ISSN 2634-1379

The Herpetological Journal

Volume 32, Number 2
April 2022

Published by the British Herpetological Society

British Herpetological Society

The Herpetological Journal is published quarterly by the British Herpetological Society and is issued free to members. Articles are listed in Current Awareness in Biological Sciences, Current Contents, Science Citation Index and Zoological Record. Applications to purchase copies and/or for details of membership should be made to the Hon. Secretary, British Herpetological Society, The Zoological Society of London, Regent's Park, London, NW1 4RY, UK. Instructions to authors are printed inside the back cover. All contributions should be addressed to the Scientific Editor.

Front cover: Alpine Newt *Ichthyosaura alpestris*, see article on page 51. (© Frank Vassen / CC BY 2.0)

Herpetological Journal

Volume 32, Number 2, 2022

Contents

Volume 32, Number 1 was published on 1 January 2022

Volume 32 (April 2022), 51-58

Herpetological Journal FULL PAPER

https://doi.org/10.33256/32.2.5158

Published by the British Herpetological Society

Effects of aquatic and terrestrial habitats on the skin microbiome and growth rate of juvenile alpine newts *Ichthyosaura alpestris*

Christopher J. Michaels

79 Bradbourne Park Road, Sevenoaks, Kent, TN13 3LQ

Cutaneous bacterial communities can be crucial in modulating amphibian-pathogen interactions, but are highly sensitive to environmental conditions. Many amphibians, in particular salamandrid newts, may inhabit aquatic or terrestrial habitats after metamorphosis. These different conditions can alter the cutaneous bacterial communities of animals and so affect both the susceptibility of individuals to disease and their potential to transmit pathogens to others. Furthermore, different environments may influence the fitness of individuals through impacts on growth rates. I investigated the impact of aquatic and terrestrial environments on the cutaneous bacterial communities and growth rates in the alpine newt (*Icthyosaura alpestris*). This species is invasive in the UK and has been reported as carrier of amphibian pathogenic chytrid fungus. I show that aquatic animals, although growing faster, present less diverse communities, lacking in species that inhibit *Batrachochytrium dendrobatidis (Bd) in vitro*. My data suggest that aquatic and terrestrial phases in amphibians may influence their susceptibility to disease and I suggest that this likely impacts the way in which pathogens, especially *Bd*, spread in the environment.

Keywords: Amphibians; cutaneous bacterial communities; microbiomes; *Bd*; captivity, *Batrachochytrium dendrobatidis*, mutialistic bacteria, *ex situ* conservation

INTRODUCTION

Amphibians are globally threatened with extinction, with more than two-thirds of species in danger (Wren et al., 2015). Emerging infectious diseases, including chytridiomycoses caused by two different *Batrachochytrium* species (Longcore et al., 1999; Martel et al., 2013) have been identified as major and currently irreversible threats (Wren et al., 2015). In order to develop mitigation measures for these diseases, it is important to understand both the biology of the pathogen and that of the host. In particular, the importance of the host-associated skin microbiota is becoming increasingly clear (Jervis et al., 2021). Bacterial communities may inhibit pathogenic growth either by competition or through the active production of toxic compounds (Belden & Harris, 2007; Brucker et al., 2008a; b; Becker & Harris, 2010; Bates et al., 2018; Kueneman et al., 2019; Jervis et al., 2021). Therefore, the microbiome plays an important part in determining host-pathogen interactions, as well as the potential for amphibians to transport pathogens between and within habitats (Bletz et al., 2013).

Amphibians have complex life cycles often involving an aquatic larval phase followed by metamorphosis into a terrestrial form, which subsequently matures to breed. In salamander species, juveniles (post-metamorphosis) of some newt species may mature under aquatic (as metamorphs or paedomorphs) or terrestrial conditions. The skin microbiome is acquired from the environment to which animals are exposed (Fitzpatrick & Allison, 2014), as well as from other animals and influenced by abiotic factors (Ruthsatz et al., 2020), and even subtle differences in environmental parameters can influence the bacterial communities associated with amphibians, as well as the fitness of animals themselves (Loudon et al., 2013; Antwis et al., 2014a; Michaels et al., 2014; Kueneman et al., 2019). Furthermore, microbiome quality is frequently associated with amphibian health in the context of both disease processes (e.g. Bates et al., 2019; Jervis et al., 2021) and environmental impacts in captivity (e.g. Antwis et al., 2014a; b; Michaels et al., 2014) and in the wild (e.g. Kueneman et al., 2019). The radically different environments to which terrestrial and aquatic animals are exposed have the potential to profoundly influence cutaneous bacterial communities (Jervis et al., 2021).

Amphibian conservation is dependent, for many species, on *ex situ* assurance colonies (Wren et al., 2015), but it has previously been shown that symbiotic or mutualistic relationships between bacteria and the skin of amphibians are sensitive to the captive environment (Loudon et al., 2013; Antwis et al., 2014; Michaels et al., 2014; Becker et al., 2014; Passos et al., 2018; Harrison et al., 2019; Michaels & Preziosi, 2020). The effects of rearing environments and of aquatic and terrestrial phases on these communities in salamanders, however, are poorly known.

Correspondence: Christopher J. Michaels (c.j.michaels44@gmail.com)

Here, I investigate the effect of different environments (aquatic and terrestrial) on animal fitness, measured as growth rate (Michaels et al., 2014), and on the skin microbiome of captive alpine newts (*Icthyosaura alpestris*) reared in aquatic and terrestrial environments. I also use *in vitro* challenges between isolated bacteria and *Batrachochytrium dendrobatidis* (*Bd*) to predict effects of environment on susceptibility to infection by the fungus. This information may be used to guide *ex-* and *in-situ* management of amphibians and their diseases, as well as inform translocations between the two.

METHODS

Study animals and experimental conditions

F1 captive-bred *Ichthyosaura alpestris apuana* were obtained as eggs from a private breeder and were the progeny of a co-housed group of three males and three females laid over a period of three days. All animals used in the study hatched within three days of one another and larvae were reared to metamorphosis in a growth chamber at air temperature of 17 °C (day) and 15 °C (night) under a 12:12 photoperiod. Larvae were reared in large, bare-bottom aquaria thickly planted with live, growing plants (*Egeria densa* and *Fontinalis antipyretica*), which were filtered with air-driven sponge filters. Hatchling larvae were initially fed on *Artemia salina nauplii* and then on *Daphnia* spp., bloodworms (*Chironomidae*) and whiteworms (*Enchytraeus albidus*) until metamorphosis.

After metamorphosis, animals were maintained in groups of three in plastic vivaria (Faunarium 'large', Exo Terra – 370 x 220 x 250 mm) and were allocated alternately to terrestrial (n = 30) or aquatic (n = 30) conditions. Newts were maintained in these environments for the duration of the 12 month study. Terraria (housing terrestrial animals) had a substrate made from coir coco-fibre (Wiggly Wigglers, UK), peat compost (B&Q, UK), rinsed silver sand (B&Q, UK), fine orchid bark (Monkfields Nutrition, UK) and crushed beech leaves in a 10:10:2:2:1 ratio. Upturned plastic plant saucers with an entrance hole cut in the rim were provided as hides and both substrate and hides were covered with a layer of live moss. Terraria were planted with *Tradescantia* sp. Substrate was not changed throughout the study as its biological capacity to break down waste, along with live plants, preserved hygienic conditions. Aquaria had a substrate of organic aquatic mulm derived from decomposed plant matter, similar to a natural pond, which was present from the start of the study, were filtered using an air-driven sponge filter and thickly planted with *E. densa* and *F. antipyretica*. 10% partial water changes were performed weekly to maintain water quality. Experimental animals were broadcast fed on whiteworms and chopped earthworms.

Individual animals were identified using photographs of the markings on the ventro-lateral surface (similar to Mettouris et al., 2016, but by eye rather than computer matching, which was feasible due to the smaller number of individuals to distinguish per tank, i.e. three animals). Two animals in aquatic conditions died during the course of the study. These animals were replaced with non-experimental individuals to maintain equal stocking density.

Morphometrics

Animals were photographed against a scale and SVL (snout-vent length) and CL (caudal length) were measured in millimetres using the freeware ImageJ (NIH; http://imagej.nih.gov/ij/) at metamorphosis and at subsequent three-month intervals up to twelve months. Proportional CL was calculated as the CL divided by the SVL of newts; this was collected in order to look for differences in the size of the tail, the primary swimming organ, between terrestrial and aquatic newts.

Bacterial communities

Bacterial communities were collected at month ten from nine newts per treatment group (n = 9, N = 18) with only one newt sampled per tank; all individuals were removed and a random one was selected for sampling. Sterile nitrile gloves were worn throughout handling and changed between newts to minimise cross-contamination. Newts were rinsed twice on their dorsal and ventral surfaces using sterile bottled water to remove any transient (i.e. non-symbiotic) bacteria from their skin (Lauer et al., 2007). Newts were then swabbed 20 - 25 times to collect cutaneous bacterial communities using sterile Eurotubo swabs (Deltalab, Rubi, Spain). The dorsal and ventral regions of the body were swabbed separately to maximize coverage and bacterial growth. Swabs were placed into 1.5 ml sterile screw-top tubes containing 1 ml of 0.8% w/v $NaCl_2$ solution to facilitate subsequent culturing methods. Care was taken to ensure newts were not harmed during this process, and individuals were monitored for two weeks post-swabbing for signs of distress or injury in response to the swabbing (skin lesions, inappetence or condition loss, excessive skin secretions, or other unusual behaviour), of which none was observed.

Tubes containing swabs were vortexed to dissociate bacteria from the swab. The swab was removed and serial dilutions were constructed up to 10-1 by pipetting 100 ul into 900 ul of 0.8 % w/v $NaCl_2$, and dilutions of 100 and 10-1 were plated out on R2A agar media (Lab M Ltd., United Kingdom) and incubated at 15-17 °C (the same temperature at which the newts were maintained). Pilot studies showed these dilutions gave plates with an intermediate amount of growth that was most suitable for assessing the bacterial community (R.Antwis, unpublished data). New morphologically distinct bacteria colonies ('morphotypes') were counted three and seven days after plating, after which negligible new colony growth was observed. Bacterial counts were summed for the two counts (days three and seven), multiplied by the dilution factor of 10 where necessary, and counts were then averaged across the two dilutions.

Representative colonies of each morphotype were streaked out on R2A agar until a pure culture was obtained. Bacterial species were identified using 16S rDNA sequencing with universal primers 27F (5'-GTGCTGCAGAGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CACGGATCCTACGGGTACCTTGTTACGACT-3') (Webster et al., 2003). 16s RNA fragments were obtained through colony PCR amplification using the Platinum PCR SuperMix (Invitrogen, Life Technologies) according to the manufacturer's instructions. DNA fragments were amplified by PCR using the following cycling parameters: 95 °C for 2 minutes followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s, with a final extension step of 5 minutes at 72 °C. Prior to purification and sequencing, PCR products were checked for the correct length with gel electrophoresis. PCR products were purified with the GenElute™ PCR Clean-up Kit (Sigma-Aldrich®), and sequenced at the DNA Sequencing Facility, University of Manchester, UK. A consensus sequence was obtained by combining the forward and reverse sequences in DNA Dynamo Sequence Analysis Software© (BlueTractorSoftware Ltd., UK). Consensus sequences were then blasted against the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify each morphotype to genus level.

***In vitro Bd* challenges**

Pure cultures of each bacterial morphotype were used to conduct *in vitro Bd* challenges similar to Harris et al. (2006). The *Bd* strain GPL SFBC 014 (isolated from *Epidalea* (formerly *Bufo*) *calamita*, UK by Peter Minting in 2010) was grown in TGhL liquid media at 18 °C until maximum zoospore production was observed. Approximately 3 ml of the *Bd* zoospore culture was then spread across 1 % tryptone, 1 % agar plates and dried in a sterile hood. Bacterial cultures were streaked on either half of the plate (two per plate), inverted and incubated at 18 °C. Bacterial streaks were then scored for the presence or absence of a zone of inhibition, indicated by a markedly reduced or absent growth of *Bd*.

Statistical analyses

Aquatic and terrestrial newt SVL and proportional CL at day 0 (i.e. immediately prior to the start of the experiment) was compared using two-sample two-tailed T-tests. Newt SVL and proportional CL data were analysed using linear mixed-effect models in the lme4 and lmertest packages (Bates et al., 2015) in R using RStudio (R Core Team, 2021). Newt individual, nested within Tank, was used as a random factor to control for the repeated measures and nested elements of the design. Time and environment were used as explanatory variables. Prior to analysis, model assumptions were confirmed by visually inspecting residuals using the ggResidpanel function in R (Goode & Rey, 2019).

Differences in the overall bacterial community composition according to environment (terrestrial vs. aquatic), surface (dorsum vs. ventrum) and their interaction were analysed using the Adonis function of the vegan package in R using RStudio© (Oksanen et al., 2020). This function was used to compute a permutational multivariate ANOVA on the overall bacterial community structure (subsequently referred to as the Adonis analysis), using a Bray-Curtis distance matrix derived from the abundance of each morphotype (Antwis et al., 2014a; b; Michaels et al., 2014). As surface had no effect on the bacterial community (see Results), the data for the dorsum and ventrum of each newt were combined for subsequent analyses. The effect of environment on species richness (the number of different morphotypes on each individual) and total abundance (total number of cultured bacteria for each individual) were analysed using t tests in JMP 10®. Differences in the abundance of two bacterial strains that inhibited the *Bd* in *in vitro* challenge assays were analysed using t-tests in JMP 10®.

RESULTS

Effects of rearing condition on morphometrics

Neither SVL (t_{57}=0.40, p=0.69), nor proportional CL (t_{57}=1.5, p=0.14) differed between treatments at the start of the study. There was a significant effect of environment, such that aquatic newts grew larger than terrestrial animals ($F_{1, 17.9}$ = 15.7, p = 0.001; Fig. 1). SVL increased [mean(SD)] by 48(15) % and 35(12) % in aquatic and terrestrial newts, respectively, over the course of the study. Aquatic newts also had significantly proportionately longer tails than terrestrial newts ($F_{1, 18.3}$ = 128.54, p<0.001); mean (standard deviation) CL:TTL ratio at month 12 was 0.47(0.02) and 0.44(0.02) in aquatic and terrestrial newts, respectively (Fig. 1).

Effects of environment on cutaneous bacterial communities

A total of 11 different morphotypes (GenBank accession numbers KC853151-KC853154 and KF444805- KF444808; Table 1) were cultured from newts, with a range of 3 to 9 morphotypes per individual. All morphotypes were isolated from animals in both the terrestrial and aquatic environments, although the proportion of individuals hosting each morphotype was generally lower for newts in the aquatic set-ups (Table 1). Three bacteria could not be identified due to poor sequence data.

The results of the Adonis analysis showed there was a highly significant difference in the overall bacterial community associated with the skin of newts maintained in a terrestrial environment compared to those kept in an aquatic environment ($F_{3, 32}$ = 25.695, p = 0.001), but no significant effect of surface (dorsum or ventrum; $F_{3, 32}$ = 0.827, p = 0.480) or the interaction between surface and environment ($F_{3, 32}$ = 0.744, p = 0.509) were detected. Newts kept in a terrestrial environment supported a significantly greater bacterial abundance (t_{17} = 6.119, p < 0.001; Fig. 2) and significantly greater species richness (t_{17} = 3.714, p < 0.001; Fig. 3).

Two of the 11 morphotypes successfully inhibited *Bd* in the *in vitro* challenges; *Arthrobacter* sp. (KC853151) and *Chryseobacterium* sp. (KF444806). Both of these were isolated from all or nearly all individuals in both environmental groups (see Table 1), although the total abundance of each bacterium was significantly higher on the skin of newts maintained in a terrestrial environment (*Arthrobacter* sp., $t_{1,8}$ = 3.83, p = 0.003; *Chryseobacterium* sp., $t_{1,8}$ = 2.13, p = 0.030; Fig. 4).

DISCUSSION

Here I show that newts reared in an aquatic environment support a simpler and less abundant bacterial community than their terrestrial counterparts. Although bacterial communities are acquired through interactions with the environment (Belden & Harris, 2007; Banning et al., 2008; Walke et al., 2011; Daskin & Alford, 2012), the rinsing

Table 1. Bacteria morphotypes isolated from skin swabs of 18 alpine newts (*Ichthyosaura alpestris apuana*) and their potential anti-*Bd* activity. Proportion of aquatic and terrestrial newts carrying each type of bacterium is provided.

Species	Anti-Bd activity	Proportion terrestrial newts (n=9)	Proportion aquatic newts (n=9)
Arthrobacter sp. (Ia1)	Yes	1.0	0.89
Unidentified (Ia2)	No	1.0	1.0
Erwinia sp. (Ia3)	No	0.78	0.14
Chryseobacterium sp. (Ia4)	Yes	1.0	1.0
Flavobacterium sp. (Ia5)	No	0.56	0.14
Lysobacter sp. (Ia6)	No	0.78	0.14
Unidentified (Ia7)	No	0.89	0.14
Rhizobium sp. (Ia8)	No	0.66	0.28
Flavobacterium sp. (Ia9)	No	0.44	0.33
Unidentified (Ia10)	No	0.28	0.89
Shewanella sp. (Ia16)	No	0.28	0.14

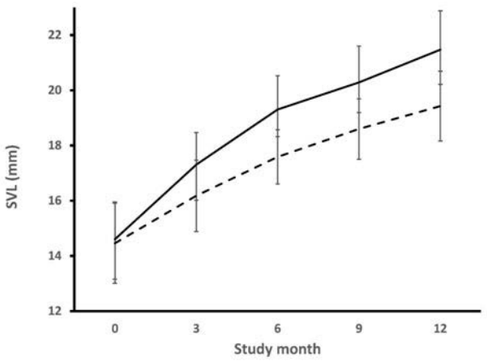

Figure 1. Growth of aquatic (solid line) and terrestrial (dashed line) alpine newts over the duration of the study. Error bars represent +/- 1SEM.

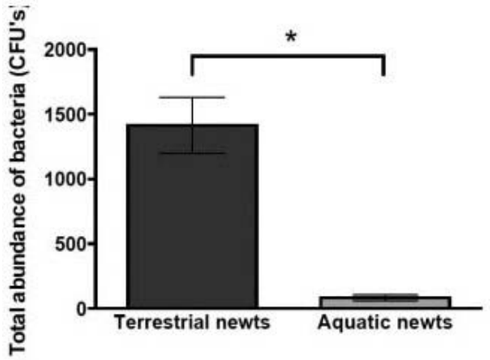

Figure 2. Total abundance of bacteria isolated from skin swabs of 9 terrestrial and 9 aquatic newts. Error bars represent +/- 1SEM.

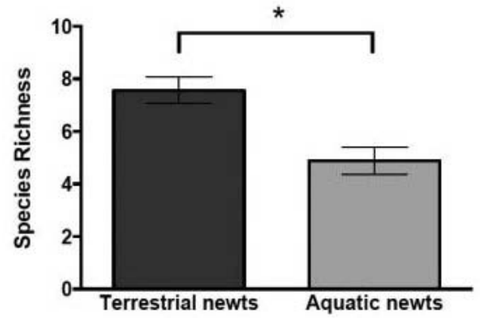

Figure 3. Mean species richness of skin bacterial communities isolated from skin swabs of 9 terrestrial and 9 aquatic newts. Error bars represent +/-1SEM.

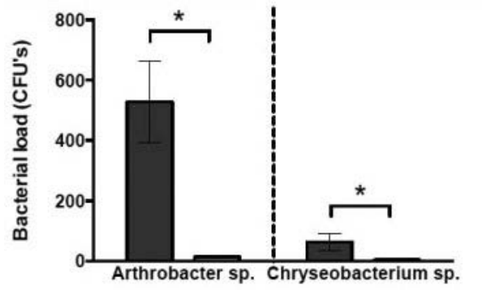

Figure 4. Prevalence of anti-Bd bacteria (*Arthrobacter* sp. and *Chryseobacterium* sp.) isolated from skin swabs of 9 terrestrial (left-hand column in each pair) and aquatic (right-hand column) newts. Error bars represent +/-1SEM.

part of the swabbing protocol is designed to remove transient environmental bacteria (Lauer et al., 2007). Simple differences in the bacterial abundance of aquatic and terrestrial environments, if these existed (we did not measure this), are therefore unlikely to have directly accounted for the difference in cutaneous communities detected, unless complex interactions inhibited or promoted the growth of certain bacteria. Therefore, the difference found in the skin microbiome between newts from aquatic and terrestrial environments may reflect the more textured and less frequently sloughed skin of terrestrial newts (pers. obs.), which may offer more niches and less disturbance for bacterial colonisation, as well as abiotic impacts of the differing environments on bacterial survival and growth.

More diverse and numerous cutaneous bacterial communities may be more robust and offer more protection against pathogens (Matos et al., 2005; Belden & Harris, 2007; van Elsas et al., 2012; Eisenhauer et al., 2013). I report in this study that the dermal bacterial communities harboured by newts included two species that inhibited the growth of *Bd in vitro*; *Arthrobacter* sp. and *Chryseobacterium* sp., and aquatically reared newts hosted lower abundances of these *Bd*-inhibiting bacteria than terrestrially reared newts. Higher density of bacteria on terrestrial newts may facilitate anti-pathogenic activity through quorum sensing, or due to minimum inhibitory concentrations of peptides required to suppress pathogen activity (Miller & Bassler, 2001; Brucker et al., 2008a; b).

The dermal bacterial communities harboured by newts included two species that inhibited the growth of *Bd in vitro*; *Arthrobacter* sp. and *Chryseobacterium* sp., and aquatically reared newts hosted lower abundances of these *Bd*-inhibiting bacteria than terrestrially reared newts. *Bd* is thought to be carried between ponds by *I.alpestris*, particularly where it has been introduced in the UK (Fisher & Garner, 2007; Duffus & Cunningham, 2010) where the strain of *Bd* used in this study originated. The presence of *Bd*-inhibiting bacteria on both phases of the newts (albeit in varying degrees) may help to explain the ability of this species to carry *Bd* infection without succumbing to disease. If aquatic phase alpine newts have less complex bacterial communities with a reduced capacity to inhibit *Bd*, this may increase the potential for alpine newts to communicate disease to other species present at breeding sites, as well as rendering them more susceptible to infection by *Bd*. However, given the effects of captivity on natural bacterial communities in amphibians in comparison to wild populations (Loudon et al. 2013; Antwis et al., 2014; Becker et al., 2014; Michaels & Preziosi, 2020), it is unclear whether wild animals will show similar differences in the bacterial communities associated with aquatic and terrestrial individuals.

In other amphibians, the number of individuals infected with *Bd* increases during breeding assemblages in water bodies (Kriger & Hero, 2007; Voordouw et al., 2010; Minting, 2012), as does the infection load of individual animals (Retallick et al., 2004). My data may suggest an additional mechanism for this (reduction in *Bd*-resistant microbiome under aquatic conditions), but this inference cannot be robustly made as I did not study animals transitioning between environments, either following metamorphosis or returning to the water to breed. Moreover, my data does not address the speed at which bacterial communities adjust in response to the change between aquatic and terrestrial habitats and this may be important in inferring epidemiological implications. In addition, although it is known that moving amphibians from the field to captivity influences microbiome (e.g. Becker at al., 2014; Bates et al., 2019), further work is required to investigate the impact of translocation into the wild on cutaneous bacterial communities of amphibians, and whether patterns established in captivity are maintained in the wild. My data correlates with those presented by Daversa et al. (2018), who showed that routine switching of alpine newts between terrestrial and aquatic habitats across their reproductive cycle reduced *Bd* growth, and heavily infected animals spent more time on land.

I also found that aquatic juveniles grew larger than terrestrial efts over the course of the twelve month experiment. This is similar to field observations of another newt species, *Notophthalmus viridescens* (Healy, 1973) and may reflect higher metabolic rates in the aquatic phase (Kristín & Gvoždík, 2013), coupled with reduced energetic cost of aquatic locomotion (Shaffer et al., 1991). This hypothesis is supported by the proportionately longer tails of aquatic newts, which is likely also an adaptation to aquatic locomotion, as seen in adults other newts (Treer & Treer, 1995). More rapid juvenile growth is linked to increased adult size in a number of amphibians (Jørgensen, 1986; Semlitsch et al., 1988; Altwegg & Reyer, 2003), which has been shown to have important impacts on survivorship (Clarke, 1974; Kusano, 1981; Bardsley & Beebee, 1998; Altwegg & Reyer, 2003) and reproductive success (Jørgensen, 1986; Briggs, 2013; Yeager & Gibbons, 2013). There is therefore strong potential for lifelong effects of juvenile environment on body size and therefore on fitness. Captive populations of newts are often reared aquatically as efts, where possible, due to the ease of feeding (aquatic newts will take defrosted frozen foods and pellets; pers. obs.; Pasmans et al. (2014)) and of cleaning aquaria. It is therefore important to understand the impacts of this practice on the fitness of populations that are candidates for translocation. Rearing newts aquatically may therefore improve both the efficiency of breeding programmes and the potential for reintroduction success in terms of survivorship and reproductive output. However, according to my data, this may come at the cost of reduced disease resistance through a reduction in cutaneous bacterial community diversity and abundance. This trade-off is in contrast with work in anurans (Ogilvy et al., 2012; Antwis et al., 2014, Michaels et al., 2014), which has found associations between factors of captive husbandry that improve growth rates as well as the diversity of cutaneous bacterial communities. This highlights the importance of using multiple biological measures to assess the effects of different captive environments on fitness traits of amphibians.

In this study I have shown the environment in which juvenile newts develop has significant effects on growth rates and cutaneous bacterial communities. Although bacterial culturing methods are known to underestimate

species richness and bacterial abundance (reviewed in Amann et al., 1995), genetic methods were outside of the feasible scope of this study. Nevertheless, these data provide an insight into the effects of environment on dermal bacterial communities that are likely to transcend to the non-culturable parts of the community. These differences may influence survivorship, adult size and reproductive fitness, as well as the part played by *I. alpestris* in disease spread, and as such have important implications for both *ex-* and *in-situ* conservation.

ACKNOWLEDGEMENTS

The author would like to thank Rachael Antwis and Trent Garner for their support with bacterial culture methods and *Bd* challenges, Peter Minting for providing the *Bd* isolate used in this study and Beatrice Gini. This project was funded by a Natural Environmental Research Council PhD studentship to CJM.

Ethical statement

All methods used in this study were non-invasive and did not require a UK Home Office Licence. The University of Manchester Ethics Committee approved this study prior to commencement.

REFERENCES

Altwegg, R. & Reyer, H.U. (2003). Patterns of natural selection on size at metamorphosis in water frogs. *Evolution* 57, 872-882.

Amann, R.I., Ludwig, W. & Schleifer, K.H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiological Reviews* 59, 143-169.

Antwis, R.E., Haworth, R.L., Engelmoer, D.J.P., Ogilvy, V., Fidgett, A.L. & Preziosi, R.F. (2014a). *Ex situ* diet influences the bacterial community associated with the skin of red-eyed tree frogs (*Agalychnis callidryas*). *PLoS ONE* 9, e85563 - e85563. Doi:10.1371/journal.pone.0085563.

Antwis, R.E., Garcia, G., Fidgett, A.L. & Preziosi, R.F. (2014b). Tagging frogs with passive integrated transponders causes disruption of the cutaneous bacterial community and proliferation of opportunistic fungi. *Applied and Environmental Microbiology* 80: 4779-4784.

Banning, J.L., Weddle, A.L., Wahl, G.W., Simon, M.A., Lauer, A., Walters, R.L. & Harris, R.N. (2008). Antifungal skin bacteria, embryonic survival, and communal nesting in four-toed salamanders, *Hemidactylium scutatum*. *Oecologia* 156, 423-429. Doi:10.1007/s00442-008-1002-5.

