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Threats of the emerging pathogen *Batrachochytrium salamandrivorans* (*Bsal*) to Italian wild salamander populations

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Abstract. The salamander killing fungus *Batrachochytrium salamandrivorans* (*Bsal*), recently introduced from Asia, is threatening salamander populations in different parts of Europe. In fact, this pathogen is rapidly spreading in Central Europe and has been also introduced into NE Spain. Of special concern are those regions with an exceptionally high salamander diversity such as Italy, where 19 salamander species are present most of which are strictly endemic. In this study, we update the information on the presence of *Bsal* in Italian wild salamanders, by adding samples from two presumptive outbreak sites, one on the island of Sardinia and one on continental Italy (Liguria). In addition, we reviewed the potential susceptibility of all the Italian salamander species on the basis of laboratory experimental infection trials, or from the tested susceptibility of the phylogenetically nearest species, according to the literature. Overall, 15 skin swabs from three species (*Speleomantes sarrabusensis*, *Speleomantes strinatii* and *Salamandra salamandra*) collected in the two presumptive *Bsal* outbreak sites were analysed by quantitative molecular methods, but none gave positive results. The majority of Italian salamander species and almost all of the endemic ones showed a high susceptibility to *Bsal* infection. Therefore, even if the presence of *Bsal* in Italian salamander populations has not been proven yet, the entire salamander fauna is highly threatened and preventive management actions should be taken. The need for strict biosecurity protocols on the international trade of captive or wild amphibians and for the implementation of preventive measures during field activities to limit the introduction and spread of the *Bsal* pathogen is again stressed.

Keywords. Amphibians, biosecurity, chytridiomycosis, conservation, emerging infectious diseases, mitigation, real-time PCR.

INTRODUCTION

The chytrid fungi *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*) are amphibian pathogens that were introduced in Europe from East Asia, probably through the international trade (O'Hanlon et al., 2018; Laking et al., 2019). The continuous spreading of these emerging pathogens is now threatening wild amphibian populations in different

parts of Europe (Scheele et al., 2019; Bosch et al., 2021). In particular, the salamander killing fungus *Bsal* is a real threat to European salamanders, because many species are extremely susceptible to this infection and, therefore, highly vulnerable (Martel et al., 2014). The pathogenic effect of *Bsal* is caused by its zoospores that penetrate the skin cells of adult salamanders, breaking innate immunological defences, and producing tissue erosions and deep ulcerations that may disrupt the host infected

skin respiratory and rehydration functions (Martel et al., 2013; Grogan et al., 2020). In this way, *Bsal* infections are capable of causing severe illness or even death of the infected amphibian hosts (Martel et al., 2013, 2014). In fact, *Bsal* is constantly expanding from the Netherlands, where it was first detected and where it caused a population collapse in the local fire salamander *Salamandra salamandra* population (Martel et al., 2013). Currently, *Bsal* has been confirmed in about 80 localities surrounding Bunderbos, in the Netherlands, where it was first detected (Martel et al., 2013), and also in Belgium and Germany (e.g., Spitzen-van der Sluijs, 2016; Lötters et al., 2020; Schmeller et al., 2020; Thein et al., 2020). *Bsal* has also been detected in 2018 in Catalonia (Northeastern Spain), where it has infected the marbled newt *Triturus marmoratus* and caused a local mass mortality event of this species (Martel et al., 2020), while its presence in North Central Spain has not been yet confirmed (Bosch et al., 2021). Therefore, to date, the presence of *Bsal* in Europe has been confirmed in the wild in four countries: The Netherlands, Belgium, Germany and Spain. The emergence and the diffusion of *B. salamandrivorans* through Europe, apparently facilitated by humans transferring and introducing in the wild environment captive amphibians, is worrying and requests urgent prevention measures and monitoring actions to prevent further loss of amphibian diversity (Thomas et al., 2019). In particular, the high salamander diversity of the southern European peninsulas, such as Italy and Spain, appears at risk. Italy alone hosts 19 species of urodeles (Sindaco and Razzetti, 2021), many of which are endemic, such as the entire genus *Salamandrina*, the Italian newt *Lissotriton italicus*, the Sardinian brook newt *Euproctus platycephalus*, and seven species of cave salamanders belonging to the genus *Speleomantes* (Sindaco and Razzetti, 2021). However, to date, only two studies screened *Bsal* in Italian salamanders (Grasselli et al., 2019, 2021). These studies analysed by means of real-time PCR (qPCR) 136 skin swabs from 6 species of Italian wild salamanders and 53 from non-native individuals that were bred in private collections (Grasselli et al., 2019). The results from these studies were that none of the 189 Italian salamander swabs gave positive results. However, recent events raised concerns on the possibility of *Bsal* outbreaks in Italian populations. In the summer of 2021, a mass mortality of *Speleomantes sarrabusensis* was observed in Sardinia by the wildlife photographer Emanuele Biggi while, more recently (in spring 2022), a living fire salamander bearing skin lesions from Liguria was photographed by Michael Fahrback and reported to the authors by Frank Pasmans from Ghent University. These observations were compatible with the suspected infection of *Bsal* on

Italian amphibians but needed robust confirmation by molecular, and histological methods or by both. Therefore, the aim of this study was twofold: i) to expand the current knowledge about the presence of *Bsal* in wild salamander populations in Italy, adding the molecular data obtained from new skin samples collected from the two *Bsal*-suspected outbreaks and ii) to assess the susceptibility to *Bsal* of all Italian salamanders, on the basis of published experimental studies or, in the absence of experimental evidence, from their phylogenetic affinities. This information will be needed to better plan *Bsal* mitigation actions and also to guide strategic conservation and management efforts on the national territory of Italy.

MATERIALS AND METHODS

Origin of skin swab samples, DNA extraction and quantitative PCR

We obtained skin swabs from two areas, one in Southern Sardinia and one in NW Italy. In Southern Sardinia (Monte Sette Fratelli), we sampled a protected underground site that hosts the largest known population of *Speleomantes sarrabusensis*. This site is closed by a gate and only an authorised person has access, however, in this location several dead cave salamanders were observed from July to August 2021, by different herpetologists. Skin swabs were collected a few days after the first mortality record (August 2021) from all the living *S. sarrabusensis* individuals (two individuals), plus one recently dead individual; a fourth swab was obtained from a *Hyla sarda* corpse found at the same site (Supplementary material, Fig. S1).

In the province of Genova (Liguria, NW Italy), we collected twelve swabs in April and May 2022 along a small stream or in an adjacent artificial cave from living fire salamanders (*Salamandra salamandra*) and cave salamanders (*Speleomantes strinatii*) where an individual of *Salamandra salamandra* with suspect skin lesions was photographed (Supplementary material, Fig. S1). In both sites, all skin swabs were obtained with a standardised protocol used in previous *Bd* and *Bsal* studies on Italian salamanders (Costa et al., 2021; Grasselli et al., 2019, 2021). Sterile cotton swabs were rubbed 30 times on the skin of different parts of the salamander's body and were preserved in individual sterile plastic tubes at 4 °C until extraction (Spitzen-van der Sluijs et al., 2016). DNA was extracted in 200 µl of Prepman ULTRA (Thermo Fisher Scientific Technologies, Monza, Italy). Samples were then analysed for *Bd* and *Bsal* DNA using a duplex qPCR, targeting the ITS1 rRNA gene of *Bd* and 5.8S rRNA gene of *Bsal*, as described by Blooi et al. (2013). All samples

were run in duplicate, together with standard curves obtained from suspensions of known numbers of *Bd* and *Bsal* zoospores (kindly provided by An Martel and Frank Pasmans) in the same plate, and results were expressed in genome equivalents (GEs) according to Thomas et al. (2018).

Assessing Italian salamanders' *Bsal* susceptibility

The susceptibility of Italian salamanders to *Bsal* infection was estimated by collecting all the published data on laboratory experimental infection trials on the same salamander species. In absence of such experimental evidence, the susceptibility was inferred from the phylogenetically nearest species, adopting a precautionary approach and thus selecting the more threatening outcome. Species susceptibility was estimated as “High” if the experimental infection had a lethal outcome, “Moderate” if the gravity of infection was dose-dependent and “Low” if the infected salamander was able to clear infection or remained asymptomatic.

Moreover, we used the results of Beukema et al. (2018, Table 2) as an index of potential niche overlap between *Bsal* and Italian salamanders. Beukema et al. (2018) estimated both native and invasive ecological niches of *Bsal* and of all native European salamanders using three different ordination methods (see Material and Methods in Beukema et al., 2018). The statistical overlap between both *Bsal* native and invasive ecological niches and the current niche of Italian salamanders was then calculated (Beukema et al., 2018). In the present study, we counted all significant overlaps between Italian salamanders and *Bsal* native and invasive niche. Therefore, there were several possible outcomes per species, ranging from 0/6 if all overlaps were non-significant, to 6/6 if all the six niches overlapped significantly. This “overlap score” may be used as a gross indication of the environmental compatibility between each Italian species and the salamander killing fungus *Bsal*.

In this study, we used species nomenclature following the most recent checklist of Italian Herpetofauna (Sindaco and Razzetti, 2021), and we revised the available data on the 19 native Italian species. We also report information for one subspecies of alpine salamander (*Salamandra atra aurorae*), that is listed as a priority taxon in annex II of the European Union (EU) Directive “Habitats” 92/43/CEE. According to Art. 1 letter (h) of this Directive, Italy has a particular responsibility for the conservation of this endemic taxon that shows an extremely small distributional range limited to the Eastern Alps.

RESULTS

DNA testing

We analysed one skin swab from *Hyla sarda*, three *Speleomantes sarrabusensis*, five *Speleomantes strinatii* and seven *Salamandra salamandra*. None of these swabs gave *Bd* or *Bsal*-positive results. Therefore, the possible *Bsal* infection in the Sardinian (prevalence 0; 95% confident limits 0.00-0.60), and Ligurian (prevalence 0; 95% confident limits 0.00-0.30) sites were not confirmed, but given the very small samples analysed, a high level of uncertainty concerning these results remains. To date, the number of Italian wild salamanders screened for *Bsal* is 151, obtained from nine species (Table 1).

Bsal susceptibility

Ten of the nineteen (53%) salamander species found in Italy were infected experimentally in laboratory trials with *Bsal*, thus providing direct evidence of their potential susceptibility to the pathogen (Table 2). In the case of the cave salamanders (genus *Speleomantes*), three species were infected in laboratory experiments by Martel et al. (2014): *S. genei*, *S. strinatii* and *S. imperialis*. The two former species resulted highly susceptible (i.e., *Bsal* infection was lethal), while the latter was able to clear the infection, thus showing low susceptibility. In this study, following the precautionary approach, we considered all non-tested *Speleomantes* species to be highly susceptible, as already done by Gilbert et al. (2020).

Overall, the majority of Italian salamander species, 15 out of 19 (79%), were shown or inferred to possess

Table 1. Italian salamanders tested for *Batrachochytrium salamandrivorans*. ^a and ^b indicate presence of samples from the non-confirmed *Bsal* outbreaks in Liguria (2022) and Sardinia (2021), respectively. This table is an expanded version of Grasselli et al. (2019).

Species or subspecies	Sample (N)	<i>Bd</i> positive	<i>Bsal</i> positive
<i>Euproctus platycephalus</i>	3	0	0
<i>Ichthyosaura alpestris</i>	76	1	0
<i>Lissotriton italicus</i>	22	0	0
<i>Salamandra atra aurorae</i>	3	0	0
<i>Salamandra salamandra</i> ^a	11	0	0
<i>Salamandrina terdigitata</i>	14	0	0
<i>Speleomantes sarrabusensis</i> ^b	3	0	0
<i>Speleomantes strinatii</i> ^a	5	0	0
<i>Triturus carnifex</i>	14	3	0
Total	151	4	0

Table 2. Susceptibility of Italian salamander species to *Bsal* infection, according to the literature or inferred from phylogenetic related species. *Bsal* susceptibility was updated from Gilbert et al. (2020). The overlap score was calculated from Table 2 of Beukema et al. (2018). * not reported in Beukema et al. (2018).

Species	Italian endemic	IUCN Status	Habitat Directive	<i>Bsal</i> susceptibility	Comments	Reference	Overlap score
<i>Euproctus platycephalus</i>	yes	EN	IV	High	Lethal, laboratory tested	Martel et al., 2014	1/6
<i>Ichthyosaura alpestris</i>	no	LC	--	Moderate	Dose-dependent laboratory tested	Martel et al., 2014	3/6
<i>Lissotriton italicus</i>	yes	LC	IV	High	Lethal, laboratory tested	Martel et al., 2014	0/6
<i>Lissotriton vulgaris</i>	no	LC	--	Moderate	Dose-dependent, laboratory tested	Bates et al., 2019	1/6
<i>Proteus anguinus</i>	no	VU	II/IV	Low	Asymptomatic, laboratory tested	Li et al., 2020	2/6
<i>Salamandra atra</i>	no	LC	IV	High	Inferred from congeneric species		2/6
<i>Salamandra atra aurorae</i>	yes		II*/IV	High	Inferred from congeneric species		-*
<i>Salamandra lanzai</i>	no	VU	IV	High	Inferred from congeneric species		0/6
<i>Salamandra salamandra</i>	no	LC	--	High	Lethal, laboratory tested	Martel et al., 2013, 2014	2/6
<i>Salamandrina perspicillata</i>	yes	LC	II/IV	High	Lethal, laboratory tested	Martel et al., 2014	1/6
<i>Salamandrina terdigitata</i>	yes	LC	II/IV	High	Inferred from congeneric species		1/6
<i>Speleomantes ambrosi</i>	yes	NT	II/IV	High	Inferred from congeneric species		3/6
<i>Speleomantes flavus</i>	yes	VU	II/IV	High	Inferred from congeneric species		0/6
<i>Speleomantes genei</i>	yes	VU	II/IV	High	Lethal, laboratory tested	Martel et al., 2014	1/6
<i>Speleomantes imperialis</i>	yes	NT	II/IV	Low	Clears infection, laboratory tested	Martel et al., 2014	1/6
<i>Speleomantes italicus</i>	yes	NT	II/IV	High	Inferred from congeneric species	Martel et al., 2014	1/6
<i>Speleomantes sarrabusensis</i>	yes	VU	II/IV	High	Inferred from congeneric species	Martel et al., 2014	1/6
<i>Speleomantes strinatii</i>	no	NT	II/IV	High	Lethal, laboratory tested	Martel et al., 2014	3/6
<i>Speleomantes supramontis</i>	yes	EN	II/IV	High	Inferred from congeneric species		0/6
<i>Triturus carnifex</i>	no	LC	II/IV	High	Inferred from congeneric species		3/6

high susceptibility to *Bsal* infection, while two (11%) had moderate and two (11%) had low susceptibility (Table 2). When focusing on endemic salamanders, 10 out of 11 (91%) have, or were inferred to have, high susceptibility, the only exception being *S. imperialis* (Table 2). Finally, only four species showed no niche overlap with *Bsal* pathogen. Three of them were salamanders found exclusively in the Mediterranean bioclimatic region (i.e., *Lissotriton italicus*, *Speleomantes flavus* and *Speleomantes supramontis*), while the fourth one (*Salamandra lanzai*) lives at high altitudes in the Alps. On the other hand, four species showed a relatively high overall niche overlap score with *Bsal*: *Ichthyosaura alpestris*, *Speleomantes ambrosii*, *Speleomantes strinatii* and *Triturus carnifex*. The two latter also had a high experimental susceptibility towards the infection with the pathogen (Martel et al., 2014).

DISCUSSION

The diffusion of *Bsal* infection is a dramatic threat to salamander diversity in Central Europe and Spain (Martel et al., 2014; Bosch et al., 2021), and recently two potential outbreak sites have been recorded in Italy. How-

ever, none of our qPCR samples from these sites provided positive results. Clearly, these partial results cannot exclude the presence of *Bsal*-infected individuals in the studied sites or elsewhere, because of the very small sample size tested. The mass mortality event reported in Sardinia is particularly concerning, because the cave salamander population lives in a high-altitude protected site, where environmental stressors should be absent. Therefore, local Authorities in charge of the site management were immediately alerted and the water quality inside the site is monitored to report any further critical condition and to report further changes as well as to mitigate a possible spread of any kind of pathogen, as indicated by Thomas et al. (2019). In the Ligurian site, no mass mortality was observed, and a monitoring programme is regularly taking place as a preventive mitigation measure (Salvidio, unpublished data).

