus 1 (no physical contact), Physical stimulus and Stimulus 2 (no physical contact). Asterisk indicates that the results show a clear difference in the frequency of behaviours in relation to the type of predatory stimulus among each species (*** $p < 0.001$; ** $p < 0.01$).

It was observed that there is a clear difference in the probability of snakes displaying active behaviours as the intensity of the stimulus varies (Fig. 1). Moreover, a change in the behavioural pattern in response to the same stimulus was observed after undergoing the physical contact stimulus ($z = 7.647$; $p < 0.001$). The chance of rattlesnakes exhibiting active behaviours after physical contact is approximately five times higher than before the more stressful stimulus ($z = 9.185$; $p < 0.001$). In the experiments with Stimulus 1, the four most common behaviours were head-turning (21.34%), striking (16.85%), coiling (15.16%) and fleeing (14.04%). In the Physical stimulus, the most performed behaviours were biting (19.62%), head turning (16.82%), inspection (13.40%) and striking (11.21%). In Stimulus 2, the most common behaviours were striking (38.34%), head turning (14.38%), fleeing (12.78%) and coiling (10.32%).

As predicted, the snakes exhibited a change in defensive behaviour in response to different stimuli. It is known that other snakes also respond differently to various types of stimuli (Herzog et al., 1989; Scudder & Chiszar, 1977; Arnold & Bennett, 1984) described something similar for *Thamnophis radix*, where the snakes became more aggressive when the simulated predator attacks were more severe. These observations suggest that individuals make behavioural decisions based on different contexts, assessing costs and benefits when confronted by a predator

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(Endler, 1986). Furthermore, another aspect that can influence defensive behaviour is the physiological state in which the snake finds itself. The baseline plasma level of corticosterone, a stress-related hormone in snakes, is associated with a higher propensity to attack. However, this behaviour has not been correlated with the magnitude of the snake's corticosterone increase (Herr et al., 2017). It would be of great interest to conduct hormonal analyses on the animals before and after physical confrontations to observe the relationship between the animal's hormonal state and behavioural changes.

Furthermore, we observed a change in behaviour patterns in response to the same stimulus after undergoing a more perilous predatory encounter. Stimulus 1 and Stimulus 2, being the same stimulus and not involving high risk, could have led to habituation in animals and reduction in their active behaviours, as observed in Glaudas (2004). However, this was not the case. The animals increased the display of active defensive behaviours. We deduce that this effect could have been due to the fact that the animals had experienced a more dangerous encounter.

Snakes possess the ability to adjust their defensive behaviour according to discrimination between high and low-risk threats (Glaudas et al., 2006). If a previous experience with a potential predatory stimulus suggests that the risk of predation is low or non-existent, the best strategy might involve not engaging in active defense (e.g. striking) and instead minimising associated costs by investing in more passive defenses to avoid confrontation. Conversely, if the risk of predation is very high, the cost of defense might be trivial compared to the individual's need to maximise its survival. Therefore, factors such as risks and previous experiences with a predator could influence an individual's ultimate decision, as may have been the case with *C. durissus*.

An unexamined factor that could have effects is the degree of muscle exhaustion the animals experienced. Arnold & Bennett (1984) assessed *T. radix* and found that the snakes exhibited fleeing behaviour when encountering the investigator until high levels of lactate were reached. Then, they changed their behaviour, adopting one of their defensive displays. This suggests that *C. durissus* might indeed possess defensive plasticity according to the type of predator and the level of danger they present. However, more studies are necessary that involve more individuals of different sexes and ages. In addition, investigating whether muscle fatigue could also be a factor that influences defensive behaviour would be beneficial.

Therefore, we present evidence that the South American rattlesnake *C. durissus* alters its defensive behaviour in response to the degree of perceived threat, suggesting an adaptation in assessing each predatory situation and responding with varying degrees of defensiveness. This research provides insights into the behavioural plasticity and the variation in defensive responses of *C. durissus* in the face of perceived threats. The study demonstrates that the snakes adapt their reactions contingent upon the intensity of the threat, suggesting a sophisticated capacity for risk assessment and behavioural modification. This contributes to an enhanced comprehension of the underlying mechanisms of animal behaviour in prey-predator interaction contexts.

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Larval morphology, vocalisations, and phylogenetic position of *Crossodactylus dantei* (Anura: Hylodidae): new insights into a little-known anuran species

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Aspects of the biology and natural history of the endemic anuran *Crossodactylus dantei* remain unknown even almost 30 years since its description. In this study, we describe its larval stage, along with the advertisement and territorial calls, and assess its phylogenetic positioning based on a fragment of the 16S rRNA mitochondrial gene. The larval stage of *C. dantei* follows the morphological pattern of Hylodidae, corroborating the relatively uniform larval morphology of the family. However, it is still possible to observe characters that distinguish *C. dantei* from other species in the genus, such as the elliptical body in dorsal view, nostrils located closer to the snout than to the eyes, an elongated pre-nasal arena, and the absence of taenia tecti medialis and taenia tecti transversalis in the chondrocranium. Its advertisement call is characterised by a sequence of about 23 pulsed notes, with ascending amplitude modulation, and has a mean duration of 2.5 seconds, with a dominant frequency of 3356 Hz. The territorial call has a mean duration of 23 seconds, a dominant frequency of 3160 Hz, and two distinct types of notes. Phylogenetic analysis indicated the existence of two main lineages in the genus. *Crossodactylus dantei* was recovered with high statistical support as a sister species to the clade composed of *C. gaudichaudii* and *C. timbuhy*. Furthermore, the group formed by these three species was recovered as the sister lineage of *C. trachystomus*. In summary, the information presented here represents a significant advance in the knowledge of this enigmatic and rare species of the northern Atlantic Forest, providing baseline data for future ecological and evolutionary studies of this unique frog.

Keywords: tadpole, oral anatomy, chondrocranium, calls, phylogeny, Atlantic Forest

INTRODUCTION

The family Hylodidae encompasses diurnal anuran amphibians that primarily inhabit flowing freshwater environments in the Atlantic Rainforest (Caldart et al., 2012). Its geographical distribution ranges from north-eastern to southern Brazil and northern Argentina (Caldart et al., 2012; Laia & Rocha, 2012). Currently, this family comprises a total of 48 recognised species, classified into four genera: *Crossodactylus* Duméril & Bibron, 1841; *Megaelosia* Miranda-Ribeiro, 1923; *Hylodes* Fitzinger, 1826; and *Phantasmarana* Vittorazzi, Augusto-Alves, Neves-da-Silva, Carvalho-e-Silva, Recco-Pimentel, Toledo, Lourenço & Bruschi, 2021. Among these, *Crossodactylus* stands as the second most diverse, with 13 species distributed from north-eastern Brazil to the province of Misiones in Argentina (Frost, 2023).

Crossodactylus demonstrates a strong association with habitats characterised by mountainous and rocky terrain (Pimenta et al., 2014), presenting significant challenges for research efforts. Notably, some species were last collected during the latter part of the 20th century (*C. boulengeri* (De Witte, 1930); *C. dispar* Lutz, 1995; *C. grandis* Lutz, 1951, and possibly others), raising concerns about their potential extinction (Pimenta et al., 2014). Their taxonomic relationships remain problematic, and a limited number of studies have been conducted on *Crossodactylus* phylogenetics, where only a small subset of the current recognised species within the genus were included, i.e. *C. caramaschii* Bastos & Pombal, 1995; *C. gaudichaudii* Duméril & Bibron, 1841; *C. schmidti* Gallardo, 1961; *C. trachystomus* (Reinhardt & Lütken, 1862); and *C. werneri* Pimenta, Cruz & Caramaschi, 2014 (e.g. Toscano et al., 2019; Vittorazzi et al., 2021; de Sá et al., 2022).

Consequently, our understanding of other biological aspects pertaining to this group remains deficient, hindering conservation efforts. The acoustic repertoire

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of the genus is under-studied, where advertisement calls have only been documented for little more than half of the *Crossodactylus* diversity (i.e. *C. caramaschii*; *C. cyclospinus* Nascimento, Cruz & Feio, 2005; *C. franciscanus* Pimenta, Caramaschi & Cruz, 2015; *C. gaudichaudii*, *C. schmidti*, *C. timbuhy* Pimenta, Cruz & Caramaschi; *C. trachystomus* and *C. werneri*). Additionally, only seven species have documented tadpole morphologies, primarily limited to external features (i.e. *C. caramaschii*, *C. cyclospinus*, *C. gaudichaudii*, *C. schmidti*, *C. timbuhy*, *C. trachystomus* and *C. werneri*); detailed accounts of the buccopharyngeal cavity, a set of features critical to feeding ecology (Wassersug, 1976; Candioti, 2007), have been recorded for merely three species (*C. caramaschii*, *C. gaudichaudii* and *C. trachystomus*); while data about the larval skeleton, i.e. the chondrocranium and hyobranchial apparatus, both of significant systematic and phylogenetic value (e.g. Larson & de Sá, 1998; Vera Candioti, 2007), exists for just one species (*C. gaudichaudii*).

Crossodactylus dantei Carcerelli & Caramaschi, 1993 is an endemic anuran species found in the municipality of Murici, State of Alagoas, north-east Brazil. This species is the only Hylodidae known to occur in the northern portion of the Atlantic Forest (Vieira et al., 2023). Its occurrence is restricted to a small range of only 8 km² and its presence has only been recorded in two streams. For over two decades (1988–2012) there were no recorded sightings of this species, and except for the adult description, there is no data about its biology or natural history. Currently, the IUCN Red List of Threatened Species designates *C. dantei* as Data Deficient (IUCN, 2004).

Herein, we provide the first description of larval morphology, and the advertisement and territorial calls of *C. dantei*; in addition, we assess its phylogenetic positioning.

MATERIALS & METHODS

Tadpole morphology

We analysed twelve tadpoles at stages 25, 37 and 44 (Gosner, 1960) housed at the Herpetological Collection of the Museu de História Natural of the Universidade Federal de Alagoas (MHNUFAL 12051–54). The specimens were collected at the species' type locality (Mata da Bananeira, 9° 12'00" S, 35° 52'00" W, DATUM WGS84; 551 m a.s.l., Estação Ecológica de Murici, municipality of Murici, Alagoas State, Brazil), anesthetised with 5% lidocaine, then fixed and preserved in 10% formalin (commercial grade).

For the external morphology, 28 measurements were taken. These measurements followed McDiarmid & Altig (1999) for total length (TL), body length (BL), maximum tail height (MTH), tail length (TaL), tail muscle height (TMH), and tail muscle width (TMW); Lavilla & Scrocchi (1986) for body width at eye level (BWE), body width at nostril level (BWN), dorsal gap of the oral disc width (DGO), extranarial distance (EnD), extraorbital distance (EoD), eye diameter (ED), eye-nostril distance (END), intranarial distance (InD), intraorbital distance (IoD), maximum body height (MBH), maximum body width (MBW), narial diameter (ND), oral disc width (ODW), snout-nostril distance (SND), snout-spiracle distance (SSD) and spiracle-posterior body distance (SPD); Grosjean (2005) for dorsal fin height (DFH) and ventral fin height (VFH); Rodrigues et al. (2017) for spiracle length (SL) and vent tube length (VTL); Pinheiro et al. (2012) for dorsal fin insertion angle (DFIA) and Altig & Johnson (1989) for angular orientation of the oral disc (ODAO). Measurements were taken using an ocular micrometer installed on a Coleman® XTB/2B stereomicroscope, with the exception of total length, which was measured using a digital caliper with an accuracy of 0.1 mm. The insertion angle of the dorsal fin and the angular orientation of the oral disc were measured using ImageJ v. 1.50i software, through photos captured with a digital camera coupled with a stereomicroscope. Terminology followed McDiarmid & Altig (1999).

Descriptions of the buccopharyngeal cavity were based on two tadpoles at stage 25 (MHNUFAL 12054), which were dissected following Wassersug (1976) to expose the buccal roof and floor. Subsequently, they were stained with methylene blue and lugol solution, observed and characterised under a stereomicroscope. Terminology followed Wassersug (1976; 1980).