Bardsley, L. & Beebee, T. (1998). Interspecific competition between *Bufo* Larvae under conditions of community transition. *Ecology* 79, 1751-1759.

Bates, D., Mächler, M., Bolker, B. & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* 67: 1–48. Doi: 10.18637/jss.v067.i01.

Bates, K.A., Clare, F.C., O'Hanlon, S., Bosch, J., Brookes, L., Hopkins, K., McLaughlin, E.J., Daniel, O., Garner, T.W., Fisher, M.C. & Harrison, X.A. (2018). Amphibian chytridiomycosis outbreak dynamics are linked with host skin bacterial community structure. *Nature Communications*, 9, 1-11.

Bates, K.A., Shelton, J.M., Mercier, V.L., Hopkins, K.P., Harrison, X.A., Petrovan, S.O. & Fisher, M.C. (2019). Captivity and infection by the fungal pathogen *Batrachochytrium salamandrivorans* perturb the amphibian skin microbiome. *Frontiers in Microbiology* 10: 1834.

Becker, M.H. & Harris, R.N. (2010). Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. *PloS ONE* 5, e10957. Doi:10.1371/journal.pone.0010957.

Becker, M. H., Richards-Zawacki, C. L., Gratwicke, B. & Belden, L. K. (2014). The effect of captivity on the cutaneous bacterial community of the critically endangered Panamanian golden frog (*Atelopus zeteki*). *Biological Conservation* 176, 199-206.

Belden, L.K. & Harris, R.N. (2007). Infectious diseases in wildlife: the community ecology context. *Frontiers in Ecology and the Environment* 5, 533-539. Doi:10.1890/060122.

Bletz, M.C., Loudon, A.H., Becker, M.H., Bell, S.C., Woodhams, D.C., Minbiole, K.P.C. & Harris, R.N. (2013). Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecology Letters* 16, 807-820. Doi:10.1111/ele.12099.

Briggs, V.S. (2013). Do big dads make big babies? Paternal effects on larval performance in red-eyed treefrogs of Belize (*Agalychnis callidryas, A. moreletti*). *The Herpetological Journal* 23, 131-138.

Brucker, R.M., Harris, R.N., Schwantes, C.R., Gallaher, T.N., Flaherty, D.C., Lam, B.A. & Minbiole, K.P.C. (2008a). Amphibian chemical defense: antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. *Journal of Chemical Ecology* 34, 1422-1429. Doi:10.1007/s10886-008-9555-7.

Brucker, R.M., Baylor, C.M., Walters, R.L., Lauer, A., Harris, R.N., Minbiole, K.P.C. (2008b). The identification of 2,4-diacetylphloroglucinol as an antifungal metabolite produced by cutaneous bacteria of the salamander *Plethodon cinereus*. *Journal of Chemical Ecology* 34, 39-43. Doi:10.1007/s10886-007-9352-8.

Clarke, R. D. (1974). Postmetamorphic growth rates in a natural population of Fowler's toad, *Bufo woodhousei fowleri*. *Canadian Journal of Zoology* 52, 1489–1498.

Daskin, J.H. & Alford, R.A. (2012). Context-dependent symbioses and their potential roles in wildlife diseases. *Proceedings of The Royal Society B* 279, 1457-1465. Doi:10.1098/rspb.2011.2276.

Daversa, D.R., Manica, A., Bosch, J., Jolles, J.W. & Garner, T.W. (2018). Routine habitat switching alters the likelihood and persistence of infection with a pathogenic parasite. *Functional Ecology* 32, 1262-1270.

Duffus, A.L. & Cunningham, A.A. (2010). Major disease threats to European amphibians. *The Herpetological Journal* 20, 117-127.

Eisenhauer, N., Schulz, W., Scheu, S., Jousset, A. & Pfrender, M. (2013). Niche dimensionality links biodiversity and invasibility of microbial communities. *Functional Ecology* 27, 282-288. Doi:10.1111/j.1365-2435.2012.02060.x.

Fisher, M.C. & Garner, T.W. (2007). The relationship between the emergence of *Batrachochytrium dendrobatidis*, the international trade in amphibians and introduced amphibian species. *Fungal Biology Reviews* 21, 2-9.

Fitzpatrick, B.M. & Allison, A.L. (2014). Similarity and differentiation between bacteria associated with skin of salamanders (*Plethodon jordani*) and free-living assemblages. *FEMS Microbiology Ecology* 88, 482-494.

Goode, K. & Rey, K. (2019). ggResidpanel Tutorial and User Manual. https://goodekat.github.io/ggResidpanel-tutorial/tutorial.html. Accessed 10/02/2021.

Harris, R.N., James, T.Y., Lauer, A., Simon, M.A. & Patel, A. (2006). Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. *EcoHealth* 3, 53-56.

Harrison, X.A., Price, S.J., Hopkins, K., Leung, W., Sergeant, C. & Garner, T.W. (2019). Diversity-stability dynamics of the amphibian skin microbiome and susceptibility to a lethal viral pathogen. *Frontiers in Microbiology* 10, 2883.

Healy, W.R. (1973). Life history variation and the growth of juvenile *Notophthalmus viridescens* from Massachusetts. *Copeia* 1973, 641-647.

Jervis, P., Pintanel, P., Hopkins, K., Wierzbicki, C., Shelton, J.M., Skelly, E., Rosa, G.M., Almeida-Reinoso, D., Eugenia-Ordoñez, M., Ron, S. & Harrison, X. (2021). Post-epizootic microbiome associations across communities of neotropical amphibians. *Molecular Ecology* 30, 1322-1335.

Jørgensen, C.B. (1986). External and internal control of patterns of feeding, growth and gonadal function in a temperate zone anuran, the toad *Bufo bufo*. *Journal of Zoology* 210, 211-241.

Kriger, K.M. & Hero, J.M. (2007). Large-scale seasonal variation in the prevalence and severity of chytridiomycosis. *Journal of Zoology* 271, 352-359.

Kristín, P. & Gvoždík, L. (2013). Aquatic-to-terrestrial habitat shift reduces energy expenditure in newts. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology* 321, 182-188.

Kueneman, J.G., Bletz, M.C., McKenzie, V.J., Becker, C.G., Joseph, M.B., Abarca, J.G., Archer, H., Arellano, A.L., Bataille, A., Becker, M. & Belden, L.K. (2019). Community richness of amphibian skin bacteria correlates with bioclimate at the global scale. *Nature Ecology and Evolution* 3, 381–389 (2019). https://Doi.org/10.1038/s41559-019-0798-1.

Kusano, T. (1981). Growth and survival rate of the larvae of *Hynobius nebulosus tokyoensis* Tago (Amphibia, Hynobiidae). *Researches on Population Ecology* 23, 360-378.

Lauer, A., Simon, M.A., Banning, J.L., André, E., Duncan, K. & Harris, R.N. (2007). Common cutaneous bacteria from the eastern red-backed salamander can inhibit pathogenic fungi. *Copeia* 2007, 630-640.

Longcore, J.E., Pessier, A.P. & Nichols, D.K. (1999). *Batrachochytrium dendrobatidis* gen. et sp. Nov., a chytrid pathogenic to amphibians. *Mycologia* 91: 219-227.

Loudon, A.H., Woodhams, D.C., Parfrey, L.W., Archer, H., Knight, R., McKenzie, V. & Harris, R.N. (2013). Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). *The ISME Journal* 8, 830-840.

Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M. C., Woeljes, A., Bosman, W., Chiers, K., Bossuyt, F. & Pasmans, F. (2013). *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. *Proceedings of the National Academy of Sciences* 110, 15325-15329.

Matos, A., Kerkhof, L. & Garland, J.L. (2005). Effects of microbial community diversity on the survival of *Pseudomonas aeruginosa* in the wheat rhizosphere. *Microbial Ecology* 49, 257-264. Doi:10.1007/s00248-004-0179-3.

Mettouris, O., Megremis, G. & Giokas, S. (2016). A newt does not change its spots: using pattern mapping for the identification of individuals in large populations of newt species. *Ecological Research* 31: 483-489.

Michaels, C.J., Antwis, R.E. & Preziosi, R.F. (2014). Impact of plant cover on fitness and behavioural traits of captive red-eyed tree frogs (*Agalychnis callidryas*). *PloS ONE* 9, e95207.

Michaels, C.J. & Preziosi, R.F. (2020). Clinical and naturalistic substrates differ in bacterial communities and in their effects on skin microbiota in captive fire salamanders (*Salamandra salamandra*). *Herpetological Bulletin* 151, 10-16.

Miller, M.B. & Bassler, B.L. (2001). Quorum sensing in bacteria. *Annual Reviews in Microbiology* 55, 165-199.

Minting, P. (2012). An investigation into the effects of *Batrachochytrium dendrobatidis* (*Bd*) on natterjack toad (*Bufo calamita*) populations in the UK (Doctoral dissertation, University of Sussex).

Ogilvy, V., Preziosi, R.F. & Fidgett, A.L. (2012). A brighter future for frogs? The influence of carotenoids on the health, development and reproductive success of the red-eye tree frog. *Animal Conservation* 15, 480-488.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, M.P., Stevens, H.H., Szoecs, E. & Wagner, H. (2020). vegan: Community Ecology Package. R package version 2.5-7. https://CRAN.R-project.org/package=vegan.

Pasmans, F., Bogaerts, S., Janssen, H., Sparreboom, M. (2014). Molche und Salamander - halten und züchten. Natur und Tier Verlag, Münster.

Passos, L.F., Garcia, G. & Young, R.J. (2018). Comparing the bacterial communities of wild and captive golden mantella frogs: Implications for amphibian conservation. *PloS ONE* 13, p.e0205652.

R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Retallick, R.W., McCallum, H. & Speare, R. (2004). Endemic infection of the amphibian chytrid fungus in a frog community post-decline. *PLoS Biology* 2, e351.

Ruthsatz, K., Lyra, M.L., Lambertini, C., Belasen, A.M., Jenkinson, T.S., da Silva Leite, D., Becker, C.G., Haddad, C.F., James, T.Y., Zamudio, K.R. & Toledo, L.F. (2020). Skin microbiome correlates with bioclimate and *Batrachochytrium dendrobatidis* infection intensity in Brazil's Atlantic Forest treefrogs. *Scientific Reports* 10: 1-15.

Semlitsch, R., Scott, D. & Pechman, J. (1988). Time and size at metamorphosis related to adult fitness in *Ambystoma talpoideum*. *Ecology* 69, 184-192.

Shaffer, H.B., Austin, C.C. & Huey, R.B. (1991). The consequences of metamorphosis on salamander (*Ambystoma*) locomotor performance. *Physiological Zoology* 64, 212-231.

Treer, T. & Treer, D. (1995). Morphometric changes in the

smooth newt (*Triturus vulgaris*) during aquatic phase. *Croatian Journal of Fisheries: Ribarstvo* 53, 151-159.

van Elsas, J.D., Chiurazzi, M., Mallon, C.A., Elhottova, D., Kristufek, V. & Falcão Salles, J. (2012). Microbial diversity determines the invasion of soil by a bacterial pathogen. *Proceedings of the National Academy of Sciences* 109, 1159-1164. Doi:10.1073/pnas.1109326109.

Voordouw, M.J., Adama, D., Houston, B., Govindarajulu, P. & Robinson, J. (2010). Prevalence of the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis*, in an endangered population of northern leopard frogs, *Rana pipiens*. *BMC Ecology* 10, 6.

Walke, J.B, Harris, R.N., Reinert, L.K., Rollins-Smith, L.A. & Woodhams, D.C. (2011). Social immunity in amphibians: evidence for vertical transmission of innate defenses. *Biotropica* 43, 396-400. Doi:10.1098/rspb.2010.2118.

Webster, G., Newberry, C.J., Fry, J.C. & Weightman, A.J. (2003). Assessment of bacterial community structure in the deep sub-seafloor biosphere by 16S rDNA-based techniques: a cautionary tale. *Journal of Microbiological Methods* 55, 155-64.

Wren, S., Angulo, A., Meredith, H., Kielgast, J., Dos Santos, M. & Bishop, P. (Eds). (2015). Amphibian Conservation Action Plan. April 2015. IUCN SSC Amphibian Specialist Group. https://www.iucn-amphibians.org/resources/acap/.

Yeager, C.R. & Gibbons, M.E. (2013). Maternal provisioning trade-off strategies of *Agalychnis callidryas*. *Journal of Herpetology* 47, 459-465.

Accepted: 10 January 2022

Herpetological Journal FULL PAPER

https://doi.org/10.33256/ 32.2.5969

Published by the British Herpetological Society

How does the feeding behaviour of the common forest toad *Rhinella henseli* (Anura: Bufonidae) vary in space and time? Trophic ecology, chemical and antimicrobial activity

Douglas da Silva Huning[1], Fabiana Tonial[2], Mateus Oliveira[3], Noeli Zanella[1], Júlia de Moraes Brandalise[4], Kielli Guerra[4], Natália Ficanha[1] & Carla Denise Tedesco[1]

[1] Graduate Program in Environmental Sciences, University of Passo Fundo, Passo Fundo, Rio Grande do Sul, Brazil.

[2] Institute of Biological Sciences, University of Passo Fundo, Passo Fundo, Rio Grande do Sul, Brazil.

[3] Sintropica environmental consultancy, Capela de Santana, Rio Grande do Sul, Brazil.

[4] University of Passo Fundo, Passo Fundo, Rio Grande do Sul, Brazil.

Studies in trophic and chemical ecology, in particular in amphibians, have gained increasing attention in recent years, given that this is the vertebrate group that has suffered the greatest decline in recent years, caused by the degradation of natural ecosystems and emerging diseases. The assessment of food preferences and prey availability between areas and seasons provides important parameters for the understanding of the population dynamics of leaf-litter toads. The study of the secretions of the parotoid macroglands of these toads also provides insights into the role of these secretions in fighting frog pathogens and their potential applications to combat pathogens that are harmful to humans. In the present study, we describe the trophic ecology of *Rhinella henseli* (Lutz, 1934), and the variation in its diet between seasons and areas. We also attempt to identify the chemical composition of the secretions of the parotoid macrogland found in the parotoid glands and test their potential antimicrobial activity. We sampled two toad populations in the Atlantic Forest of southern Brazil. The composition of the diet was analysed by season (warm vs. cool) and study area, with the prey items being identified to genus, whenever possible, and classified using the Index of Relative Importance. The parotoid secretions were removed manually from the parotoid glands and analysed via HPLC-MS/MS. We ran microdilution and agar plug diffusion tests to assess antimicrobial activity. The principal prey of these toads are large ants, primarily *Pachycondyla* sp., which vary in abundance between seasons and, to a lesser extent, between areas. We identified 21 chemical compounds, primarily steroidal bufadienolides. One of the populations presented a subset of 14 of these 21 compounds, reflecting the variation in their spatial distribution. These compounds presented anti-pathogenic properties against *Candida albicans* and, to a lesser extent, *Staphylococcus saprophyticus*. Our results indicate that the diet of *R. henseli* varies significantly between areas and seasons, as do the secretions of their parotoid macroglands between areas. The toxins exhibit antimicrobial activity, although the compounds must be tested in isolation to confirm this.

Keywords : Ecochemistry, Niche breadth, Ground-dwelling toads, Ants, Bioprospecting

INTRODUCTION

The trophic ecology of a species can provide important insights into how its populations fluctuate over time and in space (López et al., 2015). Anurans have a broad trophic ecology, with opportunistic characteristics related to the availability and detectability of prey in their immediate environment (Duellman & Trueb, 1986). The amphibian diet varies considerably among different species, regions, periods of the year, niches, and varying prey availability. Sympatric species, such as *Leptodactylus luctator*, *Boana pulchella*, *Rhinella granulosa* and *Physalaemus gracilis*, may vary considerably in their principal prey items, which are, in this specific case, beetles, flies, ants, and springtails, respectively (Rosa et al., 2002). Even sympatric species of the genus *Rhinella* with similar trophic niches may exhibit substantial differences in the frequency and abundance of their principal prey items (Sabagh & Carvalho-e-Silva, 2008).

Leaf-litter amphibians feed primarily on ants, beetles, mites, larvae, cockroaches, and other invertebrates found on the forest floor (Isacch & Barg, 2002; Ferreira & Teixeira, 2009). Many of these amphibians are toxic and obtain their alkaloids mainly from ants and mites (Savitzky et al., 2012). The spatial and temporal variation in the diets of these species is reflected in significant differences in the composition of their chemical secretions, which indicates considerable plasticity in these interactions (Daly et al., 2007; Saporito et al., 2007). Toads of the

Correspondence: Douglas da Silva Huning (Douglas.huning@gmail.com)

genus *Rhinella* feed primarily on formicids, followed by coleopterans (Clarke, 1974). Despite this, the principal constituents of the chemical secretions of this genus are endogenous, and include steroidal bufadienolides and indole alkaloids (Gao et al., 2010).

The study species, *R. henseli* (Lutz, 1934) (Anura: Bufonidae), belongs to the *Rhinella crucifer* group, and is found in the subtropical forests of the southern extreme of the Atlantic Forest domain, in southern Brazil. This species is generally found in the leaf litter of Mixed Ombrophilous Forest or *Araucaria* pine forest (Lutz, 1934). *R. henseli* is considered to be a specialist of forested environments, and is thus vulnerable to the many different types of human impact that affect the native forests of Brazil.

Determining the variation in chemical and biological activity is fundamental to the understanding of pathogen-host and prey-predator relationships, as well as the prospection of compounds of medical interest (Sales et al., 2015). Compounds such as bufadienolides, alkaloids, and peptides are known to have potential as antimicrobial agents, which may be useful to combat drug-resistant pathogens (Cunha et al., 2005, Sales et al., 2017). Previous research has shown that amphibian skin secretions, in addition to halting infections in the animal itself, can be used to control bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (De Medeiros et al., 2019), fungi, including *Candida albicans* and *Candida krusei* (Sales et al., 2015), protozoa, e.g. *Leishmania chagasi*, and *Trypanosoma cruzi* (Tempone et al., 2008), viruses (Vigerelli et al., 2014), and tumor cells (Sciani et al., 2013).

Given these considerations, we tested two hypotheses: (i) *R. henseli* is specialised in the predation of ants, although the composition of its diet varies between seasons (and areas) due to fluctuations in the availability of prey, which may limit selectivity, and (ii) the secretions of the parotoid macrogland of *R. henseli* have potential antimicrobial properties against yeasts and pathogenic bacteria. The present study describes the *R. henseli* diet in two areas of subtropical forest during the two local climatic seasons. We also investigated which chemical compounds were present in the parotoid secretions, in particular bufadienolides, and whether the crude extract is sufficient to inhibit pathogenic microorganisms.

METHODS

Study area

We sampled two *R. henseli* populations between April and December 2019 in two protected areas of the Atlantic Forest domain, in mixed ombrophilous or *Araucaria* pine forest. The local climate is humid subtropical, with a mean annual rainfall of 1,700 mm. The rains are well distributed throughout the year and the mean annual temperature is around 18 °C (Backes, 1999). We sampled the *R. henseli* populations by active nocturnal and diurnal searches, as well as pitfall traps.

Population A was sampled in the Passo Fundo National Forest (Figure 1) or FLONA-Passo Fundo (28°19'17" S, 52°11'24" W, 693 m a.s.l.) in the municipality of Mato Castelhano, in the state of Rio Grande do Sul, southern Brazil. Population B was sampled in the Sertão Municipal Natural Park, 34 km northwest of the FLONA-Passo Fundo in the municipality of Sertão, Rio Grande do Sul (28°02'31" S, 52°13'28" W, 650 m a.s.l.).

Data collection

The pitfall traps were checked every morning (after 24 hours), with the stomach contents being removed immediately. In the case of the individuals captured by active searching, the stomach contents were removed within two hours. The contents were collected by stomach flushing (Solé et al., 2005), after which, we released the animals back into the wild. We considered each toad to be a sample unit for analysis. We classified the food items collected from the toads' stomach to order, family, or genus, whenever possible, and arranged them by season (warm vs. cool) and study area. We used a Permutational Multivariate Analysis of Variance (PERMANOVA) to verify whether the composition of the diet of the toads varied significantly between types of capture (i.e., active search vs. pitfall traps). As no significant variation was found between procedures, we pooled the data for analysis.

We determined the number (N), volume (V), and the frequency of occurrence (FO) of each item in both absolute terms and as percentages. The volume was determined by the displacement of water in a graduated cylinder (Hyslop, 1980). The analysis of dietary electivity was conducted only on the data from area A, for which data are available on the abundance of arthropods (Chicheleiro et al., 2021). In this area, arthropods were sampled monthly using 2-L plastic bottle traps, which were buried in the ground and filled with alcohol 70 % and detergent.

Analysis of the diet

We evaluated the importance of each prey category in the *R. henseli* diet using the Percentage Index of Relative Importance (%IRI), calculated by the formula %IRI = (%N + %V) * %FO, where **%**N = the percentage of the number of each category of the diet, **%**V = the percentage of prey volume, and **%**FO = the percentage frequency of occurrence of the prey items (Pinkas et al., 1971; Hart et al., 2002).

We used Jacob's Electivity Index (D) to assess whether the toads were selecting prey or capturing them in proportion to their availability in the environment, based on the equation: D = Rk – Pk / (Rk + Pk) – (2. Rk.Pk), where k = the food category, P = the proportion of this category in the diet, and R = the proportion of the category in the environment. This index evaluates the contribution of each prey category to the diet in comparison with its availability in the environment, with values above 0.2 indicating that a prey is being selected actively by the animal (Jacobs, 1974; Hayward et al., 2011). This test was applied only to the dietary data from area A, given the availability of data on the abundance of prey at this site (see above).

We determined the breadth of the trophic niche by calculating Levin's Trophic Niche Amplitude Index (Krebs, 1999), which is given by the formula: B = $1/\sum jP^2ij$, where:

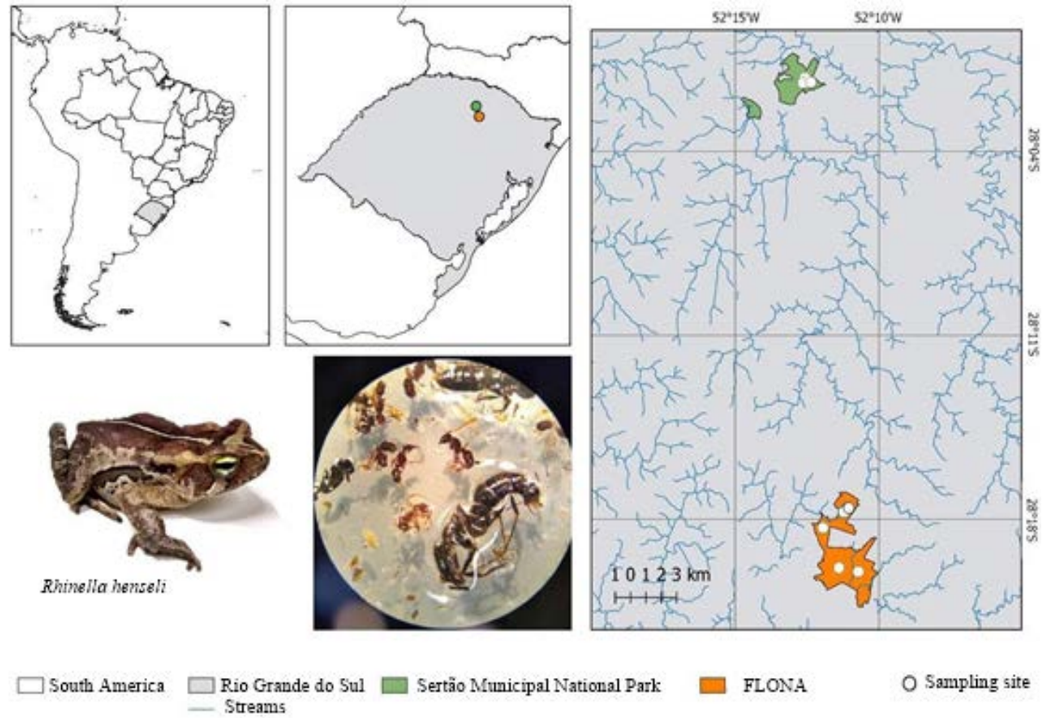

Figure 1. Location of the *R. henseli* (Anura: Bufonidae) populations sampled in the Atlantic Forest of southern Brazil and Sertão Municipal Natural Parkand Passo Fundo National Forest (FLONA Passo Fundo) in Rio Grande do Sul state, Brazil.

B = Levin's index, and Pij = the proportion of food resource j in the diet of species **i**. This index was standardized as: Bsta **=** B-1/n-1, where B = Levin's index, and n = the total number of food resources used. The values of Bsta vary from 0 to 1, depending on the degree of specialisation of the species, with values close to zero indicating a specialist diet, whereas values close to 1 indicate a generalist diet.

We used a PERMANOVA, based on the Bray-Curtis dissimilarity index, to verify the variation in the composition of the diet between populations and seasons, according to Clarke (1993) and Anderson (2001). We defined the climatic seasons as cool (mean temperature = 20.4 ºC) and warm (mean temperature = 26.7 °C). Here, we used a data matrix in which each individual was a sample, with the %IRI of the prey categories being calculated for each frog. We compared the frogs from the two areas, using data only from the warm season, and then compared the two seasons, with data from area A only (Clarke, 1993; Anderson, 2001).

The collection and analysis of the secretions of the *Rhinella henseli* parotoid macrogland

In both areas, chemical secretions were collected during the warm season. To collect these secretions, we pressed the parotoid glands of the toads manually or with tweezers. This produced a whitish, sticky substance, which we scraped off using a spatula. This secretion was stored in 5- mL Eppendorf tubes at a temperature of -20 °C.

The samples were analysed chromatographically using an HPLC-MS/MS system in SRM (Selected Reaction Monitoring) and ESI (Electrospray Ionization) in the positive and negative modes. The mobile phase consisted of a water:acetonitrile solution (v/v, 9:1) in the isocratic mode at a flow rate of 0.3 mL min-1 for 30 min, using an Agilent Zorbax GF-250 (250 mm x 4.6 mm, 4 µm) column. The injection volume of each sample was 10 µL. The capillary voltage was maintained at 2 kV and the temperature of the chosen solvent gas was 600 °C. Four spectrometric methods were used to analyse the components of the chemical secretions: 1 - scanning the precursor ion (100–1000 m/z) in positive and negative modes; 2 - monitoring the most intense ions (Selected Ion Monitoring – SIM) in positive and negative modes; 3 - scanning the product ions (100 – m/z IonPrec.) in positive and negative modes, and 4 - monitoring selected reactions (SRM) of the most intense products. The data obtained from the spectrometric analysis of the crude extract of the secretions of the *R. henseli* parotoid macroglands were compared with the findings of previous studies of other species of the family Bufonidae (Zhao et al., 2014; Zhang et al., 2016; Schmeda-Hirschmann et al., 2014; 2016; Petroselli et al., 2018).

Antimicrobial activity tests

We mixed the chemical secretions obtained from the toads

from the two study areas for the antimicrobial tests. The extracted material was diluted in physiological solution at concentrations of 1 mg/mL, 10 mg/mL, 50 mg/mL, and 100 mg/mL and was then homogenised for five minutes. We tested the activity of the secretions against nine strains of microorganism: *E. coli* (ATCC 2592), *Staphylococcus saprophyticus* (ATCC 15305), *Enterococcus faecalis* (NEWP 0012), *Salmonella typhimurium* (NEWP 0028), *Bacillus subtilis* (ATCC 6633), *Enterobacter aerogenes* (NEWP 0122), *Streptococcus pyogenes* (NEWP 0015), *S. aureus* (NEWP 0038), and *C. albicans* (NEWP 0031). The microorganism suspensions were prepared by the depletion technique in Petri dishes containing Mueller Hinton agar, which were incubated at 36 °C for 24 hours. The colonies were then diluted in Mueller Hinton broth to a concentration of 108 Colony Forming Units (CFU)/mL in the case of the bacteria and 106 CFU/mL for the yeast, by comparing the samples with the turbidity visual standard reference of 0.5 in the MacFarland scale.