Our review of species susceptibility already conducted by Beukema et al. (2018) and Gilbert et al. (2020) shows that the great majority of Italian salamanders, and in particular many endemic taxa, are potentially highly vulnerable to *Bsal* infection. Indeed, the endemic genus *Salamandrina* and the many species belonging to the genus *Speleomantes* have been experimentally proven highly vulnerable to this infection. In addition, all Ital-

ian species with few exceptions share a relatively high ecological niche overlap score with *Bsal* (Beukema et al., 2018 synthesized in Table 2 of this study), suggesting that, even in the Mediterranean region, salamanders inhabiting humid and cool microhabitats, such as *Speleomantes strinatii* and *Triturus carnifex* are exposed to a high risk. Concerning the EU priority micro-endemic subspecies of Alpine salamander, *Salamandra atra auro-rae*, Beukema et al. (2018) did not calculate niche overlap, while a high susceptibility to *Bsal* was inferred from the experimental data on other *Salamandra* subspecies (Gilbert et al., 2010). Given the high conservation concern of this taxon possessing a restricted range of occurrence (Romano et al., 2018), a demographic monitoring project has been implemented by the local province of Trento starting in 2017, while skin swabs have been collected from different sites in August 2022 (Romano, pers. com. September 2022).

Active disease surveillance and large-scale monitoring of populations of the most threatened species should be the ideal preventive strategy, but the costs associated with these activities would be clearly prohibitive (Bosch et al., 2021). Therefore, a more cost-effective policy should integrate passive reporting and intervention in the cases of confirmed mass mortality events combined with strong preventive measures, as already suggested by Thomas et al. (2019). These measures should include testing for *Bsal* throughout the amphibian international commerce to exclude the unintentional introduction of fungal pathogens from captive amphibians legally traded. Moreover, controlling and eradicating invasive populations of alien amphibians that could act as *Bsal* intermediate hosts should be considered and realised, when possible. These preventive eradications should be undertaken especially for species that act as intermediate hosts and are associated with the spread of amphibian chytrid pathogens, such as the African clawed frog *Xenopus laevis* and the North American bullfrog *Lithobates catesbeianus* (Pasmans et al., 2017). Moreover, it is pivotal that professional and amateur herpetologists always implement strict biosecurity measures during field activities, to avoid the spread of the pathogen across distant study areas, and the existence of online information about biosecurity precautions, such as <http://bsaleurope.com/>, should be better advertised.

Finally, herpetologists are not the only potential drivers of the spread of these pathogens, because unfortunately they can be spread by any kind of people performing outdoor activities. A broad dissemination of the issues of wildlife pathogens, and of the protocols to limit them, remains a fundamental preventive action (e.g., <http://www-9.unipv.it/webshi/images/files/All.%20II%20-%20CHITRIDOMICOSI.pdf>; <http://bsaleurope.com/>).

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <<http://www-262.9.unipv.it/webshi/appendix/index.html>> manuscript number 13279

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Age estimation and body size of the Parsley Frog, *Pelodytes caucasicus* Boulenger, 1896 from Lake Borçka Karagöl, Turkey

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Abstract. In this study, we described age structure, body size, body mass and the relationships among these parameters for a population of *P. caucasicus* from Lake Borçka Karagöl, Artvin, Turkey. The mean SVL with standard error was $45.87 \text{ mm} \pm 0.55$ (range: 39.98-50.28 mm) and the mean weight with standard error $8.81 \text{ g} \pm 0.39$ (range: 6.10-11.47 g) in females whereas $48.16 \text{ mm} \pm 0.45$ (range: 43.64-54.78 mm) and 11.32 ± 0.25 (range: 9.56-14.80 g) in males, respectively. We found a significant male-biased difference reflecting sexual dimorphism and statistically significant positive relationships between these variables. According to the results, the age ranged between 2-5 years in females and 2-6 years in males. The mean age distributions significantly differed between the sexes (females: $3.28 \text{ years} \pm 0.19$; males: $3.94 \text{ years} \pm 0.20$). The mean ages and maximal ages were found identical to the previously reported results from Turkey, but the mean ages were higher than in Georgian populations. Von Bertalanffy growth models demonstrated similar curves, and the growth rate was faster up to 3 years in both sexes. To conclude, this study was the first to determine age structure and growth patterns in Borçka Karagöl population and weight data for *P. caucasicus* was presented for the first time in the literature.

Keywords. Amphibia, skeletochronology, weight, Von Bertalanffy, growth rate.

INTRODUCTION

The determination of individual age is essential for studies like demographic and life history, including developmental biology and population dynamics of a species. This provides the researchers basic ecological data related to population structure such as sexual maturity time, the age structure, growth rate, and life span. Moreover, this is a parameter essential to infer the life history traits of a species and to compare it to other species (de Buffrénil et al., 2021; Ma et al., 2022). In this context, skeletochronology is an effective and reliable method for estimating the growth and age of many amphibian species as the growth of amphibians is not independent of environmental conditions (Guarino et al., 2019; Üzümlü et al., 2020). In addition, the method can be also applied to animals

such as mammals (Nacarino-Meneses et al., 2016), lizards (Beşer et al., 2019), turtles (Guarino et al., 2020), and even fossils (de Buffrénil et al., 2021). Skeletochronology calculates the lines involving the formation of calcium carbonate, called “annual rings” or “growth markers” in bone tissues (Castanet, 1994; Ma et al., 2022). For this, the diaphyseal region of their long bones, which show weaker vascularity, provides the best result for calculating the age of individuals (Castanet et al., 1993). Rozenblut and Ogielska (2005) showed that lines of arrested growth (LAGs) are most complete in the middle part of the phalangeal diaphysis in European water frogs and thus pointed out that the middle part of the long bone is optimal for age studies. Moreover, the skeletochronology helps to calculate the lifespan of the population in amphibians, explain the sexual size dimorphism, and

reveal the differences between the sexes in terms of age and size. Also, the knowledge based on the skeletochronological studies tends to show population dynamics (Peng et al., 2021).

Pelodytes caucasicus Boulenger, 1896, the Caucasian parsley frog, is a native species of the Caucasus fauna. The species is distributed throughout northwest Azerbaijan, Georgia (southwest and South Ossetia), Russia (Krasnodar district), and Turkey (Blacksea region) (Zazanashvili et al., 2012; Litvinchuk and Kidov, 2018; Çiçek et al., 2019). The species is considered as near threatened because of natural and anthropogenic pressures (Ananjeva et al., 2009; Iskanderov, 2009; Kaya et al. 2009), so it is recommended that public campaigns should be conducted to raise the awareness for this endemic relict species in the border of Georgia and Turkey (Tarkhnishvili and Kaya, 2009). From this aspect, it is important to reveal the population dynamics of *P. caucasicus* in a new population based on skeletochronology. Although the age structure of *P. caucasicus* was reported by two studies in Georgia (Gokhelasvili and Tarkhnishvili, 1994; Chubinishvili et al., 1995), there is only single study in the border of Turkey (Erişmiş et al., 2009). Given the importance of skeletochronological information to better understand the population dynamics, ecological and evolutionary processes, we aimed to present the age structure of the Borçka Karagöl population for the first time and compare the results with the previous studies. We also provided weight data for the first time in this species, as well as assessed the relationship between this trait and SVL for age and growth rate.

MATERIAL AND METHODS

Fieldwork

We sampled 50 specimens (18 ♀♀, 32 ♂♂) during the breeding season (2013) from Lake Borçka Karagöl territory, in the vicinity of Artvin, Turkey (Fig. 1). The collected frogs were anesthetized with MS222. Snout-vent length (SVL) was measured at the nearest 0.01 mm using a digital calliper and the weight was noted using the nearest 0.01 g an electronic balance for each sample. To count growth lines, the longest toe of the left hindlimb was clipped and preserved in 70% ethanol. After the sampling procedure, all frogs were released at the site of capture. Sex determination was possible due to the presence of secondary sexual characters, such as nuptial pads on the forearm, and presence of vocal sacs in males.

The standard skeletochronology procedure (Castanet and Smirina, 1990) was applied to calculate the number of the lines of arrested growth (LAGs). After removing

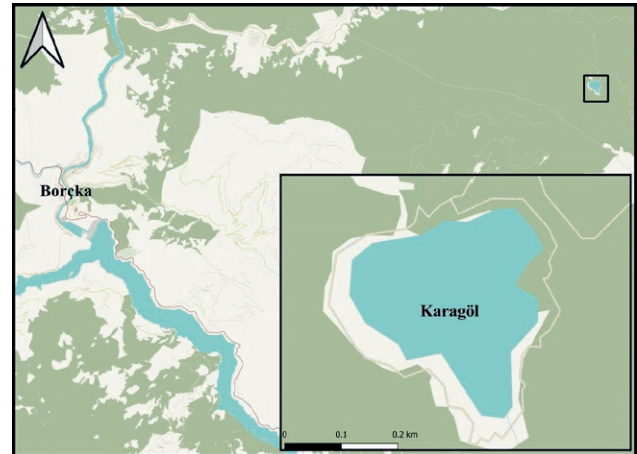


Fig. 1. Lake Borçka Karagöl and surrounding region.

soft tissues, the phalanges were washed in tap water, then decalcified in 5% nitric acid for 2 hours. Lastly, the samples were washed in distilled water overnight to ensure the removal of nitric acid. The 18 μ m cross-sections were obtained from the diaphyseal part of each phalanx using a cryostat (Thermo Shandon Cryotome, Germany) and stained with Ehrlich Haematoxylin approximately for 15 minutes. The stained cross-sections were treated with glycerol to control under a light microscope. The images of selected sections were acquired using Olympus BX51 microscope with an integrated camera to estimate the precise age of each specimen. The number of LAGs was counted by two researchers (S. Gül and N. Özdemir). The distance between two adjacent LAGs is a well-known indicator demonstrating individual growth in a given year (Kleinenberg and Smirina, 1969; Kurnaz et al. 2018). Following previous studies (Özdemir et al., 2012; Üzümlü et al., 2014), the endosteal resorption was evaluated by comparing the diameters of eroded marrow cavities with the diameters of non-eroded marrow cavities in sections from the youngest specimens.

Statistical analyses

To summarize data and present basic features of the measurements we calculated descriptive statistics using the *psych* package (Revelle, 2019). The normality assumption of the variables was checked using the Kolmogorov-Smirnov test. Since the variables followed a normal distribution ($P > 0.05$), we used parametric tests in downstream analyses. The homogeneity of variances was compared using Levene's Test in the *car* (Fox and Weisberg, 2019) package. We utilized the student t-test to compare the mean differences of the variables between males and

females. Thereafter, Pearson's product-moment correlation test was used to estimate the relationships among SVL, weight, and age.

The relationship of SVL and weight was analysed using a linear regression model. Additionally, we used an ANCOVA test to explore the patterns of SVL and weight between sexes, with age as the covariate. Thereafter, post-hoc tests were applied using the *emmeans* package (Lenth, 2021) under Bonferroni correction (estimated marginal means: aka least-square means or adjusted means).

We estimated growth curve models under the typical Von Bertalanffy's equation modified by Beverton and Holt (1957): $L_t = L_\infty \{1 - \exp[-k(t - t_0)]\}$ where L_t is the expected or average length at the time (or age) t , L_∞ is the asymptotic average length, k is the so-called Brody growth rate coefficient and t_0 is a modelling artifact that is said to represent the time or age when the average length was zero. To estimate parameter values and run the analyses *FSA* (Ogle et al., 2021), *FSAdata* (Ogle, 2019), *FSAsim* (Ogle, 2020) and *nlstools* (Baty et al., 2015) packages were used following the guide "fishR Vignette" prepared by Derek Ogle (2013). To visualize growth curve, we also added a hypothetical individual to the dataset by the reference of Erişmiş et al. (2009) under the presented parameters: SVL_0 at metamorphosis is fixed to mean 20.15 and t_0 (age at metamorphosis) is 0.3 year.

Statistical analyses were carried out using the *stats* package. Data visualization was performed using *ggplot2* (Wickham, 2016), *ggpubr* (Kassambara, 2020), and *ggally* (Schloerke et al., 2021) packages. All analyses were run in R Programming Language (R Core Team, 2020).

RESULTS

We successfully aged 50 individuals using skeletochronology technique. Ages ranged between 2-5 years in females, and 2-6 years in males. Descriptive statistics of both sexes and age groups are presented in Table 1.

The variances were found homogenous for SVL ($F_{1,48} = 0.2926$, $P = 0.5911$), weight ($F_{1,48} = 1.5006$, $P = 0.2266$) and age ($F_{1,48} = 1.9470$, $P = 0.1693$). According to the Student t-test results, statistically significant differences were found between sexes in all variables (Fig. 2). Males were significantly heavier ($t = 5.6509$, $df = 48$, $P < 0.001$) and larger ($t = 3.1545$, $df = 48$, $P < 0.01$) than females. The mean age was also found male-biased ($t = 2.2053$, $df = 48$, $P < 0.05$). A significantly positive correlation was found between SVL and age, SVL and weight for both sexes, but the correlation between weight and age was significant only in males. The correlation coefficients and p-values are presented in Fig. 3. The constructed linear regression model following the

Table 1. The summary table of descriptive statistics based on the dataset.

Variable	Females				Males			
	N	Mean \pm SE	Min	Max	N	Mean \pm SE	Min	Max
<i>All Specimens</i>								
SVL (mm)	18	45.87 \pm 0.55	39.98	50.28	32	48.16 \pm 0.45	43.64	54.78
Weight (g)	18	8.81 \pm 0.39	6.10	11.47	32	11.32 \pm 0.25	9.56	14.80
Age (years)	18	3.28 \pm 0.19	2.00	5.00	32	3.94 \pm 0.20	2.00	6.00
<i>2 Years Specimens</i>								
SVL (mm)	3	43.54 \pm 1.83	39.98	46.09	3	44.58 \pm 0.49	43.64	45.26
Weight (g)	3	7.22 \pm 0.55	6.18	8.05	3	10.38 \pm 0.22	10.11	10.81
<i>3 Years Specimens</i>								
SVL (mm)	8	46.07 \pm 0.71	43.42	49.26	9	47.18 \pm 0.59	44.27	49.72
Weight (g)	8	9.55 \pm 0.62	6.10	11.47	9	11.12 \pm 0.37	10.05	13.28
<i>4 Years Specimens</i>								
SVL (mm)	6	46.02 \pm 0.51	44.20	48.00	9	47.81 \pm 0.60	45.08	51.12
Weight (g)	6	8.44 \pm 0.53	6.70	10.05	9	10.72 \pm 0.41	9.56	13.63
<i>5 Years Specimens</i>								
SVL (mm)	1	50.28	50.28	50.28	9	49.37 \pm 0.47	47.13	51.95
Weight (g)	1	9.97	9.97	9.97	9	11.68 \pm 0.38	10.48	13.90
<i>6 Years Specimens</i>								
SVL (mm)	-	-	-	-	2	54.08 \pm 0.71	53.37	54.78
Weight (g)	-	-	-	-	2	14.65 \pm 0.15	14.50	14.80

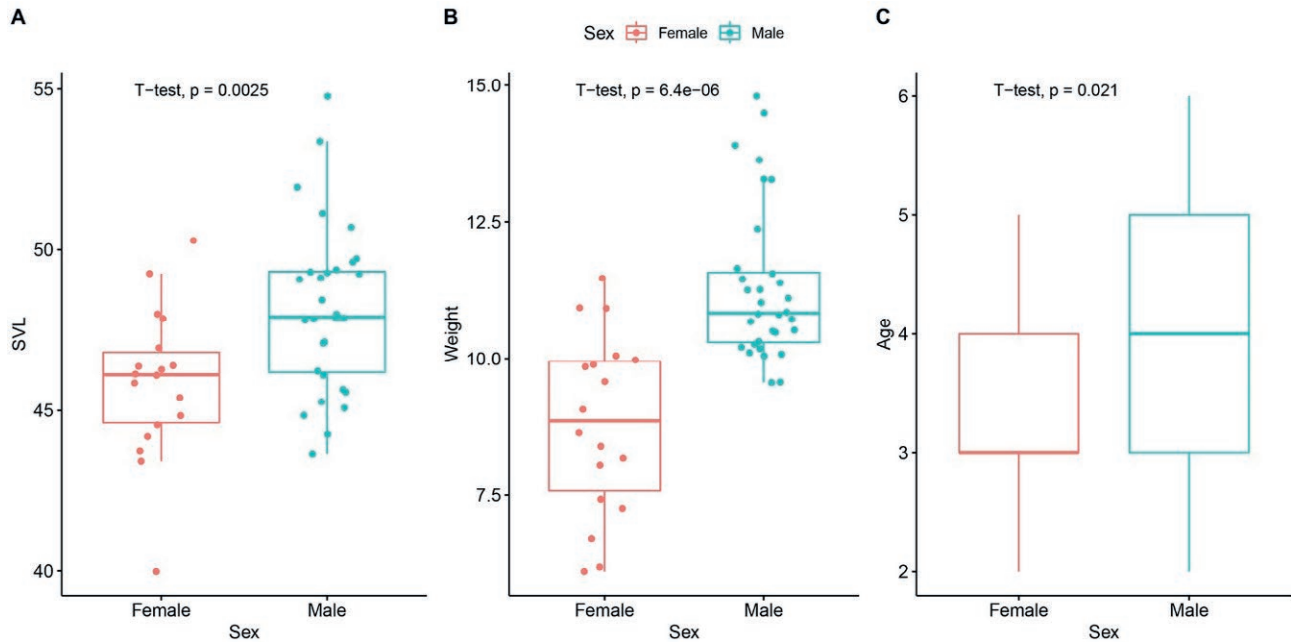


Fig. 2. The boxplots are representing the differences between the sexes (A: SVL, B: weight, C: age). The associated p-values were shown on the relevant plots.

correlations also presented significant equations between SVL and weight (males: $F_{1,30} = 42.54$, $R^2 = 0.59$, $P < 0.001$; females: $F_{1,16} = 11.23$, $R^2 = 0.41$, $P < 0.01$).