Descriptions of the larval skeleton were based on two tadpoles at stage 25 (MHNUFAL 12051, 12054). Specimens were cleaned and double-stained for bone and cartilage following the protocol of Dingerkus & Uhler (1977). They were observed and characterised under a stereomicroscope. Terminology followed Larson & de Sá (1998) and Haas (2003).

Bioacoustics data

Field expeditions were conducted between December 2012 and December 2013 at the species' type locality. On 31 May 2013 a male was observed calling above partially submerged leaves at the edge of a small permanent stream. The calls were recorded using a Marantz® PMD 660 digital recorder coupled with a Sennheiser® K6/ME66 directional microphone from a distance of about 1 m between 11:00 h – 13:00 h (air temperature = 24.2 °C, water temperature = 23.4 °C, and relative humidity = 90%). The recordings were sampled at 44 kHz and 16 bits resolution.

Data analysis was performed using the software Raven Pro 1.6.4 for Windows (Cornell Lab of Ornithology, Bioacoustics Research Program, 2023). Spectrograms were created using Seewave package version 2.2.0 (Sueur et al., 2008) in the software R version 4.2.1 (R Core Team, 2022), with Hann window, FFT size of 512 points, and 90% overlap. The calls were classified based on observations of the frogs' social context in the field (Wells, 2007). The temporal and spectral parameters analysed included: calls per minute, call duration, intercall interval, notes per call, note duration, pulses per note, internote interval and dominant frequency; terminology follows Köhler et al. (2017).

Call voucher specimens were deposited in the herpetological collection of the Museu de História Natural, Universidade Federal de Alagoas (MHNUFAL 11934). The acoustic recordings are housed in the Fonoteca Zoológica Gabriel Skuk of the Museu de História Natural (MHNUFAL-SOM 054–056).

Figure 1. *Crossodactylus dantei* tadpole (MHNUFAL 17664) at stage 25 (Gosner, 1960). **(A)** Lateral, **(B)** dorsal and **(C)** ventral views (scale bar = 10 mm); **(D)** oral disc (scale bar = 1 mm).

Molecular data

Genomic DNA was extracted from the tail muscle tissue of a tadpole collected at the type-locality (MHNUFAL 17664), utilising the Phenol/Chloroform method (Sambrook et al., 1989). Extracted DNA was quantified and purity was assessed through a Loccus® L-Quant spectrophotometer. Amplification of a 418-base pair fragment of the mitochondrial 16S rRNA gene was performed via Polymerase Chain Reaction (PCR) employing An16SF and An16SR primers (Lyra et al., 2017). The presence of amplicons was verified through electrophoresis in a 1% agarose gel stained with Sybr® Safe and visualised under ultraviolet light on a Vilber Lourmat® transilluminator. Subsequently, the sample was purified with isopropanol and sent to ACTGENE - Molecular Analysis (Alvorada, Brazil) for forward sequencing.

The sequenced material was edited with BioEdit v. 7.0.9 (Hall, 1999) and aligned using the software MAFFT v7.490 (Katoh & Standley, 2013) with representative samples of all Hylodidae and Alsodidae genera (sensu Grant, 2019) available in GenBank. Additionally, a sequence of *Cycloramphus boraceiensis* (Heyer, 1983) was used to root the phylogeny. The final molecular dataset comprised a total of 170 individuals, representing 50 species (See Table S1 in supplementary material for GenBank accession numbers). The best evolutionary model was determined using PartitionFinder 2.1.1 (Lanfear et al., 2012), considering all search algorithms, branches as connected, and using the Bayesian information criterion as the selection method. Two independent Bayesian Inference analyses were performed using MrBayes

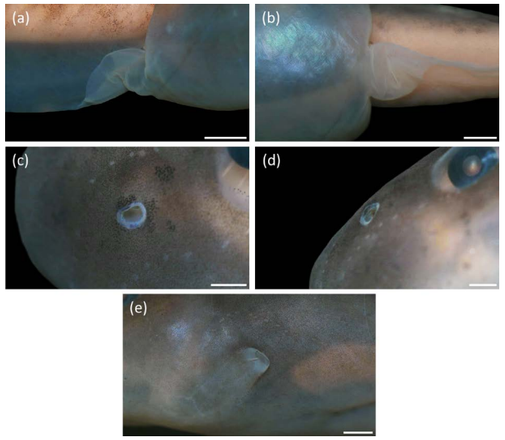

Figure 2. *Crossodactylus dantei* tadpole (MHNUFAL 12054) at stage 25 (Gosner, 1960; scale bar = 1 mm). Vent tube in **(A)** lateral and **(B)** ventral views; left nostril in **(C)** dorsolateral and **(D)** lateral views; **(E)** spiracle in lateral view.

3.2 software (Ronquist et al., 2012), with ten million generations and sampling every 1000 generations. A majority consensus tree was generated to obtain the final topology after discarding 25% of the initial trees. The Kimura-2-parameter evolutionary model (K2P; Kimura, 1980) was utilised, with complete gap deletion, to calculate the genetic distances both within and between species using the MEGA X software (Kumar et al., 2018).

RESULTS

Tadpole external morphology

Total length 43.7 ± 4.7 mm (34.3–48.8 mm). Body elliptical in dorsal and ventral views (MBW/BL = 0.49–0.65), globular-depressed in lateral view (MBH/MBW = 0.66–0.90), ventral contour convex, constriction at spiracle level present (Fig. 1A–C). Body length about 38% of total length (BL/TL = 0.35–0.42), greatest height varying between middle or posterior third of body, greatest width in the middle third of body. Tip of snout circular in lateral and dorsal views. Eyes dorsal, laterally oriented (ED/BWE = 0.12–0.21), occupying 50% of intraorbital distance (ED/IOD = 0.37–0.71), and representing 15% of maximum body width (ED/MBW = 0.12–0.19). Nostrils elliptical, dorsally located, directed laterally or anterodorsally, bearing a small flap at the medial margin; represents 16% of the intranarial distance (ND/InD = 0.10–0.23), 5% of the maximum body width (ND/MBW = 0.04–0.07), and 7% of body width at nostril level (ND/BWN = 0.05–0.10), maximum length parallel to longitudinal body axis, located closer to snout than eyes (END/SND = 0.75–1.36), openings leveled with surrounding surface (Fig. 2C & D). Spiracle single, sinistral, located below the midline at the middle third of the body (SPD/SSD = 0.62–0.88), visible in dorsal and ventral views, posterodorsally directed, inner wall presents as a small ridge, external wall ending anteriorly

to the inner wall (Fig. 2E). Vent tube dextral, attached to the ventral fin, with dextral membrane displaced dorsally and anteriorly, opening smooth (Fig. 2A & B). Tail length approximately 62% of total length (TaL/TL = 0.57–0.64), maximum height at the middle third of the tail, maximum height exceeding maximum body height (MBH/MTH = 0.67–0.99), tail tip varying between narrowly rounded to pointed. Tail musculature robust, maximum height approximately half of the maximum tail height (TMH/MTH = 0.38–0.55), progressively thinner as it approaches the posterior end, myosepta distinct along its entire length. Dorsal fin height greater than ventral fin height (VFH/DFH = 0.65–0.84), less than half of the maximum tail height (DFH/MTH = 0.33–0.45), maximum height in its middle third; originates at the body-tail junction at a narrow angle (DFIA = 2.90°–6.40°), continuing almost parallel to the tail musculature, it has a semicircle outline up to the posterior third, where it abruptly converges to the tail tip. Ventral fin begins at the ventral end of the body, maximum height in the anterior third, semicircle outline that slightly converges towards the end of the tail. Oral disc equivalent to 39% of the maximum body width (ODW/MBW = 0.33–0.50), ventrally positioned (ODAO = 20.14°–26.17°), laterally emarginated; bordered by a single row of marginal papillae (12 papillae/mm), except for its anterior margin, which presents a dorsal-medial gap equivalent to 53% of oral disc width (DGO/ODW = 0.46–0.60); papillae have conical or rounded tips and no pigmentation; submarginal papillae present, irregularly distributed along the sides of the oral disc (Fig. 1D). Labial tooth row formula (LTRF) 2(2)/3(1), P3 20% smaller than the other rows and P2 with medial indentation, A2 and P1 with medial gaps (30% of the length of A1 and 20% of the length of P2, respectively). Jaw-sheaths pigmented; upper jaw-sheath arch shaped with finely serrated margins, bearing a medial projection; lower jaw-sheath 'V' shaped and robust. A summary of tadpoles' measurements is provided in Table 1.

Colouration in preservative: anterior portion of the dorsum beige, transitioning to a dark brown in the middle third, peribranchial and abdominal regions translucent, with light brown/grey at sides; shapeless melanophores scattered irregularly across the initial portion of the anterior third of the body, which crosses the body dorsally up to the base of the tail. Fins translucent, with brown musculature on its anterior third, that becomes translucent as it approaches the end of the tail; shapeless melanophores are irregularly scattered throughout the tail, except for the anterior and middle thirds of the ventral fin. At the end of metamorphosis, stage 44, the dorsum and laterals of the body become brown, the ventral side area milky white, and the melanophores disappear from the body, remaining only on the tail.

Lateral line system (Fig. 3): supraorbital line composed of 12–21 stitches beginning posteromedially to the eyes and converging dorsally towards the snout, contouring the nostril and ending above the oral disc; infraorbital line composed of 15–20 stitches that starts posteromedially to the eyes, just below the beginning of the supraorbital line, and runs anterolaterally towards the snout, ending

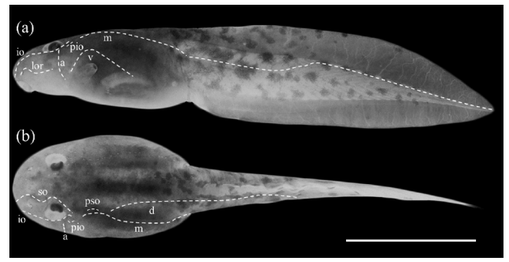

Figure 3. Lateral line system of *Crossodactylus dantei* (scale = 10 mm). Abbreviations: a = angular line; d = dorsal line; io = infraorbital line; lor = oral longitudinal line; m = medial line; pio = posterior infraorbital line; pso = posterior supraorbital line; so = supraorbital line; v = ventral line.

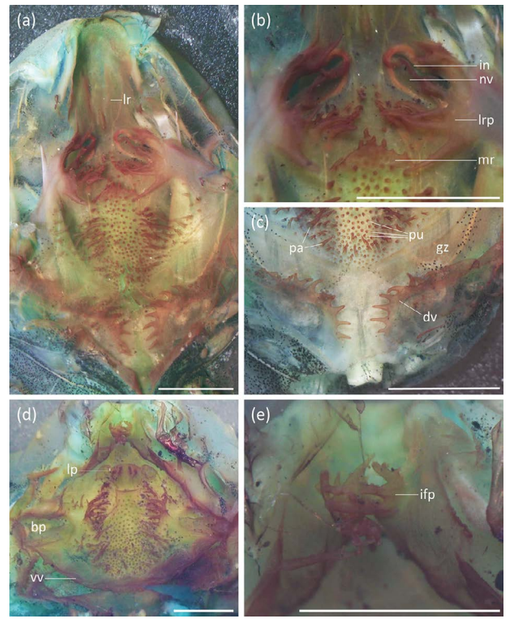

Figure 4. Buccal roof **(A–C)** and floor **(D–E)** of *Crossodactylus dantei* (MHNUFAL 12054) at stage 25 (Gosner, 1960; scale bar = 1 mm). Abbreviations: bp = buccal pockets; dv = dorsal velum; gz = glandular zone; ifp = infralabial papillae; in = internal nare; lp = lingual papillae; lr = longitudinal ridges; lrp = lateral ridge papillae; mr = median ridge; nv = narial valve; pa = papillae; pu = pustules; vv = ventral velum.

above the oral disc; posterior supraorbital line composed of 2–7 stitches that occurs posterior to the supraorbital line; posterior infraorbital line composed of 3–4 stitches located posterolaterally to the infraorbital line; angular line composed of 9–13 stitches that starts laterally to the infraorbital line, then runs ventrally, ending at the plane of the spiracle; oral longitudinal line has 7–12 stitches that starts laterally to the oral disc and runs laterally through the body until near the nostrils; ventral line

Table 1. Measurements (in mm; DFIA and ODAO in degree) of *Crossodactylus dantei* tadpoles (lots MHNUFAL 12051, 12052, 12053, 12054). Data presented as mean ± standard deviation (range). Stages according to Gosner (1960).