We assessed antimicrobial activity by microdilution and the agar plug diffusion method. The microdilution test was conducted on a plate with 96 puddles, each with a final volume of 150 µL, with 90 µL of Mueller Hinton broth, 10 µL of the microbial inoculum, and 50 µL of each concentration of the parotoid secretion. The plates were heated to 36 ºC in an oven and stained with 10 µL of MTT ([3- (4,5-dimethylthiazol-2yl) -2,5-diphenyl tetrazolium bromide]) after 24 h. Chloramphenicol (1 mg/mL) was used as the positive control for the bacteria, and nystatin (100.000 UI/mL) for the yeast. The negative control was formed by the inoculum with the addition of Muller-Hinton broth to a total volume of 150 µL.

The agar plug diffusion test was run in triplicate in Petri dishes containing Mueller Hinton agar, in which small puddles were made using a sterile test tube. In each dish, the suspension of the microorganisms, prepared as described above for the previous test, was added to the agar with a swab using the four-way spreading technique. A 50 µL aliquot of the chemical secretion at the respective concentration was added to each puddle. Saline was used as the negative control, while the positive controls were chloramphenicol (1 mg/mL) for the bacteria and nystatin (100,000 IU/mL) for the yeast. The plates were incubated at 36 °C for 24 h, following which, we evaluated the microbial growth around the puddles. We classified the results as (Fig. 2): (i) inhibitory (formation of a halo with no growth of the colony around the puddle), (ii) non-inhibitory (normal growth of the colony around the puddle) or (iii) reduction (reduced growth of the colony around the well in comparison with other puddles of the same plate).

RESULTS

Diet of *Rhinella henseli*

We collected data on the diet of 82 toads, of which 66 were collected in Area A (16 during the cool season and 50 during the warm season) and 16 in Area B (warm season). We did not detect any significant variation in the composition of the diet of the toads captured by active searching or pitfall

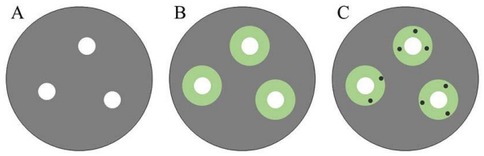

Figure 2. The figure demonstrates the hypothesised results for the agar plug diffusion method. Grey color: fungal and bacterial growth in the agar. White color: 50 µl wells containing the parotoid macroglands secretions. Green color: inhibition zones of microbial growth around the wells. **A)** No microbial inhibition. **B)** Microbial inhibition around wells containing frog secretions. **C)** reduction of microbial growth around the wells.

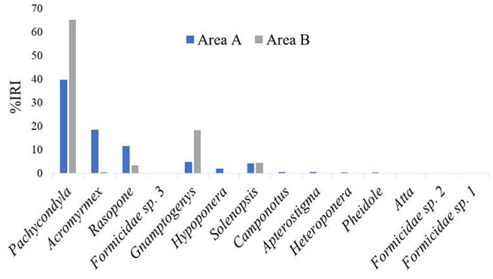

Figure 3. Percentage index of relative importance of ant genera consumed by *R. henseli* in two areas of Atlantic Forest in southern Brazil. This comparison only includes the data from the warm season. Note that in area B the genus *Pacycondylais* dominant, while in area A the values are better distributed among the prey.

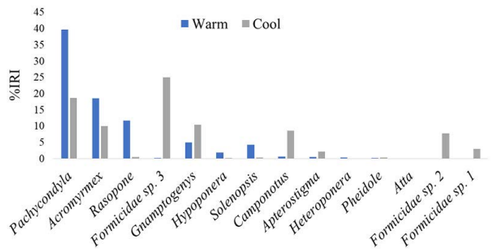

Figure 4. Percentage index of relative importance of ant genera consumed by *R. henseli* in two seasons in the Atlantic Forest of southern Brazil. This comparison only includes the data from the area A. Note that in the cold season, the most consumed genus becomes Formicidae sp. 3, including other prey absent in the warm season. In the warm season *Pachycondyla* has high %IRI values.

traps (p = 0.07), which allowed us to pool these data. The *R. henseli* stomachs contained six insect orders, including six families of the order Coleoptera and 15 genera of the

Table 1. Percentage Index of Relative Importance (%IRI), niche breadth, and electivity indices for the insect taxa recorded in the diets of two populations of *R. henseli* in the *Araucaria* pine forest, in the south of the Atlantic Forest. Due to sampling factors (see Methods), the seasonal variation in the diet was verified only at FLONA Passo Fundo (A).

Prey	FLONA Passo Fundo (A)		Sertão (B)	Electivity
	Hot	Cool	Hot	
Hymenoptera: Formicidae				0.722
Pachycondyla	**39.72**	**18.69**	**65.39**	
Acromyrmex	**18.55**	10.08	0.63	
Rasopone	**11.69**	0.49	3.43	
Formicidae sp. 3	0.25	**25.01**	0	
Gnamptogenys	4.92	**10.49**	**18.41**	
Hypoponera	1.92	0.25	0.05	
Solenopsis	4.27	0.34	4.39	
Camponotus	0.64	8.61	0.09	
Apterostigma	0.49	2.22	0	
Heteroponera	0.38	0	0.16	
Pheidole	0.28	0.37	0	
Atta	0.02	0	0	
Formicidae sp. 2	0	7.76	0.08	
Formicidae sp. 1	0	2.95	0	
Coleoptera				-0.696
Elateridae	4.61	0	0.45	
Carabidae	6.17	0	0.22	
Curculionidae	3.89	6.25	1.73	
Scarabidae	1.47	0	0.11	
Staphylinidae	0.5	6.51	4.85	
Paussidae	0.26	0	0	
Larvas	3.0	1.83	0	
Aranae	0.1	0.04	0.21	-0.621
Hemiptera	01	0	0.6	-0.728
Othoptera	0.008	0	0.03	-0.719
Niche breadth (Bsta)	**0.51**	**0.49**	**0.23**	

* Values in bold script indicate the principal prey taxa, that is, the prey most consumed in the respective area or season.

hymenopteran family Formicidae (Table 1).

The niche breadth recorded in area A (0.55) was more than the double that of area B (0.23) in the same season, although the composition of the diet did not vary significantly between the two populations (p = 0.07). The *R. henseli* diet was based on ants of the genus *Pachycondyla* during the warm season in both populations. However, there was some variation in the composition of the diet between areas (Table 1), with the toads in area A consuming primarily *Pachycondyla* sp. (%IRI = 38.99) and *Acromyrmex* sp. (%IRI = 18.98), while those in area B consumed mostly *Pachycondyla* sp. (%IRI = 65.39), with some *Gnamptogenys* sp. (%IRI = 18.41) (Figure 3).

Although niche breadth was similar between seasons in Area A (Bsta: warm = 0.51; cool = 0.49), we recorded significant differences between seasons in the consumption of food items of the class Insecta (F = 8.83; p = 0.001), and the orders Coleoptera (F = 3.85; p = 0.003) and Hymenoptera (F = 2.47; p = 0.02). In the cool season, larger %IRI values were recorded for Formicidae sp. 3 (%IRI = 39.72), *Acromyrmex* sp. (%IRI = 18.55), and *Rasopone* sp. (%IRI = 11.69), whereas in the warm season, the most important prey were *Pachycondyla* sp. (%IRI = 39.72), *Acromyrmex* sp. (%IRI = 18.55), and *Rasopone* sp. (%IRI = 11.69) (Figure 4). Regardless of the area or season, the prey most consumed by *R. henseli* were larger (in volume and length) than those ingested least frequently. Jacob's Electivity Index (D = 0.722) indicated that the toads are selecting ants (Formicidae) actively in their diet (Table 1).

Parotoid macrogland chemical composition

The parotoid secretion of *R. henseli* is composed mainly of steroidal bufadienolides, which were classified as bufogenins and bufotoxins. Twenty-one compounds were identified tentatively, including marinobufagin and telocinobufagin (Figure 5), with marinobufagin and resinobufagin representing the main bufadienolides, derived from arginine diacids (Table 2). It is interesting to note that seven of these 21 tentative compounds (Marinobufagin or desacetylcinobufagin, Azelayl arginine, 3-(N-glutaryl argininyl) marinobufagin, 3-(N-azelayl argininyl) bufalin, 3-(N-azelayl argininyl) marinobufagina, 3-(N-suberoyl argininyl) hellebrigenina, and 3- (N -suberoyl argininyl) -telocinobufagin) were present only in the individuals from area A. This variation occurred mainly in the bufadienolides derived from the arginine diacids.

Antimicrobial activity of parotoid secretions

The crude extract of the *R. henseli* secretions, at a concentration of 100 mg/mL, inhibited the growth of the yeast *C. albicans* and reduced the development of the bacterium *S. saprophyticus*. This antimicrobial activity was detected by the agar plug diffusion method.

DISCUSSION

Our data clearly indicates an ant-feeding niche in *R. henseli*. The Electivity Index indicated that *R. henseli* feeds preferentially on ants. These toads are not selecting a specific genus of ants, but are consuming the largest

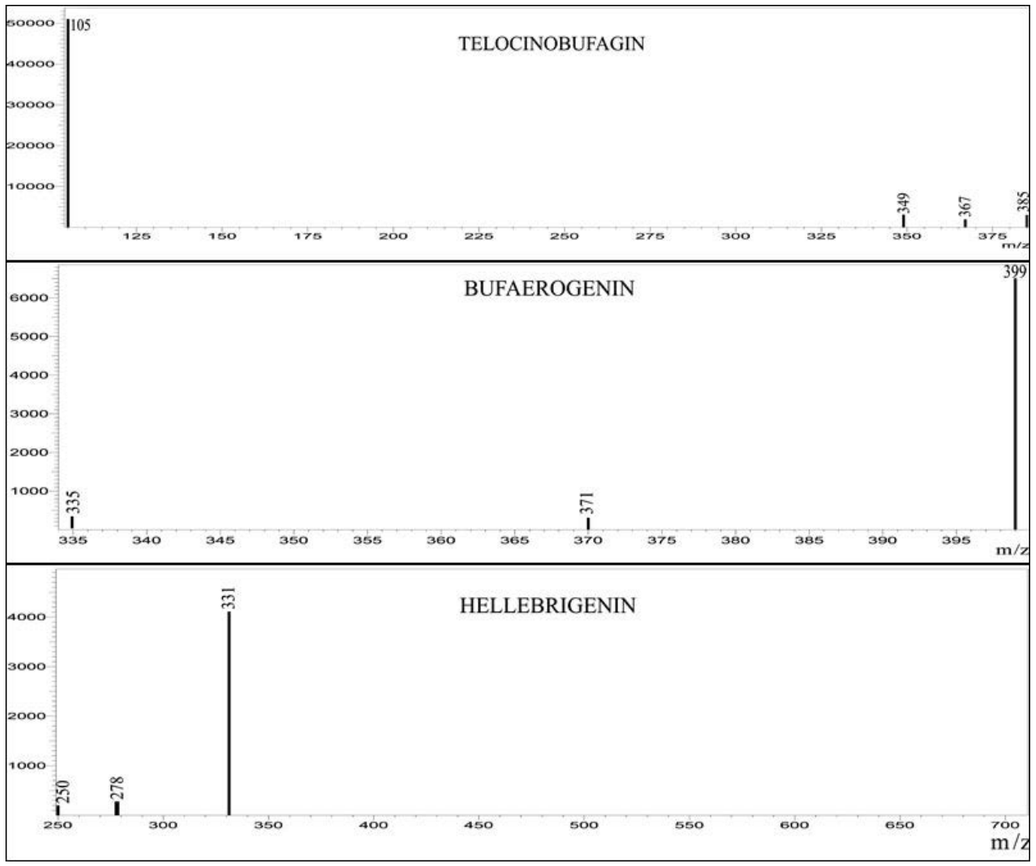

Figure 5. Compounds tentatively identified from precursor ions and fragment ions (M+H)+. Telocinobufagin and Hellebrigenin are recovered in the literature as compounds with activity against pathogens.

and likely most visible ants (i.e. *Pachycondyla, Rasopone, Gnamptogenys,* Formicidae sp. 3) (McElroy & Donoso, 2019). This is consistent with an optimal foraging strategy, in which large prey are consumed preferentially due to their relatively greater nutritional value in comparison with the energetic costs of searching and handling (Emlen, 1966; Mcelroy & Donoso, 2019). In this case, the composition of the diet of these toads is determined both by the active foraging mode of these toads and the availability of large prey in the leaf litter, in particular, the predation of the large ants found on the floor of the *Araucaria* pine forest. The consumption of ants and beetles may also account for the fact that we found no significant differences in the diet between active searching and pitfall traps, given that the relatively thick chitinous exoskeleton of these insects persists for longer in the stomachs.

It is important to note, however, that the opportunistic habits of most *Rhinella* species lead them to consume the most available prey in the environment, as observed in *Rhinella schneideri*, which preyed primarily on insect larvae in the Brazilian Cerrado savanna (Batista et al., 2011). The size of the toad may also determine its consumption of different types of prey, with *Rhinella* species smaller than *R. henseli* tending to consume more mites in the leaf-litter (Rosa et al., 2002), while larger species may prey more on beetles (Sabagh et al., 2012).

No significant variation was found in the composition of the diet between areas, considering the same (warm) season, although the %IRI values recorded in Area A were more evenly distributed, in particular among *Pachycndyla* sp., *Acromyrmex* sp. and *Rasopone* sp., whereas the toads in Area B relied more on *Pachycondyla* sp., followed by *Gnamptogenys* sp. The differences between areas in the relative abundance of each type of ant may be associated with a number of different factors. Field observations indicate that the composition of the plant community, canopy cover, litter abundance, and subsoil density are different between areas A and B. Area A has been a well-managed forest reserve since its inception, whereas area B has been subject to incursions by cattle and the poorly-controlled exploitation of its natural resources. Differences in the structure of the leaf litter of the forest floor may lead to differences in the structure of the local ant community (McGlynn et al., 2009), as well as spatial variation in the niche partitioning between specialist and generalist ant species, a heterogeneous distribution that may account

Table 2. Prospective compounds identified in the toxin of two populations of *R. henseli*, based on the comparison with the spectrometric data obtained from previous studies of toads of the family Bufonidae

Area A	Area B	[M+H]+	Prod 1	Prod 2	Prod 3	Tentative identification
X	X	303.3	158	116	112	Adipyl arginine[2]
X	X	317	158	112		Pimeloyl arginine[1]
X	X	331	250	175	158	Suberoyl arginine[1]
X	X	387	105			Bufalin[3]
X	--	401	253	151		Marinobufagin or desacetylcinobufagin[3,4]
X	X	403	385	105		Telocinobufagin[1]
X	X	405	203	188.2		Unknown
X	X	417	399		335	Bufarenogin [1]
X	X	425	385			Unknown
X	X	441	401			Unknown
X	X	455	415			Unknown
X	X	480	439			Unknown
X	X	485	311	203		Unknown
X	X	563	130	102		Unknown
X	X	632.8	317.2	331	303	[2M + H]+ Pimeloyl arginine[2]
X	X	660.8	331.2			Suberoyl arginine [2M + H]+[2]
X	X	663	332			Unknown
X	X	683.7	353	331		3-(N-pimeloyl argininyl) resibufogenin[2]
X	X	685.7	667.5	303.1		3-(N-adipoyl argininyl) marinobufagin[2]
X	X	687.7	669.6	303.2		3-(N-adipoyl argininyl) telocinobufagin[2]
X	--	688.5	345.3			Azelayl arginine[2]
X	X	697.7	331.2	369		3-(N-suberoylargininyl) resibufogenin[2]
X	--	701.6	683.6	317.2		3-(N-glutaryl argininyl) marinobufagin[2]
X	X	711.8	615	297		3-(N-azelaylargininyl) resibufogenin[2]
X	X	713.2	693	297		3-(N-suberoylargininyl) marinobufagin (Marinobufotoxin)[2]
X	--	713.8	694	331		3-(N-azelaylargininyl) bufalin[2]
X	--	715	697.6	331.2		3- (N - suberoylargininyl) -telocinobufagin(telocino bufatoxin); 3- (N - pimeloyl argininyl) -bufarenogin; arenobufagin or hellebrigenin[1]
X	--	727.6	331.3	278.1		3-(N-azelaylargininyl) marinobufagin[2]
X	--	729.2	331.3	278		3-(N-suberoylargininyl) hellebrigenin[2]

*x = present;-- = absent.

[1] (Petroselli et al., 2018); [2] (Schmeda-Hirschmann et al., 2016); [3] (Schmeda-Hirschmann et al., 2014); [4] (Zhang et al., 2016).

for at least some of the variation in the diet of the toads (Spiesman & Cumming, 2008; Tavella et al., 2018).

We found evidence of seasonal variation in the composition of the diet of *R. henseli*, as well as in the abundance and frequency of each prey type. This may have been related to the intrinsic characteristics of each type of prey, such as their thermal tolerance, which may vary considerably among species (Bishop et al., 2016), and fluctuations in the availability of food resources between seasons (Cook et al., 2011; Gomes et al., 2014). Even though the structure of the ant community shifted between seasons, they remained a key resource during the coolest part of the year, whereas only one of the six coleopteran families were present in the diet at this time of year.

We expected the toads to have a broader niche in the cool season as a result of an increase in opportunistic foraging due to the reduction in the availability of prey during this period, although the results of the analyses did not confirm this. This may be associated with the fact that, in some communities of ground-dwelling ants, while species density and composition may vary between seasons, the activity of these insects does not cease altogether (Deblauwe & Dekoninck, 2007). Overall, the niche amplitude may have been determined by distinct mechanisms in the two seasons, with the greater availability of prey in the warm (and rainy) season determining an increase in the number of taxa consumed through individual specialisation, whereas in the cool (dry) season, the decrease in the availability of prey may have driven the number of taxa exploited, to guarantee meeting the energy needs of the toads (Camargo et al., 2020).

The chemical composition of the secretions of the parotoid macrogland recorded in the present study is similar to that of other bufonids of the genus *Rhinella* (Schmeda-Hirschmann et al., 2014; 2016; Mailho-Fontana et al., 2018; Petroselli et al., 2018; De Medeiros et al., 2019), being composed primarily of steroidal bufadienolides. A number of other compounds, such as alkaloids, biogenic amines, and antimicrobial peptides, have already been identified in other species of the *R. crucifer* group (Daly et al., 2005), although they were not evaluated in the present study.

Different species and populations of amphibians may present differences in the composition of their chemical secretions. The chemical compounds of the toads from Area B were a subset of those recorded in Area A, in particular in terms of the bufadienolides derived from arginine diacids. This may have been related to either genetic variation between the populations or, possibly, phenotypic variation determined by specific environmental factors (Hayes et al., 2009; Chen et al., 2013; Wu et al., 2017; Hovey et al., 2018). *Pachycondyla* sp. was also the most important taxon in the diet of *Rhinella ornata*, and the chemical composition of the parotoid secretions of this toad was altered by the removal of this ant from its diet (França, 2015; Moskowitz et al., 2020). Further research is needed to confirm whether a similar scenario is found in *R. henseli*.

In the specific case of the bufonid toads, bioactivity has already been described against a number of different types of microorganism, including bacteria (Sales et al., 2017), protozoa (De Medeiros et al., 2019), viruses (Vigerelli et al., 2014), trematodes (Calhoun et al., 2016), and tumor cells (Gao et al., 2011; Lu et al., 2018), using both the crude extract and its isolated components. Our findings indicate that the chemical secretions of *R. henseli* contain compounds that control the growth of microorganisms, given that the extract inhibited the yeast *C. albicans* and reduced the growth of the gram-positive bacterium *S. saprophyticus*. Previous studies have also shown that bufadienolides extracted from *Rhinella* toads are toxic to *S. aureus*, *P. aeruginosa* and *Escherichia coli* (De Medeiros et al., 2019), and may also initiate the action of other antibiotics (Sales et al., 2015; 2017).

We identified some steroidal bufadienolides in the *R. henseli* secretions, including marinobufagin and telocinobufagin, which are known to have antimicrobial activity against *S. aureus* (De Medeiros et al., 2019) and *E. coli* (Cunha et al., 2005). This is in addition to resibufogenin, which is known to have anti-tumor activity (Lu et al., 2018), and hellebrigenin, which is antileishmanial and antitripanosomal (Tempone et al., 2008).

The results of the present study draw attention to the biological potential of the chemical compounds produced by wild species. It is possible that thousands of substances are lost to local extinction even before they are identified, isolated, and tested. We would recommend that scientists explore this potential through partnerships, extracting chemical compounds from samples collected in the wild, and sending them for analysis and antimicrobial testing. Due to logistical issues, we were unable to sample the diet of population B during the cool season, in addition to not collecting parotoid secretions in either climatic season. This may be a limiting factor in our research given that it was impossible to analyse more clearly the role of seasonality in the composition of the venom and in the diet of the frogs in the two areas during the cool season. Even so, *R. henseli* can be considered to be an ant-feeding specialist, with the composition, frequency, and abundance of its prey varying in both space and time, but with the predominance of large, leaf-litter ants persisting in all cases. The spatial variation observed in the composition of the chemical secretions of this species may be due to genetic factors, although the potential influence of dietary variables has yet to be tested. The secretions of the parotoid macroglands of *R. henseli* appear to be promising in terms of their bioactivity, and new assays with the isolated components may confirm an even greater effectiveness at low concentrations.

ACKNOWLEDGEMENTS

We thank CAPES (Coordination of Higher Education Personnel Training) for a masters scholarship and the Graduate Program in Environmental Sciences (PPGCiamb) at the University of Passo Fundo (UPF), which supported the study. We thank Nelson Bandeira for the chromatographic analysis, Élinton Rezende for field logistics, Caroline Ribeiro for the stomach content analysis, Juciela Chincheleiro for secondary data, and the interns at the Ecology and Herpetology laboratories for their support in the field.

Authors' contribution

Douglas da Silva Huning: Conceptualisation (Equal); Data collection (Lead); Sample processing (Equal); Methods (Lead); Writing - original draft (Lead); Writing - proofreading and editing (Lead). Fabiana Tonial: Conceptualisation (Lead); Sample processing (Supporting); Methods (Lead); Writing - proofreading and editing (Equal); Supervision (Lead); Project administration (Lead); Resources (Lead). Mateus Oliveira: Data analysis (Lead); Writing - proofreading and editing (Equal). Noeli Zanella: Writing - proofreading and editing (Lead); Resources (Equal). Júlia de Moraes Brandalise: Data collection (Equal); Sample processing (Equal); Writing - proofreading (Support). Kielli Guerra: Methods (Equal); Sample processing (Equal). Natália Ficanha: Data collection (Support); Collection of secondary data (Lead); Writing - proofreading (Support). Carla Denise Tedesco: Conceptualisation (Lead); Methods (Lead); Writing - proofreading and editing (Equal); Supervision (Lead); Project administration (Lead); Resources (Lead).

Ethical statement

The research was submitted to the Ethics Committee on the use of animals at the University of Passo Fundo and approved with the number 004/2019. The study was also registered in the Biodiversity Authorization and Information System (Sisbio) under number 68671-1.

Financing statement

Douglas da Silva Huning and Natália Ficanha: Financing through postgraduate scholarships CAPES (Coordination for the Improvement of Higher Education Personnel).
Fabiana Tonial and Carla Denise Tedesco: Financing of consumables and analyses by the Ecology and Microbiology Laboratories of the University of Passo Fundo.

Conflicts of interest

The authors declare having no competing interests.

REFERENCES

Anderson, M.J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecology* 26, 32-46.

Batista, R.C., De-Carvalho, C.B., Freitas, E.B., Franco, S.C., Batista, C.C., Coelho W.A. & Faria, R.G. (2011). Diet of *Rhinella schneideri* (Werner, 1894) (Anura: Bufonidae) in the Cerrado, Central Brazil. *Herpetology Notes* 4, 17-21.

Backes, A. (1999). Condicionamento climático e distribuição geográfica de *Araucaria angustifolia* (Bertol.) Kuntze no Brasil – II. *Pesquisas, Série Botânica* 49, 31-51.

Bishop, T.R., Robertson, M.P., Gibb, H., Rensburg, B.J.V., Braschler, B., Chown, S.L., Foord, S.H., Munyai, T.C., Okey, I., Tshivhandekano, P.G., Werenkraut, V. & Parr, C.L. (2016). Ant assemblages have darker and larger members in cold environments. *Global Ecology and Biogeography* 25, 1489-1499.

Calhoun, D.M., Woodhams, D., Howard, C., LaFonte, B.E., Gregory, J.R. & Johnson, P.T.J. (2016). Role of Antimicrobial Peptides in Amphibian Defense Against Trematode Infection. *EcoHealth* 2, 383–391.

Camargo, F.M., Reis, G.G., Camargo, A.C.L., Nardoto, G.B., Kneitel, J.M. & Vieira, E.M. (2020). Seasonal isotopic niche of a rodent: High between-individual variation but no changes in individual niche width during the rich-resource period. *Biotropica* 00, 1-10.

Chicheleiro, J., Santos-Pereira, M., Luza, A.L., Huning, D.S., Zanella, N. (2021). Effects of natural forest and tree plantations on leaf-litter frog assemblages in southern Brazil. *Austral Ecology* 46, 1.

Chen, J., Rashid, T., Feng, G., Zhao, L., Oi, D. & Drees, B.B.M. (2013). Defensive chemicals of tawny crazy ants, *Nylanderia fulva* (Hymenoptera: Formicidae) and their toxicity to red imported fire ants, *Solenopsis invicta* (Hymenoptera: Formicidae). *Toxicon* 76, 160–166.

Clarke, R.D. (1974). Food Habits of Toads, Genus *Bufo* (Amphibia: Bufonidae). *The American Midland Naturalist* 90, 140-147.

Clarke, K.R. (1993). Non-parametric multivariate analysis of changes in community structure. *Australian Journal of Ecology* 18, 117-143.

Cook, S.C., Eubanks, M.D., Gold, R.E. & Behmer, S.T. (2011). Seasonality directs contrasting food collection behavior and nutrient regulation strategies in ants. *PLoS ONE* 6, 1-8.

Cunha, G.A., Schwartz, C.A., Resk, I.S., Murta, M.M., Lemos, S.S., Castro, M.S., Kyaw, C., Pires Jr, O.R., Leite, J.R.S., Bloch Jr, C. & Schwartz, E.F. (2005). Antimicrobial activity of the bufadienolides marinobufagin and telocinobufagin isolated as major components from skin secretion of the toad *Bufo rubescens*. *Toxicon* 45, 777–782.

Daly, J.W., Spande, T.F. & Garraffo, H.M. (2005). Alkaloids from amphibian skin: A tabulation of over eight-hundred compounds. *Journal of Natural Products* 68, 1556–1575.

Daly, J.W., Wilham, J.M., Spande, T.F., Garraffo, H.M., Gil, R.R., Silva, G.L. & Vaira, M. (2007). Alkaloids in bufonid toads (Melanophryniscus): Temporal and geographic determinants for two Argentinian species. *Journal of Chemical Ecology* 33, 871–887.

Deblauwe, I. & Dekoninck, W. (2007). Spatio-temporal patterns of ground-dwelling ant assemblages in a lowland rainforest in southeast Cameroon. *Insectes Sociaux* 54, 343-350.