According to the ANCOVA results, the effect of age on the intersexual differences was found significant in SVL ($F_{1,47} = 39.8215$, $P < 0.001$) and weight ($F_{1,47} = 8.6144$, $P < 0.01$). Post-hoc test indicated that the mean SVL score, with age as a covariate, was significantly different between sexes (males: 47.8 ± 0.33 ; females: 46.6 ± 0.44 ; $P < 0.05$). Additionally, a statistically significant difference was found in terms of the weight between both sex with the age covariate (males: 11.2 ± 0.25 ; females: 9.06 ± 0.34 ; $P < 0.001$).

Growth curves estimated by Von Bertalanffy's model fit adequately described the relationship between age and SVL, and the curves indicated similar shapes in both sexes (Fig. 4). The final models were found statistically significant for all parameters ($P < 0.01$). According to the constructed models, the estimated asymptotic SVL was not higher than maximum recorded SVL values (Males: 54.78 mm; Females: 50.28 mm). The growth parameters were presented in Table 2.

DISCUSSION

The sexual size dimorphism (SSD) is an observable characteristic in animals, and it is known that the

direction of size dimorphism in most of the amphibians is female biased (Kupfer, 2007). Shine (1979) noted that females are larger than males in 61% of urodeles and 90% of anurans. The female biased SSD is generally explained by the fecundity advantage hypothesis (Shine, 1989; Andersson, 1994) when the selection is supporting large females due to the larger energy storage capacity for reproduction and more offspring production. However, a male biased SSD is relevant to the dominance in contests of strength, the extent of endurance, mate choice and higher sperm competition success in animal kingdom (Hudson and Fu, 2013). The male-biased SSD corresponding to 10% amphibian species is especially associated with territoriality behaviours and male-male competitions (Nali et al., 2014).

The family Pelodytidae is including five different species from the single genus *Pelodytes* distributing in Western Europe especially in the Iberian Peninsula, Caucasia, and north-eastern Turkey (Amphibiaweb, 2022). The previous studies have reported the female biased sexual size dimorphism in *Pelodytes* species inhabiting in Iberia (Talavera, 1990; Escoriza, 2017). For instance, Esteban et al. (2004) noted the larger average body length in females (43.31 mm) than males (36.32) mm in a northern Spain population of *Pelodytes punctatus* species. Diaz-Rodríguez et al. (2017) have described the presence of four valid species inhabiting along Western Europe by integrating molecular and morphological data. Regard-

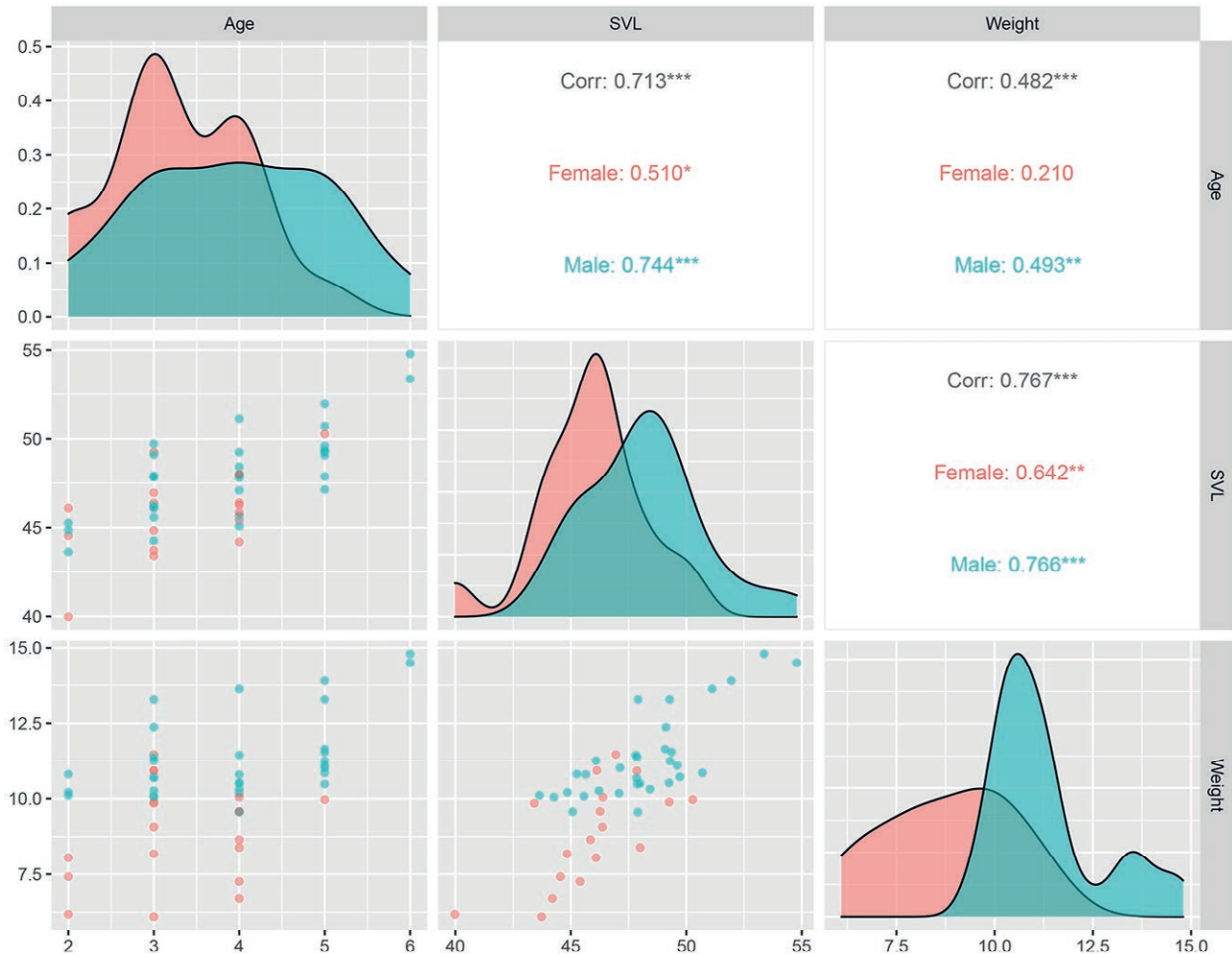


Fig. 3. The matrix is demonstrating the correlation coefficients, scatterplots, and density plots of binary variables. Corr values indicate the correlation coefficients (r). The significance level of correlation coefficients was represented with asterisk (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

ing their morphological measurements, all the species (*P. punctatus*, *P. hespericus*, *P. ibericus* and *P. atlanticus*) were characterized by larger average body length in females. However, our results revealed that unlike Iberian species, *P. caucasicus* males have a larger and heavier body than females. Our results are also consistent with the findings presented in previous studies. For example, Erişmiş et al. (2009) have comprehensively assessed the age structure and growth in *Pelodytes caucasicus* from Uzungöl, Turkey and. The mean SVL of adult males with SD were reported as 47.16 ± 2.87 mm ($n = 44$; range 41.48–52.58 mm), and significantly smaller in females (45.79 ± 2.29 mm; $n = 31$; range 40.28–50.62 mm). Arıkan et al. (2007) noted the range of SVL in sexually mature males between 45.06–52.08 mm, and 46.70–49.62 mm in mature females in Uzungöl population. Yildirimhan et al. (2009)

also recorded the mean SVL 50.6 ± 3 mm (range: 43–57 mm) in the population of Çaykara, Trabzon including 47 males, 7 females. Erişmiş et al. (2009) emphasized that the bigger size of older males in their study may deviate the results linked to SSD, but the size differences could also be derived from the biotic or abiotic selective pressures in poikilotherm animals. They validated their findings based on the former studies conducted in climatically different regions in Caucasia (Gokhelashvili and Tarkhnishvili, 1994; Chubinishvili et al., 1995) and they suggested male biased SSD is the species characteristic of *P. caucasicus*. The common SSD pattern in anura is generally female biased (Monnet and Cherry, 2002). Recently, Pincheria-Donoso et al. (2021) have investigated the SSD of amphibian species in global scale. As a result, they observed 90.8% female biased SSD 7.5% male-biased SSD

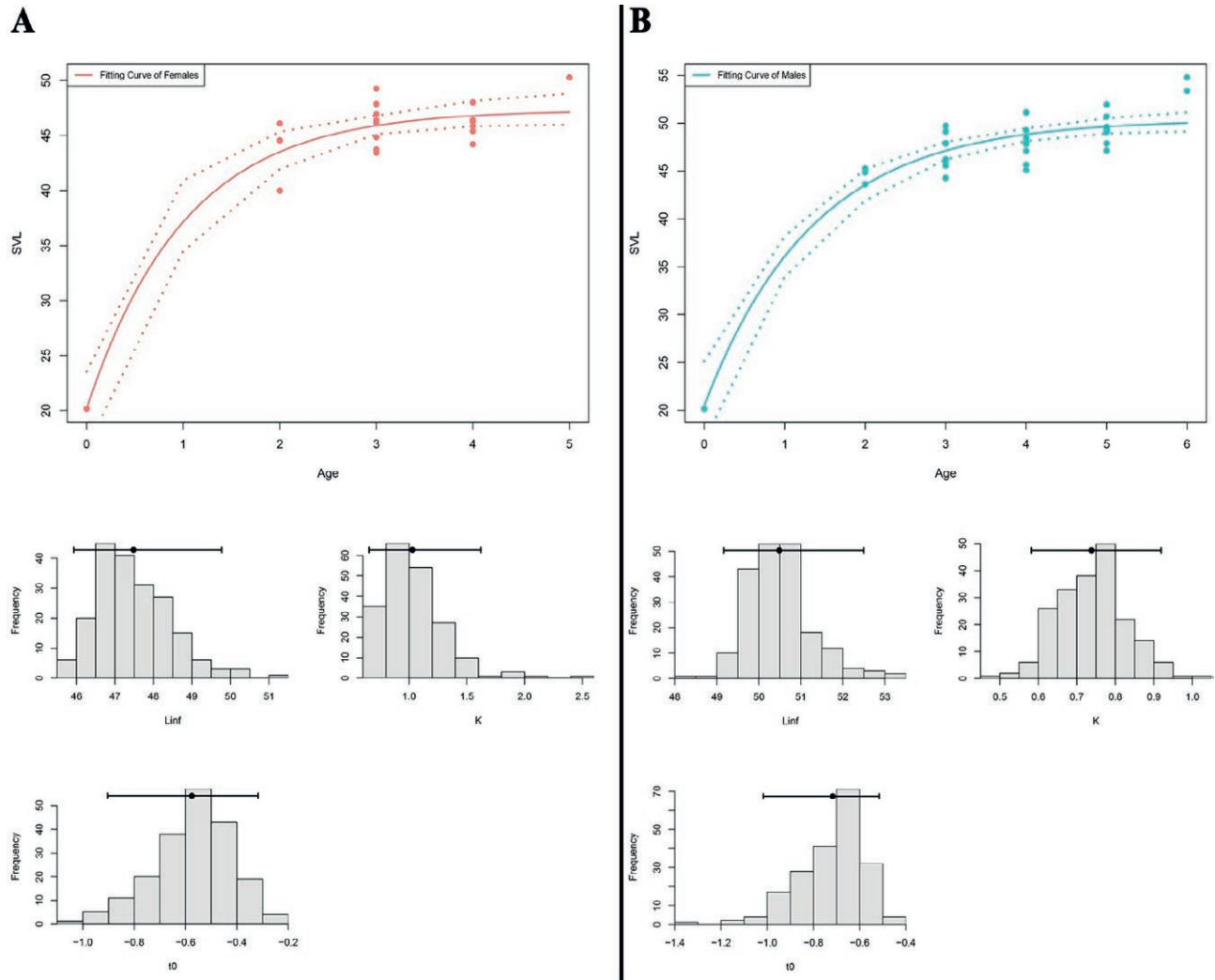


Fig. 4. Graphical visualization of Von Bertalanffy's growth curves and parameter optimization histograms. Dotted lines are representing 95% confidence intervals of fitted curves (A: Females; B: Males).

Table 2. The constructed final models of Von Bertalanffy's growth curves and associated parameters

	Estimated parameters				
	L_{∞}	CI	K	CI	t_0
Females	47.32	45.93-49.77	0.98	0.48-1.47	-0.56
Males	50.38	49.16-52.49	0.73	0.58-0.91	-0.70

and 1.7% monomorphic in anurans. However, Han and Fu (2013) determined male biased SSD in 19 different anuran families distributing in six distinct continents and along tropical and temperate habitats. Moreover, constrained habitats and microhabitats can affect the female

size and fecundity, and it can be resulted with more similar body size of both sexes as observed in Hylid frogs (Silva et al., 2020). From this aspect, it can be suggested that the directional difference of SSD between the Iberian species and Caucasian *P. caucasicus* may cause due to different ecological preferences in response to their habitats. The Iberian Peninsula which has complex orographic structure is surrounded by Atlantic Ocean and Mediterranean Sea. The Mediterranean coast and surrounding areas show dry and warm/hot summers, and mild and wet winters. The Atlantic coasts are characterized by oceanic type of climate, milder but humid winters and cooler/wetter summers, without large temperature variations. Lastly, the inner areas which is represented by continental climate type have hot and dry summers,

and cold and humid winters with large-scale precipitation, but also semi-arid areas extremely low precipitation and very hot temperatures especially in summer season (Carvalho et al., 2021) which are corresponding to the distribution of Iberian *Pelodytes* taxa (see Diaz Rodriguez et al., 2017). However, Caucasus Ecoregion has quietly high mean annual rainfall in the southwestern part over 2000 mm in the coastal area of the Black Sea. The mean annual temperature in the South Caucasus part of the Black Sea coast around 15 centigrade degree (Zazanashvili, 2009). Besides, the distribution of *P. caucasicus* species is restricted to the humid subtropics in Caucasia and Turkey with Colchic vegetation type (Tarkhnishvili, 1996; Iskanderov, 2009; Beşir and Gül, 2019). Gül (2014) also noted that the species prefers wet and warm microhabitats in Turkey. Additionally, the amount of precipitation is known as one of the most important factors constructing the distribution of *P. caucasicus* (Lukina and Koneva, 1996; Litvinchuk and Kidov, 2018). Pincheria-Donoso et al. (2021) proposed that the underlying impact of geographical variation in climatic pressures can shape large-scale patterns of SSD in synergy with natural and sexual selection such as intensification of fecundity selection to shorten breeding season in anurans. They also implied that the different selection forms can be shaped by macroecological factors climate, geographical gradients and temperature seasonality which are triggering the evolution of life-history traits associated with fecundity. Therefore, we suggested that the ecological preferences of *P. caucasicus* are potential reason causing male-biased SSD comparing to the representatives of Iberian Peninsula. Radojčić et al. (2002) also revealed similar SSD pattern in the *Bombina* species. They noted that *Bombina bombina* showed male-biased SSD while the larger body size of females was observed in *B. variegata* subspecies, and the differences were associated with reproductive behaviours and possible ecological differences. This pattern can also be observed in *Pelobates syriacus* (Bülbul et al., 2020 and references therein), *Rana arvalis* populations (Glandt and Jehle, 2008) and *Rana nigrovittata* which has similar ecological needs with *P. caucasicus* such as streams in shaded forest environments (Khonsue et al., 2000). On the other hand, the male-male competition was reported in *Pelodytes* species (Pargana, 2003; Marquez et al., 2004 and unreported mating ball of *P. caucasicus* observed by S. Gül). Therefore, it should be also taken into consideration that sexual selection which maximizes the fitness in reproductive traits may be an alternative force to describe the SSD pattern in our study as reported in different anuran species e.g., *Paa spinosa* (Gen-Yu et al., 2010) and *Hypsiboas atlanticus* (Camurugi and Juncá, 2013). SSD can be driven by life-history traits, so the accuracy of the

age determination is critical to estimate these characteristics. The post-metamorphic terrestrial growth is a major part of total growth (over 90%) in amphibians (Werner, 1986) and the variation in terrestrial growth rates and age at maturity is playing an important role in the intersexual adult size variability (Marangoni et al., 2012). Furthermore, the age determination of amphibians yields crucial information on the demographic features such as size at sexual maturity growth, longevity, and growth rate (Duellman and Trueb, 1994; Otero et al., 2017; Baraquet et al., 2021). In this study, we found that the age is ranged between 2-6 years. The mean age is 3.28 years in females and 3.94 years in males. Our constructed Von Bertalanffy's growth curves adequately fitted age/SVL relationship. The models demonstrated similar curve shape for both sexes, but the growth coefficient was higher in females.