Measurements	Stage 25 (N = 8)	Stage 37 (N = 2)	Stage 44 (N = 2)
Total length	43 ± 5 (34.3-48.8)	46.8 ± 1.7 (45.6-48.1)	23.4 ± 0.2 (23.3-23.6)
Body length	16.1 ± 2.1 (13.1-18.6)	18.5 ± 1.3 (17.6-19.4)	15.5 ± 0.1 (15.4-15.5)
Maximum body width	9.2 ± 1.4 (6.5-10.7)	11 ± 0.3 (10.8-11.2)	5.9 ± 0.4 (5.6-6.3)
Body width at eyes level	8.7 ± 1 (7.2-10)	9.9 ± 0.7 (9.4-10.4)	6.4 ± 0.4 (6.1-6.6)
Body width at the nostril level	6.6 ± 0.7 (5.4-7.5)	6.9 ± 0.1 (6.8-7)	2.9 ± 0.1 (2.8-2.9)
Maximum body height	7.2 ± 1.2 (5.1-8.5)	9.7 ± 0.1 (9.6-9.8)	5.5 ± 0.5 (5.1-5.9)
Extraorbital distance	6.1 ± 0.8 (5.4-7.4)	6.7 ± 0.2 (6.5-6.8)	6.3 ± 0.5 (5.9-6.6)
Intraorbital distance	3 ± 0.4 (2.3-3.7)	3 ± 0.2 (2.8-3.1)	5.1 ± 0.4 (4.8-5.3)
Eye diameter	1.3 ± 0.2 (1-1.6)	2.1 ± 0.1 (2-2.2)	1.7 ± 0 (1.7-1.7)
Extranarial distance	3.8 ± 0.5 (3-4.5)	4.4 ± 0 (4.4-4.4)	3 ± 0.1 (2.9-3)
Intranarial distance	3 ± 0.6 (2.1-3.9)	3.6 ± 0.4 (3.3-3.8)	2.5 ± 0.1 (2.4-2.5)
Narial diameter	0.5 ± 0.1 (0.3-0.8)	0.5 ± 0 (0.5-0.5)	0.3 ± 0.1 (0.2-0.3)
Eye-nostril distance	1.6 ± 0.2 (1.2-2)	1.6 ± 0.1 (1.5-1.6)	1.4 ± 0.1 (1.3-1.4)
Snout-nostril distance	1.8 ± 0.3 (1.1-2.1)	1.7 ± 0.3 (1.5-1.9)	0.6 ± 0.1 (0.5-0.6)
Snout-spiracle distance	9.7 ± 0.9 (8.2-11)	10.2 ± 0.9 (9.5-10.8)	
Spiracle-posterior body distance	7.4 ± 1.1 (5.5-8.8)	8.8 ± 0.9 (8.1-9.4)	
Spiracle length	1.7 ± 0.5 (1.2-2.5)	2.1 ± 0.1 (2-2.1)	
Vent tube length	2.5 ± 0.4 (2-2.9)	3.5 ± 0.7 (3-4)	
Oral disk width	3.7 ± 0.4 (3-4.2)	4.1 ± 0.1 (4-4.2)	
Width of the dorsal gap of the oral disk	2 ± 0.3 (1.7-2.6)	2.1 ± 0.3 (1.9-2.3)	
Tail length	26.9 ± 3.2 (21.2-31.7)	28.4 ± 3 (26.2-30.5)	8 ± 0.1 (7.9-8.1)
Maximum tail height	9.2 ± 1.3 (7.1-10.4)	10.2 ± 0.5 (9.8-10.5)	3 ± 0.4 (2.7-3.2)
Tail muscle height	4.8 ± 1.1 (2.9-6.3)	5.7 ± 0.5 (5.3-6)	4.5 ± 0.1 (4.4-4.5)
Tail muscle width	4.2 ± 0.8 (2.9-5.4)	5.4 ± 0 (5.4-5.4)	3 ± 0.4 (2.7-3.2)
Dorsal fin height	3.4 ± 0.6 (2.5-4)	4.1 ± 0.4 (3.8-4.3)	
Ventral fin height	2.6 ± 0.4 (2-3)	3 ± 0.2 (2.8-3.1)	
Dorsal fin insertion angle	4.3° ± 1.3° (2.9°-5.6°)	5.2° ± 1.6° (4°-6.4°)	12.1° ± 4.8° (8.7°-15.6°)
Oral disk angular orientation	23.2° ± 2.7° (20.14°-25.42°)	25.9° ± 0.3° (25.6°-26.1°)	

composed of 18–23 stitches that starts on the anterior portion of the body at the plane of the spiracle, runs posterodorsally and then converges ventrally, in a way to circle the spiracle, ending posteriorly to it but at the same plane where the line started; dorsal line composed of 19–25 stitches, starts at eyes level and posteriorly to the medial line of the body, converges dorsally, and continues alongside the base of the dorsal fin until the middle third of the tail; medial line starts posteriorly to the posterior infraorbital line, 16–21 stitches runs dorsally across the body until it reaches the tail, at that point the stitches occur close to the axis of the tail myotomes, until the middle third, where it converges to the base of the dorsal fin and runs through its posterior third, number of stitches at the tail indistinguishable.

Buccopharyngeal cavity

Buccal roof (Fig. 4A–C): buccal roof triangular, longer than wide; prenarial arena almost twice as long as wide, with three longitudinal ridges, two larger located laterally, running through the posterior third to the middle third of the arena, and a smaller one located between the two larger ones. Choanae elongated and slit-shaped, located at an angle of 45° from the transverse plane, positioned at a distance of about 30% from the jaw sheath to the esophagus; anterior wall tall and robust, bearing one to two pairs of pustules and two pairs of filiform papillae, which present as a big pair over a smaller one; posterior wall tall and robust; narial valve visible, covering almost the entire nostril; maximum length of choanae and internarial distance representing about 30% and 15%, respectively, of the total width of the buccal roof. Postnarial arena length about 40% of the prenarial arena length, with almost 25 postnarial conical papillae, arranged as an inverted 'V' that runs from the central area to close to the lateral ridge papillae; three additional small postnarial papillae, arranged in a triangular shape located anteriorly to the median ridge. Median ridge crescent-shaped, located at a distance of about 45% from the jaw sheath to the esophagus, with six to eight secondary projections along its anterior edge. A pair of lateral ridge papillae present, with five hand-shaped secondary projections pointing towards the centre of the postnarial arena. Buccal roof arena (BRA) U-shaped, delimited anteriorly by the median ridge, laterally by about 25 smooth conical papillae of varied sizes, and posteriorly by about ten small, smooth and sparsely distributed conical papillae; BRA surface covered by a large amount of pustulations; about ten pairs of irregularly scattered papillae on the lateral portions of the buccal roof. Glandular zone distinct, arch-shaped, interrupted medially, wider in its lateral

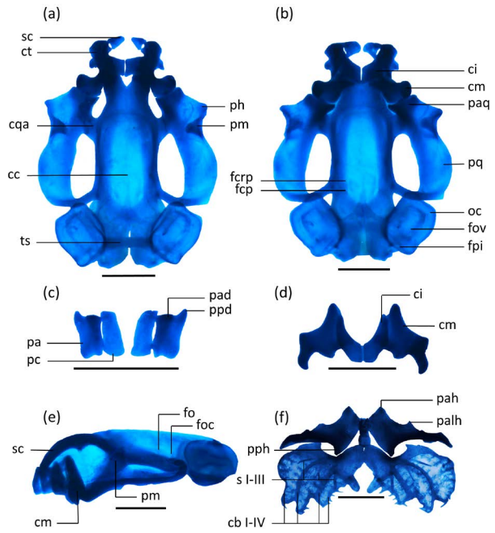

Figure 5. Skeleton of *Crossodactylus dantei* tadpole (MHNUFAL 12051, 12054) at stage 25 (Gosner, 1960; scale bar = 1 mm). Chondrocranium in **(A)** dorsal, **(B)** ventral, and **(E)** lateral views; **(C)** cartilago suprarostralis and **(D)** cartilago infrarostralis in frontal view, **(F)** hyobranchial apparatus in dorsal view. Abbreviations: cb I–IV = ceratobranchials I–IV; cc = cavum crani; ci = cartilago infrarostralis; cm = cartilago Meckeli; cqa = comissura quadratocranialis anterior; ct = cornua trabeculae; fcp = foramen caroticum primarium; fcrp = foramen craniopalatinum; fo = foramen opticum; foc = foramen oculotomotorium; fov = fenestra ovalis; fpi = foramen perilymphaticum inferior; oc = otic capsule; pa = pars alaris; pad = processus anterior dorsalis; pah = processus anterior hyalis; palh = processus anterolateralis hyalis; paq = pars articularis quadrati; pc = pars corporis; ph = processus hyalis; pm = processus muscularis; ppd = processus posterior dorsalis; pph = processus posterior hyalis; s I–III = spicules; pq = palatoquadrate; sc = suprarostral cartilage; ts = tectum synoticum.

portion, about 3% of the total length of the buccal roof. Dorsal velum about 6% of the buccal roof's total length, interrupted medially, with eight to nine large finger-shaped projections on each side.

Buccal floor (Fig. 4D & E): buccal floor triangular, slightly wider than long. Two pairs of infralabial papillae, the largest pair hand shaped and bear about 6 projections, they are arranged in a way that allow them to intertwine with the projections from the opposite pair; the smaller pair composed by conical papillae, located posteroventrally to the largest pair and covered by its projections. Lingual anlage bearing four conical lingual papillae of similar sizes distributed in an arc shape. Buccal floor arena (BFA) ellipsoid, surrounded by about 70 conical papillae that point towards the centre of the arena, arranged in 'U' shape, with abundant papillae on its lateral portion, with only around ten small papillae scattered on its posteromedial portion, largest papillae hand-shaped, bear about five finger-like projections, and found medially on the lateral portion; BFA surface covered by a large number of pustules. Buccal pockets oriented transversely, slit-shaped, about 15% of the size of the buccal floor and with three to four pairs of conical prepocket papillae. Ventral velum about 3% of the total length of the buccal floor, bears about eight conical projections on its margin, and a small irregular projection on its medial portion.

Larval skeleton

Chondrocranium elliptical in dorsal view and depressed in lateral view, higher at the level of pars articularis quadrati and wider in the middle third of palatoquadrate (Fig. 5A & E).

Suprarostral cartilage divided into pars alaris and pars corporis, both united by cartilaginous tissue dorsally and ventrally, forming a large foramen centrally; pars alaris quadrangular, wide and robust, processus posterior dorsalis flat and triangular, processus anterior dorsalis small and robust; pars corporis flat and rectangular, with no connections between them (Fig. 5C). Cornua trabeculae V-shaped and appears from planum trabeculare anticum, corresponding to 25% of chondrocranium length; processus lateralis trabeculae conspicuous at the posteroventral portion of each trabeculae; planum trabeculare anticum wider than long. Cranial floor occluded by a thin layer of cartilage, foramina carotica primaria and craniopalatina visible at its posterior portion, the latter larger than the former; orbital cartilage partially chondrified, foramina opticum and oculomotorium small. Tectum synoticum approximately 5% of chondrocranium length. Taenia tecti marginalis confluent with otic capsules.

Otic capsules quandrangular, about 30% of chondrocranium length; fenestra ovalis square-shaped, about 15% of chondrocranium length, situated in the medial portion of the otic capsules. Crista parotica inconspicuous, bearing a small processus anterolateralis. Arcus occipitalis protruding posterolaterally from the otic capsules. Foramen jugulare and foramen perilymphaticum present at the posteromedial portion of the otic capsules, the latter much larger than the former. A notch, representing 20% of chondrocranium length, marks the insertion point of notochord into the planum basale.