De Medeiros, D.S.S., Bego, T.B., Santos, A.P.A., Pontes, A.S., Moreira-Dill, L.S., Matos, N.B., Zuliani, J.P., Stábeli, R.G., Teles, C.B.G., Soares, A.M., Sperotto, A.R.M., Moura, D.J., Saffi, J., Caldeira, C.A.S., Pimenta, D.C. & Calderon, L.A. (2019). Biochemical and biological profile of parotoid secretion of the Amazonian *Rhinella marina* (Anura: Bufonidae). *BioMed Research International* 2019, 1-15.

Duellman, W.E. & Trueb, L. (1986). *Biology of Amphibians*. McGraw-Hill, New York.

Emlen, J.M. (1966). The Role of Time and Energy in Food Preference. *The American Naturalist* 100, 611–617.

Ferreira, R.B. & Teixeira, R.L. (2009). Feeding pattern and use of reproductive habitat of the Striped toad *Rhinella crucifer* (Anura: Bufonidae) from Southeastern Brazil. *Acta Herpetologica* 4, 125–134.

França, J.M.S. (2015). A composição do veneno do sapo-cururuzinho muda de acordo com a sua dieta? *Dissertação de mestrado*. Universidade Federal de Alfenas, Alfenas, Minas Gerais.

Gao, H., Zehl, M., Leitner, A., Wu, X., Wang, Z. & Kopp, B. (2010). Comparison of toad venoms from different *Bufo* species by HPLC and LC-DAD-MS/MS. *Journal of Ethnopharmacology* 131, 368-376.

Gao, H., Popescu, R., Kopp, B. & Wang, Z. (2011). Bufadienolides and their antitumor activity. *Natural Product Reports* 28, 953–969.

Gomes, E.C.F., Ribeiro, G.T., Souza, T.M.S. & Souza-Souto, L. (2014). Ant assemblages (Hymenoptera: Formicidae) in three different stages of forest regeneration in a fragment of Atlantic Forest in Sergipe, Brazil. *Sociobiology* 61, 250–257.

Hart, R.K., Calver, M.C. & Dickman, C.R. (2002). The index of relative importance: an alternative approach to reducing bias in descriptive studies of animal diets. *Wildlife research* 29, 415-421.

Hayes, R.A., Crossland, M.R., Hagman, M., Capon, R.J. & Shine, R. (2009). Ontogenetic variation in the chemical defenses of cane toads (*Bufo marinus*): Toxin profiles and effects on predators. *Journal of Chemical Ecology* 35, 391–399.

Hayward, M.W., Hayward, G.J., Tambling, C.J. & Kerley, G.I.H. (2011). Do lions *Panthera leo* actively select prey or do prey preferences simply reflect chance responses via evolutionary adaptations to optimal foraging? *PLoS ONE* 6, 1-6.

Hovey, K.J., Seiter, E.M., Johnson, E.E. & Saporito, R.A. (2018). Sequestered Alkaloid Defenses in the Dendrobatid Poison Frog *Oophaga pumilio* Provide Variable Protection from Microbial Pathogens. *Journal of Chemical Ecology* 44, 312–325.

Hyslop, E.J. (1980). Stomach contents analysis - a review of methods and their application. *Journal of Fish Biology* 17, 411–429.

Isacch, J.P. & Barg, M. (2002). Are bufonid toads specialized ant-feeders? A case test from the Argentinian flooding pampa. *Journal of Natural History* 36, 2005–2012.

Jacobs, J. (1974). Quantitative Measurement of Food Selection: A Modification of the Forage Ratio and Ivlev's Electivity Index. *Oecologia* 14, 413–417.

Krebs, C.J. (1999). Ecological Methodology. 2 ed. – Benjamin Cummings, California, USA.

Levings, S.C. (1983). Seasonal, Annual, and Among-site Variation in the Ground Ant Community of a Deciduous Tropical Forest: Some Causes of Patchy Species Distributions. *Ecological Monographs* 53, 435–455.

López, J.A., Scarabotti, P.A. & Ghirardi, R. (2015). Amphibian trophic ecology in increasingly human-altered wetlands. *Herpetological Conservation and Biology* 10, 819-832.

Lu, Z., Xu, A., Yuan, X., Chen, K., Wang, L. & Guo, T. (2018). Anticancer effect of resibufogenin on gastric carcinoma cells through the phosphoinositide 3-kinase/protein kinase B/glycogen synthase kinase 3β signaling pathway. *Oncology Letters* 16, 3297–3302.

Lutz, A. (1934). Notas sobre espécies Brasileiras do gênero *Bufo*. *Memórias do Instituto Oswaldo Cruz* 28, 111-148.

Mailho-Fontana, P.L., Antoniazzi, M.M., Sciani, J.M., Pimenta, D.C., Barbaro, K.C. & Jared, C. (2018). Morphological and biochemical characterization of the cutaneous poison glands in toads (*Rhinella marina* group) from different environments. *Frontiers in Zoology* 15, 1–15.

Mcelroy, M.T. & Donoso, D.A. (2019). Ant Morphology Mediates Diet Preference in a Neotropical Toad (*Rhinella alata*). *Copeia* 107: 430-438.

McGlynn, T.P., Fawcett, R.M. & Clark, D.A. (2009). Litter Biomass and Nutrient Determinants of Ant Density, Nest Size, and Growth in a Costa Rican Tropical Wet Forest. *Biotropica* 41, 234-240.

Moskowitz, N.A., Dorritie, B., Fay, T., Nieves, O.C., Vidoudez, Z., Fisher, E.K., Trauger, S.A., Coloma, L.A., Donoso, D.A. & O'Connell, L.A. (2020). Land use impacts poison frog chemical defenses through changes in leaf litter ant communities. *Neotropical Biodiversity* 6, 75-87.

Petroselli, G., Raices, M., Jungblut, L.D., Pozzi, A.G. & Erra-Balsells, R. (2018). MALDI-MS argininyl bufadienolide esters fingerprint from parotoid gland secretions of *Rhinella arenarum*: Age, gender, and seasonal variation. *Journal of Mass Spectrometry* 53, 465–475.

Pinkas, L., Oliphant, M.S. & Liverson, I.L.K. (1971). Food habits of albacore, bluefin tuna, and bonito in California waters. Calif. Dep. Fish Game, Fish. Bull., 152p..

Rosa, I., Canavero, A., Maneyro, R., Naya, D.E. & Camargo, A. (2002). Diet of four sympatric anuran species in a temperate environment. *Boletín de la Sociedad zoológica del Uruguay* 13, 12–20.

Sabagh, L.T. & Carvalho-e-Silva, A.M.P.T. (2008). Feeding overlap in two sympatric species of *Rhinella* (Anura: Bufonidae) of the Atlantic Rain Forest. *Revista Brasileira de Zoologia* 25, 247-253.

Sabagh, L.T., Carvalho-e-Silva, A.M.P.T. & Rocha, C.F.D. (2012). Diet of the toad *Rhinella icterica* (Anura: Bufonidae) from Atlantic Forest Highlands of southeastern Brazil. *Biota Neotropical* 12, 258-262.

Sales, D.L., Oliveira, O.P., Cabral, M.E.S., Dias, D.Q., Kerntopf, M.R., Coutinho, H.D.M., Costa, J.G.M., Freitas, F.R.D., Ferreira, F.S., Alves, R.R.N. & Almeida, W.O. (2015). Chemical identification and evaluation of the antimicrobial activity of fixed oil extracted from *Rhinella jimi*. *Pharmaceutical Biology* 53, 98–103.

Sales, D.L., Morais-Braga, M.F.B., Santos, A.T.L., Machado, A.J.T., Filho, J.A.A., Dias, D.Q., Cunha, F.A.B., Saraiva, R.A., Menezes, I.R.A., Coutinho, H.D.M., Costa, J.G.M., Ferreira, R.S., Alves, R.R.N. & Almeida, W.O. (2017). Antibacterial, modulatory activity of antibiotics and toxicity from *Rhinella jimi* (Stevaux, 2002) (Anura: Bufonidae) glandular secretions. *Biomedicine and Pharmacotherapy* 92, 554–561.

Saporito, R., Donnelly, M.A., Norton, R.A., Garraffo, H.M., Spande, T.F. & Daly, J.W. (2007). Oribatid mites as a major dietary source for alkaloids in poison frogs. *PNAS* 104, 8885-8890.

Savitzky, A.H., Mori, A., Hutchinson, D.A., Saporito, R.A., Burghardt, G.M., Lillywhite, H.B & Meinwald, J. (2012). Sequestered defensive toxins in tetrapod vertebrates: principles, patterns, and prospects for future studies. *Chemoecology* 22, 141–158.

Schmeda-Hirschmann, G., Quispe, C., Theoduloz, C., Sousa Jr, P.T. & Parizotto, C. (2014). Antiproliferative activity and new argininyl bufadienolide esters from the "cururú" toad *Rhinella* (*Bufo*) *schneideri*. *Journal of Ethnopharmacology* 155, 1076–1085.

Schmeda-Hirschmann, G., Quispe, C., Arana, G.V., Theoduloz, C., Urra, F.A. & Cárdenas, C. (2016). Antiproliferative activity and chemical composition of the venom from the Amazonian toad *Rhinella marina* (Anura: Bufonidae). *Toxicon* 121, 119–12.

Sciani, J.M., De-Sá-Júnior, P.L., Ferreira, A.K., Pereira, A., Antoniazzi, M.M., Jared, C. & Pimenta, D.C. (2013). Cytotoxic and antiproliferative effects of crude amphibian skin secretions on breast tumor cells. *Biomedicine and Preventive Nutrition* 3, 10–18.

Solé, M., Beckmann, O., Pelz, B., Kwet, A. & Engels, W. (2005). Stomach-flushing for diet analysis in anurans: An improved protocol evaluated in a case study in *Araucaria* forests, southern Brazil. *Studies on Neotropical Fauna and Environment* 40, 23–28.

Spiesman, B.J. & Cumming, G.S. (2008). Communities in context: The influences of multiscale environmental variation on local ant community structure. *Landscape Ecology* 23, 313–325.

Tavella, J., Alvarez Pringles, A.P. & Cagnolo, L. (2018). Determinants of ant species spatial distribution in habitats from central Argentina. *Community Ecology* 19, 300–310.

Tempone, A.G., Pimenta, D.C., Lebrun, I., Sartorelli, P., Taniwaki, N.N., Andrade Jr, H.F., Antoniazzi, M.M. & Jared. C. (2008). Antileishmanial and antitrypanosomal activity of bufadienolides isolated from the toad *Rhinella jimi* parotoid macrogland secretion. *Toxicon* 52, 13–21.

Vigerelli, H., Sciani, J.M., Jared, C., Antoniazzi, M. M., Caporale, G.M.M., Silva, A.C.R. & Pimenta, D.C. (2014). Bufotenine is able to block rabies virus infection in BHK-21 cells. *Journal of Venomous Animals and Toxins Including Tropical Diseases* 20, 1–8.

Wu, M.C., Chang, Y.W., Lu, K.H. & Yang, E.C. (2017). Gene expression changes in honey bees induced by sublethal imidacloprid exposure during the larval stage. *Insect Biochemistry and Molecular Biology* 88, 12–20.

Zhang, Y., Jin, H., Li, X., Zhao, J., Guo, X., Wang, J., Guo, Z., Zhang, X., Tao, Y., Liu, Y., Chen, D. & Liang, X. (2016). Separation and characterization of bufadienolides in toad skin using two-dimensional normal-phase liquid chromatography × reversed-phase liquid chromatography coupled with mass spectrometry. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences* 1026, 67–74.

Zhao, H., Wu, X., Wang, H., Gao, B., Yang, J., Si, N. & Bian, B. (2014). Determination of eight bufadienolides in the skin of BUFO *Bufo gargarizans cantor* and *Bufo melanostictus schneider* using HPLC coupled with triple quadrupole mass spectrometry. *Journal of Liquid Chromatography and Related Technologies* 37, 1163–1175.

Accepted: 11 January 2022

Please note that the Supplementary Material for this article is available online via the Herpetological Journal website: https://thebhs.org/publications/the-herpetological-journal/volume-32-number-2-april-2022

Herpetological Journal FULL PAPER
https://doi.org/10.33256/32.2.7079
Published by the British Herpetological Society

Reproductive ecology of the Amaral's Blind Snake *Trilepida koppesi* in an area of Cerrado in south-eastern Brazil

Rebeca Stella Khouri[2,4] Bruno Ferreto Fiorillo[1,3,5], Henrique Bartolomeu Braz[2], Jorge Henry Maciel[1], Selma Maria Almeida-Santos[2] & Marcio Martins[1]

[1]Departamento de Ecologia, Instituto de Biociências, Universidade de São Paulo, Rua do Matão, Travessa 14, 101, 05508-090 São Paulo, SP, Brazil.

[2]Laboratório de Ecologia e Evolução, Instituto Butantan, Av. Vital Brazil, 1500, 05503-900 São Paulo, SP, Brazil.

[3]Programa de Pós-Graduação em Ecologia Aplicada, Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, 13418-900 Piracicaba, São Paulo, Brazil.

[4]Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo, Rua do Matão, Travessa 14, 101, 05508-090 São Paulo, SP, Brazil.

[5]Herp Trips, 18230-000, RPPN Trápaga, São Miguel Arcanjo, São Paulo, Brazil.

Studies on reproductive biology have largely contributed to the understanding of snake ecology. However, detailed reproductive data are scarce for many groups, particularly blind snakes. Here, we describe the reproductive biology of *Trilepida koppesi* (Leptotyphlopidae), a widely distributed species in the savannas of south-central Brazil. We describe its macro- and microscopic reproductive anatomy, female reproductive cycle, potential clutch size, seasonal activity, and sexual dimorphism of a population from south-eastern Brazil. Males have plurilobulated testes. Spermiogenesis occurs in early spring (October), when gonads and kidneys show a textured surface, the sexual segment of the kidney is hypertrophied, and the *ductus deferentia* are opaque and packed with sperm. Females have only the right oviduct, which shows developed epithelium and uterine glands in spring. Mating likely occurs in spring (October–December), and females store sperm in infundibular receptacles until ovulation between late spring and early summer. Potential clutch size ranges from three to five eggs. Females grow larger than males. The synchrony between spermiogenesis and mating defines the male cycle as prenuptial, which is considered the ancestral state of Squamata. These results agree with the hypothesis of conservative parameters for the group.

Keywords: reproductive morphology, sexual dimorphism, Scolecophidia, clutch size, female sperm storage

INTRODUCTION

Studies on reproductive biology have largely contributed to the understanding of snake ecology, considering that a significant portion of snake activity is related to their reproductive cycle (Pizzatto et al., 2007a and 2007b; Nilson, 2011). However, anatomical and histological information about reproductive cycles remain scarce for several groups, particularly blind snakes (Scolecophidia) (Shea, 2001; Khouri et al., 2020). Few studies have addressed the reproductive anatomy of scolecophidians and how it is associated with their natural history (Khouri et al., 2020). Snakes usually exhibit a generalised anatomical pattern in reproductive organs, with a pair of oviducts and ovaries and a single or bifurcated vaginal pouch in females (Siegel et al., 2012), and a pair of testes, kidneys, and *ductus deferentia* in males (Trauth & Sever, 2011). However, some scolecophidians present important anatomical variations, such as the lack of the left oviduct (Fox & Dessauer, 1962; Khouri et al., 2020) and vaginal pouch (Siegel et al., 2011).

In addition to morphological traits, histological data are also helpful for understanding key traits in snake reproductive biology (Almeida-Santos et al., 2014). For instance, female sperm storage is a major feature in snake reproduction, as it allows the temporal dissociation between copulation and ovulation and, consequently, the evolution of different reproductive phenologies (Sever & Hamlett, 2002; Siegel et al., 2011). Female sperm storage has been suggested to occur in many snake species (Birkhead & Møller, 1993). However, some of these suggestions (particularly those implying long-term sperm storage) may alternatively be cases of parthenogenesis (Booth & Schuett, 2011). True female sperm storage can be reliably demonstrated using standard histological techniques. Sperm storage in infundibular receptacles has been suggested to occur in some scolecophidians (Fox & Dessauer, 1962; Siegel et al., 2011), but it has only recently been confirmed (Khouri et al., 2020).

The leptotyphlopid *Trilepida koppesi* (Amaral, 1955) is a typical species of the savannas of south-central South America (the Cerrado), where it is widely distributed (Passos et al., 2006; Nogueira et al., 2010; Nogueira et al., 2019). Although the species is known in several localities (there are 41 occurrence records in the snake distribution database of Nogueira et al., 2019), its natural history remains poorly known (Sawaya et al., 2008). This species is believed to be oviparous, with females producing a relatively small clutch size (five to seven eggs) from spring (Sawaya et al., 2008). However, there is no detailed information about its reproductive anatomy and cycle. Furthermore, the habitat of *T. koppesi* (the open vegetation types of the Cerrado) has been increasingly degraded throughout its range (Sano et al., 2010; Bonanomi et al., 2019). Thus, knowing the reproductive biology of this species may also help in planning conservation actions. Anatomical descriptions of scolecophidians are still needed (Martins et al., 2019), and this knowledge may be helpful to group or separate evolutionary lineages (Passos et al., 2006; Pinto et al., 2015). Reproductive data also show both specific and general patterns that may contribute to our understanding of the evolution of snake reproduction in general (Nilson, 2011; Almeida-Santos et al., 2014).

We hypothesise that *T. koppesi* shares traits with other scolecophidian species, such as female-biased sexual size dimorphism and seasonal reproduction. As a member of a basal clade, we also hypothesise that *T. koppesi* shows particularities that might be useful to understand the evolution of the reproductive strategies of the ancestor of snakes. To explore our hypotheses, we provide novel information on the reproductive biology of a population of *T. koppesi* in an area of Cerrado, in south-eastern Brazil. Specifically, we describe reproductive anatomy, female reproductive cycle, potential clutch size, seasonal activity, and sexual dimorphism.

Correspondence: Rebeca Stella Khouri (sk.rebeca@gmail.com)

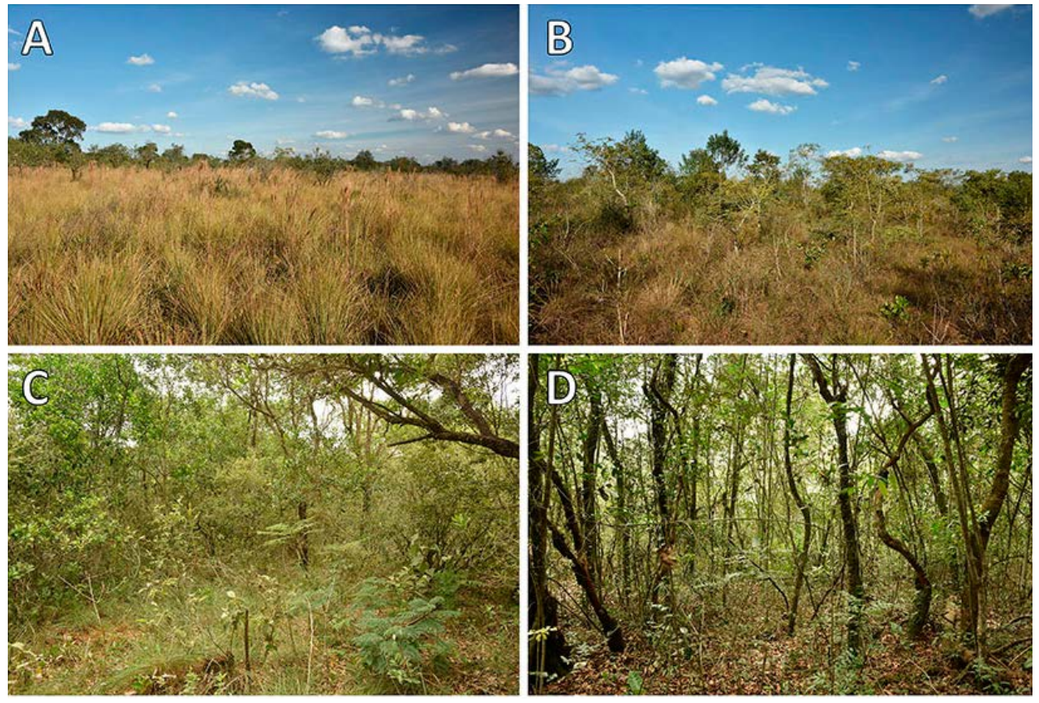

Figure 1. Natural vegetation types of Santa Bárbara Ecological Station where *T. koppesi* was sampled. **A)** *Campo sujo* (grassy scrubland). **B)** *Campo cerrado* (grassy scrubland with scattered trees). **C)** *Cerrado sensu stricto* (dense savanna). **D)** *Cerradão* (cerrado woodland).

MATERIALS & METHODS

Study Site

Snakes were collected at the Santa Bárbara Ecological Station (SBES), municipality of Águas de Santa Bárbara, state of São Paulo, south-eastern Brazil (approximate coordinates: 22°48' S, 49°13' W; 600–680 m a.s.l.). The SBES has a total area of 3,223 ha (Melo & Durigan, 2011) and contains different Cerrado vegetation types, from *campo sujo* (grassy scrubland) to *cerradão* (cerrado woodland; Fig. 1), and some small areas of semideciduous forests. The climate type is humid subtropical (Cwa in the Köppen's classification; Peel et al., 2007). Temperature averages 17 °C in the coldest months and 24 °C in the warmest months (range: 3.4 to 35.2 °C), with occasional frosts in autumn and winter. Annual rainfall varies from 1010 to 2051 mm (mean = 1454.2 mm), with marked dry and rainy seasons from autumn to winter (April to September; monthly mean = 70.2 mm) and from spring to summer (October to March; monthly mean = 172.1 mm), respectively (data for 1995-2014 at Manduri, SP, 20.3 km from our study area; CIIAGRO, http://www.ciiagro.sp.gov.br/).

Fieldwork

We conducted fieldwork monthly from August 2016 to July 2018. Snakes were collected through time-constrained searches (Campbell & Christman, 1982; Martins & Oliveira, 1998), pitfall traps with drift fences (Greenberg et al., 1994; Cechin & Martins, 2000; Mendes et al., 2015), and opportunistic encounters (i.e., all snakes found in situations other than searching activities). Time-constrained searches occurred mainly at night (18.00–23.00) in open vegetation types. We performed 1,248 person-hours of searches in different vegetation types. We installed three units of pitfall traps with drift fences in four vegetation types: *campo cerrado*, *campo sujo*, *cerrado sensu stricto* and *cerradão* (Fig. 1). Each unit of pitfall traps corresponded to two 40 m trap lines, 60 m apart. Each trap line had four 100 L plastic buckets every 10 m, connected by a 60 cm-high plastic fence. The buckets were perforated at the bottom to avoid the accumulation of rainwater. The fence was buried 10 cm into the soil and attached to wooden stakes (see Cechin & Martins, 2000; Sawaya et al., 2008). To prevent dehydration of the captured animals, we placed a styrofoam (20 × 20 × 5 cm) supported by wooden sticks and a small water plate inside each bucket to provide shelter and moisture (Sawaya et al., 2008). We installed 12 trap units (24 lines, 96 buckets, and 960 m of fences) and kept them open for ten consecutive days every month. Thus, sampling by pitfall traps occurred during 240 non-consecutive days, corresponding to 23,040 bucket-days. Snakes were collected under SISBIO (50658-1) and COTEC (SMA nº 260108–011.518/2015) scientific collection permits. Specimens were euthanised by intracelomic injection of lidocaine, fixed in 10 % formaldehyde, and preserved in 70 % ethanol for later examination. Specimens were deposited in the Museu de Zoologia da Universidade de São Paulo and the Instituto Butantan (Appendix A).

Macroscopic data

To obtain reproductive information, we dissected 24 specimens (10 females and 14 males) collected during fieldwork. The dissected females were collected in October 2016 (n = 5), December 2016 (n = 3), and February 2017 (n = 1). All dissected males were collected in October 2016. We sexed individuals by direct observation of the reproductive tract. Males were considered adults if they had enlarged testes, convoluted *ductus deferentia*, or sperm in the reproductive tract (Shine, 1977a). Females were considered adults if they had pleated oviducts, vitellogenic follicles, oviductal eggs, or sperm in the reproductive tract (Shine, 1977b). In males, we measured (1) the length, width, and thickness of the testes and the major lobule, (2) the length of the kidneys, and (3) the width of the *ductus deferentia* in the distal portion (close to the cloaca) using a Mitutoyo analogical caliper. We calculated testis volume per individual using the ellipsoid volume formula: $V = (2/3)\pi abc$, where a = length, b = width, and c = thickness (Pleguezuelos & Feriche, 1999). In females, we recorded (1) the gross morphology of the right infundibulum, glandular uterus, and nonglandular uterus, (2) the number of ovarian follicles, and (3) the diameter (at the longitudinal axis) of the largest follicle (using a Mitutoyo manual caliper; to the nearest 0.02 mm). Because we found no gravid females (see Results), we estimated clutch size by counting the number of ovarian vitellogenic follicles (Almeida-Santos et al., 2014; Braz et al., 2019). Thus, we treated this estimate as potential clutch size. To test for sexual dimorphism, we measured snout-vent length (SVL), tail length, body mass, body width, head length (from the base of the maxilla to the tip of the snout), head height (from the base of the maxilla to the top of the head). We measured SVL using a ruler (to the nearest 1 mm), body mass using a Pesola spring scale (to the nearest 0.1 g), and tail length, head length, and head height using a Mitutoyo digital caliper (to the nearest 0.01 mm).

Histological analyses

Reproductive structures were described following the nomenclature proposed by Siegel et al. (2011) and Trauth & Sever (2011). We collected samples of the right side of the reproductive tract for histological analysis. In males, we collected samples of the kidney, testis, and distal portion of the *ductus deferens*. The stage of the seminiferous tubules was classified according to Goldberg & Parker (1975). In females, we collected the oviduct and two different sized follicles to determine the vitellogenic stage. Tissue samples were processed for paraffin's standard method (Junqueira et al., 1979). Histological sections were cut at 5 µm using a Leica microtome, mounted on glass slides, and stained with hematoxylin and eosin. Histological sections were photographed and measured using Olympus Cell Sens Standard software and an Olympus BX51 microscope with a DP73 lens (Olympus Corporation, Japan). We estimated the seminiferous epithelial height, seminiferous tubule diameter, sexual segment of the kidney (SSK) epithelial height, and SSK diameter by taking five random measurements for each individual. Values for all measured variables were averaged to obtain a mean value per individual. We characterised female reproductive cycles (Mathies, 2011) and described male traits for the season in which they were collected.

Data analysis

The annual activity pattern of *T. koppesi* was inferred from the frequency of adult males, adult females, and juveniles recorded monthly. A Sexual Size Dimorphism (SSD) index was calculated as the mean SVL of the larger sex divided by the mean SVL of the smaller sex minus one. This index is conventionally expressed as positive when females are the larger sex and negative when males are larger (Lovich & Gibbons, 1992). We tested for intersexual differences in mean adult SVL with a Student's t-test. We tested for sexual dimorphism in head length, head height, tail length, body width and body mass using analysis of covariance (ANCOVA), with SVL as the covariate. For these analyses, we used only the measures from dissected individuals to avoid bias due to sexing errors in non-dissected specimens. Data are provided in supporting information Data 1, and the R script used in the analysis is provided in supporting information Script 1.

RESULTS

Seasonal activity

We found 121 individuals of *T. koppesi*. Most snakes (91 %) were found from late winter to late spring (September to December), and no snake was found between autumn and mid-winter (April to August; Fig. 2). Adult males were collected between late winter and late spring (September to December) but were more abundant in spring (97.67 %, October-December; Fig. 2). Adult females were collected between late winter and late summer (September to March) but were more abundant in spring (82.5 %, October-December; Fig. 2). Juveniles were collected between late winter and mid-summer (September-February) but were more abundant in spring (88.23 %, Fig. 2).