Previously, Gokhelasvili and Tarkhnishvili (1994) studied the age distribution of the reproductive population of *P. caucasicus* in Borjomi canyon during two consecutive years (1992-1993). According to their results, the mean ages were 2.96 and 2.74 for males, 2.74 and 3 for females, respectively. They also reported the dominance of young specimens in the reproductive portion of the populations and a very-high annual mortality rate index (0.78-0.83). The males reached maximum of 6 years, and 4 years in females, which is in accordance with what Gokhelasvili and Tarkhnishvili (1994) reported. In the following year, Chubinishvili et al. (1995) studied certain aspects of the population ecology of *P. caucasicus*. They reported sexual maturity between 2-3 years. Contrary to their findings, the mean age is found approximately one year above the one measured in the Borçka Karagöl population.

Erişmiş et al. (2009) reported the mean age of males and females 3.61 ± 0.9 years and 3.03 ± 0.7 years, respectively and the age structure difference is statistically significant ($P < 0.05$). The oldest male was 5 years while the female was 4 years. The sexual maturity is reached at two years of age. We also obtained approximate values from the mean SVL and age (see table 1). The differences observed in SVL and age between sexes were also statistically significant. Given the identical results for Uzungöl and Borçka Karagöl populations, we can assume that there is a separation between Georgian and Turkish populations related to age structure. Erişmiş et al. (2009) also calculated a survival rate at 0.78 in males and 0.76 in females, and these figures can be taken as a reference to explain the high mortality rate noted by Gokhelasvili and Tarkhnishvili (1994). Based on the literature, the maximum age reported in the genus *Pelodytes* is 10 years for females and 8 years for males in *Pelodytes punctatus* species (Esteban et al., 2004). Additionally, the mean

age (Burgos: 1.71 years \pm 1.41 years, Valencia: 3.82 years \pm 1.22 years) and mean SVL (Burgos: 34.36 mm \pm 2.22 mm, Valencia: 36.41 mm \pm 2.50 mm) of males in the study of Esteban et al. (2002) were lower than our mean values. The authors noted that males were aged between 1-6 years old, and the age structure was highly skewed corresponding 50% of the sample of males being 1 year old. This situation can cause uncertainties when age/SVL relationships in the genus *Pelodytes*. In this study, we also did not age any individual in 1 year old from Lake Karagöl population. However, it is known that the low average age and high proportion of younger individuals might be associated with rapid decrease of local population of the species such as *Rana porosa* (Misawa et al., 2002) and Esteban et al. (2002) pointed that this pattern is more relevant to conservation status due to anthropogenic pressures in the Burgos population.

According to the growth curves estimation of Erişmiş et al. (2009), asymptotic SVL and growth coefficient were very similar between the sexes (Males: SVL_{max}: 53.42 mm \pm 1.01 mm; K = 0.42 \pm 0.03; Females: SVL_{max} = 52.04 mm \pm 0.75 mm; K = 0.38 \pm 0.01). In both sexes, the estimated asymptotic SVL was slightly higher than the maximum SVL record and the age-specific growth rate under the 3 years reported faster than higher ages. On the contrary, the estimated SVL_{max} values were lower, but the estimated K values were higher in our constructed growth model. From this aspect, we think that the more bell-shaped curve of our models is likely due to lack of the data from one-year individuals, and this may affect parameter fitting. The second reason is likely the preferred formula equation differences. The common point of the models is a remarkable decrease of growth rate after 3 years following sexual maturity in both sexes.

Data on body mass is limited in amphibians, but Santini et al. (2018) emphasized that weight data can be used to assess ecological and evolutionary processes such as dispersal distance, reproduction, population abundance and energy intake and it was also highly correlated with SVL in various anuran families (Bufonidae, Hylidae, Myobatrachidae, Ranidae). Moreover, they said that rapid body mass rise is associated with SVL rise for terrestrial and semi-aquatic species because allometric relationships between length and mass vary in amphibian species based relevant to the different habitat preferences. Lastly, they suggested the geometric similarity hypothesis (Hill, 1950) is fitting these assumptions better because if two organisms are geometrically similar their linear dimensions can be made equal by multiplying those of one of them by a constant. In this study, we contributed to the literature by first recorded weight data of *P. caucasicus* species. In the genus *Pelodytes* the available weight data was presented

by Esteban et al. (2002) for male *P. punctatus* specimens (Burgos: 2.36 g \pm 0.28 g, Valencia: 3.71 g \pm 0.58 g). Contrary to the mean weight in our male specimens, Spanish populations have lower values in parallel with mean SVL and age differences. The linear regression model and ANCOVA results also indicated the allometric relationship between SVL and weight, and the weight differed both between sexes and among age groups. Habitat preference of *P. caucasicus* are terrestrial and freshwater systems (Kaya et al., 2009), thus our findings are suggesting the geometric similarity hypothesis and habitat preference effect proposed by Santini et al. (2018) to describe the relationship between SVL and weight. In addition, Xiong et al. (2020) noted that age is affecting the intersexual difference on the weight for the Shangcheng Stout Salamander *Pachyhynobius shangchengensis*. From this aspect, the synchronized rise of SVL with aging may contribute to the intersexual difference associated with weight in *P. caucasicus* species. Camurugi and Juncá (2013) said that male-biased sexual size dimorphism corresponding to length and weight is unusual pattern and among frogs and even if it is generally described by male-male competition, the reasons are not fully understood yet. They also described the male-biased SSD as synapomorphic characteristic in *Hypsiboas punctatus* species group. Monnet and Cherry (2002) indicated that *Bufo achalensis*, *Rana cascadae*, *R. nigrovittata* species showed male-biased SSD because males were older sex as we determined that the mean age of *P. caucasicus* males. This pattern is causing due to delayed maturity which is determinant factor when larger body is contributing to mating success and males are not accelerating the growth to breed earlier than females. Tarkhnishvili (1993) said that the lower fecundity, smaller eggs, the volume of clutch and smaller body are the differences in spawning mood associated with female body size, and they are causing to rapid maturation for the species including *Pelodytes caucasicus* as a strategy to shorten the period between generations and to increase the number of adult individuals. Therefore, we assume that the male biases SSD observed in weight data may also occur in response to the life-history and reproductive traits.

To sum up, we determined age structure and growth patterns in Borçka Karagöl population. Future studies can comprehensively investigate the relationship between these variables and ecological conditions, life-history traits, and reproductive characteristics.

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Patterns of acoustic phenology in an anuran assemblage of the Yungas Andean forests of Argentina

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Abstract. Breeding seasons in anurans are usually noticed by their advertisement calls, which stand as the main signal emitted by males during their adult life. These calls are species-specific signals with multiple information and can be used to monitor anuran populations over extended time periods. Applying a Passive Acoustic Monitoring method (PAM), we described the acoustic breeding phenology of an anuran assemblage along an altitudinal elevation range in the Yungas Andean forests of Argentina. In addition, we propose a new classification scheme for their acoustic phenological strategies, based on the male's calling records throughout an entire year. Also, we assessed the temporal and spectral niche overlap by the anuran species recorded. The assemblage was active throughout the entire year, with a higher concentration of calls recorded during the spring-summer season. We describe five distinct Acoustic Breeding Strategies based on the calling patterns of the recorded species. Temporal niche overlap was higher in the spring-summer season and in the lowest study site. The use of a PAM as a tool to monitor the advertisement calls in anurans communities could be a reliable technique to obtain different information about the species' acoustic phenology and the temporal use of the acoustic communication channel.

Keywords. Acoustic breeding strategies, acoustic phenology, advertisement calls, anurans, calling guilds, Passive Acoustic Monitoring.

INTRODUCTION

Advertisement calls are the main signals emitted by anurans during the breeding season (Wells, 2007). These species-specific features are mainly expressed by males, with some exceptions in females (e.g., the bullfrog *Lithobates catesbeianus*) of certain species, and voiceless males like *Rhinella gallardoi* (Emerson and Boyd, 1999; Carrizo, 1992). Advertisement calls are considered key factors for mate selection (Márquez and Verrell, 1991; Gerhardt and Huber, 2002; Wells and Schwartz, 2007) and are used by receivers according to their temporal and spectral characteristics, both for identification and location of their

potential pairs, as well as to recognize the quality of the emitter (Gerhardt and Schwartz, 2001; Mason, 2007). Thus, these signals are used in mate choice behaviour, transmitting several messages simultaneously (Candolin, 2003). In addition, due to their "species-specific" nature, these are used as a taxonomic character for species description and identification (Köhler et al., 2017). Thus, advertisement calls have been widely used in long-term monitoring programs (Bridges and Dorcas, 2000; Dorcas et al., 2009; Llusia, 2013; Márquez et al., 2014; Measey et al., 2016). The surveying of advertisement calls has proven to be a useful technique for detection and monitoring of anuran species at large spatial scales (de Solla et

al., 2006), as well as for assessing population changes over time (Buckley and Beebe, 2004; Pieterse et al., 2006). These kinds of surveys can also provide valuable information about threatened species that breed in narrow time windows or display sporadic calling activity (Williams et al., 2013; Willacy et al., 2015; Akmentins and Boullhesen, 2020).

The breeding phenology of anurans, monitored through their advertisement calls, can be explored at different time scales, mainly in regions where seasonality shapes the extent, the start, and ending of their breeding activity (Weir and Mossman, 2005). Thereby, different species may have distinct daily and seasonal calling patterns (Cook et al., 2011; Yoo et al., 2012). Studying these calling patterns, can serve as a tool to define the core calling periods of the species recorded (Lemckert and Mahony, 2008), for which it is considered of utmost importance to increase the detection probability in anuran survey programs. The use of automated recording devices for passive acoustic monitoring techniques (PAM) can add valuable information such as the acoustic breeding patterns, as several species of anurans may breed simultaneously in different reproductive sites (Nelson and Garcia, 2017; Duarte et al., 2019; Ulloa et al., 2019; Pérez-Granados et al., 2020).

Amphibians are suffering alterations in their phenological patterns because of the increasingly frequent extreme climatic events caused by the global climate crisis (Lanno and Stiles, 2020). This is particularly concerning for the species distributed throughout the biodiversity hotspot of the tropical Andes of South America (Myers et al., 2000), which are severely affected by the consequences of climate change (Burrowes, 2008). In addition, in times of the global diversity crisis, amphibians are considered the most affected groups among terrestrial vertebrates (Stuart et al., 2004; IUCN, 2023).

Based on the temporal pattern of their breeding phenology, anurans have been historically classified into two discrete groups, as proposed by Wells (1977): explosive breeders and prolonged breeders. Although there is a behavioural continuum between these two extremes (Wells, 1977; 2007). Several works focused on better describing the breeding acoustic strategies that can be found in anuran species (Chen et al., 2023; Donnelly and Guyer, 1994; Forti et al., 2022; Huang et al., 2001; Prado et al., 2005; Prasad et al., 2022; Bertoluci and Rodrigues, 2002) but there is still missing information mainly for Neotropical anurans. Continuous monitoring of anuran advertisement calls along different spatial-temporal scales, can provide valuable information about the different breeding strategies displayed by each species within an assemblage (Moreira et al., 2007).

The acoustic niche hypothesis (Krause, 1987) proposes that each individual elaborating messages through sound in each environment will present a partition in its spectral and temporal features, to avoid being masked by others (Krause, 1993). In species-rich assemblages from different clades, different strategies are expected to be present to avoid the competition in the acoustic communication channel (Bertolucci and Rodrigues, 2002; Herrick et al., 2018; Klump and Gerhardt, 1992). One way to minimize competition for the acoustic space is made effectively by segregating the niche in its temporal and spectral dimensions (Both and Grant, 2012; Sinsch et al., 2012; Guerra et al., 2020, Lima et al., 2019). The temporal segregation of the advertisement calls can be fundamental for the constitution of large anuran assemblages, mainly in breeding areas where several species vocalize simultaneously (Drewry and Rand, 1983; Schwartz and Wells, 1983; Bertolucci and Rodrigues, 2002; Duarte et al., 2019).

The Yungas ecoregion is one of the most biodiverse environments in Argentina (Brown et al., 2006) harbouring up to 40 species of anurans (modified from Lavilla and Heatwole, 2010). These subtropical montane forests are characterized by a steeped altitudinal gradient described by phytogeographic stratum (Grau and Brown, 2000). The anuran assemblages that inhabit the subtropical montane forest of the Parque Nacional Calilegua (PNC) within the Yungas ecoregion, was reported to present a wide range of temporal and spatial breeding patterns ranging from opportunistic to prolonged breeders (Vaira, 2002). However, these records of breeding activity were obtained based on regular monthly surveys lasting from 3 to 5 days carried out through active searches by visual and acoustic sampling (Vaira, 2002). The anuran assemblage of the PNC is composed by a few endemic species to the Yungas ecoregion and by numerous species with a wide distribution in Argentina and other South American countries (Lavilla, 2001; Vaira et al., 2017). In addition, a low number of studies aimed to describe and understand the complete acoustic breeding phenology over an entire year in neotropical anurans (Bertoluci and Rodrigues, 2002; Prado et al., 2005; Saenz et al., 2006). Therefore, there are still wide gaps of information about the daily and annual patterns of calling activity and the breeding acoustic phenology of the anuran species that conforms different assemblages inhabiting the Yungas forests.

A recent study suggests the use of PAM as an effective technique to monitor the species of anurans in the Yungas forests (Boullhesen et al., 2021). In addition, with the implementation of a PAM program, researchers were able to discover hidden behavioural insights of phenological activity, such as the nocturnal calling activity of a

frog species inhabiting the Yungas forests, that was previously considered as a strictly diurnal species (Pereyra et al., 2016).

In this study, we describe the daily and annual patterns of calling activity of the anuran assemblages inhabiting the Yungas Andean Forests using PAM. Furthermore, we propose a new classification scheme for the acoustic breeding strategies used by different species of Neotropical anurans. Also, we aim to explore the occupation level of the acoustic communication channel and to determine the temporal overlap of the acoustic niche of this subtropical anuran assemblage.