Palatoquadrate wide, expanding laterally in its posterior portion, with a dorsal curvature that connects to the orbital cartilage through the processus ascendens; lateral margin convex in dorsal view, and flat in lateral view, subocular arch with a smooth margin; processus ascendens attached to chondrocranium at the orbital cartilage, below the level of foramen oculomotorium (low suspensorium). Palatoquadrate attached to chondrocranium anteriorly through commissura quadratocranialis anterior; processus quadratoethmoidalis inconspicuous; processus pseudopterygoideus absent; processus antorbitalis inconspicuous; processus muscularis quadrati small,

Table 2. Acoustic traits of the advertisement and territorial calls of *Crossodactylus dantei*. Data presented as mean ± standard deviation (range).

Call traits	Advertisement call	Territorial call	Note A	Note B
Calls	14	3		
Calls/minute	0.85	0.15		
Call duration (s)	2.5 ± 0.13 (2.3–2.7)	23.16 ± 6.5 (18–30.5)		
Intercall interval (s)	61 ± 39 (15–143)			
Notes/call	22.8 ± 1.2 (21–25)	26.6 ± 11.5 (15–38)	16.3 ± 8 (0–25)	10.3 ± 3.7 (6–13)
Note duration (ms)	44 ± 18 (5.2–90.6)	134.9 ± 84 (33.8–324.8)	72.4 ± 15.8 (33.8–105.3)	233.6 ± 42 (94–324.8)
Pulses/note	15.2 ± 6.75 (2–35)	20.5 ± 7.82 (7–38)	25 ± 7.7 (10–38)	13.2 ± 1.9 (7–17)
Internote interval (ms)	70.4 ± 20.3 (17.3–190)	390.3 ± 281.1 (94–1141.2)		
Dominant frequency (Hz)	3356 ± 564 (1636–6546)	3160 ± 635 (2239–4134)	3452 ± 616 (2239–4134)	2690 ± 401 (2239–3617)

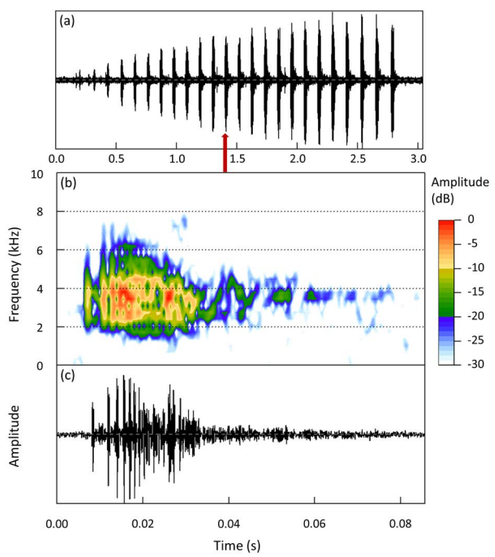

Figure 6. **(A)** Oscillogram of the advertisement call of *Crossodactylus dantei* (MHNUFAL 11934), followed by **(B)** a spectrogram and **(C)** corresponding oscillogram of one the notes identified by a red arrow.

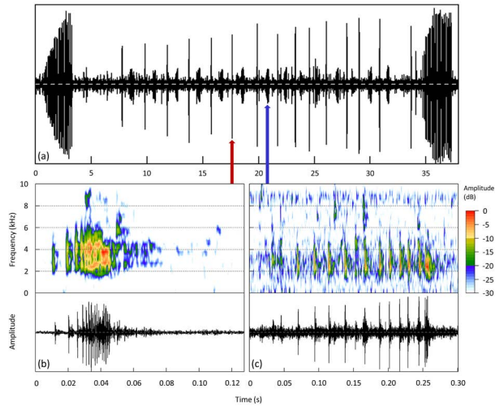

Figure 7. **(A)** Oscillogram of a territorial call emitted amid advertisement calls by *Crossodactylus dantei* (MHNUFAL 11934), followed by a spectrogram and corresponding oscillogram of **(B)** atype A note (identified by a red arrow) and **(C)** a type B note (identified by a blue arrow).

triangular, with rounded tip, curved medially, pointing towards processus antorbitalis; facies articularis hyalis relatively large and rounded, located at the posterior portion of the connection between the processus muscularis quadrati and the palatoquadrate. Pars articularis quadrati square-shaped and robust, forming the anterior portion of the palatoquadrate, with a ventral curvature.

Cartilago meckeli robust, transversely oriented, at an angle of approximately 80° to the main axis of the body; processus retroarticularis robust, with a curvature that embraces the pars articularis quadrati; processus dorsomedialis larger than processus ventromedialis. Cartilago infrarostralis sickle-shaped, longer than wide, fused medially by a thin layer of connective tissue, which can be U-shaped in dorsal view (Fig. 5B & D).

Ceratohyal elongated, oriented perpendicularly to the chondrocranium; processus anterior hyalis larger than processus anterolateralis hyalis, both triangular; processus posterior hyalis large, with a rounded margin; copula anterior absent; pars reuniens small and square-shaped; copula posterior small, longer than wide; processus urobranchialis inconspicuous. Hypobranchial plates ovoid, fused medially, posterior margins diverging in an inverted 'U' shape; processus anterior branchialis large, triangular, medially pointed; ceratobranchials confluent with the hypobranchial plate, ceratobranchialis I and II smaller than the others; spicules evident; processus branchialis 'open' (Fig. 5F).

Vocalisations

The advertisement call of *C. dantei* is formed by a sequence of notes emitted as short energy bursts in a brief time interval, each interspersed with well-defined silences (Fig. 6). The call exhibits an ascendant amplitude modulation, in which the duration and intensity of the notes progressively increase over time, occasionally slightly fading before the end. Each note consists of partially fused pulses, where the first pulse is often followed by a short silent period. In a single recording

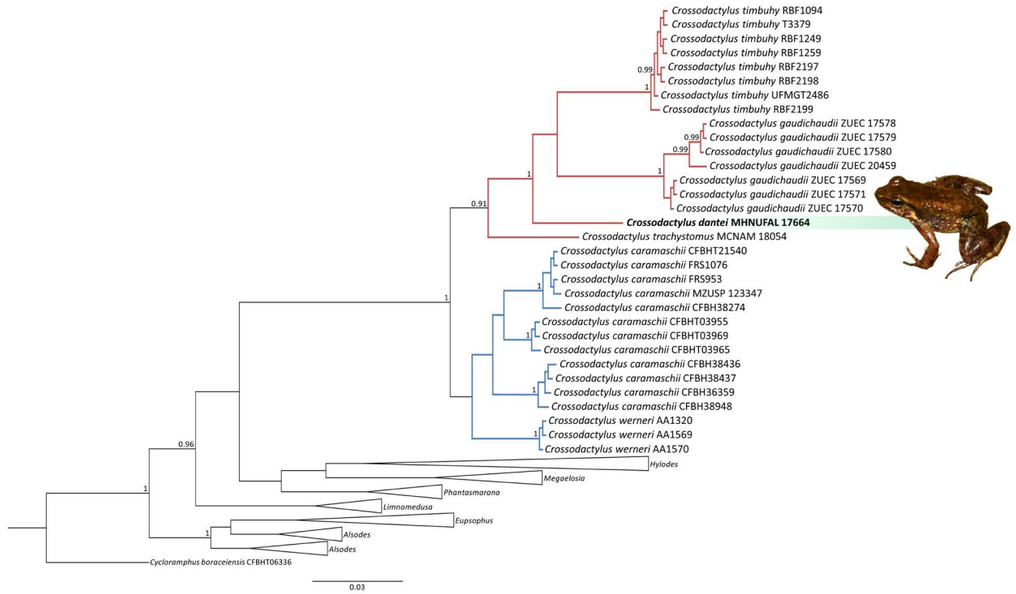

Figure 8. Bayesian Inference phylogram of the 16S rRNA Mitochondrial gene fragment for Hylodidae and Alsodidae families with nodes showing posterior probabilities > 0.90. Coloured branches represent different clades within *Crossodactylus*.

Table 3. Genetic distances between and within (intra) species belonging to the *Crossodactylus* genera. Estimated using Kimura 2-parameters evolutionary model (K2P) with complete deletion of gaps in the 16S rRNA mitochondrial gene fragment.

K2P	*C. dantei*	*C. gaudichaudii*	*C. caramaschii*	*C. timbuhy*	*C. trachystomus*	*C. werneri*	Intra
C. dantei	-						-
C. gaudichaudii	0.095	-					0.01
C. caramaschii	0.097	0.101	-				0.03
C. timbuhy	0.092	0.116	0.088	-			0
C. trachystomus	0.092	0.100	0.080	0.109	-		-
C. werneri	0.115	0.126	0.062	0.105	0.081	-	0

of nearly 17 minutes, 14 instances of an advertisement call were registered (Table 2). Each call lasted 2.5 ± 0.13 s (2.3–2.7 s) and consisted of 22.8 ± 1.2 (21–25) notes, each lasting 44 ± 18 ms (5.2–90.6 ms) and separated by silent intervals of 70.4 ± 20.3 ms (17.3–190 ms). The dominant frequency was 3356 ± 564 Hz (1636–6546 Hz). Harmonics may be discernible, but the recording quality precluded definitive identification of their precise spectral characteristics. The call can be classified as a "pulse repetition" according to Beeman's (1998) sound categories.

A distinct vocalisation was also recorded, which was categorised as territorial call based on a combination of acoustic analysis and field observations of social context. It consists of a complex call characterised by the presence of two different note types: one short, with partly fused pulses (type A); and the other long, with pulses separated by silent intervals (type B). These notes tend to occur in an alternating manner, interspersed by long intervals of silence, with ascendant amplitude modulation. Three territorial calls were recorded across three different recordings that totaled approximately 19 minutes, one of these calls was emitted amid a series of advertisement calls (Fig. 7, Table 2). The territorial call lasts 23.16 ± 6.5 s (18–30.5 s) and is composed of 26.6 ± 11.5 (15–38) notes, of which 16.3 ± 8 (0–25) are type A and 10.3 ± 3.7 (6–13) type B. The dominant frequency was 3160 ± 635 Hz (2239–4134 Hz), with type B notes exhibiting a lower mean frequency [2690 ± 401 Hz (2239–3617 Hz)] than type A notes [3452 ± 616 Hz (2239-4134 Hz)].

Phylogenetic position

The final alignment resulted in a matrix of 756 base pairs. The GTR+G evolutionary model was selected by PartitionFinder as the most appropriate for our dataset. Phylogenetic analysis recovered the genus *Crossodactylus* as monophyletic, with two major sister clades (PP = 1; GD = 0.09). The first clade consisted of *C. timbuhy*, *C. gaudichaudii*, *C. dantei* and *C. trachystomus*; while the second clade comprised *C. caramaschii* and *C. werneri*

(Fig. 8). In the first clade, *C. trachystomus* was recovered as the most external species, while *C. dantei* was found to be a sister species to a clade (PP = 1) composed of *C. gaudichaudii* (GD = 0.09) and *C. timbuhy* (GD = 0.09). Refer to Table 3 for genetic divergence values.

DISCUSSION

Tadpole morphology

Crossodactylus larvae have a highly conserved body plan, which may result from analogous ecological pressures exerted by their specialised microhabitat (Caramaschi & Kisteumacher, 1989; Toscano et al., 2019). Typically, these tadpoles occupy the backwaters of fast-flowing streams, that are characterised by sandy or muddy substrates, rocks and abundant tree litter. These species exhibit benthic and cryptic habits, often swimming near the bottom and using the substrate, leaves, rocks and crevices as sources of camouflage and shelter (e.g. Caramaschi & Kisteumacher, 1989; Silva-Soares et al., 2015; Toscano et al., 2019; de Sá et al., 2022; Lacerda et al., 2022). The external morphology of *Crossodactylus* tadpoles is typically characterised by an ovoid or elliptical body in dorsal view, rounded snout, eyes and nostrils dorsally or dorsolaterally positioned, a short sinistral spiracle posterodorsally oriented, dextral vent tube, oral disc ventrally positioned, jaw sheaths robust, LTRF 2(2)/3(1), colouration brown or beige with melanophores scattered along the tail.