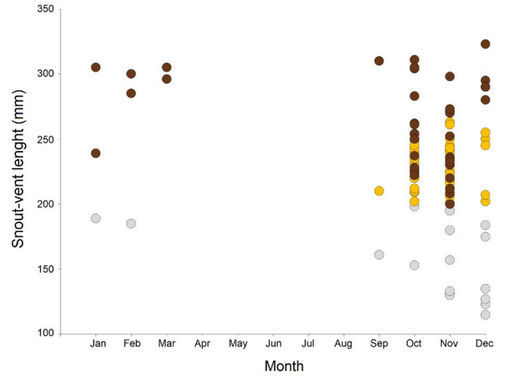

Figure 2. Temporal variation of snout-vent length of individuals of *T. koppesi* observed at the Santa Bárbara Ecological Station. Juveniles: grey circles; Females: dark brown; Males: yellow circles.

Male reproductive tract

All dissected males were collected in October (early spring). Thirteen out of fourteen dissected males were sexually mature. All males had paired reproductive structures, with the right organs located more cranially than the left ones. The single juvenile male examined (SVL = 153 mm) had undeveloped gonads and smooth kidneys. All adult males had elongated, plurilobulated testes (with 3-7 lobes, supporting information Table S1) with slightly textured surfaces (Fig. 3A). The right testis volume in adults averaged 40.53 ± 10.25 mm^3 (range = 26.89–63.47 mm^3), and the left testis averaged 40.23 ± 14.13 mm^3 (range = 4.59–71.78 mm^3). The seminiferous tubules were either in spermiogenesis (n = 3, Fig. 3C) or early regression (n = 5, Fig. 3D). Almost all individuals had testicular lobules with seminiferous tubules at the same spermatogenic stage. The exceptions were two males that showed one of the lobules smaller than the others. In these cases, the larger lobules had seminiferous tubules in early regression, while the smaller lobules had regressed seminiferous tubules (Fig. 3B). The kidneys were highly textured in ten adult males (SVL = 154–198 mm), slightly textured in two males (SVL = 231–238 mm), and smooth in one male (SVL = 254 mm). All adult males had hypertrophied SSKs with acidophilic and basophilic secretions in the lumen (Fig. 3E). The width of the right *ductus deferens* averaged 0.61 ± 0.11 mm (range = 0.48–0.90 mm). The *ductus deferentia* of all adult males were packed with sperm, which contained secretion granules with an unidentified function (Fig. 3F). The seminiferous tubule diameter, SSK diameter, and epithelial thickness were slightly larger in individuals in spermiogenesis than in early regression (supp. information Table S2). However, the diameter and epithelial thickness of the *ductus deferentia* were larger in individuals with testes in early regression (supp. information Table S2).

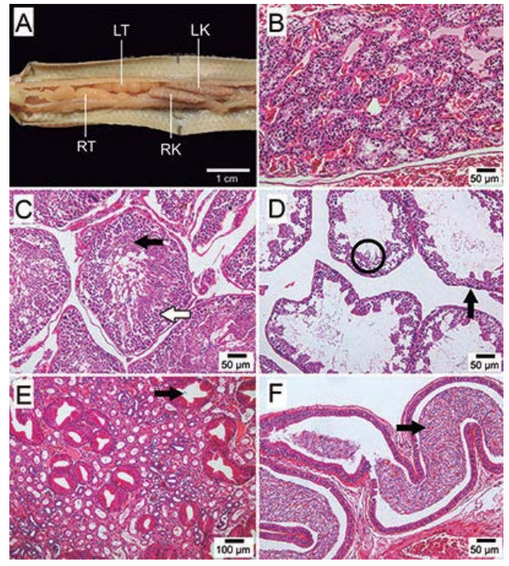

Figure 3. Reproductive anatomy and histology of male *T. koppesi* from Santa Bárbara Ecological Station. **A)** Anterior reproductive system, with plurilobulated testes; LK: left kidney, RK: right kidney, LT: left testis; RT: right testis. **B)** Regressed seminiferous tubules. **C)** Seminiferous tubules in spermiogenesis; white arrow: spermatocyte I cells; black arrow: spermatocyte II cells. **D)** Seminiferous tubules in early regression; black arrow: Sertoli cells, black circle: sperm. **E)** Hypertrophied SSK; black arrow: basophilic secretion. **F)** *Ductus deferens* with sperm; black arrow: unknown substance among the sperm.

Female reproductive tract

All females (n = 10) dissected were sexually mature; these females were collected in spring (October, n = 5; December, n = 3) and summer (February, n = 1). Data on the collection season of a vitellogenic female was missing, so the characteristics of this individual are not detailed below, although it was analysed anatomically. The female reproductive tract consisted of a pair of ovaries and a single oviduct, present only at the right side (Fig. 4A). The infundibulum was pleated and opaque

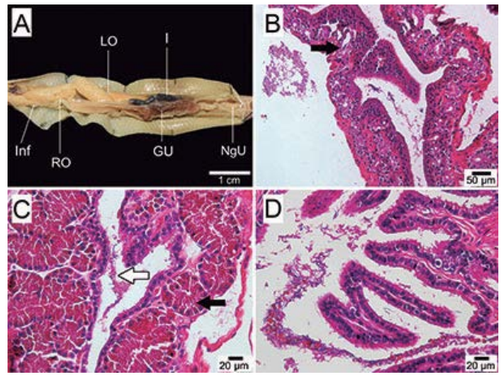

Figure 4. Reproductive anatomy and histology of female *T. koppesi* from Santa Bárbara Ecological Station. **A)** Overview of the reproductive system of a vitellogenic female; I: intestine, Inf: infundibulum, GU: glandular uterus, LO: left ovary, NgU: non-glandular uterus, RO: right ovary. **B)** Infundibulum epithelium and receptacles; black arrow: sperm stored in the receptacle. **C)** Glandular uterus; black arrow: uterine glands, white arrow: sperm. **D)** Nonglandular uterus.

in most females (n = 9) but pleated and translucent in one vitellogenic female from spring. All females (n = 8) collected in spring had infundibular sperm receptacles with highly ciliated epithelial cells (Fig. 4B). Three of these females had sperm in the receptacles; in all cases, sperm were parallelly aligned (Fig. 4B). The female collected in summer lacked infundibular sperm receptacles. The glandular uterus was pleated in the posterior region in three females from spring (December) and smooth in the remaining ones (n = 6). All females had some secretion in their glandular uterus, four of them (October = 2, December = 2) with sperm (Fig. 4C). Uterine glands were recrudescent in early spring (October, ranging from 77–118 µm), larger in late spring (December, 116–180 µm), and regressed in summer (February) (supp. information Table S3). The gross morphology and epithelium of the nonglandular uterus showed no seasonal variation, with ciliated cells in all individuals (Fig. 4D). Five females (October = 2, December = 3) had sperm in the lumen of the nonglandular uterus, and three of them with granules with similar histological staining to those found in the male's *ductus deferentia*.

The smallest follicle showing vitellogenic granules (determined by histology) measured 6.7 mm (Fig. 4A). Therefore, we considered all follicles larger than 6 mm as vitellogenic and follicles smaller than 6 mm as non-vitellogenic. Vitellogenic females collected in October (early spring) had follicles with 6.4–10.2 mm diameter, and vitellogenic females in December (late spring) had follicles with 16.5–21.6 mm diameter. Non-vitellogenic females were collected in October (follicle diameter = 4.1 and 4.9 mm; n = 1) and February (follicle diameter = 3.1–3.5 mm ; n = 1). We found no gravid female. Potential clutch size (estimated by the number of vitellogenic follicles) ranged from one to five follicles (mean = 3.9 ± 1.4 follicles; supp. information Table S1). However, most (6 out of 8) females had either four or five vitellogenic follicles.

Table 1. Sexual dimorphism in morphological traits of *T. koppesi*. Values in bold indicate significant differences at P < 0.05.

Trait	Effect	*t/F*	df	*P*
SVL (mm)	sex	5.852	21	**< 0.0001**
Tail length (mm)	slopes	0.098	1, 19	0.757
	sex	24.114	1, 20	**< 0.0001**
Head length (mm)	slopes	0.048	1, 19	0.828
	sex	0.302	1, 20	0.589
Head height (mm)	slopes	1.422	1, 19	0.248
	sex	0.014	1, 20	0.908
Body width (mm)	slopes	1.821	1, 12	0.202
	sex	1.609	1, 13	0.227
Body mass (g)	slopes	1.914	1, 19	0.183
	sex	7.256	1, 20	**0.014**

Sexual dimorphism

Adult females (n = 10) ranged from 225-311 mm SVL (285.0 ± 25.4 mm), and adult males (n = 13) ranged from 198-254 mm SVL (232.6 ± 17.6 mm). Adult females were significantly larger and heavier than adult males, but adult males had relatively longer tails than adult females (Table 1). The SSD index was 0.225. We found no sexual dimorphism in head length, head height, and body width (Table 1).

DISCUSSION

Seasonal activity

T. koppesi is endemic to the Cerrado and typical of open habitats (see Passos et al., 2006; Nogueira et al., 2010; Pinto & Curcio, 2011; Pinto & Fernandes 2012). Scolecophidian snakes are usually small and slender, which may result in low thermal inertia (Christian et al., 2006). Parpinelli & Marques (2008) suggested that the lower temperatures in autumn and winter may explain the lower detectability of *L. beui* on the surface during this period. This hypothesis may also explain the surface inactivity we observed in *T. koppesi* between autumn and winter. The higher surface activity observed in spring coincides with the timing of mating in *T. koppesi*. In many snake species, the encounter rate of individuals increases during the mating season (Pizzatto et al., 2007).

Reproductive anatomy and cycles

Our results show that male *T. koppesi* are sexually active at least in spring (October), as evidenced by

sperm production, sperm in the *ductus deferentia*, and hypertrophied SSKs. The occurrence of individuals with regressed seminiferous tubules in spring suggests that individual males show a discontinuous cycle (Mathies, 2011). Discontinuous sperm production has also been observed in at least two scolecophidians: the typhlopids *Anilios nigrescens* from Australia (Shea, 2001) and *Amerotyphlops brongersmianus* from Brazil (Khouri et al., 2020). Unfortunately, the lack of males and histological data from months other than October prevents us from inferring reproductive seasonality at the population level. Nevertheless, given the apparent inactivity on the surface and the low temperatures between mid-autumn and mid-winter (no specimen was collected between May and August), testes are likely inactive during this period. Spermatogenesis is seasonal in the typhlopids *A. nigrescens* and *A. brongersmianus*; however, sperm production peaks in summer – autumn in *A. nigrescens* and winter in *A. brongersmianus* (Shea, 2001; Khouri et al., 2020). In a Brazilian anomalepidid (*Liotyphlops beui*), Parpinelli & Marques (2015) suggested that spermatogenesis is continuous, but their suggestion lacks microscopic confirmation. Our data for *T. koppesi* suggest that the timing of spermatogenesis varies among the scolecophidians studied so far.

The presence of sperm in the lumen of the vagina and nonglandular uterus suggests recent mating (Siegel et al., 2011). Thus, our finding of sperm in these regions suggests that *T. koppesi* copulates at least in spring. The synchrony between spermiogenesis and mating defines male spermatogenesis as prenuptial (Saint-Girons, 1982; Aldridge et al., 2020), which seems to be a basal characteristic in squamates (Aldridge et al., 2020). Mating in *T. koppesi* occurs with females either in early or late vitellogenesis. Consequently, females must store sperm in their reproductive tract for a short time until ovulation, which likely occurs in late spring-early summer. In snakes, female sperm storage occurs in crypts in the nonglandular uterus, infundibular glands, or both (Siegel et al., 2011). In *T. koppesi*, we found no crypts that could serve as sperm storage receptacles. Thus, we hypothesise that the nonglandular uterus does not function for sperm storage. In contrast, we found sperm stored in infundibular glands (sperm receptacles) of various females collected in spring. Infundibular glands have been reported in leptotyphlopids and typhlopids, but their function as sperm receptacles in these scolecophidian families has been questioned because no sperm had been observed in them (Fox & Dessauer, 1962; Siegel et al., 2011). However, sperm storage in the infundibular glands was recently confirmed in the typhlopid *A. brongersmianus* (Khouri et al., 2020). Our finding of infundibular sperm storage in another scolecophidian family (Leptotyphlopidae) suggests that this feature appeared early in snake evolution.

We suggest that female reproduction in this population is seasonal, with vitellogenesis and egg-laying occurring in spring – early summer. In a geographically close population (Itirapina, ~160 km), female *T. koppesi* were hypothesised to lay eggs in late spring (December) (Sawaya et al., 2008). We found no gravid female or egg-laying to confirm this hypothesis, but we suspect that female *T. koppesi* oviposit mostly in summer (January-February). Female squamates typically retain eggs in the oviducts after ovulation and lay them with partially developed embryos (mainly at limb bud stages; Shine, 1983; Blackburn, 1995). The duration of retention varies interspecifically but may last ≥ two weeks (Andrews & Mathies, 2000). We lack data on the embryo stage at oviposition for *T. koppesi*, but other scolecophidians appear to retain eggs longer than other snakes (Erasmus & Branch, 1983; Shine & Webb, 1990; Kamosawa & Ota 1996; Sandoval et al., 2020; see also Blackburn, 1995 to compare with other snakes). In the typhlopid *Indotyphlops braminus* from Japan, oviposition occurs about a month after ovulation (Kamosawa & Ota, 1996). Since preovulatory females in our study occurred only in December (late spring), we suggest that female *T. koppesi* likely lay eggs from early to mid-summer (January – February). Female reproductive seasonality (with egg-laying concentrated in summer) also occurs in many other scolecophidians and snake groups, including tropical species (Shine & Webb, 1990; Kamosawa & Ota, 1996; Webb et al., 2000; Webb et al., 2001; Ávila et al., 2006; Mathies, 2011; Parpinelli & Marques, 2015). By laying eggs in early to mid-summer, hatchings are likely to occur between mid-summer and early autumn, based on the incubation period (30–70 days) in other scolecophidians (Sandoval et al., 2020).

We suggest that the textured kidney found in *T. koppesi* reflects SSK development (Figs. 3A and 3E) and can be considered an indicator of sexual maturity. This is because the SSK development is related to male sexual activity and mating (Aldridge et al., 2011). The relationship between the SSK development and the textured aspect of the kidneys was also observed in the typhlopid *A. brongersmianus* (Khouri et al., 2020). The semen shows granules both in the *ductus deferentia* and the oviducts. These granules exhibit an acidophilic aspect in other squamates (Burtner et al., 1965; Sever & Hopkins, 2005). However, histochemical studies have yet to be conducted to clarify the function of these secretions in scolecophidians, although they have a glycoprotein and mucoprotein nature in other snake groups (e.g., Rojas et al., 2013; Silva et al., 2020). Our finding that female *T. koppesi* show only the right oviduct agrees with previous observations in other scolecophidians (Fox & Dessauer, 1962).

Individuals of *T. koppesi* were found mostly in spring (Fig. 2), when most individuals are sexually active. In this period, there is a higher search activity for mates by males, which would increase the encounter rate (Pizzatto et al., 2007b). In spring, male and female *T. koppesi* also have more food content (M. Martins & B.F. Fiorillo, unpublished data), which may indicate that the snakes are optimising their foraging to store body fat and minimising exposure to predators, as seen in other Scolecophidians (Saint-Girons, 1982; Webb et al., 2000). The seasonal activity pattern observed here (Fig. 2) is similar to that observed in another conspecific population (Sawaya et al., 2008), where the species was most active on the surface in spring.

Sexual maturity, sexual dimorphism, and clutch size

As in other scolecophidians, males reach sexual maturity at smaller body sizes than females (Shine & Webb, 1990; Webb et al., 2000; Webb et al., 2001; Parpinelli & Marques, 2015). Scolecophidian species with mean SVL similar to *T. koppesi* (males = 220 mm, females = 250 mm) achieve sexual maturity at smaller SVLs than we found for our study species (smallest adult male = 198 mm, smallest adult female = 225 mm). Shine & Webb (1990) found that males and females of *Anilios affinis* reach sexual maturity with 172 and 206 mm SVL, respectively (average SVL in males = 206 mm; females = 252 mm), and Ávila et al. (2006) found that male and female *A. brongersmianus* reach sexual maturity with 180 and 211 mm SVL, respectively (average SVL in males = 227.2 mm; females = 241.9 mm). This may indicate that *T. koppesi* mature at larger SVLs than other scolecophidians or that our sampling was insufficient to capture the variation in body size at maturity in this population.

Female *T. koppesi* were larger and heavier than males, as in other scolecophidians (Webb et al., 2001; Cox et al., 2007) and alethinophidians (Shine, 1994). Sexual size dimorphism is often attributed to sexual and natural selection (Shine, 1994). The female-biased SSD index of *T. koppesi* (0.225) resembles those reported in many snake species lacking male combat. Indeed, such behaviour has never been reported in scolecophidians (Shine, 1978, 1994; Shine & Webb, 1990; Webb et al., 2000; Parpinelli & Marques, 2015). In the absence of male combat, female-biased SSD is attributed to selection for increased fecundity because larger females tend to produce larger clutches (Shine, 1994). Unfortunately, we could not test for such a relationship in this population of *T. koppesi*, but clutch size increases with maternal body size in many scolecophidians with female-biased SSD (Shine & Webb, 1990; Webb et al., 2001).

Longer tails in males are a common feature in snakes, including scolecophidians (Shine & Webb, 1990; Webb et al., 2000; Parpinelli & Marques, 2015), and are usually attributed to the presence of hemipenes and associated muscles (King, 1989; Shine et al., 1999). However, this condition may also be advantageous in tail wrestling during courtship (King, 1989; Shine et al., 1999). There is no record of reproductive aggregation in *T. koppesi*, but such behaviour has been reported in some scolecophidians (McCoy, 1960; Shine & Webb, 1990; S. M. Almeida-Santos, unpublished data). Thus, male scolecophidians may also benefit from larger tails in reproductive aggregations. The lack of sexual dimorphism in head size agrees with results for other scolecophidians (Webb et al., 2000). In snakes, sexual dimorphism in head size may reflect sex divergences in dietary niche (Shine, 1993). Thus, similarity in head size between the sexes may reflect the consumption of similar-sized food items.

Clutch size of *T. koppesi* was estimated by counting vitellogenic follicles (3-5) and is smaller than what was reported for a nearby conspecific population (5-7 eggs; Sawaya et al., 2008) and other similar-sized scolecophidians such as the typhlopids *A. affinis* (mean SVL = 252 mm) and *A. brongersmianus* (mean SVL = 241 mm) (3 and 4-5 eggs, respectively; Shine & Webb, 1990; Ávila et al., 2006). Inferring clutch size from vitellogenic follicles may sometimes overestimate clutch size because not all the vitellogenic follicles may be ovulated (Almeida-Santos et al., 2014). Nevertheless, our clutch size estimate is still smaller than that reported by Sawaya et al. (2008). Small clutches are common in small-sized snakes (including scolecophidians) and likely reflect space constraints within the female's body since clutch size tends to be correlated with body size (Shine & Webb, 1990; Webb et al., 2000). Small clutches also seem to be characteristic of several unrelated fossorial snakes (e.g., Marques & Puorto, 1998; Balestrin & Di-Bernardo, 2005; Braz et al., 2014; Braz et al., 2019).

Here, we show that female *T. koppesi* reproduce seasonally, with ovulation and egg-laying occurring in the warmer seasons, as observed in other basally split snakes. We also show that females lack the left oviduct, like other scolecophidians. The patterns of sexual dimorphism observed here may help to understand other aspects of scolecophidian reproductive ecology, such as breeding aggregations. Although male and female *T. koppesi* are reproductive in spring (when most individuals are active on the surface), we found some reproductive asynchrony between the sexes. Therefore, females store sperm in infundibular glands, a strategy rarely reported in scolecophidians. Thus, our study shows how scolecophidians still need to be studied, and the novelties we found can help to understand several other aspects of the biology and evolution of this group.

ACKNOWLEDGEMENTS

We thank Giordano Novak Rossi for suggestions on earlier versions of the manuscript. Fieldwork was conducted with the help of Ana L.M.R. Santos, Ana Paula Carmignoto, Gabriel Sampaio, Gabriel Sonoda, Carolina Farhat, Carlos Abrahão, Claudio Marino, Gabriella Leal, Giovana Felício, Jairo Roldan, John U. Rosas, Nathany Biela, Paula Rocha, Rafael C. Menegucci, Rafaela Pereira, Ricardo Santa Maria, Solimary García-Hernandez, Vinícius Gabriel, and Paula Rocha. We thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for research grants (#2013/50741-7, #2013/50741-7, #2015/21259-8, #2018/14091-1 and #2020/12658-4). MM and SMA-S thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for research fellowships (#306961/2015-6 and #310357/2018-7; respectively).

Authors' contribution

Marcio Martins (MM) and Bruno Ferreto Fiorillo (BFF) planned the study; BFF and Jorge Henry Maciel collected the specimens and field data, BFF and Henrique Bartolomeu Braz (HBB) analysed the data; Rebeca Stella Khouri (RSK) collected reproductive data, interpreted histological slides, led the writing of the manuscript. BFF, HBB, MM, RSK, and Selma Maria Almeida-Santos contributed writing to the manuscript. All authors approved the final version of this manuscript for publication.

Ethical statement

Snakes were collected under SISBIO (50658-1) and COTEC (SMA nº 260108–011.518/2015) scientific collection permits.

REFERENCES

Aldridge, R.D., Jellen, B.C., Siegel, D.S. & Wisniewski, S.S. (2011). The Sexual Segment of the Kidney. In: *Reproductive biology and phylogeny of snakes*, Aldridge, R. D. & Sever D. M., Eds. CRC Press. Boca Raton, USA, p. 477-509.

Aldridge, R.D., Siegel, D.S., Goldberg, S.R. & Pyron, R.A. (2020). Seasonal timing of spermatogenesis and mating in squamates: a reinterpretation. *Copeia* 108, 231-264. Doi:10.1643/CH-19-230.

Almeida-Santos, S.M., Braz, H.B., Santos, L.C., Sueiro, L.R., Barros, V.A., Rojas, C.A. & Kasperoviczus, K.N. (2014). Biologia reprodutiva de serpentes: recomendações para a coleta e análise de dados. *Herpetologia Brasileira* 3, 14-24.

Amaral, A. (1955). Contribuição ao conhecimento dos ofídios neotrópicos: Descrição de duas espécies novas de cobra-cega (fam. Leptotyphlopidae). *Memórias do Instituto Butantan* 26, 203-205.

Andrews, R.M. & Mathies, T. (2000). Natural history of reptilian development: constraints on the evolution of viviparity. *Bioscience* 50, 227-238. Doi:10.1641/0006-3568(2000)050[0227:NHORDC]2.3.CO;2.

Ávila, R.W., Ferreira, V.L. & Souza, V.B. (2006). Biology of the blindsnake *Typhlops brongersmianus* (Typhlopidae) in a semideciduous forest from central Brazil. *The Herpetological Journal* 16, 403-405.

Balestrin, R.L. & Di-Bernardo, M. (2005). Reproductive biology of *Atractus reticulatus* (Boulenger, 1885) (Serpentes, Colubridae) in southern Brazil. *The Herpetological Journal* 15, 195-199.

Birkhead, T.R. & Møller, A.P. (1993). Sexual selection and the temporal separation of reproductive events: sperm storage data from reptiles, birds and mammals. *Biological Journal of the Linnean Society* 50, 295-311. Doi:10.1111/j.1095-8312.1993.tb00933.x.

Blackburn, D.G. (1995). Saltationist and punctuated equilibrium models for the evolution of viviparity and placentation. *Journal of Theoretical Biology* 174, 199-216. Doi:10.1006/jtbi.1995.0092.

Bonanomi, J., Tortato, F.R., Santos, R., Penha, J.M., Bueno, A.S. & Peres, C.A. (2019). Protecting forests at the expense of native grasslands: Land-use policy encourages open-habitat loss in the Brazilian Cerrado biome. *Perspectives in Ecology and Conservation* 17, 26-31. Doi:10.1016/j.pecon.2018.12.002.

Booth, W. & Schuett, G.W. (2011). Molecular genetic evidence for alternative reproductive strategies in North American pitvipers (Serpentes: Viperidae). Long-term sperm storage and facultative parthenogenesis. *Biological Journal of the Linnean Society* 104, 934-942. Doi:10.1111/j.1095-8312.2011.01782.x.

Braz, H.B., Kasperoviczus, K.N. & Almeida-Santos, S.M. (2014). Reproductive ecology and diet of the fossorial snake *Phalotris lativittatus* in the Brazilian Cerrado. *The Herpetological Journal* 24, 49-57.

Braz, H.B., Kasperoviczus, K.N. & Guedes, T.B. (2019). Reproductive biology of the fossorial snake *Apostolepis gaboi* (Elapomorphini): a threatened and poorly known species from the Caatinga region. *South American Journal of Herpetology* 14, 37-47. Doi:10.2994/SAJH-D-17-00116.1.

Burtner, H.J., Floyd, A.D. & Longley, J.B. (1965). Histochemistry of the "sexual segment" granules of the male rattlesnake kidney. *Journal of Morphology* 116, 189-195. Doi:10.1002/jmor.1051160204.

Campbell, H.W. & Christman, S.P. (1982) Field techniques for herpetofaunal community analysis. In: *Herpetological Communities: A Symposium of the Society for the Study of Amphibians and Reptiles and the Herpetologists' League* Scott Jr., N.J., Ed. U.S. Fish & Wildlife Service Wildlife Research Report 13. Washington, USA, p. 193-200.

Cechin, S.Z. & Martins, M. (2000). Eficiência de armadilhas de queda (pitfall traps) em amostragens de anfíbios e répteis no Brasil. *Revista Brasileira de Zoologia* 17, 729-740.

Christian, K.A., Tracy, C.R. & Tracy, C.R. (2006). Evaluating thermoregulation in reptiles: an appropriate null model. *The American Naturalist* 168, 421–430. Doi: 10.1086/506528.

Cox, R.M., Butler, M.A. & John-Alder, H.B. (2007). The evolution of sexual size dimorphism in reptiles. In: *Sex, Size and Gender Roles: Evolutionary Studies of Sexual Size Dimorphism*. Fairbairn, D.J., Blanckenhorn, W.U. & Szekely, T., Eds., Oxford University Press. New York, USA, p. 38–49.

Erasmus, H. & Branch, W.R. (1983). Egg retention in the South African blind snake *Typhlops bibronii*. *Journal of Herpetology* 17, 97-99. Doi:10.2307/1563794.

Fox, W. & Dessauer, H.C. (1962). The single right oviduct and other urogenital structures of female *Typhlops* and *Leptotyphlops*. *Copeia* 1962, 590-597. Doi: 10.2307/1441184.

Goldberg, S.R. & Parker, W.S. (1975). Seasonal testicular histology of the colubrid snakes, *Masticophis taeniatus* and *Pituophis melanoleucus*. *Herpetologica* 31, 317-322.

Greenberg, C.H., Neary, D.G. & Harris, L.D. (1994). A comparison of herpetofaunal sampling effectiveness of pitfall single-ended and double ended funnel traps used with drift fences. *Journal of Herpetology* 28, 319-324. Doi:10.2307/1564530.

Junqueira, L.C.U., Bignolas, G. & Brentani, R.R. (1979). Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *The Histochemical Journal* 11, 447-455. Doi: 10.1007/BF01002772.

Kamosawa, M. & Ota, H. (1996). Reproductive biology of the brahminy blind snake (*Ramphotyphlops braminus*) from the Ryukyu archipelago, Japan. *Journal of Herpetology* 30, 9-14. Doi:10.2307/1564700.