MATERIALS AND METHODS

Study sites and data collection

A PAM was carried out for 12 months (from September 2017 to August 2018) along an altitudinal gradient in the PNC, being the most representative portion of the Yungas ecoregion in Jujuy province, Argentina. The PNC harbours the phytogeographic strata described for the Yungas forests as well as different environments where anurans breed (Vaira, 2002). The study was carried out in the three main forest types described for the ecoregion: Premontane Lowland Forests (400 - 700 m a.s.l.) which is a semideciduous environment with a marked seasonality, the understory consists of dense bushes, herbs, ferns and lianas; Lower Montane Forests (700 - 1300 m a.s.l.) dominated by evergreen trees such as *Juglans australis*, *Cedrella balansae* and *Enterolobium contortisiliquum*; the Upper Temperate Montane Forests (1500 - 2500 m a.s.l.) which is a primary old forest dominated by trees from Myrtaceae family (Grau and Brown, 2000). The two upland forest types have markedly more moisture than the lower forest. Three automated recording units Song Meter 4 (Wildlife Acoustics Inc., Concord, Massachusetts), one per site were installed and programmed to record 3 minutes per hour (24/7) (Shirose et al., 1997; Márquez et al., 2014). Recordings were done in MONO channel using in-built low noise microphones and stored in 32GB SDXC Flash Cards in .WAV format. The recorders were placed at 1.5 m above ground in three sites: Premontane Forest (PF) (23°45'16.84"S; 64°50'59.35"W, 650 m a.s.l.), at the edge of a permanent pond with an approximate area of 1114 m². The Lower Montane Forest (LMF) (23°41'36.84"S; 64°52'5.04"W, 1125 m a.s.l.), at the edge of a permanent stream and in Upper Montane Forest (UMF) (23°40'28.56"S; 64°53'44.15"W, 1750 m a.s.l.), attached to a tree near temporary ponds. Recorders were visited monthly to data download and battery replace. These locations are

representative to the breeding areas used by the anuran assemblage of the region (Vaira, 2002).

Data Analysis

13,485 recordings were listened corresponding to one day per week (= 224.75 hours) from the three study sites together throughout a year-round monitoring. Recordings were inspected manually by a trained specialist in anurans call recognition of Yungas forests (MB) in Raven Pro 1.5 (Bioacoustics Research Program, 2014) using a window type = Hann, DFT size = 512 samples, and overlap = 50%. For the general description of the acoustic phenology of the anuran species recorded, the monitored year was divided in two seasons (six months each) marked by the regional climate as follows: A) spring-summer season, corresponding to the period from September 2017 to March 2018. B) fall-winter season, corresponding to the period from March 2018 to September 2018.

To describe the annual acoustic phenology, we used the classification of core calling periods proposed by Lemckert and Mahony (2008). This considers the core calling period for each species as the time-period containing > 90 % of the calling events. For these classifications we considered the species with a total of ≥ 50 calling events records only. With the data of the calling events per-day recorded throughout the year-round study we conducted a bottom-up hierarchical cluster analysis using vegan and cluster R packages. For this analysis euclidean distance and complete method were employed after correlation checking of the cophenetic distance obtained with the original data used (>0.90). This analysis was implemented in free software R.

To describe the daily and annual vocal activity of each species we adapted the classification proposed by Bridges and Dorcas (2000) as follows: 0 = no male vocalizing; 1 = one male vocalizing; 2 = multiple males vocalizing with the possibility of occasionally distinguishing single calls; 3 = multiple males vocalizing but unable to distinguish single calls. We considered each advertisement call detected from a recording as a "calling event", since we could not assign a distinct call to an individual. Circular statistics was employed to describe and analyse the daily calling patterns (Jammalamadaka et al., 2001; Pewsey et al., 2013). Rayleigh test was applied to explore whether the population of circular data, from which a sample is drawn, differs from randomness (Wilkie, 1983).

To explore temporal niche overlap in calling activity, we computed the Pianka (Pianka, 1973) and Czekanowski indices (Feinsinger et al., 1981) using the TimeOverlap program (Castro-Arellano et al., 2010; Guerra, 2020). The Czekanowski index or Proportional Similarity Index

varies between 1 (widest amplitude of the niche where the population exploits the resources in proportion to their availability) and 0 (where the population specializes in the rarest state of a resource and skip the other items). Whereas the Pianka index can return values less than zero (allowing for a coexistence between species) or greater than one (promoting a competition between species).

To characterize the use of the acoustic communication channel in anurans recorded, the methodology proposed by Emmrich et al. (2020) was used according to their acoustic calls features where:

Guild A = unmodulated simple call

Guild B = modulated simple call

Guild C = unmodulated pulsed call

Guild D = modulated pulsed call

Guild E = unmodulated pulsed multi-note call

Guild F = uniform modulated pulsed multi-note call

Guild G = non-modulated different multi-notes call

Guild H = modulated different multi-notes call

These guilds groups where previously visualized using clean calls from each species recorded with the *seewave* package in R using a Hanning type windows, 75 % overlap and 1024 sample size (Fig. S1).

RESULTS

Annual Calling Periods

A total of 3318 calling events of anuran species were recorded in the three study sites within the Calilegua National Park (Fig. 1). We detected calling activity throughout the entire year (Fig. 1), with a minimum of one species vocalizing in the driest months of July and August and a maximum of 16 species vocalizing simultaneously during December (Fig. 1). Most of the calls were recorded during the hottest and rainy period (spring-summer seasons), with peaks of calling activity during November, December, and January (Tables 1 and 2).

Boana riojana was the only species recorded throughout the year-round monitored and presented high records in the fall-winter period (Fig. 1). Meanwhile, the rest of the species from Hylidae family were recorded calling over the spring-summer period (Fig. 1-2). Most of the species from Leptodactylidae family where recorded calling at spring-summer periods, with the summer period having the highest core calling periods. However, *Pleurodema borellii* presented peaks of calling activity in the fall-winter period (Fig. 1). The two toads of the genus *Rhinella* were registered calling during the early spring-summer period. Meanwhile *Melanophryniscus rubriventris*

was recorded vocalising in the spring-summer, but was also detected in the early dry-cold fall-winter period (Fig. 1). The direct development-frogs from Strabomantidae family presented calling records concentrated in the spring-summer period, mainly during the summer season (Fig. 1-2). The only arboreal specie from Phyllomedusidae family (*P. boliviana*) presented calling records concentrated in the summer season (Fig. 1-2).

Acoustic Breeding Strategies

The cluster analysis revealed five different acoustic breeding strategies (Fig. 3): continuous breeders, prolonged non-seasonal breeders, prolonged seasonal breeders, prolonged non-regular breeders, and sporadic seasonal breeders.

A continuous breeding strategy was found in males of *Boana riojana*, who vocalized during all months of the year. A prolonged non-seasonal breeding pattern was observed in *Pleurodema borelli*, which started calling in spring-summer and continued through the fall-winter period. Prolonged seasonal breeders were clustered into two subgroups: prolonged-regular breeders called evenly spaced throughout the breeding season (spring - summer period), presenting many calling events records; prolonged non-regular breeders had a calling activity spaced throughout the summer season. Sporadic seasonal breeders were found in nine species belonging to five different families, which called sporadically during the spring-summer period only (Fig. 3).

Daily calling patterns

Species of the family Bufonidae presented highly contrasting daily calling patterns. Species of the genus *Rhinella* presented a mainly crepuscular and nocturnal calling pattern; *R. arenarum* presented a peak of activity at 20:00 h, while *R. diptycha* vocalised mainly between 20:00 h and 21:00 h (Rayleigh = 0.77; P = 0.0002; Rayleigh = 0.93; P = 0.0001) (Table 1, Fig. 4). Meanwhile, *Melanophryniscus rubriventris* was mainly a diurnal species, with peaks of vocal activity at 06:00 h and 18:00 h (Rayleigh = 0.52; P < 0.0001 (Table 1, Fig. 4).

The species of the family Hylidae at the PF site recorded a mainly crepuscular-nocturnal vocal activity, although sporadic calling activity was detected during the day. *Boana riojana* was recorded at PF and at the LMF sites, at the PF site presented a mainly nocturnal calling activity, with peaks of vocal activity at 01:00 h and 03:00 h; meanwhile, at the LMF site, with a higher number of records, presented a mainly nocturnal calling pattern,

Species	2017					2018								Total
	September	October	November	December	January	February	March	April	May	June	July	August		
<i>Boana riojana</i>	44	237	211	214	152	51	22	4	82	1	35	66	1093	
<i>Dendropsophus nanus</i>	8	16	14	36	51	35	14	1					175	
<i>Leptodactylus aepyta</i>		5	16	33	19								73	
<i>L. elenae</i>		10	32	48	45			2					137	
<i>L. fuscus</i>			22	33	5								60	
<i>L. gracilis</i>	2	3	4	1	1								12	
<i>L. macrosternum</i>			31	51	28	15							125	
<i>L. latinasus</i>	1	32	27	33	19	4							116	
<i>Melanophryniscus rubriventris</i>			1	111	187	81							380	
<i>Oreobates barituensis</i>	6	27	74	36	16	2	17						178	
<i>O. berdemenos</i>	38	86	87	85	10		1	3	2	4			312	
<i>Pleurodema borellii</i>		28	41	6	9	7	59	39					189	
<i>Phyllomedusa boliviana</i>		6	24	40	36	5							111	
<i>Physalaemus cuqui</i>		14	37	63	60	41	12						227	
<i>Rhinella dypticha</i>	1	6											7	
<i>Scinax nasicus</i>				12		4							16	
<i>Scinax fuscovarius</i>	1	13	18	31	9	6							78	
<i>Trachycephalus typhonius</i>			6	6	1								13	
Total	101	483	645	803	648	301	125	49	84	5	35	66	3302	

Fig. 1. Total number of calling events recorded for anuran species in the Parque Nacional Calilegua spanning the three sites together. Grey squares indicate calling activity. Dark squares indicate core calling periods.

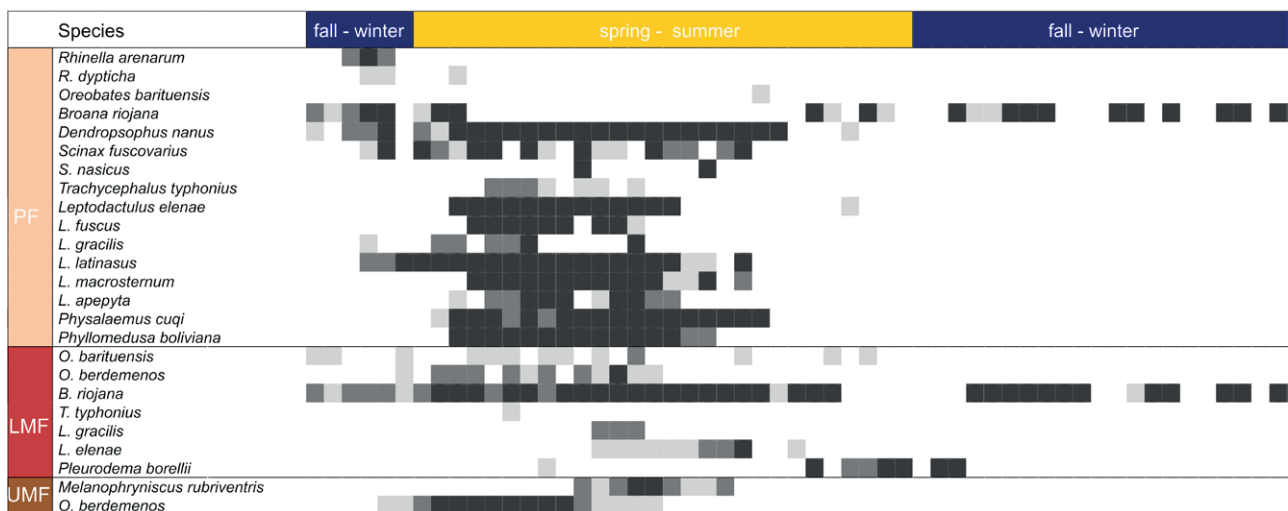


Fig. 2. Records of calling activity in the anuran assemblage of the Parque Nacional Calilegua. Black cells = maximum call events registered (3). Grey cells = medium calling events registered (2). Light cells = minimum callings events registered (1). PF = premontane forest; LMF = lower montane forest; UMF = upper montane forest.

but also with diurnal vocalizations (Table 1, Rayleigh = 0.8; $P < 0.0001$). *Dendropsophus nanus* presented a crepuscular-nocturnal vocal activity, with separated activity peaks at 20:00 h, 21:00 h, and 05:00 h (Rayleigh = 0.65; $P < 0.0001$). *Scinax fuscovarius* presented a calling pattern mainly crepuscular and nocturnal, with a peak of records at 22:00 h and 06:00 h, but also presented vocalizations

during the day at 14:00 and 17:00 h (Rayleigh = 0.42, $P < 0.0001$). By the other hand, *S. nasicus* presented a more sporadic and nocturnal vocal activity, with peaks at 22:00 h and 23:00 h (Rayleigh = 0.8, $P = 0.005$). *Trachycephalus typhonius* had a mainly crepuscular activity, with a peak of activity at 20:00 h, and was also recorded calling during the day at 14:00 h (Rayleigh = 0.59, $P = 0.007$).

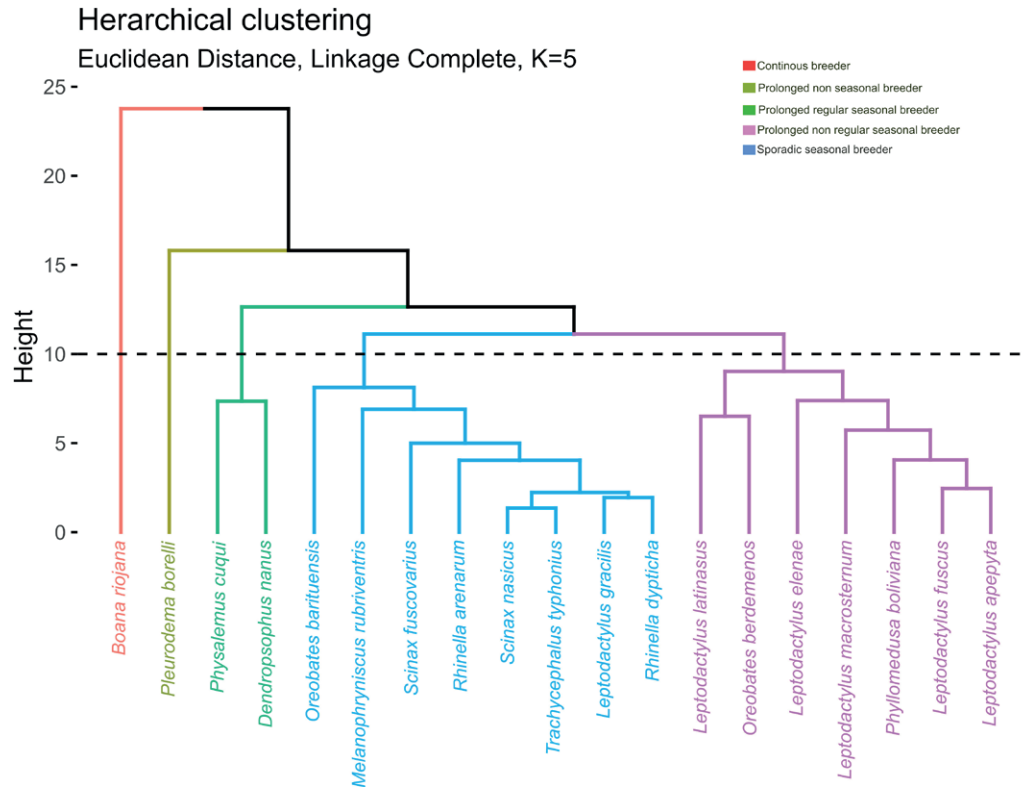


Fig. 3. Dendrogram showing the bottom-up hierarchical cluster analysis in the species recorded at the Parque Nacional Calilegua. Colours represent the five acoustic breeding strategies obtained.

Table 1. Circular statistics for the anuran species recorded in the study sites. Mu = trigonometric moment; rho = length; sd = standard deviation; cos = cosine; sin = sine; p = order; n = number of calling events; Rayleigh = Rayleigh uniformity test; P value = confidence value.