Only *Crossodactylus cyclospinus*, *C. dantei* and *C. gaudichaudii* exhibit an elliptical body shape in dorsal view (Nascimento et al., 2005; Silva-Soares et al., 2015), with the remaining species in the genus presenting ovoid bodies; except for *C. schmidti*, whose dorsal body has been described as "subovoid" (Faivovich, 1998). Among those with an elliptical body shape, *C. dantei* stands out for being the only one with its nostrils positioned closer to the snout than the eyes. Furthermore, *C. dantei* and *C. cyclospinus* have an emarginated oral disc, while only *C. cyclospinus* possesses an incised upper jaw sheath. Consequently, *C. dantei* can be distinguished from *C. cyclospinus* by its non-incised upper jaw sheath, from *C. gaudichaudii* by its emarginated oral disc, and from the remaining species in the genus by its elliptical body shape in dorsal view. In lateral view, *C. dantei* has a considerably more elongated and depressed body compared to its congeners; however, certain specimens may bear a resemblance in lateral view to *C. gaudichaudii* due to a similar body shape (Silva-Soares et al., 2015).

Another shared feature in *Crossodactylus* species is the presence of a distinct lateral line system. Francioni & Carcerelli (1993) reported the absence of a distinct lateral line system in *C. gaudichaudii*, although Silva-Soares et al. (2015) did not explicitly mention the presence of a lateral line system in this species (as *C. aeneus*), the system seems discernible in the provided images, suggesting that *C. gaudichaudii* has a distinct lateral line system.

Crossodactylus gaudichaudii, *C. trachystomus* and *C. caramaschii* are the only ones with documented buccopharyngeal anatomy (Haas, 2003; Weber & Caramaschi, 2006; Toscano et al., 2019). Although these species display remarkable similarities, distinct traits can still be observed in *C. dantei*. Notably, *C. dantei* exhibits three longitudinal ridges in the prenarial arena, whereas other species either lack visible structures (*C. trachystomus*) or possess inconspicuous ones (e.g. a single tiny pustulation in *C. caramaschii*, a faint longitudinal ridge in *C. gaudichaudii*). Moreover, we reported the occurrence of two pairs of filiform papillae on the choanae anterior wall of *C. dantei*, whereas the remaining species either have one pair (*C. gaudichaudii*, *C. trachystomus*) or none (*C. caramaschii*). Weber & Caramaschi (2006) suggested that the morphology of the ventral velum constitutes a crucial taxonomic trait for the genus, probably due to its variations. Curiously, the ventral velum of *C. dantei* bears striking resemblance to that of *C. gaudichaudii.*

The chondrocranial morphology was only reported for *C. gaudichaudii* (Silva-Soares et al., 2015). In fact, descriptions of larval skeletons are rare in the family Hylodidae, and to date, they only exist for *Hylodes meridionalis* (Mertens, 1927), described by Nogueira-Costa & Wachlevski (2015), *H. ornatus* (Bokermann, 1967), described by Bilate et al. (2012), and *H. pipilans* Canedo & Pombal, 2007, described by Nogueira-Costa et al. (2017). It has been observed that the chondrocranium of this family is highly comparable across species, indicating the conservative nature of this characteristic within this family, as suggested by Nogueira-Costa & Wachlevski (2015). Despite the limited number of larval skeleton characterisations, trait variation was still observed between *Hylodes* and *Crossodactylus*. *Hylodes* possesses a pair of adrostral cartilages, which are absent in *Crossodactylus*; the processus muscularis is more extensively developed in *Hylodes* compared to *Crossodactylus*; and the processus anterolateralis hyalis is more developed in *Crossodactylus* compared to *Hylodes* (Nogueira-Costa & Wachlevski, 2015; Bilate et al., 2012; Silva-Soares et al., 2015). Among *Crossodactylus*, the absence of taenia tecti medialis and taenia tecti transversalis in *C. dantei* differentiates it from *C. gaudichaudii*, in which these features are present (Silva-Soares et al., 2015).

Vocalisations

The advertisement call of *C. dantei* follows the pattern observed in non-mute Hylodidae, as a trill with short, high-pitched, quickly repeated notes. This structure is attributed to the presence of a noisy environment generated by fast-torrent streams, which has driven the evolution of high-pitched calls that effectively surpass background noise (Haddad & Giaretta, 1999; Lingnau & Bastos, 2007). Notably, *C. dantei* exhibits an ascending amplitude modulation, a characteristic shared with other *Crossodactylus* species such as *C. trachystomus*, *C. franciscanus*, *C. werneri* and *C. timbuhy* (Pimenta et al., 2008; 2015; Vidigal et al., 2018; Lacerda et al., 2022); however, the emission of isolated notes between calls, which has been reported in other *Crossodactylus* species (Pimenta et al., 2015; Vidigal et al., 2018), was not observed in *C. dantei.* The advertisement call of *C. dantei* can be distinguished from those of its congeners by the presence of a distinct set of extreme traits that are unique to this species. *Crossodactylus dantei* (21–25 notes) and *C. werneri* (12–25 notes) have the lowest number of notes per call (Vidigal et al., 2018). *Crossodactylus dantei* (5.2–90.6 ms) and *C. gaudichaudii* (40–50 ms) produce the longest notes on average (Weygoldt & Silva, 1992). Additionally, *C. dantei* has the shortest advertisement call in the genus. The mean call frequency is comparable to that of *C. werneri*, the species with the lowest-frequency call (Vidigal et al., 2018); considering the similarity in snout-vent length (SVL) between both species, these results align with the conclusions of Augusto-Alves et al. (2021), who established a correlation between the dominant frequency of advertisement calls and SVL in Hylodidae.

Territorial calls have been documented for *C. gaudichaudii, C. cyclospinus, C. schmidti, C. werneri* and *C. timbuhy* (Weygoldt & Silva, 1992; Nascimento et al., 2005; Caldart et al., 2012; Vidigal et al., 2018; Lacerda et al., 2022). Of these, only *C. dantei* and *C. timbuhy* have been observed to emit two distinct notes in an alternating pattern during territorial behaviour. Both calls consist of a long note, with ascending amplitude modulation and pulses separated by silent intervals, and a short note, with partially fused pulses. However, it is possible to differentiate the territorial call of *C. dantei* from *C. timbuhy* based on the shorter mean duration of its long note (*C. dantei* = 233.6 ms vs. *C. timbuhy* = 400 ms), and the lower dominant frequency of both short and long notes (*C. dantei* χ = 3.4 kHz and χ = 2.6kHz vs. *C. timbuhy* χ = 3.7 kHz and χ = 3.4 kHz).

Phylogenetic position

Despite being constructed based on a single-gene dataset, we present one of the most comprehensive phylogenetic trees for the genus *Crossodactylus* to date. Our analyses revealed a basal split within the genus, resulting in the formation of two major clades.

Our findings differed from previous work (Toscano et al., 2019; Vittorazzi et al., 2021; de Sá et al., 2022) that recovered *C. trachystomus* as sister taxon of *C. caramaschii* + *C. werneri*, instead, we found strong statistical support (PP = 0.91) for *C. trachystomus* as sister taxon to a clade composed of *C. dantei* + *C. gaudichaudii* + *C. timbuhy*.

Crossodactylus caramaschii and *C. werneri* were recovered as sister species, as previously reported (Toscano et al., 2019; Vittorazzi et al., 2021; de Sá et al., 2022). Additionally, *C.caramaschii* is structured into three distinct mitochondrial lineages (GD = 0.03), suggesting that this taxon may represent a species complex (Fabri, 2013; Toscano et al., 2019).

Recently, Portik et al. (2023) developed a comprehensive phylogenetic hypothesis for anurans, based on more than 5000 species, including six representatives of the genus *Crossodactylus*. In comparison to the present study, its molecular data matrix incorporated *C. schmidti* instead of *C. dantei*, and multiple loci instead of just one. The resulting final topology highlights a basal dichotomy between *C. schmidti* and the other species of the genus, which in turn, exhibit a topology similar to that presented herein.

Toscano et al. (2019), Vittorazzi et al. (2021) and de Sá et al. (2022) present *Crossodactylus* topologies that are very similar to each other but are slightly different from those obtained by Portik et al. (2023) and in the present study. Although these works use a larger molecular data set, Toscano et al. (2019) exclusively used the 16S gene. A common factor that differentiates them from the current study, as well as from Portik et al. (2023), is the absence of *C. timbuhy* and significantly smaller outgroups (For a comprehensive view of the outgroup, refer to Figure S1 in supplementary material). Whereas Portik et al. (2023) also included in their analysis the markers used in the previously cited literature, factors such as the number of species of the genus *Crossodactylus* included in the analysis, and possibly the number of species in the outgroup, seem to be important for the topology obtained by the current work.

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Feeding ecology of the Jingdong frog *Odorrana jingdongensis* from Vietnam

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The Jingdong frog *Odorrana jingdongensis* Fei, Ye & Li, 2001, is known from China and Vietnam. It is listed as Vulnerable in the IUCN Red list. Knowledge about natural history of this species is virtually lacking. As a result of our field work in Son La Province of north-western Vietnam, we provide novel data about the feeding ecology of *O. jingdongensis* based on stomach-flushing technique to obtain stomach contents without sacrificing the frogs, a total of 159 individuals (63 males and 96 females). We found a total of 21 prey categories with 682 items, comprising 663 items of invertebrates, three items of vertebrates and 16 unidentified items in the stomachs of *O. jingdongensis*. The most important (IRI) groups among the prey of *O. jingdongensis* were Coleoptera (32%), followed by insect larvae (11.10%), Araneae (9.60%), Orthoptera (7.49%), Hymenoptera (6.33%) and Lepidoptera (6.25%). There was an overlap of 78.16% in the diet between males and females, in both sexes the trophic spectrum was similar, predominantly consisting of Coleoptera, insect larvae, Lepidoptera, Araneae, Orthoptera and Hymenoptera. In addition, we also compared the diet composition between the three study sites and seasons. Prey categories varied from 16 to 18 in sites and were higher in the rainy season (19) compared with the dry season (16).

Keywords: amphibians, invertebrates, prey items, stomach contents, Son La Province

INTRODUCTION

Globally, one-third of all amphibian species are threatened with extinction and almost half are experiencing population declines (Stuart et al., 2004; IUCN, 2023). However, information on the diet ecology of many frogs is lacking. The diet of frogs depends on prey availability in the environment (Schoener, 1974; Toft, 1980; Strüssmann et al., 1984; Donnelly, 1991; Caldart et al., 2012; Ngo et al., 2014), so frogs in tropical-subtropical regions have more diverse diets than in temperate (Moseley et al., 2005; Ugarte et al., 2007; Hothem et al., 2009; Brito et al., 2013; Ngo et al., 2014). The main factors influencing the feeding ecology of frogs are food abundance, size constraints, ecological tolerances and climatic conditions (Toft, 1980; Duellman & Trueb, 1986; Kwet, 2001; Miranda et al., 2006). Thus the diet composition is crucial for a better understanding of the frogs' life history, population fluctuations and the impact of habitat modification on populations (Toft, 1980; Strüssmann et al., 1984; Donnelly, 1991; Ogoanah & Uchedike, 2011; Caldart et al., 2012; Merlita & Olga, 2013; Ngo et al., 2014; Pham et al., 2019a; 2022; Le et al., 2020). Mostly invertebrates and sometimes small vertebrates make up the diets of frogs, of which terrestrial and aquatic insects have been reported as preferred frog prey (Duellman & Trueb, 1994; Wells, 2007; Strüssmann et al., 1984; Miranda et al., 2006; Merlita & Olga, 2013; Ngo et al., 2014; Pham et al., 2019a; 2022; Le et al., 2020). Identifying the prey taxa for each species helps to clarify the impact of frogs on invertebrate control (Nakamura & Tominaga, 2021). Feeding is opportunistic with food up to mouth width being ingested (Donnelly, 1991; Wells, 2007; Merlita & Olga, 2013; Ngo et al., 2014). However, frogs only target moving prey and prefer elongated prey such as crickets or insect larvae that move across their field of vision (Merlita & Olga, 2013). Due to sexual dimorphism, the diet also differs between the sexes, both concerning the dietary composition or the number of prey consumed (Donnelly, 1991; Valderrama-Vernaza et al., 2009; Brasileiro et al., 2010; Caldart et al., 2012). In addition, frog individuals may use different ways to find food as a response to fluctuations in prey abundance (Duellman & Trueb, 1994; Caldart et al., 2012).