Khouri, R.S., Almeida-Santos, S.M. & Fernandes, D.S. (2020). Anatomy of the reproductive system of a population of *Amerotyphlops brongersmianus* from southeastern Brazil (Serpentes: Scolecophidia). *Anatomical Records* 303, 2485-2496. Doi: 10.1002/ar.24382.

King, R.B. (1989). Sexual dimorphism in snake tail length: sexual selection, natural selection, or morphological constraint? *Biological Journal of the Linnean Society* 38, 133-154. Doi: 10.1111/j.1095-8312.1989.tb01570.x.

Lovich, J. E. & Gibbons, J.W. (1992). A review of techniques for quantifying sexual size dimorphism. *Growth, Development and Aging* 56, 269-281.

Martins, M. & Oliveira, M.E. (1998). Natural history of snakes in forests of the Manaus region Central Amazonia Brazil. *Herpetological Natural History* 6, 78-150.

Martins, A., Koch, C., Pinto, R., Folly, M., Fouquet, A. & Passos, P. (2019). From the inside out: Discovery of a new genus of threadsnakes based on anatomical and molecular data, with discussion of the leptotyphlopid hemipenial morphology. *Journal of Zoological Systematics and Evolutionary Research* 57, 840-863. https://Doi.org/10.1111/jzs.12316.

Marques, O.A. & Puorto, G. (1998). Feeding, reproduction and growth in the crowned snake *Tantilla melanocephala* (Colubridae), from southeastern Brazil. *Amphibia-Reptilia* 19, 311-318. Doi:10.1163/156853898X00214.

Mathies, T. (2011). Reproductive Cycles of Tropical Snakes. In: *Reproductive biology and phylogeny of snakes*. Aldridge, R. D. & Sever D.M., Eds. CRC Press. Boca Raton, USA, p. 511-550.

McCoy, C.J. (1960). An unusually large aggregation of Leptotyphlops. *Copeia* 1960, 368.

Melo, A.C.G. & Durigan G. (2011). Plano de Manejo da Estação Ecológica de Santa Bárbara. Instituto Florestal, Secretaria do Meio Ambiente de São Paulo. Downloaded on 12 December 2020. https://smastr16.blob.core.windows.net/iflorestal/2013/03/Plano_de_Manejo_EEc_Santa_Barbara.pdf.

Mendes, D.M., Leão, R.F. & Toledo, L.F. (2015). Drift fences in traps: theoretical evidence of effectiveness of the two most common arrays applied to terrestrial tetrapods. *Natureza e Conservação* 13, 60-66. Doi:10.1016/j.ncon.2015.05.002.

Nilson, G. (2011). History of reproductive studies on snakes. *Reproductive Biology and Phylogeny of Snakes* p. 1-17, R.D. Aldridge, R.D. & Sever, D.M., Eds., USA, CRC Press.

Nogueira, C., Colli, G.R., Costa, G.C. & Machado, R.B. (2010). Diversidade de répteis Squamata e evolução do conhecimento faunístico no Cerrado. In: *Cerrado: Conhecimento Científico Quantitativo como Subsídio para Ações de Conservação*. Diniz, I.R., Marinho-Filho, J., Machado, B.R., Cavalcanti, R.B., Eds., Editora UnB. Brasília, Brasil, p. 333-375.

Nogueira C., Argôlo, A.J.S., Arzamendia, V., Azevedo, J.A., Barbo, F.E., Bérnils, R.S., Bolochio, B.E., Borges-Martins, M., Brasil-Godinho, M., Braz, H., Buononato, M.A., Cisneros-Heredia, D.F., Colli, G.R., Costa, H.C., Franco, F.L., Giraudo, A., Gonzalez, R.C., Guedes, T., Hoogmoed, M.S., Marques, O.A.V., Montigelli, G.G., Passos, P., Prudente, A.L.C., Rivas, G.A., Sanchez, P.M., Serrano, F.C., Silva, N.J., Strüssmann, C., Vieira-Alencar, J.P.S., Zaher, H., Sawaya, R.J. & Martins, M. (2019). Atlas of Brazilian Snakes: Verified Point-Locality Maps to Mitigate the Wallacean Shortfall in a Megadiverse Snake Fauna. *South American Journal of Herpetology* 14, 1-274. Doi:10.2994/SAJH-D-19-00120.1.

Passos, P., Caramaschi, U. & Pinto, R.R. (2006). Redescription of *Leptotyphlops koppesi Amaral*, 1954, and description of a new species of the *Leptotyphlops dulcis* group from Central Brazil (Serpentes: Leptotyphlopidae). *Amphibia-Reptilia* 27, 347-357. Doi:10.1163/156853806778190006.

Parpinelli, L. & Marques, O.A. (2008). Seasonal and daily activity in the Pale-headed Blindsnake *Liotyphlops beui* (Serpentes: Anomalepidae) in southeastern Brazil. *South American Journal of Herpetology* 3, 207-212. Doi:10.2994/1808-9798-3.3.207

Parpinelli, L. & Marques, O.A.V. (2015). Reproductive biology and food habits of the Blindsnake *Liotyphlops beui* (Scolecophidia: Anomalepididae). *South American Journal of Herpetology* 10, 205-210. Doi:10.2994/SAJH-D-15-00013.1.

Peel, M.C., Finlayson, B.L. & McMahon, T.A. (2007). Updated world map of the Köppen-Geiger climate classification. *Hydrology and Earth System Sciences* 11, 1633–1644. Doi:10.5194/hess-11-1633-2007.

Pinto, R.R. & F.F. Curcio (2011). On the Generic Identity of *Siagonodon brasiliensis*, with the Description of a New Leptotyphlopid from Central Brazil (Serpentes: Leptotyphlopidae). *Copeia* 1, 53–63. Doi:10.1643/CH-09-119.

Pinto, R.R. & Fernandes, R. (2012). A new blind snake species of the genus *Tricheilostoma* from Espinhaço Range, Brazil and taxonomic status of Rena dimidiata (Jan, 1861) (Serpentes: Epictinae: Leptotyphlopidae). *Copeia* 2012, 37-48. Doi:10.1643/CH-11-040.

Pinto, R.R., Martins, A.R., Curcio, F. & Ramos, L.D.O. (2015). Osteology and cartilaginous elements of *Trilepida salgueiroi* (Amaral, 1954) (Scolecophidia: Leptotyphlopidae). *The Anatomical Record* 298, 1722-1747. Doi:10.1002/ar.23191.

Pizzatto, L., Almeida-Santos, S.M., Marques, O.A.V., Nascimento, L. & Oliveira, M. (2007a). *Biologia reprodutiva de serpentes brasileiras.* In*: Herpetologia no Brasil II*. Nascimento, L.B., Oliveira, M.E., Eds., Sociedade Brasileira de Herpetologia. Belo Horizonte, Brasil, p. 201-221.

Pizzatto, L., Cantor, M., De Oliveira, J.L., Marques, O.A.V., Capovilla, V. & Martins, M. (2007b). Reproductive ecology of dipsadine snakes, with emphasis on South American species. *Herpetologica* 64, 168-179. Doi:10.1655/07-031.1.

Pleguezuelos, J.M. & Feriche, M. (1999). Reproductive ecology of the horseshoe whip snake (*Coluber hippocrepis*) in the Iberian Peninsula. *Journal of Herpetology* 33, 202-207. Doi:10.2307/1565715.

Rojas, C.A., Barros, V.A. & Almeida-Santos, S.M. (2013). The Reproductive Cycle of the Male Sleep Snake *Sibynomorphus mikanii* (Schlegel, 1837) From Southeastern Brazil. *Journal of Morphology* 274, 215–228. Doi:10.1002/jmor.20099.

Saint-Girons, H. (1982). Reproductive cycles of male snakes and their relationships with climate and female reproductive cycles. *Herpetologica* 38, 5-16.

Sandoval, M.T., Ruiz García, J.A. & Álvarez, B.B. (2020). Intrauterine and post-ovipositional embryonic development of *Amerotyphlops brongersmianus* (Vanzolini, 1976) (Serpentes: Typhlopidae) from northeastern Argentina. *Journal of Morphology* 281, 523-535. Doi:10.1002/jmor.21119.

Sano, E.E., Rosa, R., Brito, J.L.S. & Ferreira, L.G. (2010). Land cover mapping of the tropical savanna region in Brazil. *Environmental Monitoring and Assessment* 166, 113-124. Doi:10.1007/s10661-009-0988-4.

Sawaya, R.J., Marques, O.A.V. & Martins, M. (2008). Composition and natural history of a Cerrado snake assemblage at Itirapina, São Paulo State, southeastern Brazil. *Biota Neotropica* 8, 127-149. Doi:10.1590/S1676-06032008000200015.

Sever, D.M. & Hamlett, W.C. (2002). Female sperm storage in reptiles. *Journal of Experimental Zoology* 292, 187-199. Doi:10.1002/jez.1154.

Sever, D.M. & Hopkins, W.A. (2005). Renal sexual segment of the ground skink, *Scincella laterale* (Reptilia, Squamata, Scincidae). *Journal of Morphology* 266, 46-59. Doi:10.1002/jmor.10364.

Shea, G.M. (2001). Spermatogenic cycle, sperm storage, and Sertoli cell size in a Scolecophidian (*Ramphotyphlops nigrescens*) from Australia. *Journal of Herpetology* 35, 85-91. Doi:10.2307/1566027.

Shine, R. (1977a). Reproduction in Australian elapid snakes I. Testicular cycles and mating seasons. *Australian Journal of Zoology* 25, 647–653. Doi:10.1071/ZO9770647.

Shine, R. (1977b). Reproduction in Australian elapid snakes II. Female reproductive cycles. *Australian Journal of Zoology* 25, 655–666. Doi:10.1071/ZO9770655.

Shine, R. (1978). Sexual size dimorphism and male combat in snakes. *Oecologia* 33, 269-277. Doi:10.1007/BF00348113.

Shine, R. (1983). Reptilian reproductive modes: the oviparity-viviparity continuum. *Herpetologica* 1-8.

Shine, R. & Webb, J.K. (1990). Natural history of Australian typhlopid snakes. *Journal of Herpetology* 24, 357-363. Doi:10.2307/1565050.

Shine, R. (1993). Sexual dimorphism in snakes. In: *Snakes: Ecology and Behavior*. Seigel, R.A., Collins, J.T., Eds., McGraw-Hill. New York, USA, p. 49–86.

Shine, R. (1994). Sexual size dimorphism in snakes revisited. *Copeia* 1994: 326-346. Doi:10.2307/1446982.

Shine, R., Olsson, M.M., Moore, I.T., LeMaster, M.P. & Mason, R.T. (1999). Why do males have longer tails than females? *Proceedings of the Royal Society of London* 266, 2147–2151. Doi:10.1098/rspb.1999.0901.

Siegel, D.S., Miralles, A., Chabarria, R.E. & Aldridge, R.D. (2011). Female reproductive anatomy: cloaca, oviduct, and sperm storage. In: *Reproductive biology and phylogeny of snakes*. Aldridge, R. D., & Sever D. M., Eds. CRC Press. Boca Raton, USA, p. 347-409.

Siegel, D.S., Miralles, A., Trauth, S.E. & Aldridge, R.D. (2012). The phylogenetic distribution and morphological variation of the 'pouch' in female snakes. *Acta Zoologica* 93, 400-408. Doi: 10.1111/j.1463-6395.2011.00514.x.

Silva, K.M., Braz, H.B., Kasperoviczus, K.N., Marques, O.A. & Almeida-Santos, S.M. (2020). Reproduction in the pitviper *Bothrops jararacussu*: Large females increase their reproductive output while small males increase their potential to mate. *Zoology* 142, 125816. Doi:10.1016/j.zool.2020.125816.

Trauth, S.E. & Sever, D.M. (2011). Male Urogenital Ducts and Cloacal Anatomy. In: *Reproductive biology and phylogeny of snakes*. Aldridge, R.D. & Sever D.M., Eds. CRC Press. Boca Raton, USA, p. 416-480.

Webb, J.K., Shine, R., Branch, W.R. & Harlow, P.S. (2000). Life-history strategies in basal snakes: reproduction and dietary habits of the African thread snake *Leptotyphlops scutifrons* (Serpentes: Leptotyphlopidae). *Journal of Zoology* 250, 321-327. Doi:10.1111/j.1469-7998.2000.tb00776.x.

Webb, J.K., Branch, W.R. & Shine, R. (2001). Dietary habits and reproductive biology of Typhlopid snakes from Southern Africa. *Journal of Herpetology* 35, 558–567. Doi:10.2307/1565893.

APPENDIX I

Voucher specimens of *Trilepida koppesi* analysed in this study (n = 24; field numbers): MRCM290, MRCM292, MRCM294, MRCM307, MRCM308, MRCM309, MRCM310, MRCM311, MRCM312, MRCM313, MRCM314, MRCM318, MRCM320, MRCM321, MRCM323, MRCM327, MRCM328, MRCM340, MRCM355, MRCM381, MRCM382, MRCM383, MRCM457 and MRCM546.

Accepted: 27 February 2022

Herpetological Journal FULL PAPER
https://doi.org/10.33256/32.2.8084
Published by the British Herpetological Society

Flashy male Jamaican anoles *Anolis grahami* show accelerated telomere attrition

Luiza F Passos[1], Gerardo Garcia[2] & Robert Young[3]

[1]School of Biological and Environmental Sciences, James Parsons Building, Liverpool John Moores University, Liverpool, L3 3AF.

[2]Chester Zoo, Cedar House, Caughall Road, Upton by Chester, Chester CH2 1LH

[3]School of Environment and Life Sciences, Peel Building, University of Salford Manchester, Salford, M5 4WT

Secondary sexual traits have evolved through sexual selection, many species have developed signals that can indicate their level of other fitness-relevant traits such as fight ability. Previous studies have shown that male sexual signals are honest signals about quality in an intrasexual context, demonstrating a direct relationship between the signal's design and the fighting ability of its possessor. However, signals can be costly since conspicuous signals are more likely to attract predators or be energetically expensive. Here we have analysed if dewlap size and colouration were reliable signs of a male's bite force, and the physiological costs associated with larger dewlaps and intense colouration in Jamaican anoles (*Anolis grahami*). We analysed dewlap size and colouration against bite force, and telomere attrition. Our results supported the hypothesis that dewlap size and colour intensity are honest predictors of an individual's fighting potential as indicated by bite force. However, we have also found a relationship between colour intensity with higher telomere attrition rates, thereby indicating a possible cost of this trait for the individual.

Keywords: Bite force, Dewlap, Telomere, Sexual selection

INTRODUCTION

Secondary sexual traits have evolved through sexual selection, by female preference, inter-male competition or, in some cases, both (Zahavi, 1975; Berglund et al., 1996; Lailvaux & Irschick, 2007). In this context, many species have developed signals that can also indicate the level of other fitness-relevant traits such as fight ability (Emlen, 2008; Putman et al., 2018). Males can express this information in the form of colouration or ornamentation. Conspecific males interpret such signals to evaluate possible competitors, and females use this information to evaluate potential mates (Berglund et al., 1996). Traits that honestly signal fighting ability are advantageous as they can predict contest outcomes and, thus, males can avoid unwinnable physical combats and the costs associated with them (Andersson, 1994).

To establish whether a signal is reliable, the trait should be evaluated as to whether the size/shape or colouration of a secondary sexual character is predictive of ecologically relevant performance abilities (Perry et al., 2004). Different studies demonstrate that male secondary sexual signals express reliable information, demonstrating a direct relationship between the signal's design (mainly size and colour) and the fighting ability of its possessor expressed as bite force (e.g. Jennions & Backwell, 1996; Lailvaux & Irschick. 2007).

Signals inherent involve costs, more conspicuous signals (or more time devoted to signalling) are more likely to attract predators or be energetically costly to develop (Engqvist et al., 2015). One long-standing hypothesis about secondary sexual signals suggests that their honesty or reliability is related to how costly they are to produce and maintain (Lailvaux et al., 2012). An example of this is carotenoid-based colours (i.e. yellow/red spectrum), which are appropriate for honest signalling due to the costs related to pigment acquisition and the trade-offs between energetic allocation in ornaments against other metabolic processes such protection against oxidative DNA damage (de Lanuza et al., 2014).

The genus *Anolis* is characterised by having an extendible throat fan called a dewlap. The dewlap is a versatile signal structure being used in different contexts as a fundamental part of sexual/territorial display behaviours (Vanhooydonck et al., 2005). The dewlap extension is used as a threat or challenge to other males and predators (Jenssen et al., 2001) and to attract potential mates. Females show preference for males with certain dewlap characteristics and are more receptive to these males performing dewlap extensions (Greenberg & Noble, 1944; Crews, 1975). Studies have evaluated the relationship between dewlap size and fighting capacity (i.e. bite force) in anoles species with mixed results depending on the species (Vanhooydonck et al., 2005),

Correspondence: Luiza F. Passos (l.figueiredopassos@ljmu.ac.uk)

level of sexual dimorphism (Lailvaux & Irschick, 2007), territoriality (Vanhooydonck et al., 2005) and level of within-population competition (Baeckens et al., 2018).

Here we aimed to analyse if dewlap size and dewlap colouration were reliable signs of a male's bite force and if there is any physiological cost associated with these signals (using telomere attrition) in captive Jamaican anoles (*Anolis grahami*, Gray 1845). Consistent with previous studies, we expected dewlap size to be a good predictor of bite force, more importantly we predicted that there would be a biological cost associated with more intense coloured dewlaps.

METHODS

Subjects

During this experiment 10 adult males, of unknown age, were used for data collection. All animals were hand caught in Nonsuch Island, Bermuda and transported by air to Chester Zoo, under licence 16-07-05-46, after clearance from a veterinary surgeon. *A. grahami*, despite being an invasive species in Bermuda is the most observed lizard on the island (Bacon et al., 2011). Subject animals weighed on average 10.20±2.12 g and had a snout-ventral length of 6.65±0.30 cm and all individuals in this experiment exhibited breeding behaviour over the course of this study.

All the experimental methods described here were approved by the Chester Zoo's Ethics Committee, UK and conform to all regulations and laws in all relevant countries in relation to care of experimental animal subjects. Additionally, we can confirm, from our post-experimental monitoring that no animals suffered any injuries, became ill or had their survivorship negatively affected as a result of this study.

Lizard housing

Lizards were kept as a group of one male and two females in ExoTerra 60 cm x 45 cm x 90 cm screen terrariums inside an isolated and temperature-controlled room at Chester Zoo, UK. A 12 hour photo period was maintained with an average temperature of 30° C during the day and 24° C at night. Temperature and humidity (around 60 %) were monitored with a thermometer/hygrometer. Each terrarium was supplied with a basking lamp, soil substrate, and a potted plant. The terraria were sprayed daily with water, and lizards were fed live crickets 3 times a week. The side of the terrariums were covered with black plastic between adjacent terrariums to avoid visual contact between different lizard groups.

Dewlap area

To obtain a reliable measure of dewlap size, lizards were positioned sideways side against 1 cm^2 grid paper and the base of the second ceratobranchial was carefully pulled forward with a pair of forceps until completely extended (Fig. 1). Before taking a digital picture, animals were placed in such a manner that the extended dewlap was parallel to the lens of the camera (Canon PowerShot SX520HS digital camera). All measurements were made in an identical manner using the same settings on the camera. We

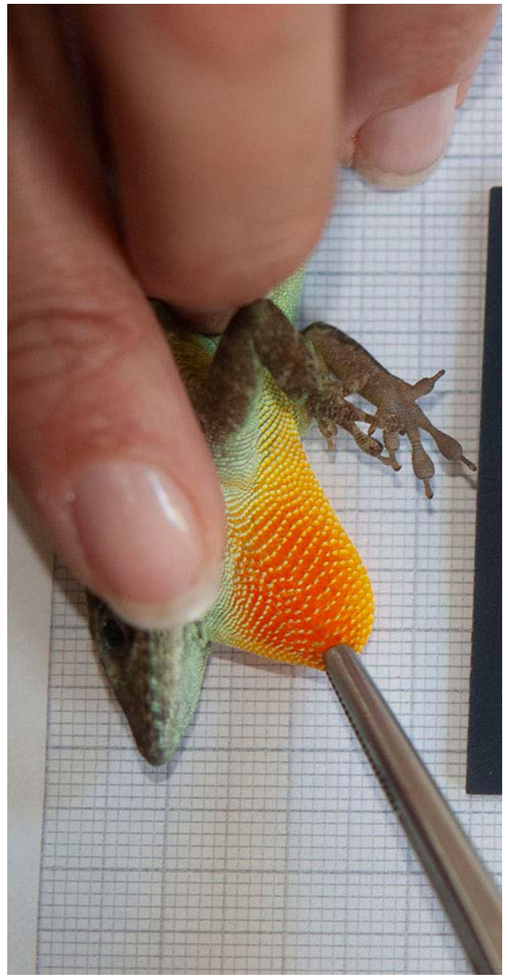

Figure 1. Jamaican anole (*Anolis grahami*) having its dewlap fully extended with the use of forceps for total area measurement.

calculated the total dewlap area for each individual using ImageJ software (Schneider et al., 2012).

Dewlap colouration

We used a USB-2000 portable diode-array spectrometer and a PX-2 xenon strobe light source (both from Ocean Optics, Dunedin, USA) to perform spectrophotometric measurements. Spectral analyses were conducted in the 300 and 700 nm range. Spectral reflectance measurements were always taken of each male from the centre of the dewlap, three measurements per lizard. Spectralon white standard measurements were taken between each individual to account for lamp drift. We calculated the colorimetric parameters using the Pavo package (Maia et al, 2013) for R studio (R Studio Team, 2015): brightness (mean reflectance across 320–700 nm), hue (wavelength corresponding to [{max reflectance−min reflectance}/2]), and red chroma (sum of reflectance from 605–700 divided by brightness).

Bite force

We induced the lizards to bite a force transducer by tapping them gently on the side of the mouth, this method resulted in a characteristic threat response where the jaws are opened maximally. Lizards were held by the researcher for immobilisation and then encouraged to bite a force sensitive resistor connected to force a transducer and charge amplifier (Kistler Inc., Winterthur, Switzerland). When the free end of the holder (i.e. bite plates) was placed between the jaws of the animal, prolonged and repeated biting resulted. The force resistor placement on the lizards' mouth was standardised for all animals. For more detailed descriptions of setup and biting procedure, see Herrel et al. (1999). The bite force of all lizards were measured five times and only the maximum value was used for analysis.

Telomere erosion

To analyse telomere erosion, animals were swabbed using a buccal swab twice over a six months interval. Swabs were placed in tubes with dry capsules for stabilisation until laboratory analysis. After a DNA extraction using a DNeasy Blood and Tissue Kit (Qiagen, Australia), a real-time quantitative PCR (qPCR) allowed the measurement of telomere length. Telomere primers to used were: Telb1 (5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3') and Telb2 (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT -3'). The 18S ribosomal RNA (18S) gene (92 bp in *Anolis*) was selected as the reference gene as it had previously been successfully used in a reptile (Plot et al., 2012). The primer sequences used were 18 S-F (5'- GAGGTGAAATTCTTGGACCGG -3') and 18 S-R (5'- CGAACCTCCGACTTTCGTTCT -3'). The qPCR was performed in a final volume of 20 µl for both telomeres and 18S. 10 ng of DNA was used per reaction, and the primers at a concentration of 250 nM. Reactions were run in triplicate for each sample. The measurement of the relative telomere length was calculated by using an adaptation of the qPCR method described by performed following protocol described by Giraudeau et al. (2016) and Dutra et al. (2020). The relative telomere length calculation consisted of the ratio of telomere repeat copy (T) to the reference gene (S; 18S) copy number (S); T/S.

Statistical analysis

Prior to statistical analyses, data on bite force was $\log_{10}$ transformed to meet the assumptions of normality and homoscedascity (Shapiro-Wilk's test with W≥ 0.95). To remove the influence of body size on the variables, we used analyses of covariance (ANCOVAs) with SVL as covariate. To test if dewlap size and colour were a good predictor of a male's bite force and telomere attrition we used ANCOVA with size as a covariate. We used RStudio version 1.2.5033 (R Studio Team, 2015) for all statistical analyses.

RESULTS

The ANCOVA analysis showed that dewlap size was positively significant for bite force ($F_{1,8}$=5.6, p=0.05). The dewlap colouration analysis was positively significant for brightness against bite force ($F_{1,8}$=10.6, p=0.01), and telomere attrition ($F_{1,8}$=7.17, p=0.03). Yellow chroma ($F_{1,8}$=8.09, p=0.02), red chroma ($F_{1,8}$=6.26, p=0.03), and hue ($F_{1,8}$=5.67, p=0.04) were positively significant for telomere attrition. All other analyses did not result in statistically significant relationships.

DISCUSSION

Our results show that dewlap size and colour intensity were honest predictors of an individual's bite force. Our analysis also demonstrated that intense dewlap colouration is associated with higher telomere attrition rates, thereby indicating a possible cost of this trait for the individual.

A. grahami are highly territorial lizards, with males defending their territories with visual displays, including dewlap extensions (Vanhooydonck et al., 2005). The reliability of such display could avoid the costly interactions physical combats may impose through bites during male-male competition (Andersson, 1994). Our results agree with previous studies showing that dewlap size is a reliable sign of combat performance, as measured by bite force. Previous studies have shown a relationship between bite force and combat success, especially in *Anolis* species with high sexual dimorphism (Lailvaux & Irschick, 2007), such as *A. grahami*. This study reinforces the idea that dewlaps, among other signals, can act as a reliable indicator of combat ability to rival individuals; thereby, avoiding potentially physically damaging fights (Lailvaux & Irschick, 2007).

The red colouration observed on dewlap of adult male *A. grahami* is associated with carotenoids (Macedonia et al., 2000) which are commonly obtained thought diet and they are an important antioxidant defence against reactive oxygen radicals (McGraw, 2005; Freeman-Gallant et al., 2011). Pigment allocation to increase brightness of colourful ornaments could reduce the availability of carotenoids for use as antioxidants and, thereby, could increase DNA damage (McGraw & Ardia, 2003), leading to telomere attrition. A pioneering study on Australian painted dragons (*Ctenophoruspictus*) demonstrated this trade-off between telomere attrition and colour maintenance for signalling in a lizard species (Giraudeau et al., 2016), similar evidence has also been found in birds (Taff & Freeman-Gallant, 2017). Our results support these findings suggesting that the maintenance of carotenoid-based colouration could lead to telomere shortening.

A central idea of life-history theory is that investment in present reproduction reduces survivorship and, consequently, future reproduction (Cox & Calsbeek 2010; Cox et al., 2010). The foundation for this hypothesis is that organisms that prioritise breeding over cellular maintenance (such as antioxidant production) often age quicker, having shorter life spans and higher rates of telomere attrition (Ricklefs & Wikelski, 2002). Physiological explanations for the trade-off are grounded in the notion that these two components compete for

limited energy and nutrients (Zera & Harshman, 2001). Laboratory and field manipulations have demonstrated this prediction with respect to different physiological processes including DNA repair and resistance to oxidative stress using different species, including *Anolis* lizards (Cox et al., 2010). Our results support this hypothesis with male anoles investing in reproduction now at the cost of accelerated telomere attrition. Male anoles, by investing in an intense dewlap colouration, increase their chances of reproduction, as it has been shown that females have a preference for intense coloured dewlaps (Sigmund, 1983). If telomere attrition is, in fact, associated with increased senescence in anole lizards, then maintaining dewlap coloration with the associated benefits in terms of reproductive success but at the expense of longevity may be favoured by increasing reproductive output. Within a polygynous mating system, males can maximise reproductive success by monopolising females through male-male contests such as dewlap displays (Orrell & Jenssen, 2002). The breeding strategy *Anolis* consists of females laying multiple clutches of single eggs during a prolonged season (Cox & Calsbeek, 2010), giving males the opportunity to sire multiple batches of offspring in one breeding season.