Species	mu	sd	rho	cos	sin	P	n	Rayleigh test	p value
<i>Boana riojana</i> (PF)	6.08°	0.66°	0.38	0.38	-0.008	2	100	0.8	<0.0001
<i>B. riojana</i> (LMF)	6.25°	0.98°	0.15	0.15	-0.01	2	337	0.6	<0.0001
<i>Dendropsophus nanus</i>	0.51°	0.92°	0.15	0.02	0.15	2	167	0.65	<0.0001
<i>Leptodactylus apepyta</i>	4.13°	0.74°	0.33	0.29	0.15	2	50	0.75	<0.0001
<i>L. macrosternum</i>	5.14°	1.01°	0.17	0.17	0.02	2	127	0.59	<0.0001
<i>L. elenae</i>	6.49°	0.86°	0.14	0.14	-0.01	2	130	0.68	<0.0001
<i>L. fuscus</i>	8.89°	0.89°	0.21	0.15	-0.14	2	59	0.66	<0.0001
<i>L. gracilis</i>	6.9°	0.85°	0.08	0.08	-0.02	2	13	0.69	0.0009
<i>L. latinasus</i>	-0.4°	0.77°	0.74	-0.07	0.73	2	115	0.74	<0.0001
<i>Melanophryniscus rubriventris</i>	12.43°	1.13°	0.05	-0.006	-0.05	2	67	0.52	<0.001
<i>Physalaemus cuqui</i>	5.77°	0.98°	0.02	0.02	0.001	2	212	0.61	<0.0001
<i>Phyllomedusa boliviana</i>	5.52°	0.64°	0.4	0.39	0.04	2	107	0.81	<0.0001
<i>Pleurodema borellii</i>	10.6°	1.35°	0.089	0.031	-0.08	2	96	0.39	<0.0001
<i>Oreobates barituensis</i>	13.85°	1.16°	0.18	-0.08	-0.016	2	31	0.5	0.0002
<i>O. berdemenos</i>	15.39°	1.53°	0.07	-0.05	-0.04	2	167	0.3	<0.001
<i>Rhinella arenarum</i>	4.22°	0.71°	0.43	0.39	0.19	2	12	0.77	0.0002
<i>R. diptycha</i>	5.93°	0.35°	0.77	0.77	0.01	2	6	0.93	0.001
<i>Scinax fuscovarius</i>	1.13°	1.3°	0.2	0.06	0.19	2	77	0.42	<0.0001
<i>S. nasicus</i>	4.23°	0.65°	0.6	0.54	0.27	2	7	0.8	0.005
<i>Trachycephalus typhonius</i>	5.38°	1.01°	0.079	0.078	0.012	2	13	0.59	0.007

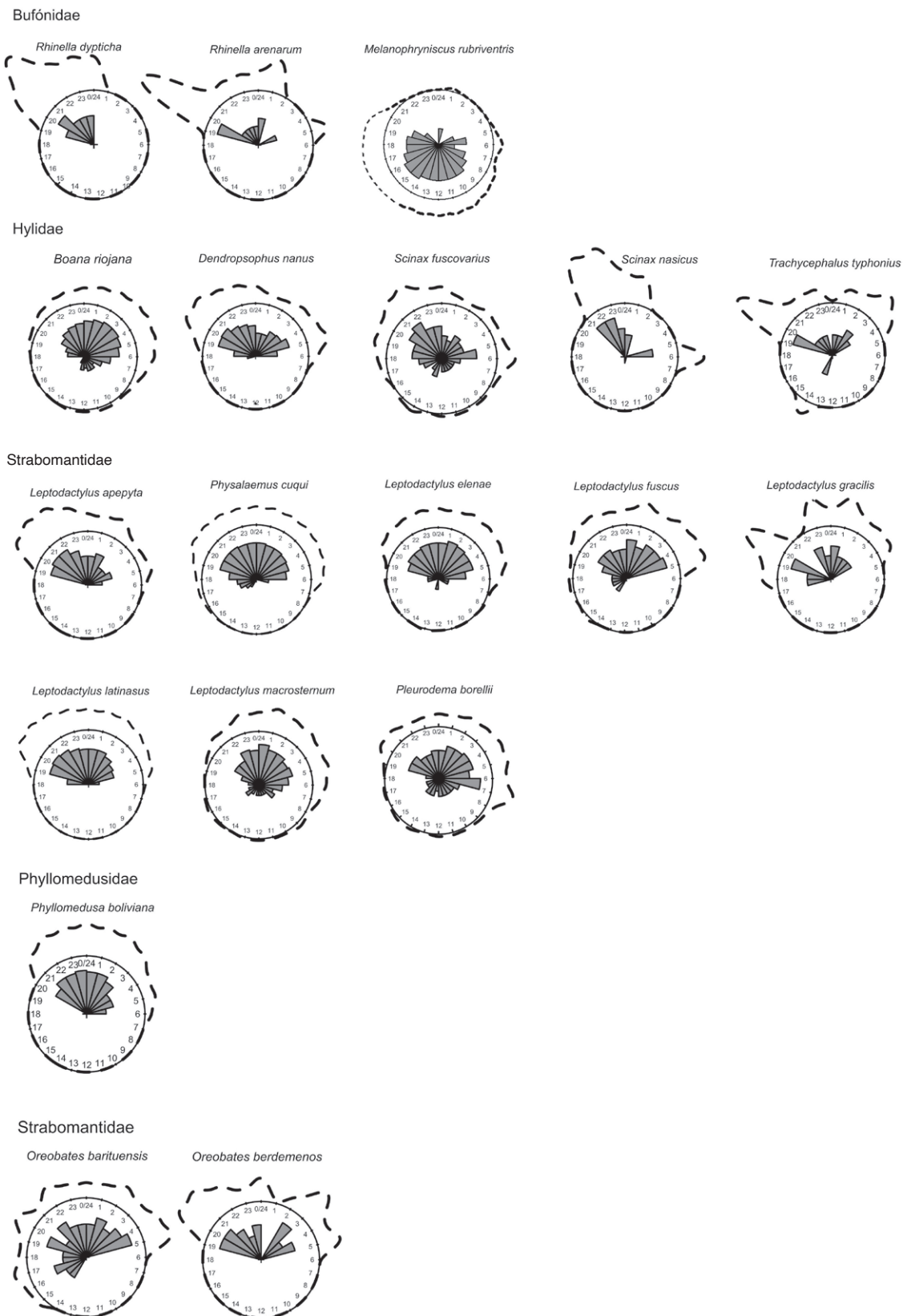


Fig. 4. Rose diagrams showing daily calling activity of recorded species in the study sites. Dotted lines = Kernel data distribution.

The species of the family Strabomantidae presented a daily vocal pattern but was mainly crepuscular-nocturnal. *Oreobates barituensis* presented a sporadic pattern during the day with records of calls from 17:00 h to 20:00 h, with a peak of vocal activity at 05:00 h (Rayleigh = 0.5; $P < 0.0002$) (Table 1, Fig. 4). *Oreobates berdemenos* presented markedly crepuscular-nocturnal vocal activity with a peak of vocal activity between 05:00 h and 06:00 h but was recorded vocalizing continuously during the 24 hs of the day (Rayleigh = 0.3; $P < 0.0001$).

Species of the family Leptodactylidae presented a mostly crepuscular-nocturnal daily vocal pattern, but records of calling activity were also detected during the day for some species. *Leptodactylus apepyta* began to vocalize at 19:00 h in the evening and peaked at 20:00 h and at 21:00 h (Rayleigh = 0.75; $P < 0.0001$). *Leptodactylus macrosternum* presented a mainly nocturnal calling activity with a peak between 24:00 and 01:00 h but also was recorded calling during the daytime, frequently between 06:00 h and 08:00 h (Rayleigh = 0.59; $P < 0.0001$). *Leptodactylus elenae* called mainly at night, presenting peaks of calling activity at 22:00 h and at 01:00 h (Rayleigh = 0.68; $P < 0.0001$). *Leptodactylus fuscus* presented a mainly nocturnal vocal activity, with sporadic peaks at 21:00 h and between 05:00 h and 06:00 h, with sporadic records during the daytime (Rayleigh = 0.66, $P < 0.0001$). *Leptodactylus gracilis* was recorded even more sporadically, mainly at twilight, with a peak at 20:00 h (Rayleigh = 0.69; $P = 0.0009$). *Leptodactylus latinasus* presented a twilight-nocturnal vocal activity, beginning to call at 19:00 h, with extensive records during the last hours of the night and a peak of calling activity at 20:00 h (Rayleigh = 0.74; $P < 0.0001$) (Table 1, Fig. 4). *Phyllomedusa boliviana*, an arboreal species, presented a strictly nocturnal vocal activity, with records of calls at 21:00 h and at 02:00 h, and a peak of calling at 22:00 h (Rayleigh = 0.81; $P < 0.0001$) (Table 1, Fig. 4).

Temporal niche overlap

The annual temporal niche overlap, and the spring-summer season overlap of the recorded species, was moderately high at PF site according to Pianka's index. According to the Czekanowski's index, the use of the temporal acoustic space was equitable (Czekanowski ~ 0.5) in the species assemblage recorded at the same site, both for the entire year and during the highest records of vocal activity in spring-summer season (Table 2). Meanwhile, the temporal niche overlap was lower in the LMF and UMF sites (Table 2), according to the Pianka's index (Table 2). In addition, the time niche overlap during the fall-winter season was moderate at LMF, according to the

Pianka's index (Table 2). According to the Czekanowski's index, a temporal niche overlap was found to be greater than that expected by chance (coincident activity) in the spring-summer period in the species that vocalize at the LMF site (Table 2).

Calling guilds

Six different calling guilds were recorded based on their advertisement calls features (Table 3; Fig. S1). *Leptodactylus apepyta*, was the only representative of the calling guild "A"; *L. elenae*, *L. fuscus*, *L. latinasus*, *Physalasmus cuqui* and *Trachycephalus typhonius*, were framed within the calling guild "B". *Rhinella arenarum* and *R. dypticha* were part of the guild "D". *Oreobates barituensis*

Table 2. Temporal acoustic niche overlap recorded in the study sites. Czekanowski index (0-1); Pianka index (0-1).

Site	Period	Czekanowski	p-value	Pianka	p-value
PF	Annual	0.51	< 0.001	0.62	< 0.001
LMF	Annual	0.26	0.003	0.37	0.005
UMF	Annual	0.38	1.12	0.42	0.041
PF	Spring-Summer	0.51	< 0.001	0.61	< 0.001
LMF	Spring-Summer	0.31	0.003	0.4	0.007
UMF	Spring-Summer	0.38	1.12	0.42	0.041
PF	Fall-Winter	0.12	0.13	0.21	0.1
LMF	Fall-Winter	0.42	0.051	0.59	0.054
UMF	Fall-Winter	-	-	-	-

Table 3. Calling guilds recorded along an altitude gradient within the Parque Nacional Calilegua, Argentina: permanent pond in the premontane forest (PF, 650 m a.s.l.), permanent stream in lower montane Forest (LMF, 1125 m a.s.l.), and upper montane forest (UMF, 1750 m a.s.l.). Total number of species recorded $n = 19$. Guild A = unmodulated simple call; Guild B = modulated simple call; Guild C = unmodulated pulsed call; Guild D = modulated pulsed call; Guild E = unmodulated pulsed multi-note call; Guild F = uniform modulated pulsed multi-note call; Guild G = non-modulated different multi-notes call; Guild H = modulated different multi-notes call.

Guilds	N° of recorded species	Percent	PF	LMF	UMF
A	1	5.26 %	1	-	-
B	5	26.31 %	5	-	-
C	-	-	-	-	-
D	2	10.52 %	2	-	-
E	1	5.26 %	1	1	-
F	8	42.10 %	7	2	1
G	2	10.52 %	1	-	1
H	-	-	-	-	-

was the only representative of the calling guild “E”. *Boana riojana*, *Dendropsophus nanus*, *L. gracilis*, *Oreobates berdemenos*, *Phyllomedusa boliviana*, *Pleurodema borellii*, *Scinax fuscovarius*, *S. nasicus* were part of the largest recorded group, the calling guild “F”. *Leptodactylus macrosternum* and *Melanophryniscus rubriventris* formed the calling guild “G”.

DISCUSSION

In this study, the calling phenology of anuran species was described at a fine timescale (hourly and daily) and quantitatively assessed along an altitudinal gradient in three Andean Montane Forest ecosystems of the Yungas ecoregion, within the PNC. This level of detail enabled us to propose a new classification scheme of acoustic breeding strategies according to the time spent by each species calling throughout a year.

Previous breeding strategies classification schemes were used to describe the breeding patterns of anuran species in the Parque Nacional Calilegua, following Duellman and Trueb (1986). Following this previous classification scheme only two breeding strategies were recorded for the entire species assemblage in this protected area (Vaira, 2002). Prado et al. (2005) described three reproductive activity patterns for a diverse anuran assemblage in a floodplain in the Pantanal region of Brazil. With the use of automatic recorders Saenz et al. (2006) were able to classify the anuran breeding activity patterns in: explosive breeders, winter breeders, summer breeders and continuous breeders depending on the core calling period recorded. Kopp et al. (2010) identified up to four distinct anuran reproductive patterns recorded by 13 visual encounter surveys in the Cerrado of Brazil. In this study, with the implementation of a PAM over an entire year we were able to identify five distinct breeding acoustic patterns and provide a novel, standardized classification framework, so that it can be tested in other ecosystems.

The great majority of the species recorded in this study showed a markedly seasonal breeding acoustic pattern, with their core calling periods centred during the spring-summer season in agreement with the warm and rainy season. These records were also reported by a previous study conducted on the same assemblage 20 years ago (Vaira, 2002). However, we now provide a detailed acoustic breeding phenology of the anuran species inhabiting three phytogeographical strata of the Yungas Andean Forest. The marked seasonal acoustic pattern might be related to the breeding behaviour present in these species that mainly use lentic waterbodies for their reproduction,

with free-living tadpoles (Vaira et al., 2002; Pereyra et al., 2018; Boullhesen et al., 2019).

Strictly sporadic seasonal breeders were found in the families Bufonidae, Leptodactylidae, and Strabomantidae, with one to four calling events per month. The calling activity recorded for sporadic-breeding species in a tropical forest of Guyana lasted between one and three consecutive nights (Gottsberger and Gruber, 2004). The records of calls from explosive breeders were in coincidence with the warm and rainy period of the study. The calling activity of explosive breeders has been documented to correlate positively with temperature and rainfall cues in tropical anuran assemblages that use ephemeral ponds (Ulloa et al., 2019). Similarly, another study described the explosive breeders to call in the rainy season in the Pantanal of Brazil (Prado et al., 2005).

Prolonged seasonal breeders were the most representative groups in the study area. In another study along an altitudinal gradient of Brazil this reproductive pattern was also predominant among anuran species whose calling activity was recorded for 6 months (Arzabe, 1999). These temporal reproductive patterns agreed with others already reported for the Yungas forests (Vaira, 2002; Akmentins et al., 2015; Pereyra et al., 2018). The species of the family Leptodactylidae are mainly prolonged breeders, calling throughout the night and with sporadic calling activity during the day in representatives of the genus: *Leptodactylus*, *Physalaemus*, and *Pleurodema* (Vaira, 2002; Camurugi et al., 2017; Boullhesen et al., 2019). However, it should be noted that certain species of the family such as *Leptodactylus fuscus* were characterized as mainly nocturnal in other ecoregions (Lucas et al., 2008; Guerra et al., 2020), although they showed an extended pattern of calling activity in daylight time recorded by a PAM in the Yungas ecoregion (Boullhesen et al., 2019). Our work provides novel information about the acoustic phenology patterns of a recently described species, *L. apepyta*, calling in the summer season showing a crepuscular-nocturnal activity where males vocalize outside subterranean nests that they built near the edge of temporary ponds (Schneider et al., 2019).