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The genus *Odorrana* Fei, Ye & Huang, 1990, currently contains 64 species that inhabit forest streams, from Japan, China and Indochina to India, Myanmar, Thailand, Malaya, Sumatra and Borneo (Frost, 2023). Despite the high level of species richness, dietary studies in the genus to date has only been undertaken in *O. chapaensis* and *O. morafkai* in Vietnam (Pham et al., 2019; Le et al., 2020).

The Jingdong frog *Odorrana jingdongensis* Fei, Ye & Li, 2001 was described from China. Subsequently, Bain et al. (2003) described a new species, *Rana* (*Odorrana*) *hmongorum*, with the holotype deriving from Lao Cai Province. Ohler (2007) stated that this latter species represents a junior synonym of *O. jingdongensis*. Currently, in Vietnam, *O. jingdongensis* is known from Dien Bien, Ha Giang, Lai Chau and Lao Cai provinces (Luong et al., 2021). This species is listed as Vulnerable due to over-collection and continued degradation and destruction of its habitat (IUCN SSC, 2022). This species is closely associated with forest streams in mixed secondary evergreen forest of larger and medium hardwoods, shrubs and arrowroot (Fei et al., 2001; Bain et al., 2003).

In this study, we provide information on the dietary ecology of *O. jingdongensis* for the first time.

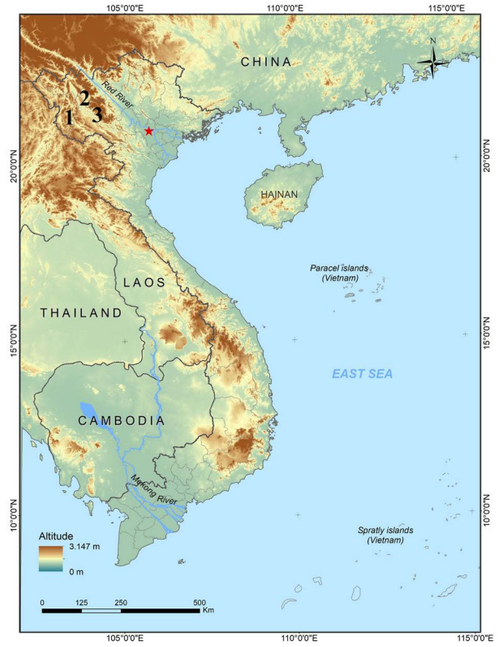

Figure 1. Map showing the survey sites in Son La Province, northern Vietnam: 1) Copia Nature Reserve; 2) Muong La Nature Reserve; and 3) Ta Xua Nature Reserve.

MATERIAL & METHODS

Field surveys were conducted in three nature reserves (NRs) of Son La Province, north-western Vietnam. The study area is characterised by a subtropical climate and seasonal monsoons with annual average rainfall ranging from 600 mm to 2000 mm, and an annual average air temperature ranging from 20 °C to 23 °C (maximum up to 40 °C and minimum -2 °C) (Nguyen, 2000). Co-ordinates were taken with a Garmin GPSMap 64SC (WGS 84 datum). Field surveys were carried out in two seasons at three sites: 1) Hua Ty Village, Co Ma Commune within Copia NR (21° 20'41.6" N, 103° 33'47.4" E, elevation 1200 m asl) from 15 to 22 April 2016 and from 13 to 16 October 2016; 2) Nam Nghiep Village, Ngoc Chien Commune within Muong La NR (21° 34'04.5" N, 104° 15'01.8" E, elevation 1580 m asl) from 26 to 30 April 2016 and from 22 to 28 October 2016, and 3) Cua Mang Village, Xim Vang Commune within Ta Xua NR (21° 20'00.2" N, 104° 25'09.9" E, elevation 1450 m asl) from 20 to 30 April 2017 (Fig. 1). The typical habitats at the three study sites were undisturbed evergreen forest, disturbed evergreen forest and secondary forest.

Frogs were collected by hand along streams transects (approximately 2.0–3.0 km in length) between the hours of 20:00 and 23:00 following the guideline approved by the American Society of Ichthyologists and Herpetologists for animal care (Beaupre et al., 2004). The surrounding habitat was mixed evergreen forest with larger and medium hardwoods, shrubs and arrowroot. The following information was recorded for each collected individual: site, date, time, elevation, temperature, relative humidity, type of vegetation and substrate type (leaf litter, moss, forest floor and leaves). Sex was determined by morphological examination based on a pair of pharyngeal subcapsular structures in males. We used a stomach-flushing technique to obtain stomach contents without sacrificing the frogs (Griffiths, 1986; Leclerc & Courtois, 1993; Solé et al., 2005). Spatula, forceps, two syringes with thread (60 ml) and the infusion tube of soft material (silicon) were used to collect prey items in the stomach of frogs, in particular for small individuals to avoid perforations of the oesophagus and stomach. Each individual was stomach-flushed only once following the guidelines approved by the American Society of Ichthyologists and Herpetologists for animal care (Beaupre et al., 2004). The water for flushing was taken from the streams where the frogs were captured and used after filtration. After stomach-flushing, frogs were monitored for vigour and body conditions and released within 30 minutes at the place of capture. Prey items were preserved in 70% ethanol. Frogs were subsequently released at the collection site after measurements of snout-vent length (SVL) and mouth width (MW) with a digital calliper to the nearest 0.01 mm and measured weight (BM) using electronic scales to the nearest 0.1 g.

Prey items were identified under a microscope (Olympus SZ 700) based on identification keys (i.e. Naumann et al., 1991; Johnson & Triplehorn, 2005; Brusca et al., 2016; Thai, 2022). The maximum length (L) and width (W) of each prey item were measured to

the nearest 0.1 mm using either a caliper or a calibrated ocular micrometer fit to a microscope. The volume (V) of the prey item was calculated using the formula for a prolate spheroid (π = 3.14; Magnusson et al., 2003):

$$V = \frac{4\pi}{3} * \left(\frac{L}{2}\right) * \left(\frac{W}{2}\right)^2$$

The index of relative importance (IRI) was used to determine the importance of each food category. This index provides a more informed estimation of prey item consumption than any of the three components alone by using the following formula (Caldart et al., 2012):

$$IRI = \frac{\%F + \%N + \%V}{3}$$

where F is the frequency of prey occurrence in stomachs and N is the total number of prey items concerning all prey items.

We used the Shannon-Wiener (H) index to estimate the diversity of the diet of frogs for each site, each season and each sex category, H is calculated from the equation:

$$H = -\sum(pi * \ln(pi))$$

where pi is the ratio of food items in the taxon to the total number of food items in the sample (Magurran, 2004; Muñoz-Pedreros & Merino, 2014).

The diet overlap between males and females was calculated by measuring the percentage overlap (Krebs 1999; Caldart et al., 2012), defined by the following equation:

$$Pjk = [\sum^{n}(\text{minimum Pij, Pik})]\ 100$$

where P_{jk} is the percentage overlap between groups j and k, P_{ij} and P_{ik} respectively represent the proportions of prey i consumed by groups j and k, and n is the total number of prey categories.

We used linear regression to examine the relationship between mouth width (MW), snout-vent length (SVL), body mass (BM) and prey size. In addition, we determined the difference between sex, sites and season.

Statistical analyses were performed with the SPSS 20.0 (SPSS Inc., Chicago, Illinois, USA) and with the significance level set to $p < 0.05$ for all analyses. Data are presented as mean ± standard deviation (SD) unless otherwise noted. We used Kendall's tau b statistics to examine the number of prey items, prey volume from frogs of different sexes, localities and season. We used one-way analysis of variance (ANOVA) to examine the size of prey items collected between sexes, among localities, and by season. We also compared the SVL and body mass of males and females using a one-way ANOVA.

RESULTS

Survey results

A total of 159 adult individuals (63 males and 96 females) of *O. Jingdongensis* were captured in Son La Province for stomach flushing, including 43 frogs from the Copia NR (19 males and 24 females); 34 from Muong La NR (15 males and 19 females); and 82 from Ta Xua NR (49 males and 33 females).

Morphological characteristics

SVL min-max: 42.0–72.5 mm; mean and SD: 56.3±7.0 mm, n=63), MW 14.4–26.7 mm (20.74 ± 2.41 mm, n=63), and body mass (BM 5.6–29.17 g, 13.98±5.5 g, n=63) in males was smaller than in females SVL 63.4–104.2 mm (82.8±10.8 mm, n=96); MW 18.0–37.1 mm (29.20±3.68 mm, n=96); BM 13.08–107.42 g, 46.24±20.22 mm, n=96) (ANOVA, all p<0.001) (Tables 1 & 2). Head longer than broad; snout round; tympanum of the males larger than in females, about two-thirds of the eye diameter; vomerine teeth developed; tongue deeply notched; discs

Table 1. Summary (Total, mean, SD and range) of the prey item number (N), width (W), length (L), and volume (V) data for *Odorrana jingdongensis* males, females and 3 communities (in mm for W and L; in mm^3 for V).

			Prey item volume				
	W	L	Minimum	Maximum	Mean	Total	N
Copia (n=43)	4.83±3.62 0.50-22.0	16.02±8.77 2.0-46.0	108.80±330.15 0.33-2093.33	1130.98±1665.67 9.42-7698.80	403.20±480.28 4.0-2093.33	1723.36±2401.32 9.42-10560.34	4.07±2.30 1-9
Muong La (n=34)	3.61±1.86 0.60-12.0	11.69±6.69 2.0-42.0	34.51±48.02 0.47-226.08	393.04±479.19 29.31-2260.80	144.55±143.87 21.98-752.73	718.77±658.61 43.96-2592.07	5.38±3.42 1-15
Ta Xua (n=82)	3.44±2.20 0.5-14.0	13.33±10.22 1.0-80.0	79.56±104.50 0.26-697.08	377.05±374.34 4.71-1641.17	189.78±155.10 4.71-697.08	635.74±665.71 4.71-3496.39	3.95±6.79 1-62
Male (n=63)	2.99±2.02 0.50-10.0	10.75±8.12 1.0-46.0	66.36±104.56 0.26-697.08	291.04±272.38 4.71-1151.33	144.39±122.38 4.71-697.08	473.74±395.50 4.71-1667.08	4.38±7.78 1-62
Female (n=96)	4.42±2.83 0.5-22.0	15.49±9.32 1.90-80.0	85.37±228.31 0.33-2093.33	776.85±1211.76 9.42-7598.80	299.14±357.50 4.0-2093.33	1258.62±1761.87 9.42-10560.34	4.23±2.60 1-15
Rainy season (n=124)	3.85±2.77 0.50-22.0	14.0±9.66 1.0-80.0	91.05±211.58 0.26-2093.33	603.16±1069.07 4.71-7598.80	255.25±322.32 4.0-2093.33	965.63±1565.93 4.71-10560.34	4.06±5.72 1-62
Dry season (n=35)	3.82±2.17 0.50-12.0	12.38±7.42 2.0-42.0	31.01±33.84 0.33-157.0	517.74±601.31 29.31-2260.8	176.09±174.15 21.98-752.73	883.87±878.43 43.96-3680.77	5.09±3.18 2-15

Table 2. Results of analysis variance (ANOVA) examining the impact season, sex and sites on the number of prey items in stomachs, volume of prey, length of prey items, and width of prey items of *Odorrana jingdongensis.*

Variables	Source	df	F	*P*
Number of prey items in stomachs	Sex	1	0.027	0.869
	Sites	2	0.924	0.399
	Season	1	1.009	0.317
Volume of prey in stomachs	Sex	1	12.130	<0.001
	Sites	2	9.484	<0.001
	Season	1	0.090	0.765
Length of prey items	Sex	1	46.708	<0.001
	Sites	2	10.569	<0.001
	Season	1	4.143	0.042
Width of prey items	Sex	1	51.867	<0.001
	Sites	2	17.839	<0.001
	Season	1	0.014	0.905
SVL	Sex	1	295.189	<0.001
HW	Sex	1	247.158	<0.001
BM	Sex	1	154.736	<0.001

of fingers wide; all fingers with grooves; feet fully webbed to disks; tibiotarsal articulation reaching the tip of snout or anterior corner of eye; dorsal skin smooth, scattered with fine identical tubercles; venter smooth; dorsum olive green or dark green; dorsal limbs olive green with dark crossbars; lip-stripe yellow, indistinct, extending across upper lip; sides of body light green scattered with brown and green marks; webbing uniformly brown; venter creamy white in females and immaculate creamy white or mottled with dark brown in males. Females with white eggs and males with velvety nuptial pads on thumbs; paired gular pouches absent in males (determination after Fei et al., 2001; Bain et al., 2003; Ohler, 2007). There were strong positive correlations between the morphological measurements (SVL and MW: r=0.933, p< 0.001; SVL and BM: r=0.937; p<0.001; MW and body mass: r=0.894, p<0.001; Fig. 2).