In summary our results support previous studies showing that dewlap size and colouration are honest signals of bite force, but that these come with physiological costs. More studies using free-ranging animals and larger sample sizes are necessary to better understand this trade-off, including additional measurements such as circulating carotenoids and reactive oxygen radicals to fully understand the link between carotenoid-based colouration and telomere attrition (i.e., longevity).

ACKNOWLEDGEMENTS

The authors are grateful to Chester Zoo, Government of Bermuda, specially Mark Outerbridge, for the chance to develop this project at their facilities and for the logistical support. LFP was in receipt of a CAPES postgraduate scholarship Proc. 6060-13-7. The authors have no conflicts of interest to declare that are relevant to the content of this article.

Availability of data and material

Data is available Doi:10.5281/zenodo.6341613

Authors contribution

LFP, GG and RJY contributed to research design. LFP and GG contributed to data collection. LFP and RJY have contributed with analysis and writing. GG has reviewed manuscript.

REFERENCES

Andersson, M. (1994). Sexual Selection. Princeton, New Jersey: Princeton University Press.

Bacon, J.P., Gray, J.A. & Kitson, L. (2011). Status and conservation of the reptiles and amphibians of the Bermuda Islands. *Conservation of Caribbean Island Herpetofaunas*, 1, 161–182. https://doi.org/10.1163/ej.9789004183957.i-228

Baeckens, S., Driessens, T., Huyghe, K., Vanhooydonck, B. & Van Damme, R. (2018). Intraspecific variation in the information content of an ornament: Why relative dewlap size signals bite force in some, but not all island populations of *Anolis sagrei*. *Integrative and Comparative Biology* 58(1), 25–37. https://doi.org/10.1093/icb/icy012

Berglund, A., Bisazza, A. & Pilastro, A. (1996). Armaments and ornaments: an evolutionary explanation of traits of dual utility. *Biological Journal of the Linnean Society* 58, 385–399.

Cox, R.M. & Calsbeek, R. (2010). Severe costs of reproduction persist in *Anolis* lizards despite the evolution of a single-egg clutch. *Evolution* 64(5), 1321–1330. https://doi.org/10.1111/j.1558-5646.2009.00906.x

Cox, R.M., Parker, E.U., Cheney, D.M., Liebl, A.L., Martin, L.B. & Calsbeek, R. (2010). Experimental evidence for physiological costs underlying the trade-off between reproduction and survival. *Functional Ecology* 24(6), 1262–1269. https://doi.org/10.1111/j.1365-2435.2010.01756.x

Crews, D. (1975). Effects of different components of male courtship behaviour on environmentally induced ovarian recrudescence and mating preferences in the lizard, *Anolis carolinensis*. *Animal Behavior* 23, 349–356.

De Lanuza, G.P.I, Carazo, P. & Font, E. (2014). Colours of quality: structural (but not pigment) coloration informs about male quality in a polychromatic lizard. *Animal Behaviour* 90, 73–81. https://doi.org/10.1016/j.anbehav.2014.01.01

Dutra,L., Souza, F.S., Friberg, I.M., Araújo, M., Vasconcellos, A.S & Young, R.Y. (2020). Validating the use of oral swabs for telomere length assessment in dogs. *Journal of Veterinary Behaviour* 40, 16-20.

Emlen, D.J. (2008). The evolution of animal weapons. Annual Review of Ecology, *Evolution and Systematics* 39,387–413.

Engqvist, L., Cordes, N. & Reinhold, K. (2015). Evolution of risk-taking during conspicuous mating displays. *Evolution* 69(2), 395–406. https://doi.org/10.1111/evo.12591

Freeman-Gallant, C.R., Amidon, J., Berdy, B., Wein, S., Taff, C.C. & Haussmann, M.F. (2011). Oxidative damage to DNA related to survivorship and carotenoid-based sexual ornamentation in the common yellowthroat. *Biology Letters* 7(3), 429–432. https://doi.org/10.1098/rsbl.2010.1186

Giraudeau, M., Friesen, C.R., Sudyka, J., Rollings, N., Whittington, C.M., Wilson, M.R. & Olsson, M. (2016). Ageing and the cost of maintaining coloration in the Australian painted dragon. *Biology Letters* 12(7), 734–738. https://doi.org/10.1098/rsbl.2016.0077

Gray, J.E. (1845). Catalogue of the Specimens of Lizards in the Collection of the British Museum. *London: Trustees of the British Museum*.

Greenberg, B. & Noble, G.K. (1944). Social behaviour of the American chameleon (*Anolis carolinensis* Voigt). *Physiological Zoology* 17, 392–439.

Herrel, A., Aerts, P., Fret, J. & De Vree, F. (1999). Morphology of the feeding system in agamid lizards: ecological correlates. *The Anatomical Record* 254, 496–507.

Jennions, M.D. & Backwell, P.R.Y. (1996). Residency and size affect fight duration and outcome in the fiddler crab *Ucaannulipes*. *Biological Journal of the Linnean Society* 57, 293–306.

Jenssen, T.A., Lovern, M.B. & Congdon, J.D. (2001). Field-testing the protandry-based mating system for the lizard, *Anolis carolinesis*: Does the model organism have the right model? *Behavioral Ecology and Sociobiology* 50(2), 162–172. https://doi.org/10.1007/s002650100349

Lailvaux, S.P. & Irschick, D.J. (2007) The evolution of performance-based male fighting ability in Caribbean *Anolis* lizards. *The American Naturalist* 170, 573-586.

Lailvaux, S.P., Gilbert, R.L. & Edwards, J.R. (2012). A performance-based cost to honest signalling in male green anole lizards (*Anolis carolinensis*). *Proceedings of the Royal Society B: Biological Sciences* 279(1739), 2841–2848. https://doi.org/10.1098/rspb.2011.2577

Maia, R., Eliason, C.M., Bitton, P.P., Doucet, S.M. & Shawkey, M.D. (2013). pavo: An R package for the analysis, visualization and organization of spectral data. *Methods in Ecology and Evolution* 4(10), 906–913. https://doi.org/10.1111/2041-210X.12069

Macedonia, J.M., James, S., Wittle, L.W. & Clark, D.L. (2000). Skin Pigments and Coloration in the Jamaican Radiation of *Anolis* Lizards. *Journal of Herpetology* 34(1), 99–109.

Mcgraw, K.J. (2005). The antioxidant function of many animal pigments: Are there consistent health benefits of sexually selected colourants? *Animal Behaviour* 69(4), 757–764. https://doi.org/10.1016/j.anbehav.2004.06.022

McGraw, K.J. & Ardia, D.R. (2003). Carotenoids, Immunocompetence, and the Information Content of Sexual Colors: An Experimental Test. *American Naturalist* 162(6), 704–712. https://doi.org/10.1086/378904

Orrell, K.S. & Jenssen, T.A. (2002). Male mate choice by the lizard *Anolis carolinensis*: A preference for novel females. *Animal Behaviour* 63(6), 1091–1102. https://doi.org/10.1006/anbe.2002.3013

Perry, G., Levering, K., Girard, I. & Garland, T. Jr. (2004). Locomotor performance and dominance in male *Anolis cristatellus*. *Animal Behaviour* 67, 37–47.

Plot, V., Criscuolo, F., Zahn, S. & Georges, J.Y. (2012). Telomeres, age and reproduction in a long-lived reptile. *PLoS ONE* 7(7), 1–6. https://doi.org/10.1371/journal.pone.0040855

Putman, B.J., Azure, K.R. & Swierk, L. (2018). Dewlap size in male water anoles associates with consistent inter-individual variation in boldness. *Current Zoology* 65(2), 189–195. https://doi.org/10.1093/cz/zoy041

R Studio Team. (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA. http://www.rstudio.com

Ricklefs, R.E. & Wikelski, M. (2002). The physiology/life-history nexus. *Trends in Ecology & Evolution* 17, 462–468.

Schneider, C.A., Rasband, W.S. & Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* 9, 671-675.

Sigmund, W.R. (1983). Female preference for *Anolis carolinensis* males as a function of dewlap color and background coloration. *Journal of Herpetology* 17(2), 137–143. https://doi.org/10.2307/1563454

Taff, C.C. & Freeman-Gallant, C.R. (2017). Sexual signals reflect telomere dynamics in a wild bird. *Ecology and Evolution* 7(10), 3436–3442. https://doi.org/10.1002/ece3.2948

Vanhooydonck, B., Herrel, A.Y., Van Damme, R. & Irschick, D.J. (2005). Does dewlap size predict male bite performance in Jamaican *Anolis* lizards? *Functional Ecology* 19(1), 38–42. https://doi.org/10.1111/j.0269-8463.2005.00940.x

Zahavi, A. (1975). Mate selection - a selection for a handicap. *Journal of Theoretical Biology* 53:205–214.

Zera, A.J. & Harshman, L.G. (2001). The physiology of life history trade-offs in animals. *Annual Review of Ecology, Evolution, and Systematics* 32, 95–12

Accepted: 10 March 2022

Herpetological Journal FULL PAPER

https://doi.org/10.33256/32.2.8592

Published by the British Herpetological Society

Embryonic morphology in two species of the *Physalaemus signifer* clade (Anura: Leptodactylidae)

Marianna Isabella Rosa Rodrigues De Oliveira[1], Jimena Grosso[2,3,4], Marcelo Felgueiras Napoli[1], Luiz Norberto Weber[1,5] & Florencia Vera Candioti[2]

[1]Universidade Federal da Bahia, Instituto de Biologia, Programa de Pós-Graduação em Biodiversidade e Evolução, Campus Universitário de Ondina, Rua Barão de Jeremoabo, s/n, Ondina, Salvador, Bahia, Brazil 40170-115.

[2]Unidad Ejecutora Lillo – CONICET-FML, San Miguel de Tucumán, Argentina.

[3]Instituto de Ciencias Marinas y Limnológicas, Universidad Austral de Chile, Valdivia, Chile

[4]Centro de Humedales Río Cruces (UACh), Valdivia, Chile.

[5]Universidade Federal do Sul da Bahia, Centro de Formação em Ciências Ambientais, BR 367 Rodovia Porto Seguro-Eunápolis, km 10, Porto Seguro, Bahia, Brazil 45810-000.

We studied the embryonic morphology of *Physalaemus camacan* and *P. signifer*, two small foam-nesting frogs endemic to the Atlantic Forest. We analysed the development of transient embryonic structures and of the larval oral disc. These embryos have features typical of most congeneric species, such as the kyphotic dorsal curvature, three pairs of gills and the configuration of hatching and adhesive glands. Main differences regarding embryos of the *P. cuvieri* clade are the larger size and yolk provision at tailbud stage, less developed external gills and an apparently novel pattern of oral marginal papilla ontogeny. While some shifts could be correlated with variant modes of oviposition, others appear to be developmental modifications not related with ecomorphological aspects.

Keywords: adhesive glands, external gills, hatching gland, Leiuperinae, oral disc.

INTRODUCTION

The Neotropical genus *Physalaemus* (Fitzinger, 1826) currently includes 50 species (Frost, 2021) grouped into two major clades (*sensu* Lourenço et al., 2015): *P. signifer* clade (with *P. nattereri* and phenetic species groups of *P. signifer* and *P. deimaticus*) and *P. cuvieri* clade (with *P. aguirrei*, *P. cicada* and the species groups of *P. biligonigerus*, *P. cuvieri*, *P. gracilis*, *P. henselii* and *P. olfersii*). These species reproduce in a wide variety of environments, like rainforests to seasonal habitats (Cei, 1980; Heyer et al., 1990), and have a broad geographic distribution across northern and central Argentina, eastern Bolivia, Paraguay, Uruguay, Brazil, the Guianas, lowlands of southern Venezuela, and llanos of south-eastern Colombia (Frost, 2021). Species of *Physalaemus* generally deposit eggs in foam nests and tadpoles develop in puddles (Lynch, 1971). The foam nests are interpreted as an adaptation to environments with sparse rainfall, high temperature, and intense solar radiation, as they are suggested to provide protection for these and many other factors (Heyer, 1969; Duellman & Trueb, 1986; Méndez-Narváez et al., 2015).

Frogs of the *P. signifer* clade are endemic to the Atlantic Forest and breed in or close to small puddles inside the forest (Pupin et al., 2010). While most species in the genus build foam nests on the water surface, several species of this clade inhabiting forested environments exhibit a tendency toward terrestrial reproduction (Pupin et al., 2010). This has been reported in *P. atlanticus*, *P. bokermanni*, *P. caete*, *P. crombiei*, *P. erythros*, *P. signifer* and *P. spiniger*, which may build their foam nests directly on the humid forest floor, tree holes, axils of bromeliads, or between leaves on the floor (reviewed in Pupin et al., 2010, 2018). *P. signifer* is distributed in the Brazilian States of Bahia, Espírito Santo, Rio de Janeiro and São Paulo; these frogs build foam nests on the forest floor near small water-bodies (Wogel et al., 2002). *P. camacan* has apparently a more restricted distribution, and it is only reported in localities of Bahia; the species is known to reproduce in small shallow ponds inside forest patches (Pimenta et al., 2005). During field-work in breeding areas, we found vocalising males and foam nests among leaves on humid soil, making this the first report of terrestrial nests for this species.

The early ontogeny in frogs has acquired a renewed interest, since comparative studies have shown the wide morphological and heterochronic variation potentially informative for interpretations about species diversification and evolution (e.g., Nokhbatolgfoghahai et al., 2005; Vera Candioti et al., 2016). In *Physalaemus*, the embryonic morphology has been explored comparatively in previous studies. Vera Candioti et al. (2011) studied the development of the oral disc, and later, Grosso et al. (2019) studied the embryonic morphology and heterochronic development

Correspondence: Marianna Isabella Rosa Rodrigues De Oliveira (oliveira.rmi@gmail.com)

in twelve species of the genus. These studies included only species from the *P. cuvieri* clade with oviposition in aquatic foam nests; therefore, information on the *P. signifer* clade is needed to complete the comparative panorama of the genus. In this study, we explore the early ontogeny in *P. camacan* and *P. signifer*, two species representative of the *P. signifer* clade with terrestrial foam nests. We describe morphological and developmental diversity in embryonic and larval characters, compare with information available for species of the *P. cuvieri* clade, and discuss our results in the context of early development in aquatic and terrestrial environments.

MATERIALS & METHODS

We analysed embryonic series of *P. camacan* and *P. signifer*, obtained from clutches and from amplectant adults collected in the field. Embryos are deposited in the amphibian collection of the Universidade Federal do Sul da Bahia (UFSB517, *P. camacan*: municipality of Itabuna, Campus from the Universidade Estadual de Santa Cruz – UESC, State of Bahia, Brazil, 14°47'46.7" S, 39°10'19.7" W; UFSB515 and UFSB516, *P. signifer*: municipality of Porto Seguro, State of Bahia, Brazil, 16°23'19.2" S, 39°10'07.5" W). In all cases foam nests were found on humid soil and placed among leaves. Vocalising males and amplectant pairs of each species were found near the nests in both locations, and no other congeneric frogs were active at that moment. Additionally, some embryos were reared until older larval stages to confirm species identity. Clutches were moved and maintained in containers with puddle water and under natural conditions of light and temperature. The specimen manipulation was carried out following the recommendations in the Guidelines for Ethical Conduct in the CEUA-UFBA protocol (43/2017). Embryos were euthanised every 6–8 h by immersion in water with lidocaine, and then preserved in 8 % formalin. We focused on the period between tailbud stage and emergence of hind limbs and complete development of the oral disc (from Stage 17 to 18 to 26; Gosner, 1960: here abbreviated as GS). We studied a total of 98 embryos of *P. signifer* (two clutches) and 49 embryos of *P. camacan* (one clutch); while the ontogenetic series of *P. signifer* is complete, the series of *P. camacan* unfortunately lacks the earliest stages (the first available embryos already had the operculum differentiated at the gill base). Specimens were examined and photographed with a stereomicroscope Leica EZ4E. Methylene blue solution was used to contrast structures such as gills, adhesive glands, and oral papillae (Wassersug, 1976). Additionally, nine embryos of *P. signifer* were dehydrated using serial dilutions of ethanol and coated with gold to perform scanning with a Zeiss Supra 55VP electron microscope at Centro Integral de Microscopía Electrónica–CIME– (CONICET, Tucumán). The images were obtained mainly from a ventral view because of the arrangement of most morphological structures. The characterisation of transient embryonic structures follows Nokhbatolfoghahai & Downie (2005, 2007, 2008) and Nokhbatolfoghahai et al. (2005). The oral disc development was described following Thibaudeau & Altig (1988) and Vera Candioti et al. (2011). The definitive configuration of the oral disc was determined by comparison with the original tadpole description (e.g., Weber & Carvalho-e-Silva, 2001; Pimenta et al., 2005). In addition, we followed Grosso et al. (2019) to register the embryo body length and area, yolk area and the extent of dorsal curvature at tailbud stage (this latter measured in lateral view, as the angle subtended by the body from a dorsal midpoint), and the length of the primary filament of first gill pair as an indicative of gill development. Measurements were taken from photographs in lateral view, using the Leica Application Suite software (V4.4.0) and areas were estimated using the software Image J.

RESULTS

The following description represents the main developmental changes in early development for both focused species and are mostly based on *P. signifer* (Figs. 1-5 and 7B); differences regarding *P. camacan* (Figs. 6 and 7A) were highlighted whenever necessary. Gosner (1960) stages are estimated from embryo general aspect and consigned when possible. Measurements are summarised in Table 1; to facilitate intrageneric comparisons, values from Grosso et al. (2019) for species in the *P. cuvieri* clade were included in Table 1.

At tailbud stage (GS17-18; Fig. 1A), embryos are about 2 mm long, unpigmented, and markedly kyphotic (average dorsal curvature ca. 97°), curved over a large subspherical yolk mass that occupies ca. 50 % of the body surface area. Later, the first gill pair buds are visible on both sides of the cephalic region (GS19; Fig. 1B). Type-C adhesive glands (*sensu* Nokhbatolfoghahai et al., 2005) differentiate and are visible as two bumps posterolateral to the stomodeum. The second pair of gills differentiate (GS19-20; Fig. 1C) and both pairs start to branch (GS20-21; Fig. 1D,E). The hatching gland is evident as revealed by ultrastructural analysis in *P. signifer*. Hatching cells are arranged in a T-shaped area, frontally and along a long dorsal line (Fig. 2A); individual cells are scattered in a discontinuous patch (Fig. 2B) and show rather short microvilli (Fig. 2C). When the tail reaches the body length, a short third gill pair develops (Fig. 2A). At this point, a slight pigmentation appears, with the first melanophores occurring dorsally on the cephalic region and the proximal tail. Gills at full development reach only the first third of the body length (Fig. 3A). Gills are branched and ciliated; the first pair is the longest and branches into 6-8 (*P. signifer*; Fig. 3A,B) or 5 (*P. camacan*; Fig. 6A,E) filaments, with the primary filament being the longest (0.43 and 0.55 mm in both species, respectively). The second pair has 5-6 (*P. signifer*) or 4-5 (*P. camacan*) filaments, and the third pair remains very short, non or scarcely branched, and almost covered by the operculum margin. Adhesive glands are conical, prominent, with small secretory cells arranged at the central region (Fig. 3A). As development progress, the operculum differentiates at the gill base (GS22-23; Figs. 1F,G, 3A and 6A), later fuse medially (Figs. 1H, 3B, and C) (before gills reach full development in *P. camacan*; Fig. 6B), and gills begin regression. The right gill is concealed

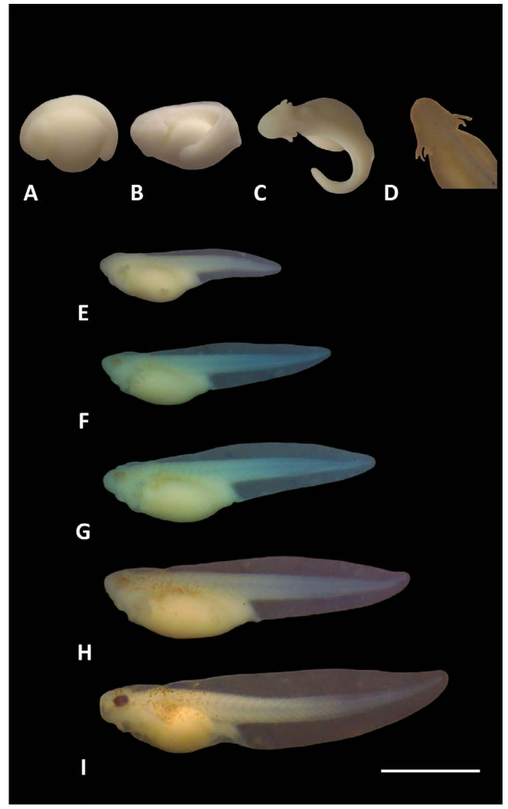

Figure 1. Developmental series of *Physalaemus signifer*. **(A)** Embryo at tailbud stage. **(B)** Differentiation of the first gill pair. **(C)** Differentiation of the second gill pair. **(D–E)** First and second gill pairs branched. **(F)** Operculum at the gill base. **(G)** Gills at full development. **(H)** Operculum medially fused. **(I)** Right gill concealed. Scale bar = 2 mm.

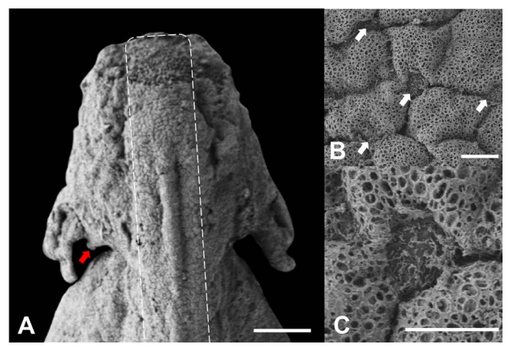

Figure 2. Hatching gland in embryos of *Physalaemus signifer*. **(A)** Dorsal view showing gland arrangement (dotted area). **(B)** Distribution of secretory (white arrows) and epidermal cells. **(C)** Individual secretory cell with microvilli. Note the short third gill pair almost covered by the developing operculum (red arrow). Scale bars = 200 µm **(A)** and 5 µm **(B, C)**.

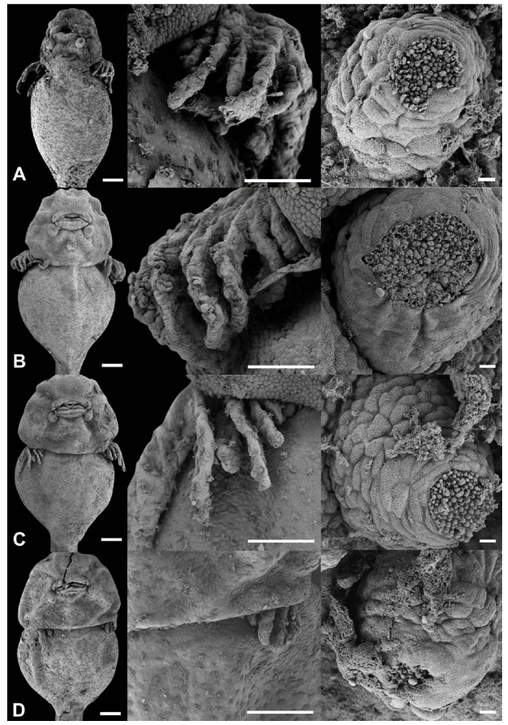

Figure 3. External gill and adhesive gland development in *Physalaemus signifer*. **(A)** Embryo with operculum at the gill base, showing details of the left gill and left adhesive gland. **(B)** Embryo with operculum medially fused, and details of right gill and left gland. **(C)** Embryo with operculum medially fused and gills starting regression, and details of right gill and right gland. **(D)** Concealment of the right gill, and details of left gill and left gland. Scale bars = 200 µm (left column), 100 µm (middle column) and 10 µm (right column).

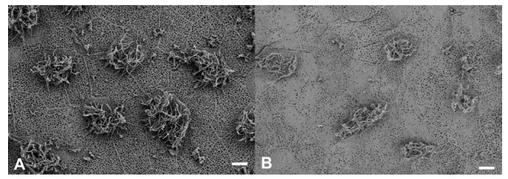

Figure 4. Body ciliation in embryos of *Physalaemus signifer*. **(A)** Maximum density of ciliated cells in the abdominal region of a specimen with operculum at the gill base. **(B)** Ciliation regressing from right gill concealment. Scale bars = 5 µm.

by the operculum (GS24; Figs. 1I and 3D), and finally the left gill regresses and the spiracle is formed (GS25; Fig. 6C,D). Limb buds differentiate before (*P. signifer*) or shortly after (*P. camacan*) the complete formation of the spiracle. Adhesive glands become less prominent throughout this lapse, and the secretory region starts to regress concomitant with the right gill regression (Fig. 3D). Body

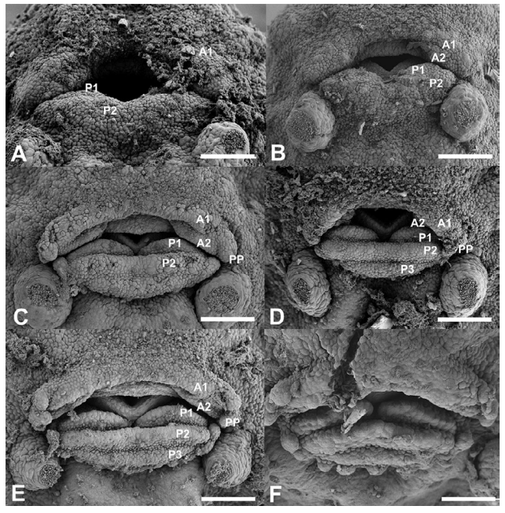

Figure 5. Development of the oral disc in *Physalaemus signifer*. **(A)** Embryo with fully developed gills, showing rows A1, P1 and incipient P2. **(B)** Embryo with operculum at the gill base showing differentiated row A2. **(C)** Embryo with operculum medially fused and developing marginal papillae (PP). **(D)** Embryo with operculum medially fused and row P3. **(E)** Embryo with regressing gills and marginal papillae progressing medially. **(F)** Embryo with right gill concealed and complete marginal papillae. Scale bars = 100 µm.

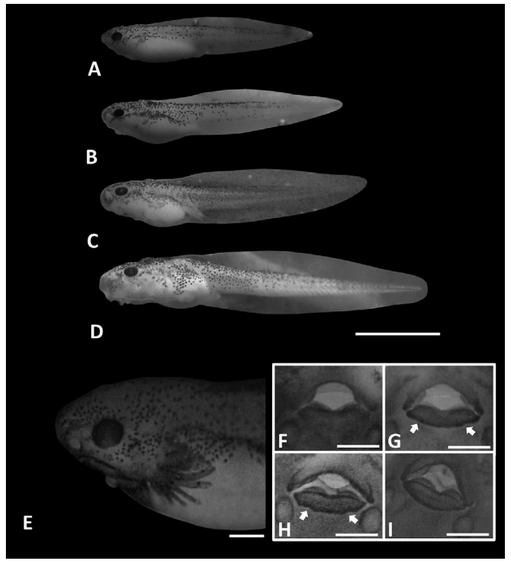

Figure 6. Developmental series of *Physalaemus camacan*. **(A)** Embryo with operculum at the gill base. **(B)** Operculum medially fused. **(C)** Gills concealed. **(D)** Hind limbs at Gosner Stage 26. **(E)** Detail of the gills in the specimen figured in **(A)**. **(F–I)** Development of the oral disc, from specimens with operculum at the gill base to gills concealed. Note the lower lip with small ventrolateral indentations (white arrows). Scale bars = 2 mm **(A–D)**, and 0.5 mm **(E–I)**.