The tree frog *Boana riojana* was the only continuous breeder recorded in the study area calling throughout every month of the year. Duellman (1970) suggested that the hylids of Central America reproduce continuously throughout the year if the environmental conditions are favourable. In addition, these records may, in turn, be linked to the characteristics of the monitored site. For example, the site LMF is characterized by abundant vegetation (evergreen forest) and a permanent mountain stream, providing optimal conditions for *B. riojana* to vocalise and breed throughout the entire year. In other

species of this genus, a continuous breeding strategy has been suggested to correlate with adult male gravid and post metamorphic females' abundance recorded throughout a year of survey (Hiert and Moura, 2010). Previous research suggested that the members of the family Hyliidae have a crepuscular-nocturnal vocal activity (Guerra et al., 2020). However, our PAM study showed that *B. riojana* also calls during daylight, thus suggesting a more plastic calling behaviour in this species. There are reports of diurnal movement in species of the genus *Boana* in temperate forests of Brazil (de Oliveira et al., 2016). Therefore, it is interesting to explore the vocal repertoire of *B. riojana*, to determine if these daytime vocalizations correspond to advertisement calls or other types of vocalizations, such as territorial interactions or rain calls (Toledo et al., 2015).

The anuran assemblage recorded in this study presented a slight temporal acoustic niche overlap (time spent calling), mainly in the site with the highest diversity of species (PF). In addition, the temporal overlap of advertisement calls detected during the spring-summer period in the LMF site is remarkable. These results are in contrast with those reported for congeneric syntopic species that reproduce in permanent water bodies in the Cerrado savanna of Brazil (Guerra et al., 2020). The high diversity of species that compose the assemblages of the Yungas forests (Lavilla and Heatwole, 2010; Vaira et al., 2017), suggests that in places where several species reproduce simultaneously, the greatest diversity of calling patterns should be found to avoid being masked by others (Bertoluci and Rodrigues, 2002; Herrick et al., 2018; Klump and Gerhardt, 1992). The classification of advertisement calls of anurans, according to their spectral parameters in guilds, enables the comparison between groups of species to be faster and easier (Emmrich et al., 2020). This variety of acoustic strategies can be seen reflected in the calling guilds diversity detected in this study, mainly in the assemblage belonging to the PF site, with a total of five calling guilds recorded. Nevertheless, the calling guild with modulated pulsed multi-note calls was the most representative of the study, and unlike other guilds detected where there is a strong phylogenetic signal, this guild is made up of species from five different families. This observed pattern may conform with the Public Information Theory where anuran species with different phylogenetic relatedness elaborate advertisement calls with similar acoustics features (Danchin *et al*, 2004; Goodale et al., 2010; Sugai et al., 2021). In this sense, the anuran species belonging to different lineages could be using the same venue for information to determine the optimal conditions to breed. Thus, enabling an inadvertent social information resource available and driving the

species to use similar acoustic traits (Danching et al., 2004; Goodale et al., 2010).

The PAM employed in this study allowed us to describe in detail the acoustic reproductive patterns in an anuran assemblage in the mountain forests of the southern Yungas of north-western Argentina. This information made it possible to extend the previous knowledge regarding the calling activity of the species, contributing quantitatively to a better knowledge of a key aspect in the life history for each species recorded (calling activity), and to add new data for several secretive species.

In addition, it was possible to better characterize the variety of calling breeding strategies of the species recorded and, describe a standardized classification scheme to be tested in other ecosystems employing PAM method. In addition, we were able to access precise information on the temporal daily and seasonal acoustic distribution of the recorded anuran assemblage. This data is valuable and may be of great importance for implementing monitoring programs of anuran diversity in the Andean regions of South America in the current context of climate crisis.

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <<http://www-9.unipv.it/webshi/appendix/index.html>> manuscript number 14050.

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Diet and trophic niche overlap of four syntopic species of *Physalaemus* (Anura: Leptodactylidae) in southern Brazil

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Abstract. Despite the current increase in studies on the diet of neotropical anurans, few of them take a comparative approach between syntopic species. The objective of this study was to compare the diet of four syntopic species of the genus *Physalaemus*. The collections occurred between November 2014 and January 2016 in a preserved area of the subtropical Atlantic Forest in southern Brazil. We compared the gastrointestinal content of 109 individuals distributed in the species *Physalaemus biligonigerus*, *P. cuvieri*, *P. gracilis* and *P. henselii*. We measured the Index of Relative Importance of each prey category and calculated trophic niche breadth using the Levins' index (Bsta) and trophic niche overlap using the Pianka's index (Ojk). We also applied compositional analysis to evaluate feeding specializations. Formicidae was the most important prey category for *P. biligonigerus* (IRI = 88.5%) and *P. gracilis* (IRI = 39.1%). For *P. henselii* and *P. cuvieri*, the most important category was Isopoda (IRI = 51.7% and 57.9%, respectively), followed by Formicidae (IRI = 34.9% and 24.8%). Isopoda was also important in the diet of *P. gracilis* (IIR = 28.6%), followed by Araneae (IIR = 22.6%). The trophic niche breadth of the four species was narrow, all smaller than 0.32, and the lowest was recorded for *P. biligonigerus* (0.04). The trophic niche overlap was higher between *P. biligonigerus* and *P. cuvieri* (96%), and between *P. gracilis* and *P. henselii* (95%). Only *P. gracilis* presented a significant level of feeding specialization. The differences in their diets suggest different uses of the resources, which could relate to different ways of exploring the microhabitat.

Keywords. Foraging, trophic niche, amphibians, behavior.

INTRODUCTION

Resources can be shared by species in three different dimensions: temporal, trophic and spatial (Pianka, 1973). The different ways that species use resources can favor their coexistence and promote a reduction of competition between them (Pianka, 1974). These differences

can be observed, for example, in the different ways the species use the microhabitat, in the variety and sizes of food items and their period of activity (Schoener, 1974; Duellman and Trueb, 1986). Thus, basic data gathering about the ecology of organisms brings a great contribution to the elaboration of hypotheses and premises regarding niche partitioning and general ecology.

Although the divergence in feeding habits favors the coexistence of sympatric organisms, closely related species tend to use resources in a similar way (Pianka, 1973) since they are similar in physiology and often in morphology and behavior as well (Heyer et al., 1990). The number of studies on the diet of Neotropical anurans has increased in recent years (Siqueira et al., 2006, Dietl et al., 2009, Rodrigues and Santos-Costa, 2014, Moser et al. 2017; Oliveira et al. 2017, Dias et al. 2018, Farina et al. 2018, Moser et al. 2019, Protázio et al. 2019, Oliveira et al. 2021, Moser et al. 2022). However, few studies focus on a comparative approach to the species' diet (Oliveira et al., 2015, Moser et al., 2017).

Trophic relationships between species comprise one of the main aspects of their life history (Duellman and Trueb, 1986; Vitt and Caldwell, 2009). Information on resource partitioning among species can help us understand some parameters about community dynamics such as niche overlap and breadth (Lawor, 1980), which are essential data to describe part of their ecological niche (Sih and Christensen, 2001).

The genus *Physalaemus* (Leptodactylidae) has about 51 species and is widely distributed in the Neotropical ecozone (Frost et al., 2006). Species of this genus are characterized by consuming predominantly ants, beetles and spiders, but having an opportunistic diet pattern (López et al., 2003; Becker et al., 2007; Santana and Juncá, 2007; Rodrigues and Santos-Costa, 2014; Oliveira et al., 2015; Moser et al., 2017; Farina et al., 2018). Possible variations in diet composition among species are generally associated with variations in prey availability since, in general, anurans have an opportunistic diet (López et al., 2003; Moser et al., 2017). Thus, the study of syntopic populations presents an excellent opportunity to evaluate differences in diet among species that have the same variety and amount of potential prey available. The objective of this study was to compare the diet of four species of *Physalaemus* (*P. biligonigerus*, *P. cuvieri*, *P. gracilis* and *P. henselii*) that occur in syntopy in a remnant of Atlantic Forest in southern Brazil.

MATERIAL AND METHODS

Study site

The study was carried out in forest habitats inserted in the Atlantic Forest domain, located in a conservation unit (Reserva Biológica Lami José Lutzenberger - RBLJL), in Porto Alegre, state of Rio Grande do Sul (30°14'08"S; 51°05'42"W), southern Brazil. The local landscape corresponds to the transition region between forest formations and subtropical restinga, being one of the closest forest

remnants to the southern boundary of the Atlantic Forest (Printes, 2002; Witt, 2013). The climate of the region is classified as subtropical humid, with an average temperature in the warmer month of 22 °C and average annual temperature of 18 °C (Maluf, 2000).

Data collection

In this study, we explored data from anurans available in herpetological collections. We had the opportunity to access a group of frogs collected accidentally by other colleagues when using pitfall traps (Campbell and Christman, 1982) for entomological sampling. As traps were constructed with 10-L buckets, they were large enough to capture small frogs. In addition, as the buckets were filled with an ethanol solution (70%), they preserved the gastrointestinal content of frogs. We had access to frogs captured from November 2014 to January 2016 and, according to Brazilian laws, since the captures were accidental, no collecting permits were needed. The accidentally caught anurans were donated to the Laboratory of Ecology of Terrestrial Vertebrates (LEVERT) by the management team of the biological reserve.

In the laboratory, the animals were dissected to remove the gastrointestinal contents (stomach and intestine), which were preserved in 70% ethanol and screened under a stereomicroscope. For each prey category, number, volume and frequency of occurrence were calculated. Volume was calculated by estimations of the area (mm²) occupied by each item with a graph paper support attached to the bottom of the Petri dish, where we evenly spread each item, maintaining a regular height of 1 mm (Hellawell and Abel, 1971). In order to calculate each item volume (V), the area value (mm²) was multiplied by its height (1 mm) (Oliveira et al., 2015). The set of prey present in each content (individual) was considered as a sample. Prey items were identified until the lowest possible taxonomic level based on Ribeiro-Costa and Rocha (2006). We were unable to reach the species level due to the high fragmentation in most of the prey items caused by the digestion process.

To improve our data interpretation, we added to our dataset data of *Physalaemus henselii* that had been previously published (Farina et al., 2018). We highlight that this non-novel dataset represents 16% of the amount of data of the current study. All analyzed individuals were collected in the same locality with the same trap model and in the same period of the year.

Data analysis

We used the Index of Relative Importance (IRI), according to Pinkas et al. (1971) to calculate the impor-

tance of each category of prey using the following equation: $IRI = (N\% + V\%) FO\%$, where $N\%$ is the relative abundance of each prey category in the diet, $V\%$ is the relative volumetric contribution of the prey in the diet, and $FO\%$ is its relative frequency of occurrence in the diet (Pinkas et al., 1971; Krebs, 1999). The higher the IRI value, the greater is the importance of a given prey category in the diet.

We constructed rarefaction curves using Past 4.03 software to estimate the sampling representativity of the prey set for each anuran species (Sanders, 1968). In this analysis, each gastrointestinal content was considered as a sample. Curves were based on Jackknife 1 estimator of species richness (Burnham and Overton, 1978, 1979) which could represent the studied populations.

The Levin's Standardized Niche Breadth Index (B_{sta}) (Krebs, 1999) ranges from 0 to 1 and is calculated according to the following equation: $B_{sta} = (B-1) / (n-1)$, where n is the number of resources registered in the diet (prey categories), and $B = 1 / \sum p_i^2$, p represents the proportion of individuals of a given prey category (i) found in the diet. Values near 0 indicate a specialist diet (narrow niche breadth), while values near 1 indicate a generalist diet (wide niche breadth). B_{sta} was used to facilitate comparisons of trophic ecology between species.

To analyze the food overlap and/or degree of similarity between species diets, we used the Pianka's Trophic Niche Overlap Index (O_{jk}) (Pianka, 1973), defined by the following equation:

$$O_{jk} = \frac{\sum_{i=1}^n p_{ij} \times p_{ik}}{\sqrt{\sum_{i=1}^n p_{ij}^2 \times \sum_{i=1}^n p_{ik}^2}}$$

where O_{jk} is the niche overlap index between the species j and k ; p_{ij} is equivalent to the proportion of the resource type i relative to the total of resources used by the species j ; p_{ik} is the proportion of resource i relative to the total of resources used by the species k ; and n is the total number of resource categories used by the species j and k . This index ranges from 0 to 1 when there is no overlap or a complete overlap between the species diets, respectively (Krebs, 1999). For this analysis, we used the program EcoSim v1.2d (Gotelli and Entsminger, 2000). Since we have samples from all species from all seasons, comparisons helped us in detect if and how feeding behavior differ between species. Possible prey preferences were evaluated by Compositional Analysis (Aebischer et al., 1993) using the R package 'adehabitatHS' (Calenge, 2013). This analysis is based on a comparison between the matrix of consumed prey and the mean frequencies of components used by all individuals of the

sampled species (Sacchi et al., 2013). The analysis use randomization tests (number of permutations = 500) to assess the significance of the ranking matrices (Aebischer et al., 1993).

RESULTS

We analyzed the gastrointestinal contents of 109 individuals (Table 1). Of these, eight were *Physalaemus biligonigerus* (containing 190 food items distributed in 11 prey categories), nine *P. cuvieri* (221 items, 13 categories), 74 *P. gracilis* (529 items, 19 categories) and 18 *P. henselii* (154 items, 10 categories) (Table 1). Only one individual of *P. cuvieri* and 10 of *P. gracilis* had no gastrointestinal content. Even though some species, such as *P. biligonigerus* (8) and *P. cuvieri* (9), had a small sample size, we recorded 11 and 13 prey categories for them, respectively. Furthermore, an estimate of 9,81 for *P. biligonigerus*, 8 for *P. cuvieri*, 74,4 *P. gracilis* and 18,9 *P. henselii* was obtained using the Jackknife 1 estimator, which indicates that even with a small N (*P. biligonigerus* and *P. cuvieri*) the samples were sufficient to represent the populations, in the same way as for *P. henselii*. However, the rarefaction curves (Fig. 1) did not stabilize, suggesting that, by adding new samples (more frogs), other prey taxa were expected to be recorded. Based on this, results must be evaluated with caution.

Formicidae was the most important prey category for *P. biligonigerus* (IRI = 88.5%) and *P. gracilis* (IRI = 39.1%). For *P. henselii* and *P. cuvieri*, the most important category in the diet was Isopoda (IRI = 51.7% and 57.9%, respectively), followed by Formicidae (IRI = 34.9% and 24.8%). Isopoda was also important in the diet of *P. gracilis* (IRI = 28.6%), followed by Araneae (IRI = 22.6%) (Table 1). The trophic niche breadth was higher for *P. henselii* ($B_{sta} = 0.32$) followed by *P. gracilis* ($B_{sta} = 0.23$). The lowest values of niche breadth were recorded for *P. biligonigerus* ($B_{sta} = 0.04$) and *P. cuvieri* ($B_{sta} = 0.13$). The overlap of trophic niche (O_{jk}) was 96% between *P. biligonigerus* and *P. cuvieri*, 95% between *P. gracilis* and *P. henselii*, 91% between *P. cuvieri* and *P. gracilis*, 83% between *P. cuvieri* and *P. henselii*, 83% between *P. biligonigerus* and *P. gracilis*, and 71% between *P. biligonigerus* and *P. henselii*.

Results from Compositional Analysis pointed out that only *Physalaemus gracilis* has a certain degree of feeding specialization ($\lambda = 0.108$; $P < 0.001$; Table 1), preying upon Araneae, Coleoptera larvae, Isopoda, Formicidae and Hymenoptera in a higher frequency than expected by chance.

Table 1. Prey categories found in the gastrointestinal contents of *Physalaemus biligonigerus*, *P. cuvieri*, *P. gracilis* and *P. henselii*. N = number of individuals, V% = total volume (in mm³) occupied by prey category, FO% = frequency of occurrence of prey category, IRI% = Index of Relative Importance.