Prey items

The number of prey items per individual was 1–62 (mean and SD: 4.29±5.27 items, n=159). Mean and SD prey item length was 13.58±9.15 mm (min-max: 1.0–80.0 mm, n=682); mean and SD prey item width was 3.84±2.63 mm (0.5–22.0 mm, n=682) in both sexes. The average dietary volume per individual was 947.63±1440.88 mm3 (min-max 4.71–10560.34 mm3, n=159).

There was no positive correlation between the SVL of frogs and the minimum prey volume (Kendall's_tau b=0.047, p=0.389) (Fig. 3a) while there were correlations between the SVL of frogs and the mean prey item volume (tau=0.216, p<0.001), maximum prey item volume (tau=0.262, p<0.001), and the total prey volume (tau=0.291, p<0.001) (Fig. 3 b,c,d). Similarly, we also found no correlation for the minimum prey volume with the MW and body mass of frogs (MW: tau=0.066, p=0.236, BM: tau=0.70, p=0.199) and there were correlations between MW and body mass of frogs with the mean prey

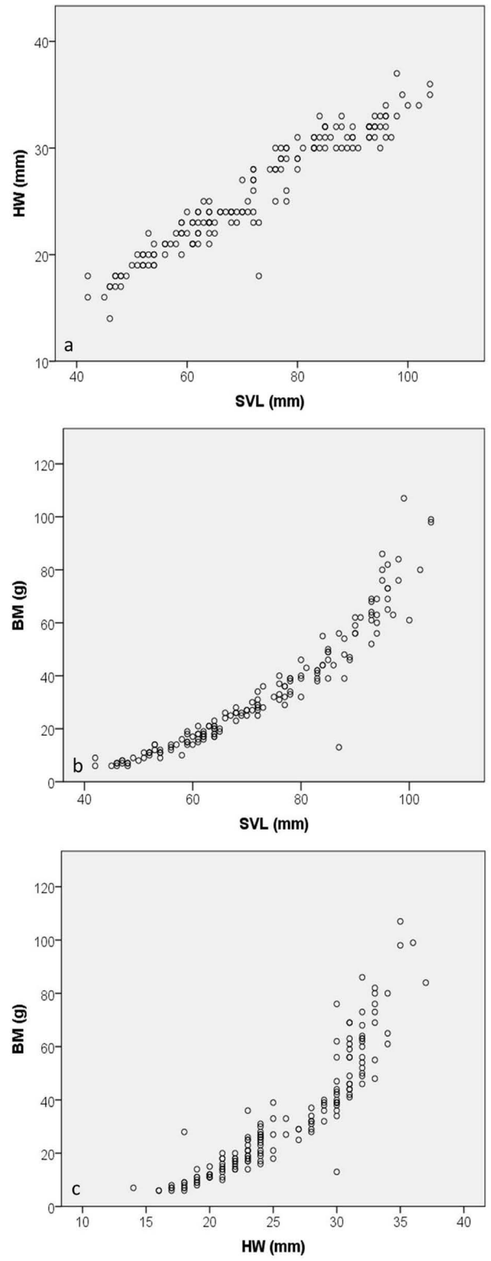

Figure 2. Dispersion diagrams from Pearson's correlations between **a)** snout-vent length and mouth width, **b)** snout-vent length and body mass, and **c)** mouth width and body mass of *Odorrana jingdongensis* in Son La Province, Vietnam.

item volume (MW: tau=0.214, p<0.001, BM: tau=0.225, p<0.001), maximum prey item volume (MW: tau=0.235, p<0.001, BM: tau=0.270, p<0.001), and the total prey volume (MW: tau=0.274, p<0.001, BM: tau=0.300, p<0.001). There were correlations between MW, BM

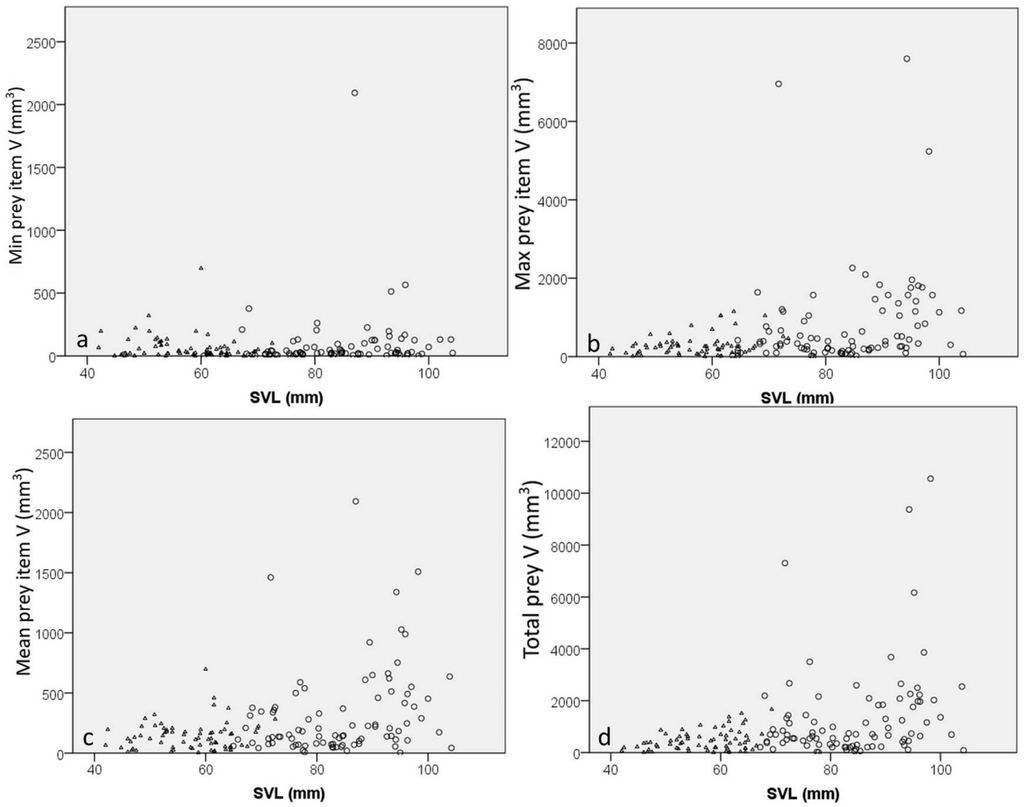

Figure 3. Relationships between the SVL of *Odorrana jingdongensis* and **a)** the minimum, **b)** maximum and **c)** the mean prey item volume, and **d)** the total prey volume. Open triangles: males; dots: females.

Table 3. Prey categories consumed by *Odorrana jingdongensis* in Son La Province, Vietnam (n=159), F) total frequency,%F) relative frequency, N) total abundance,%N) relative abundance, V) total volume (mm³),%V) relative volume; IRI) importance index.

Prey taxa	F	%F	N	%N	V	%V	IRI
Gastropoda	8	1.94	8	1.17	4,836.12	3.21	2.11
Araneae	38	9.20	43	6.30	20,013.58	13.28	9.60
Opiliones	6	1.45	7	1.03	2,383.78	1.58	1.35
Scorpiones	3	0.73	3	0.44	295.03	0.20	0.45
Polydesmida	29	7.02	31	4.55	2,038.32	1.35	4.31
Scolopendromorpha	2	0.48	2	0.29	77.45	0.05	0.28
Blattodea	9	2.18	11	1.61	9,516.82	6.32	3.37
Coleoptera	113	27.36	227	33.28	53,248.75	35.34	32.00
Dermaptera	19	4.60	26	3.81	2,943.81	1.95	3.46
Diptera	7	1.69	14	2.05	942.39	0.63	1.46
Hemiptera	10	2.42	13	1.91	1,569.74	1.04	1.79
Hymenoptera	34	8.23	58	8.50	3,376.56	2.24	6.33
Lepidoptera	29	7.02	45	6.60	7,724.66	5.13	6.25
Odonata	6	1.45	6	0.88	3,373.41	2.24	1.52
Orthoptera	32	7.75	48	7.04	11,595.99	7.70	7.49
Phasmatodea	3	0.73	3	0.44	1,196.34	0.79	0.65
Plecoptera	8	1.94	10	1.47	1,169.68	0.78	1.39
Insecta larvae	39	9.44	108	15.84	12,098.86	8.03	11.10
Amphibia	2	0.48	2	0.29	1,962.50	1.30	0.69
Reptilia	1	0.24	1	0.15	7,598.80	5.04	1.81
Unidentified	15	3.63	16	2.35	2,710.93	1.80	2.59

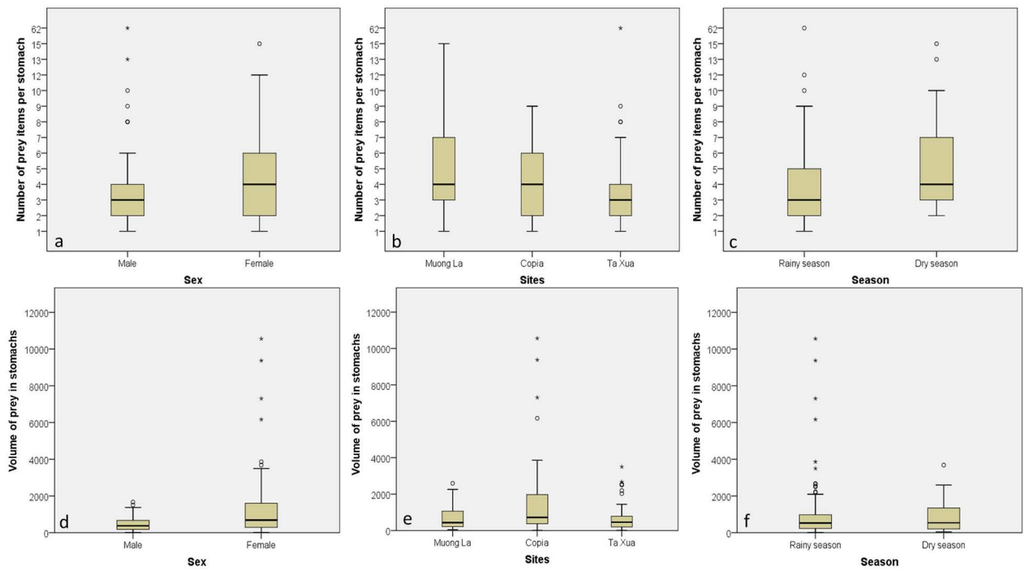

Figure 4. Boxplots representing factors that differed significantly among treatments: **a)** number of prey items of *Odorrana jingdongensis* per stomach and sex; **b)** number of prey items per stomach and sites; **c)** number of prey items per stomach and season; **d)** Prey volume per stomach and sex; **e)** prey volume per stomach and sites; **f)** prey volume per stomach and season.

and prey items: MW and width prey items: r=0.335, p<0.001; MW and length prey items: r=0.299; p<0.001; BM and width prey items: r=0.339, p<0.001; and BM and length prey items: r=0.277; p<0.001.