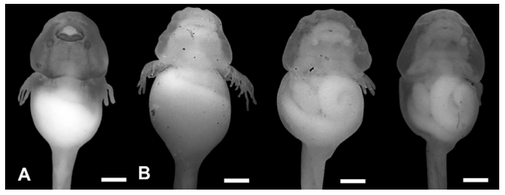

Figure 7. Development of the digestive tract. **(A)** *Physalaemus camacan*, showing the beginning of coiling in a specimen with operculum medially fused. **(B)** *Physalaemus signifer*, showing coiling progression from the stage of medially fused operculum to left gill regression. Scale bars = 0.5 mm.

ciliation also changes during the embryonic ontogeny: from large, densely arranged ciliated cells of embryos with fully developed gills (Fig. 4A), cells become smaller and sparsely disposed during gill occlusion (Fig. 4B). The development of the oral disc begins concomitant with the differentiation of the operculum, when a curved upper lip and a slightly indented lower lip are evident. Later, labial tooth ridges begin differentiation, starting with rows A1, P1, P2 (Fig. 5A) and followed by A2 (Figs. 5B and 6F). The marginal papillae appear at commissures (Fig. 5C) and row P3 differentiates as a transverse ridge distal to P2 (Fig. 5D). Marginal papillae development progresses medially (Fig. 5E), until large, rounded, widely spaced papillae surround the whole lower lip (Figs. 5F and 6I). Ventrolateral gaps are apparently not defined, but the lower lip in embryos of *P. camacan* shows two shallow, transient indentations that could be comparable (Fig. 6G,H). Keratinisation of the jaw sheaths and labial ridges (i.e. serrations and labial teeth) completes after the soft mouthparts are formed. The larval oral disc shows a C3 pattern (*sensu* Vera Candioti et al., 2011), consisting of a LTRF 2(2)/3(1) and complete lower marginal papillae. Finally, in the digestive tract, the first coils develop shortly after gills are fully developed and row P3 is differentiated in the oral disc; yolk persists after the spiracle is formed (Fig.7).

DISCUSSION

Embryonic morphology is, in general, conserved in species of *Physalaemus* (see Vera Candioti et al. 2011; Grosso et al. 2019), and, with some slight differences, the close similarity between embryos of *P. camacan* and *P. signifer* and regarding congeneric species was expected. Grosso et al. (2019) recovered some putative morphological and heterochronic synapomorphies for embryonic *Physalaemus*, but since no representatives of the *P. signifier* clade are included in that analysis, the authors highlighted that those features could indeed define the *P. cuvieri* clade. Small size (less than 2 mm), deeply kyphotic dorsal curvature, and lack of pigmentation at tailbud stage are common to most species of *Physalaemus* including the two in this study; they are shared with embryos of *Engystomops* and some widespread among other leiuperines. Also, like most congeneric species, *P. camacan* and *P. signifer* develop three pairs of gills. After inclusion of these species in a

Table 1. Measurements of embryos at tailbud stage and gill aspects for species of *Physalaemus*. Absolute values are given as average ± standard deviation (excepting the first gill filament where only the longest is consigned), and those corresponding to species from the *P. cuvieri* clade are taken from Grosso et al. (2019). Tailbud stage **(TB)**: Body length **(BL)**, Body area **(BA)**, Yolk area **(YA)**, Yolk proportion **(YP)**, Dorsal curvature **(DC)**; Gill at full development **(GFD)**: First gill filament **(FGF)**; number of specimens **(N)**. Cells with (-) indicate that embryos were not available for those measurements.

	TB						GFD	
Species	**N**	**BL (mm)**	**BA (mm²)**	**YA (mm²)**	**YP (%)**	**DC (°)**	**N**	**FGF (mm)**
P. signifer	4	2.01 ± 0.07	1.06 ± 0.07	1.12 ± 0.08	51	96.9 ± 0.01	6	0.43
P. camacan	-	-	-	-	-	-	3	0.55
P. aff. *albonotatus*	8	1.53 ± 0.1	1.13 ± 0.19	0.48 ± 0.05	42.5	66 ± 5.06	12	0.73
P. albifrons	2	1.40 ± 0.04	1.12 ± 0.1	0.38 ± 0.01	33.7	46 ± 5.66	8	0.79
P. albonotatus	7	1.50 ± 0.06	1.16 ± 0.04	0.44 ± 0.08	38	62 ± 2.98	14	0.91
P. biligonigerus	5	1.65 ± 0.06	1.3 ± 0.14	0.52 ± 0.13	40	75 ± 2.88	8	0.68
P. carrizorum	3	1.90 ± 0.06	1.94 ± 0.08	0.91 ± 0.07	46.8	80 ± 2	7	0.97
P. cicada	-	-	-	-	-	-	5	0.84
P. cuvieri	3	1.55 ± 0.12	1.86 ± 0.04	0.77 ± 0.15	41.5	63 ± 4.5	6	0.72
P. fernandezae	-	-	-	-	-	-	5	0.30
P. gracilis	-	-	-	-	-	-	8	0.75
P. henselii	-	-	-	-	-	-	3	0.25
P. riograndensis	3	1.36 ± 0.14	0.96 ± 0.17	0.46 ± 0.03	48	64 ± 2.31	15	1.04
P. santafecinus	3	1.43 ± 0.09	1.21 ± 0.1	0.57 ± 0.03	47	71 ± 2.52	10	0.69

phylogenetic analysis, this feature could maintain the status as a putative synapomorphy of *Physalaemus*, with an instance of reduction to two pairs in the *P. henselii* species group. Regarding adhesive glands, Grosso et al. (2019) recovered the morphogenetic type C as the plesiomorphic state for Leiuperinae, and accordingly, glands in *P. camacan* and *P. signifer* have the same morphology and development as those described for most *Physalaemus* and *Pseudopaludicola*. Likewise, the configuration of the hatching gland and the general morphology of hatching gland cells are like those of other *Physalaemus*. Finally, hind-limb development follows a similar pattern as in *Engystomops* + *Physalaemus* clade, with limb buds differentiating almost simultaneously with spiracle formation.

Combined with these features overall conserved at the generic level, some traits appear to be distinctive for species of the *P. signifer* clade. Species studied are almost identical in development, with some slight variations in differentiation of hind-limb buds (earlier in *P. signifer*) and medial fusion of the operculum (earlier in *P. camacan*). Main differences of these species regarding most members of the sister clade *P. cuvieri* are related to egg/embryo size, gill development and the ontogeny of the oral marginal papillae. Some of these transformations could be correlated with the different modes of oviposition, but others appear to be developmental modifications not related with ecomorphological aspects.

Previous reports highlight differences in clutch and egg sizes of species of the *P. signifer* clade as compared with congeneric species: while egg number is significantly higher in species with aquatic oviposition, egg size is larger in species with terrestrial nests (Pupin et al., 2010). Large eggs usually develop into large embryos with increased yolk provision (Salthe & Duellman, 1973). Although our small sampling prevents us from definitive conclusions, preliminary observations indicate that early embryos of *P. signifer* clade are larger and provisioned

with proportionately more yolk than known embryos of the *P. cuvieri* clade (Table 1). Persistence of yolk in the developing digestive tract is also longer than in other *Physalaemus* (Grosso et al., 2019). Yolk supply may ensure survival of embryos within the nests, and it likely represents an advantage for hatchlings in terrestrial nests that depend on being flooded or washed away to water bodies for further development (Salthe & Duellman, 1973; Pupin et al., 2010, 2018). A similar correlation between embryo size, yolk proportion, and persistence of hatchlings within the nests is reported for embryos of the *Leptodactylus fuscus* species group that develop in nests in underground galleries (Downie, 1984; Grosso et al., 2017). Interestingly, size differences between embryos of *P. signifer* and *P. cuvieri* clades persist at older embryonic stages (GS 24–26; Oliveira et al., unpubl. data) but reverse in larval and postmetamorphic periods, rendering the tadpoles and adults of *P. signifer* and *P. camacan* among the smallest in the genus (Weber & Carvalho-e-Silva, 2001; Pimenta et al., 2005). The initial investment in body growth at the expense of yolk provision, along with a likely later beginning of active feeding suspected from the yolk persistence in the digestive tract, could explain these differences in proportional size-increase from embryos to tadpoles to adults in species of *Physalaemus*. From a functional perspective, large size and yolk provision could be only essential for these embryos at first feeding in their aquatic environments, in a context of interspecific competition or uncertain availability of food resources.

Except for species of the *P. henselii* group, gills are in general well developed in *Physalaemus* (Grosso et al., 2019). Within the genus, gills are larger and more branched in embryos of *P. cicada* and species of the *P. cuvieri* group, and a relation with breeding in warm, xeric environments has been suggested for *P. cicada* and other Leiuperinae (Grosso et al., 2019). Gill size and branching is comparatively smaller in *P. camacan* and *P. signifer* here studied (Table 1) but wider sampling is needed before making generalisations at clade level and correlations with oviposition sites.

The development of the oral disc was studied in several species of the *P. cuvieri* clade, and ontogenetic patterns were summarised by Vera Candioti et al. (2011) and Grosso et al. (2019). Our study confirms that the first lower tooth ridge (P1) is the earliest to differentiate on the lower lip of species of the *P. signifer* clade, as occurs in all other known species of *Physalaemus* and *Pseudopaludicola*, but unlike *Pleurodema* (row P2 develops first). Nevertheless, development of the lower marginal papillae indicates that the scenario could be more complex than that synthesised by the cited previous studies. According to those contributions, *Physalaemus* and *Pseudopaludicola* are characterised by the occurrence (transient or maintained in larval stages) of ventrolateral gaps in the lower marginal papillae. Additionally, the five different configurations of the oral disc known for *Physalaemus* would result from common ontogenetic trajectories ending at different states, or from trajectories that differ initially in the formation of a ventral gap (see Figs. 4 and 10 in the cited papers). The oral disc development of the species here studied was expected to fit in one of two main trajectories, likely that leading to the oral configuration of *P. gracilis* with a similar larval oral disc (labial tooth row formula 2/3 plus complete marginal papillae, i.e. the C3 configuration *sensu* Vera Candioti et al., 2011). However, observations in both *P. camacan* and *P. signifer* apparently reveal a different pattern: although the small indentations of the lower lip margin could represent some variant of gaps, we never observed proper marginal papillae developing initially on the mental region, thus ventrolateral gaps cannot be undoubtedly defined as present as in other species described in that trajectory. If this novel trajectory was confirmed for the *P. signifer* clade (with more resolution in developmental series and including additional species), this would imply that the most widespread oral configuration in *Physalaemus*, the C3 configuration, develops according varied pathways that so far follow: i) a complex, recapitulatory way that includes only ventrolateral gaps as in closely related species (in *P. gracilis* of *P. gracilis* group); ii) an alternative way that includes ventral and ventrolateral gaps (in *P. carrizorum* of *P. gracilis* group); iii) a combined way that joins the development of all gaps with an early filling of ventral gap (in some specimens of *P. cicada*; for these first three, please see Fig. 10 in Grosso et al., 2019); and iv) a novel, "telescoped" version of some of these trajectories, where papillae appear to progress medially, but the mental region still exhibits some vestigial structures defining incipient ventrolateral gaps (in *P. camacan* and *P. signifer* of *P. signifer* clade). Clearly, a more exhaustive sampling of species with C3 oral discs and detailed studies of how they develop are needed to assess this subject.

In contrast to uncertain conditions characteristic of temporary ponds in open seasonal areas, more predictable environments such as ponds inside the Atlantic rainforest may favour the evolution of varied reproductive strategies (Haddad & Prado, 2005; Pupin et al., 2018). In this context, embryonic morphology and physiology could be also highly adaptive to face requirements of alternative microhabitats, and transient embryonic and larval features exhibit transformations correlated with survival and resource acquisition. Our studies in early ontogeny of species of *Physalaemus* are framed in this scenario, but further investigations, especially from ecological and experimental perspectives, are encouraged to deepen this subject.

ACKNOWLEDGEMENTS

This study was financially supported by Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB; Nº BOL0459/2017), project "Taxonomia Integrativa da Fauna de Anfíbios Anuros do Sul da Bahia" (FAPESB-NºAPP0109/2016) Agencia Nacional de Promoción de la Investigación, el Desarrollo Tecnológico y la Innovación (PICT 2017–2437 and 2018–3349), and CONICYT Concurso FONDECYT de Postdoctorado 2020 N° 3200490 to J. Grosso. Specimens were collected under permit number 60078–3 SISBIO (Código de autenticação: 0600780320181101). We deeply thank the staff of Laboratório de Taxonomia e História Natural de Anfíbios (AMPHIBIA) from the Universidade Federal da Bahia (UFBA) and Laboratório e coleção de zoologia from the Universidade Federal do Sul da Bahia (UFSB) for logistical support. MFN acknowledges the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for productivity grant (#310490/2018-9).

Authors' Contribution

Marianna Isabella Rosa Rodrigues de Oliveira: Conceptualisation; formal analysis; investigation; methodology; project administration; writing-original draft; writing review & editing. Jimena Grosso: Methodology; investigation; writing review & editing. Marcelo Felgueiras Napoli: Supervision; writing review & editing. Luiz Norberto Weber: Supervision; conceptualisation; formal analysis; methodology; resources; supervision; writing original draft. Florencia Vera Candioti: Supervision; conceptualisation; formal analysis; investigation; methodology; supervision; writing-original draft; writing review & editing.

REFERENCES

Cei, J.M. (1980). Amphibians of Argentina. *Monitore Zoologico* Italiano 2, 1–609.

Downie, J.R. (1984). How *Leptodactylus fuscus* tadpoles make foam, and why. *Copeia* 1984, 778–780.

Duellman, W.E. & Trueb, L. (1986). *Biology of Amphibians*. The John Hopkins University Press, Baltimore, USA.

Frost, D.R. (2021). Amphibian Species of the World: an Online Reference. Version 6.1 (Accessed on 5 August 2021). Electronic Database accessible at https://amphibiansoftheworld.amnh.org/index.php. American Museum of Natural History, New York, USA.

Gosner, K.L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16, 183–190.

Grosso, J., Baldo, D. & Vera Candioti, M.F. (2017). Heterochronic changes during embryonic development of neotropical foam nesting frogs (genus *Leptodactylus*). *Zoologischer Anzeiger* 266, 35–49.

Grosso, J., Baldo, D., Cardozo, D., Kolenc, F., Borteiro, C., Oliveira, M.I.R. De., Bonino, M.F., Barrasso, D.A. & Vera Candioti, F. (2019). Early ontogeny and sequence heterochronies in Leiuperinae frogs (Anura: Leptodactylidae). *PLoS ONE*, 14(6), e0218733.

Haddad, C.F.B. & Prado, C.P.A. (2005). Reproductive modes in frogs and their unexpected diversity in the Atlantic forest of Brazil. *Bioscience* 55, 207–217.

Heyer, W.R. (1969). The adaptive ecology of the species group of the genus *Leptodactylus* (Amphibia: Leptodactylidae). *Evolution* 23, 421–428.

Heyer, W.R., Rand, A.S., Cruz, C.A.G., Peixoto, O.L. & Nelson, C.E. (1990). Frogs of Boracéia. *Arquivos de Zoologia* 31, 231–410.

Lourenço, L.B., Targueta, C.P., Baldo, D., Nascimento, J., Garcia, P.C.A., Andrade, G.V., Haddad, C.F.B. & Recco-Pimentel, S.M. (2015). Phylogeny of frogs from the genus *Physalaemus* (Anura, Leptodactylidae) inferred from mitochondrial and nuclear gene sequences. *Molecular Phylogenetics and Evolution* 92, 204–216.

Lynch, J.D. (1971). Evolutionary relationships, osteology and zoogeography of leptodactyloid frogs. *Miscellaneous Publications of the Museum of Natural History of the University of Kansas* 53, 1–238.

Méndez-Narváez, J., Flechas, S.V. & Amézquita, A. (2015). Foam nests provide context-dependent thermal insulation to embryos of three leptodactylid frogs. *Physiology Biochemistry Zoology* 88, 246–253.

Nokhbatolfoghahai, M. & Downie, J.R. (2005). Larval cement gland of frogs: comparative development and morphology. *Journal of Morphology* 263, 270–283.

Nokhbatolfoghahai, M. & Downie, J.R. (2007). Amphibian hatching gland cells: Pattern and distribution in anurans. *Tissue and Cell* 39, 225–240.

Nokhbatolfoghahai, M. & Downie, J.R. (2008). The external gills of anuran amphibians: comparative morphology and ultrastructure. *Journal of Morphology* 269, 1197–1213.

Nokhbatolfoghahai, M., Downie, J.R., Clelland, A.K. & Rennison, K. (2005). The surface ciliation of anuran amphibian embryos and early larvae: patterns, timing differences and functions. *Journal of Natural History* 39, 887–929.

Pimenta, B.V.S., Cruz, C.A.G. & Silvano, L.S. (2005). A new species of the genus *Physalaemus* Fitzinger, 1826 (Anura, Leptodactylidae) from the Atlantic Rain Forest of southern Bahia, Brazil. *Amphibia-Reptilia* 26, 201–210.

Pupin, N.C., Gasparini, J.L., Bastos, R.G., Haddad, C.F.B. & Prado, C.P.A. (2010). Reproductive biology of an endemic *Physalaemus* of the Brazilian Atlantic forest, and the trade-off between clutch and egg size in terrestrial breeders of the *P. signifer* group. *Herpetological Journal* 20, 147–156.

Pupin, N.C., Haddad, C.F.B. & Prado, C.P.A. (2018). Maternal provisioning by foam-nesting frogs of the genus *Physalaemus* (Anura, Leptodactylidae) in contrasting environments. *Amphibia-Reptilia* 39, 120–125.

Salthe, S.N. & Duellman, W.E. (1973). Quantitative constraints associated with reproductive mode in anurans. In: *Evolutionary Biology of the Anurans*, 229–249. Vial, J.L. (ed.). Columbia: University of Missouri Press.

Thibaudeau, D.G. & Altig, R. (1988). Sequence of ontogenetic development and atrophy of the oral apparatus of six anuran tadpoles. *Journal of Morphology* 197, 63–69.

Vera Candioti, F., Haad, B., Baldo, D., Kolenc, F., Borteiro, C. & Altig, R. (2011). Different pathways are involved in the early development of the transient oral apparatus in anuran tadpoles (Anura: Leiuperidae). *Biological Journal of the Linnean Society* 104, 330–345.

Vera Candioti, F., Grosso, J., Haad, B., Pereyra, M.O., Bornschein, M.R., Borteiro, C., Costa, P., Kolenc, F., Pie, M.R., Proaño, B., Ron, S., Stanescu, F. & Baldo, D. (2016). Structural and heterochronic variations during the early ontogeny in toads (Anura: Bufonidae). *Herpetological Monographs* 30, 79–118.

Wassersug, R.J. (1976). Oral morphology of anuran larvae: terminology and general description. *Occasional Papers of the Museum of Natural History of the University of Kansas* 48, 1-23.

Weber, L.N. & Carvalho-e-Silva, S.P. (2001). Descrição da larva de *Physalaemus signifer* (Girard, 1853) (Amphibia, Anura, Leptodactylidae) e informações sobre a reprodução e a distribuição geográfica da espécie. *Boletim do Museu Nacional, Nova Série, Série Zoologia* 462, 1–6.

Wogel, H., Abrunhosa, P.A. & Pombal, J.P., Jr. (2002). Atividade reprodutiva de *Physalaemus signifer* (Anura, Leptodactylidae) em ambiente temporário. *Iheringia Série Zoologia* 92(2): 57–70.

Accepted: 12 March 2022

THE HERPETOLOGICAL JOURNAL SUBMISSION PROCESS

The Herpetological Journal is an international peer-reviewed publication of the British Herpetological Society, with open-access publication options. *The Journal* has a broad focus relating to behaviour, ecology, evolution, systematics, taxonomy, physiology, anatomy, functional morphology, pathology, natural history, method development and conservation of reptiles and amphibians. All articles should appeal to a general herpetological audience and have a solid grounding in natural history. We are committed to open science and avoiding unconscious biases so moving forward we will operate a double-blind peer review process.

Manuscripts that describe natural history observations, range extensions or checklists are not appropriate submissions (unless they address a bigger question) and would be better suited to our sister publication, *The Herpetological Bulletin*.

The Herpetological Journal welcomes contributions in the following categories:

- Full length research articles
- Short communications
- Reviews
- Perspectives/Opinion pieces

Files to be uploaded to the OJS system: Cover Page, Main Text, Tables, Figures, Supplementary Files.

Cover Page:
This will not be seen by peer reviewers in order to comply with a double-blind peer review process. The cover page should include Title, Authors, Author Affiliations, Ethical Statement and Author Contributions (if editors do not deem that authors contributed substantially to the research then the article will be rejected).

Main Text:
Nb. author names or affiliations should not be included in this file.

Full length research articles should be between 2,500 and 6,000 words and include the following sections: Title, Abstract (maximum 300 words), Keywords (five words that are not used in the title), Introduction, Methods, Results, Discussion, Acknowledgements, Data Accessibility, References, Figure captions, Table captions. The word limit excludes Data Accessibility and References. There are no limits to the number of figures and tables.

Short communications should be less than 2,500 words and include the following sections: Title, Abstract (maximum 250 words), Keywords (five words not used in the title), Main Text (NOT separated into Introduction, Methods, Results and Discussion), Acknowledgements, Data Accessibility, References, Figure captions, Table captions. The word limit excludes Data Accessibility, References, and Figure and Table captions. Short communications can have a maximum of ONE figure and ONE table.

Reviews are either solicited by editors or a short email enquiry should be sent to the Editor-in-Chief (bhsherpetologicaljournal@gmail.com) to enquire about the suitability of a proposed review. Reviews should be between 2,500 and 6,000 words. Section headings can be specified at the authors discretion.

Perspectives/Opinion pieces will be considered if they address a new or controversial topic/idea, or if they are comments about newly published articles in *The Herpetological Journal*. Perspectives/Opinion pieces should be a maximum of 1,500 words (excluding references) and can include ONE figure and ONE table.

Tables:
Tables should be provided in a separate Word file. Tables should be numbered in Arabic numerals, e.g. Table 1. Tables should be as simple as possible and typed double-spaced on separate sheets with a title/short explanatory paragraph above the table. Horizontal and vertical lines should be avoided, as should tables that split over more than one page or that need to be set in landscape format.

Figures:
Figures should initially be submitted in a single Word or PDF file. Graphs, line drawings and photographs should be numbered in sequence in Arabic numerals, e.g. Figure 1. If a figure has more than one part, each should be identified as (a), (b), etc. Figure captions should be included at the end of the main text. After acceptance figures should be submitted as separate image or pdf files with a minimum resolution of 300dpi and a maximum file size of 5MB.

Supplementary Data/Files:
To conform with an open science process, it will be necessary for datasets, code, supplementary figures etc. to be deposited in an online repository (e.g. https://osf.io/) and made available after publication. At the initial submission stage, at a minimum, any code and supplementary figures should be uploaded in the submission portal for review. Any new taxonomic changes should be recorded on ZooBank.

It is a fundamental condition that submitted manuscripts have not been published and will not be simultaneously submitted or published in another journal. However, as a journal we do support the submission of articles on preprint servers (e.g., bioRxiv) as long as the preprints are linked to final published articles.

By submitting a manuscript, the authors agree that the copyright for their article is transferred to the publisher if and when the article is accepted for publication. The copyright covers the exclusive rights to reproduce and distribute the article, including reprints and photographic reproductions. Permission to use images after publication will almost always be granted but must be sought in advance from the Editors.

Papers should be written in British English (including figure labels) and spelling should be that of the Oxford English Dictionary.

Times and dates should conform to the following formats: for time of day use 0900, 1000 etc; for dates

use 7 July 2017 etc. Please avoid using bold text, all caps or small caps for emphasis. If emphasis is required, use italics. Common names should be in lower case unless a proper noun is used.

All submissions must adhere to the British Herpetological Society's Ethical Policy and Guidelines, which can be found here – https://www.thebhs.org/images/stories/BHS-ethicspolicy.pdf.

Open Access Policy: *The Herpetological Journal* supports "green" open access, as outlined by the Research Councils UK, to facilitate deposition of articles e.g. at institutional repositories. *The Herpetological Journal* also offers the option of "gold" open access for individual articles (free of charge for members of the British Herpetological Society, and at an article processing charge of £97 for non-members).

REFERENCE STYLE

CITATION IN TEXT:

- Chronological then alphabetical
- Use “et al.” (not italicised) for more than two authors
- Last name (s) and year separated by comma
- Names separate by “&”
- References separated by semicolon

Ex. 1: (Heyer et al., 1988; Weygoldt et al., 1989; Eterovick et al., 2005)
Ex. 2: (Smith et al., 2004; Jones & Smith, 2008)
Ex. 3: (Smith et al., 2015)
Ex. 4: “Although Smith et al. (2008) did not include -”
Ex. 5: "- as observed by Smith & Jones (2017)"

REFERENCES

- Authors
- Last name separated from initials by comma
- Initials capitalised and separated by period (no space)
- Names separated by commas
- Last name separated by “&”

Ex.: Smith, A.H., Jones, R.D. & Lloyd, K.A.
Ex.: Smith, A.H. & Jones, R.D.

Year:

- In parentheses, followed by a full stop.

Title:

- Only first letter capitalised except book titles (in this case, All First Letters Capitalised).

Journal:

- Journal name should be written in full, italicised, followed by a comma
- Volume and pages separated by comma and ending with full stop (not italicised).

Journal article:

- Authors. (Year). Title. *Journal*, Volume (Issue), xx–xx.

Book:

- Authors. (Year). Book Title. City: Country. Xxx p.

Book chapter:

- Authors. (Year). Chapter title. In: *Book Title*, Book editor (s). (Ed./Eds.) City: Country. Xxx p.

Ex. 1:

Lebboroni, M. & Corti, C. (2006). Road-killing of lizards and traffic density in central Italy. In: *Herpetologia Bonnensis II: Proceedings of the 13th Ordinary General Meeting of Societas Europaea Herpetologica*, 81–82. Vences, M., Köhler, J., Ziegler, T. & Böhme, W. (eds). Bonn: Societas Europaea Herpetologica.

Ex. 2:

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989). Preparation and Analysis of Eukaryotic Genomic DNA. In: *Molecular Cloning: A Laboratory Manual*, 2nd Eds. Cold spring Harbor Laboratory Press, New York, USA.

Websites:

Lang, J., Chowfin, S. & Ross, J.P. (2019). *Gavialis gangeticus*. The IUCN Red List of Threatened Species 2019: e.T8966A149227430. Downloaded on 3 October 2019. http://dx.doi.org/10.2305/IUCN.UK.2019-1.RLTS.T8966A149227430.en.

All contributions should be addressed to the Scientific Editor:

Simon T. Maddock, University of Wolverhampton, UK.
E-mail: bhsherpetologicaljournal@gmail.com

Associate Scientific Editors:
Annemarieke Spitzen, Anthony Herrell, Anyelet Valencia-Aguilar, Ben Tapley, Deepak Veerappan, Diogo Borges Provete, Gabriella Bittencourt, Inga Zeisset, Jim Labisko, John Vanek, Jose Valdez, Lewis Campbell, Luis San José, Mirco Solé, Rachael Antwis, Richard Brown, Robert Jehle, Sam Cruickshank, Simon Loader.

Managing Editor:
Julie Tee (managingeditor@thebhs.org)

Advertisements:
The Herpetological Journal accepts advertisements subject to approval of contents by the Managing Editor, to whom enquiries should be addressed.