Dietary diversity

We identified 20 prey categories in the stomachs of *O. jingdongensis* and other unidentified objects. Insects formed the main food component of *O. jingdongensis*, with 11 orders (Blattodae, Coleoptera, Dermaptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, Odonata, Orthoptera, Phasmatodea and Plecoptera) and insect larvae. Other categories were other invertebrate groups (Gastropoda, Araneae, Opiliones, Scorpiones, Polydesmida and Scolopendromorpha), Amphibia (Megophrydae) and Reptilia (Scincidae) (Table 2). The highest proportion (%N) of prey items identified was Coleoptera (32.28%), followed by insect larvae (15.84%), Hymenoptera (8.5%), Orthoptera (7.04%), Lepidoptera (6.6%), and Araneae (6.3%), while the most frequently foraged (%F) prey group was Coleoptera (27.36%), followed by insect larvae (9.44%), Araneae (9.2%), Hymenoptera (8.23%), Orthoptera (7.75%), and Lepidoptera (7.02%). In the comparisons by the IRI (%), Coleoptera (32%), followed by insect larvae (11.1%), Araneae (9.6%), Orthoptera (7.49%), Hymenoptera (6.33%), and Lepidoptera (6.25%) were found to be the most important prey groups (Table 3).

Dietary differences between sexes

The number of prey items in males was 1–62 (4.38±7.78 items, n=63) and 1–15 in females (4.23±2.6 items, n=96) (Table 1, Fig. 4a), the values were not significantly different from each other (ANOVA, $F_{1, 157}$=0.027, p=0.869) (Table 2). Mean prey item length in males were 10.75±8.12 mm (1.0–46.0 mm, n=276) and 1.9–80.0 mm in females (15.49±9.32 mm, n=406), the values were significantly different from each other ($F_{1, 680}$=46.708, p<0.001). Mean prey item width in males were 2.99±2.02 mm (0.5–10.00 mm, n=276) and ranged from 0.5 to 22.0 mm in females (4.42±2.83 mm, n=406) (Table 1), the values were also significantly different between males and females ($F_{1, 680}$=51.867, p<0.001) (Table 2). The average dietary volume per individual in males was 473.74±395.5 mm³ (4.71–1667.08 mm³, n=63) and 1258.62±1761.87 mm³ (9.42–10560.34 mm³, n=96) in females (Table 1), the values were significantly different from each other ($F_{1, 157}$=12.130, p<0.001) (Table 2, Fig. 4b).

The Shannon-Wiener index of diet diversity in *O. jingdongensis* from Son La Province was H=2.14. Adult males (19 prey categories) consumed preys with slightly lower diversity than adult females (20 prey categories). The Shannon-Wiener index of prey categories of adult males (H=2.14) was also slightly lower than that of adult females (H=2.31).

There was an overlap of 78.16% in the diet between males and females. The trophic spectrum of males with the most important (IRI>5%) consisted of Coleoptera (31.93%), insect larvae (14.33%), Lepidoptera (10.12%), Araneae (9.80%), Orthoptera (6.55%), Hymenoptera (5.77%) and Polydesmida (5.58%), while the trophic spectrum of females with the most important (IRI>5) comprised Coleoptera (32.44%), Araneae (9.53%), insect larvae (9.07%), Orthoptera (7.91%), Hymenoptera (6.63%) and Lepidoptera (5.08%) (Fig. 5).

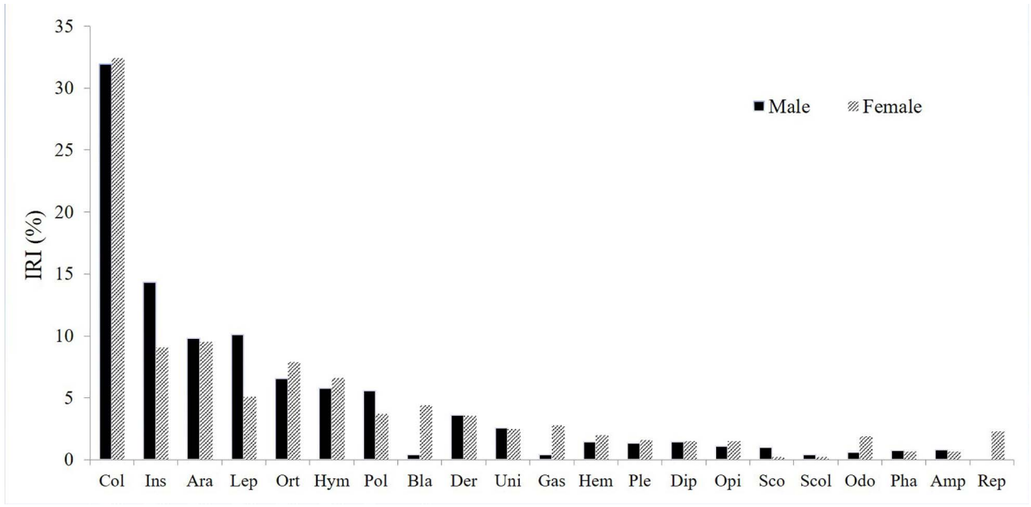

Figure 5. Importance indices (Ix) for prey categories consumed by males (black) vs. females (cross) of *Odorrana jingdongensis* in Vietnam. Coleoptera (Col), Insecta larvae (Ins), Araneae (Ara) Lepidoptera (Lep), Orthoptera (Ort), Hymenoptera (Hym), Polydesmida (Pol), Blattodea (Bla), Dermaptera (Der), Unidentified (Uni), Gastropoda (Gas), Hemiptera (Hem), Plecoptera (Ple), Diptera (Dip), Opiliones (Opi), Scorpiones (Sco), Scolopendromorpha (Scol), Odonata (Odo), Phasmatodea (Pha), Amphibia (Amp), Reptilia (Rep).

Dietary differences among sites

The number of prey items in the stomachs of frogs from Copia NR was 1–9 (4.07±2.30 items, n=43), 1–15 from Muong La NR (5.38±3.42 items, n=34), and 1–62 from Ta Xua NR (3.95±6.79 items, n=82) (Table 1, Fig. 4b), the values were not significantly different from each other ($F_{2, 156}$= 0.924, p=0.399) (Table 2). Mean prey item length of frogs from Copia NR was 16.02±8.77 mm (2.0–46.0 mm, n=175), 11.69±6.69 mm from Muong La NR (2.0–42.0 mm, n= 183), and 13.33±10.2 mm from Ta Xua NR (1.0–80.0 mm, n=324) (Table 1), the values were significantly different from each other ($F_{2, 680}$=10.569, p<0.001) (Table 2). Mean prey item width from Copia NR was 4.83±3.62 mm (0.5–22.0 mm, n=175), 3.61±1.86 mm from Muong La NR (0.6–12.0 mm, n=183), and 3.44±2.2 mm from Ta Xua NR (0.5–14.0 mm, n=324) (Table 1), the mean prey item width was significantly different between the three sites ($F_{2, 680}$=17.839, p<0.01) (Table 2). The average dietary volume per individual from Copia NR was 1723.36±2401.32 mm^3 (9.42–10560.34 mm^3, n=43), 718.77±658.61 from Muong La NR (43.96–2592.07 mm^3, n=34), and 635.74±665.71 mm^3 from Ta Xua NR (4.71–3496.39 mm^3, n=82) ($F_{2, 156}$=9.484, p<0.01) (Tables 1 & 2; Fig. 4e).

Frogs from Ta Xua NR (18 prey categories) consumed preys with a slightly higher diversity than those from Muong La NR (17 prey categories) and from Copia NR (16 prey categories). The broadest Shannon-Wiener index of diet diversity in *O. jingdongensis* was found in Muong La NR (*H*=2.41), followed by Ta Xua NR (*H*=2.1) and the narrowest dietary diversity was found in Copia NR (*H*=1.80).

Dietary differences between seasons

The number of prey items in the stomachs of frogs during the rainy season was 1–62 (4.06±5.72 items, n=124) and 2–15 during the dry season (5.09±3.18 items, n=35), the values were not significantly different from each other ($F_{1, 157}$=1.009, p=0.317) (Tables 1 & 2, Fig. 4c). The mean prey item length of frogs within the rainy season was 14.0±9.66 mm (1.0¬80.0 mm, n=504) and ranged from 2.0 to 42.0 mm within the dry season (12.38±7.42 mm, n=178). The mean prey item length was not significantly different between the rainy season and the dry season ($F_{1, 680}$=4.143, p=0.042) (Tables 1 & 2). The average dietary volume per frog individual in the rainy season was 4.71–10560.34 mm^3 (965.63±1565.93 mm^3, n=124) and 43.96–3680.77 mm^3 in the dry season (883.87±878.43 mm^3, n=35). The volume per individual was not significantly different between the rainy season and the dry season ($F_{1, 157}$=0.090, p=0.765) (Tables 1 & 2, Fig. 4f). The Shannon-Wiener index (*H*=2.14) of diet diversity in *O. jingdongensis* during the rainy season was lower than during the dry season (*H*=2.35), while prey categories in the rainy season (19) showed a higher diversity than during the dry season (16 prey categories).

DISCUSSION

Pham et al. (2019a) reported the dietary composition of *Odorrana chapaensis*, another representative of the genus *Odorrana* also from Muong La and Ta Xua NRs in Son La Province, Vietnam. Both *O. chapaensis* and *O. jingdongensis* inhabit similar environment conditions, viz. clinging on cliffs or tree branches near streams in mixed evergreen forests of larger and medium hardwoods and shrubs. They were found at elevations between 900 and 1600 m asl. The relative humidity was approximately 75–90% and the air temperature ranged from 20 to 30 °C. The prey categories of *O. jingdongensis* were slightly more diverse than in *O. chapaensis* (20 vs. 19 in the latter). Interestingly, the prey of *O. jingdongensis* was similar to that of *O. chapaensis*, with the prey categories Coleoptera, Hymenoptera, Orthoptera, Lepidoptera, Insecta larvae and Araneae being the most important prey items (Pham et al., 2019a). The slight differences in the diet between *O. jingdongensis* and *O. chapaensis* may reflect differences in foraging activity and microhabitat use. Scorpiones, Scolopendromorpha, Amphibia and Reptilia were found exclusively in the diet of *O. jingdongensis*, whereas Ephemeroptera, Mantodea and Trichoptera occurred only in the diet of *O. chapaensis* (Pham et al., 2019a). Prey of *O. jingdongensis* comprised mainly invertebrates but also small frogs and lizards which were not found in *O. chapaensis* (see Pham et al., 2019a).

On the other hand, the diet of *O. jingdongensis* mainly consisted of invertebrates, with Coleoptera, Hymenoptera, Orthoptera, Lepidoptera and Araneae being the most important prey categories, similar to the results found for *O. morafkai*, *Nanorana yunnanensis* and *Quasipaa verrucospinosa* (Ngo et al., 2014; Pham & Nguyen, 2018; Le et al., 2020). Beetles, which make up the main item in the diets of *O. jingdongensis*, *O. chapaensis*, *O. morafkai*, *N. yunnanensis* and *Q. verrucospinosa* are among the most abundant insects in the evergreen forest in Vietnam (Ngo et al., 2014; Pham & Nguyen, 2018; Pham et al., 2019a; Le et al., 2020). Other frog species in Vietnam have a different diet despite the dominance of beetles in the forerst ecosystem. For example, *Amolops shihaitaoi* and *Limnonectes nguyenorum* have a diet mostly consisting of ants (Pham et al., 2019b; Sonephet et al., 2023), and also some species of the family Microhylidae (*Microhyla butleri*, *M. heymonsi*) in north-western Vietnam have a diet consisting exclusively of ants and termites (Pham et al., 2022). In this study, we also found that there was very little difference in the diet composition of males and females, both sexes had a diverse prey spectrum, comprising Coleoptera, insect larvae, Lepidoptera, Araneae, Orthoptera, Hymenoptera (IRI>5%). Reptilia were found exclusively in the diet of females.

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