Prey categories	<i>P. biligonigerus</i> (N = 8)				<i>P. cuvieri</i> (N = 8)				<i>P. gracilis</i> (N = 64)				<i>P. henselii</i> (N = 18)			
	N%	V%	FO%	IRI%	N%	V%	FO%	IRI%	N%	V%	FO%	IRI%	N%	V%	FO%	IRI%
Acarina	1.1	0.2	12.5	0.1	2.7	0.5	22.2	0.3	5.7	0.8	18.9	1.6	11.0	2.0	50.0	6.7
Amphipoda	1.1	0.7	25.0	0.3	0.0	0.0	0.0	0.0	3.0	0.7	4.1	0.2	0.0	0.0	0.0	0.0
Araneae	1.6	1.0	37.5	0.6	5.0	4.1	55.6	2.2	18.0	13.4	55.4	22.6	5.2	3.6	22.2	2.0
Blattodea	0.0	0.0	0.0	0.0	1.8	6.1	44.4	8.7	0.6	0.6	4.1	0.1	3.9	8.4	16.7	2.1
Coleoptera	2.1	3.0	25.0	0.8	3.6	5.8	44.4	1.8	2.3	4.7	13.5	1.2	5.2	2.0	16.7	1.2
Coleoptera (larva)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0	8.5	14.9	2.2	0.0	0.0	0.0	0.0
Collembola	0.0	0.0	0.0	0.0	1.8	0.8	22.2	0.6	1.0	0.1	2.7	0.0	0.0	0.0	0.0	0.0
Dermoptera	0.5	0.1	12.5	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Diptera	1.6	1.5	25.0	0.5	2.3	1.0	22.2	0.7	1.3	0.6	8.1	0.2	2.6	0.5	16.7	0.5
Diptera (larva)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.6	1.2	1.4	0.0	0.0	0.0	0.0	0.0
Formicidae	83.7	52.1	100.0	88.5	57.5	15.8	77.8	24.8	32.5	12.0	67.6	39.1	35.1	12.1	72.2	34.9
Gastropoda	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.2	5.6	18.9	2.4	0.7	0.3	5.6	0.1
Hemiptera	0.0	0.0	0.0	0.0	0.5	0.4	11.1	0.0	1.5	1.6	9.5	0.4	0.0	0.0	0.0	0.0
Heteroptero	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	1.4	0.0	0.0	0.0	0.0	0.0
Hymenoptera	0.5	1.0	12.5	0.1	0.5	0.4	11.1	0.0	3.4	1.7	14.9	1.0	2.0	1.4	16.7	0.6
Isopoda	1.1	2.2	25.0	0.5	13.1	28.3	66.7	57.9	20.4	29.0	44.6	28.6	33.1	36.7	72.2	51.7
Isoptera	0.0	0.0	0.0	0.0	9.5	7.8	11.1	2.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lepidoptera (larva)	4.7	24.5	37.5	7.4	0.9	0.7	22.2	0.2	0.6	1.1	4.1	0.1	0.0	0.0	0.0	0.0
Odonata	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.6	2.7	0.0	0.0	0.0	0.0	0.0
Opilionida	0.0	0.0	0.0	0.0	0.5	0.6	11.1	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Orthoptera	2.1	2.3	37.5	1.1	0.5	0.1	11.1	0.0	1.0	1.6	6.8	0.2	1.3	0.6	11.1	0.2
Pseudoescorpionidae	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.5	4.1	0.1	0.0	0.0	0.0	0.0
Other	--	11.4	--	--	--	27.7	--	--	--	15.8	--	--	--	32.4	--	--
Niche breadth	0.04				0.13				0.23				0.32			

DISCUSSION

The species of this study exhibited a similar prey composition in their diets. In all of them, Formicidae and/or Isopoda were the predominant prey categories. The high relevance of ants was also recorded for other populations of *Physalaemus biligonigerus*, *P. cuvieri* and *P. gracilis* in studies of restinga habitats in southern Brazil (Oliveira et al., 2015), Atlantic Forest biome in northern Brazil (Santos et al., 2004) and Araucaria forest in southern Brazil (Moser et al., 2017). Formicidae had high importance in the diet of *P. biligonigerus*, reaching IRI values of 88.47%, while IRI of the second most important prey category was less than 8% (Lepidoptera larvae). Based on this, we argue that *P. biligonigerus* is locally a “Formicidae specialist”. Large consume of Formicidae has been previously reported in *P. biligonigerus* (Oliveira et al., 2021) as well in other *Physalaemus* (Santana and Juncá, 2007). Is hard to list environmental components that favored the dominance of ants in contents. On the

other hand, high consumption of ants involves physiological adaptations to the digestion due to the presence of formic acid, resulting in a high energy cost (Hirai and Matsui, 2002). Then, the ability to feed on ants would increase the advantages in explore the food resources in the habitat. Our sampling design did not make us able for a deeper discuss among diet specialization, niche partitioning and competition. We believe that specialization on ant consumption is a topic that deserves new studies.

Ants were also important food items for other species of the genus in different Brazilian locations, such as the Atlantic and Amazon Forests for *P. lisei* (Moser et al., 2017) and *P. ephippifer* (Rodrigues and Santos-Costa, 2014), Caatinga (Protázio et al. 2019; Oliveira et al. 2021) and in Argentina for *P. riograndensis* (López et al., 2003) and *P. albonotatus* (Falico et al., 2012).

In general, ants are considered unpalatable for several predators (Hirai and Matsui, 2000) but species of *Physalaemus* consume ants with considerable frequency (Moser et al., 2017). For some amphibians, the seques-

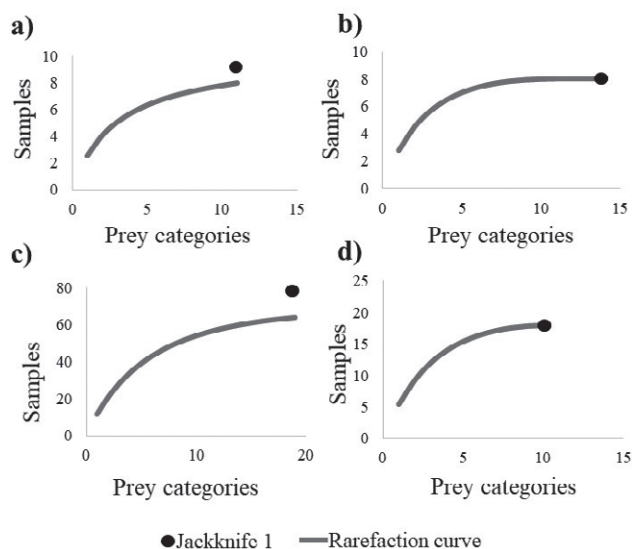


Fig. 1. Rarefaction Curves and the Jackknife 1 estimator of prey-species richness according to the number of samples (anurans): a) *Physalaemus biligonigerus*, b) *Physalaemus cuvieri*, c) *Physalaemus gracilis* and d) *Physalaemus henselii*.

tration of alkaloids is associated with the consumption of ants (Saporito et al., 2004), but no studies prove the ingestion for this purpose by *Physalaemus*. The high consumption of Formicidae may be associated with the abundance and displacement capacity of these invertebrates in the environment (Baretta, 2007), thus characterizing an opportunistic feeding behavior by the anurans. This behavior may minimize the potential competition between species, favoring their coexistence.

Also, Isopoda was one of the most important prey categories for three of the four species (*P. cuvieri*, *P. gracilis* and *P. henselii*). The relevance of Isopoda to the diet of *Physalaemus* species was poorly reported. Although the group is part of the genus' diet (Rodrigues and Santos-Costa, 2014; Moser et al., 2017), it is not frequently consumed. In the study by Leivas et al. (2018), Isopoda were not found in the diet of *P. cuvieri*, which may be associated with prey availability. We observed feed specialization for two prey groups: ants and isopoda. Both of them are social insects. Their nests offer a large number of preys which could lead to their high density in contents of frogs that are able to eat them.

The trophic niche breadth for *P. biligonigerus*, *P. cuvieri* and *P. gracilis* was equal to or similar to results found in other studies of the same species (Santos et al., 2004, Moser et al. 2017). In contrast, for *P. henselii* ($B_{sta} = 0.32$, Farina et al., 2018), trophic niche breadth was higher than for other species of this study and also of the literature, such as *P. gracilis* and *P. lisei* ($B_{sta} = 0.15$ and

$B_{sta} = 0.11$ respectively, Moser et al., 2017), *P. ephippifer* ($B_{sta} = 0.19$, Rodrigues and Santos-Costa, 2014) and *P. biligonigerus* ($B_{sta} = 0.04$, Oliveira et al., 2015), suggesting a more generalist behavior in relation to its congeners (Farina et al., 2018). In general, leptodactylids have narrower niches when compared to other families, such as hylids (Sabagh et al., 2010; Barbosa et al., 2014), revealing a great abundance of local resources that favors coexistence and high trophic niche overlap. Despite of little speculative our data suggests a reasonable degree of food specialization in for *P. biligonigerus* and *P. cuvieri*.

The predominant consumption of ants is one of the reasons for the high trophic niche overlap between the species. The phylogenetic proximity of the species may also relate to their high niche overlap (Lourenço et al., 2015), besides indicating a great prey availability in the environment, which is evidenced by the niche breadth. Also, species may be foraging in similar places, considering that species that share the same habitat tend to have a similar diet (Duellman and Trueb 1986, Guidali et al., 2000, Sabagh et al., 2010). Compositional Analysis pointed out that differently from other species, *P. gracilis* exhibited a considerable level of selectivity in its diet, preying on Araneae, Coleoptera larvae, Isopoda, Formicidae and Hymenoptera more frequently than their expected availability in the habitat. This result brings some light to the relevance of studying syntopic species to elucidate feeding adaptations.

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Screening of *Ophidiomyces ophidiicola* in the free-ranging snake community annually harvested for the popular ritual of *San Domenico e dei Serpari* (Cocullo, AQ, Italy)

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Abstract. In the Abruzzi village of Cocullo (Italy), each year, on May 1st, local snake hunters (known as *Serpari*) display colubrids, captured in the wild, to commemorate the ancient ritual of San Domenico. The ascomycete *Ophidiomyces ophidiicola* (Oo) is the causative agent of ophidiomycosis, an emerging disease with sublethal effects. Skin lesions, such as dysecdysis, edematous, crusty or necrotic scales, swellings, nodules, and ulcers, are the most common clinical manifestation of the disease. The pathogen and its associated disease are well characterized in wild snakes in North America, whereas broad screenings of free ranging wild ophidians in Europe are rare. In 2019, as part of a multi-year snake health monitoring project, all the Cocullo ophidians were carefully examined for integumentary affections and those showing signs consistent with ophidiomycosis were dry swabbed on the skin and on any visible cutaneous lesions with a single applicator. The extracted DNA underwent a broad-range panfungal PCR targeting the D1-D2 region, as well as two conventional PCRs specific to the ITS2 and IGS regions of Oo DNA. Twenty-three out of 129 snakes (13/82 *Elaphe quatuorlineata*; 7/31 *Hierophis viridiflavus*; 3/15 *Zamenis longissimus*; 0/1 *Natrix helvetica*) resulted clinically affected, but no specific Oo genomic DNA was detected by PCR. The Cocullo ritual celebration provided a unique opportunity for the first systematic testing of a large sample size of a local snake community for the monitoring of this pathogen in Italy.

Keywords. Ophidiomycosis, Snake Fungal Disease, SFD, snakes, health monitoring, Cocullo, Abruzzi (Italy).

Ophidiomyces ophidiicola (Oo) is the etiological agent of ophidiomycosis (also known as Snake Fungal Disease – SFD), a fungal infection of snakes (Lorch et al., 2015). This onygenalean fungus is resistant to various physical and chemical agents (Allender et al., 2015b), and hibernacula may represent its environmental reservoir (Camp-

bell et al. 2021). *Ophidiomyces* infection has been associated with sublethal effects on adults (Agugliaro et al., 2020; Lind et al. 2018a, b; Tetzlaff et al., 2017) and potentially lethal outcomes on newborns (e.g., Britton et al., 2019), translating into a potential impact on wild populations' fitness and a threat to conservation. This emerging

infectious disease occurs with various cutaneous signs as dysecdysis, desquamation, scales abnormalities (e.g., displacing), local skin thickening, yellowish/brownish crusts, skin ulcerations, swelling and nodules (revised by Baker et al., 2019), whereas visceral lesions are less frequently recorded. Albeit impacts on different populations seem locally divergent or controversial, Oo has been detected in free-ranging ophidians in most part of North America (Di Nicola et al., 2022). In Europe, samples deriving from wild *Coronella austriaca*, *Hierophis viridiflavus*, *Natrix helvetica*, *N. maura*, *N. natrix*, *N. tessellata*, *Vipera berus*, *V. nikolskii* and *Zamenis longissimus* from UK, Czech Republic, Switzerland, Germany, France, Austria, Hungary, Poland, Ukraine, or Italy tested positive with molecular methods (Franklinos et al., 2017; Meier et al., 2018; Schüler et al., 2022; Blanvillain et al., 2022; Marini et al., 2023), and a retrospective analysis date back the presence of the fungus in Italy and Switzerland since 1959 (Origgi et al., 2022).

In this paper we report the results of an investigation aimed at testing the presence of Oo in a snake community from Central Italy. Data were obtained by snakes captured for a religious ritual (the Catholic cult of *San Domenico* – of pagan and pre-Christian origins) in the village of Cocullo (Abruzzi). This ceremony takes place in the first days of May, and has remained unchanged for several hundred years. The main feature of this occurrence is the presence of large numbers of wild-caught snakes by local snake hunters (*serpari*) during the weeks before the events. This ritual is well known and important in Abruzzi's (Italy) culture and history, and it is closely dependent on the local ophidofauna. In recent years, the ceremony has been accompanied by some significant conservation actions by the local authorities of Cocullo, due to the increased awareness of the importance of environmental protection, in particular snake conservation. Although no decline of Cocullo's snakes' populations has been anecdotally detected in past years, some effects on the reproductive phenology of *E. quatuorlineata* and *Z. longissimus* has been observed (Filippi and Luiselli, 2003) and a few areas surrounding Cocullo are characterized by some disturbance factors for ophidofauna, including a high density of wild boars (regarding this critical issue on snakes see Filippi and Luiselli, 2002). Various species of Colubridae are involved during the celebration, in particular *Elaphe quatuorlineata* (Pellegrini et al., 2017), one of the largest and more vulnerable species of snakes in Mediterranean central Italy (Filippi and Luiselli, 2000; Filippi, 2003; Capula and Filippi, 2011), but also *Zamenis longissimus* and *Hierophis viridiflavus*. Snakes are captured by snake hunters from the 19th of March till the 30th of April every year. Since 2010, all

snakes captured by *serpari* are assessed during the 2-3 days preceding the event, that has a fixed date on May 1st (overall n = 1300 snakes were registered and checked from 2010 to 2023, Filippi and Montinaro, in prep.): a scientific committee (composed by EF and GM in collaboration with a veterinarian) records the captured species, biometric data (weight, snout-vent length [SVL]), sex, age class (juvenile, subadult, adult), site of capture of the ophidians brought by snake hunters. Moreover, PIT tags are checked or implanted, and a physical examination is carried out in addition to a swab for bacteriological analyses (processed by IZS - *Istituto Zooprofilattico Sperimentale* Abruzzo e Molise from 2015 – e.g., Filippi et al. 2010). Then, at the end of the rite, or in any case within seven days, snakes are released by *Serpari* in the same place where they were captured.

In 2019, on 29th and 30th of April, we also conducted a focused survey to assess the presence of Oo (in 2020 and 2021 the rite did not take place due to the Covid-19 pandemic). All snakes underwent an additional physical examination. During this investigation, a particular attention was given to macroscopic clinical signs consistent with ophidiomycosis (Fig. 1). Based on this, a binary value according to Hileman and colleagues (2018) was assigned to each snake: “0” (absence of signs consistent with ophidiomycosis); “1” (presence of signs consistent with ophidiomycosis). The clinical signs from the snakes categorised as “1” (clinically affected) were carefully documented, and the grade of infection severity was retrospectively calculated following the Infection Severity Score (ISS) proposed by Baker and colleagues (2019) (Table 1). A single sterile cotton-tipped applicator with wooden stick (Aptaca Spa, Canelli, Italy) was used to (dry) swab each individual belonging to the apparently affected group, and then placed in 20 ml plastic tubes (Sarstedt AG & Co. KG, Nümbrecht, Germany). The snakes were swabbed with moderate pressure 10 times along the entire dorsal surface, ventral surface, head, and, additionally, ≥ 10 times on each suspected lesion (see Di Nicola et al. 2022; Marini et al., 2023). In cases in which scales and/or lesions naturally exfoliated during swabbing (i.e., abnormal scales partially detached or scales adjacent to skin lesions) or pieces of exuviae detached (due to dysecdysis), these tissues were stored together with the swab from the same individual in the same 20 ml vial. The tubes were stored at +4 °C until shipment. Each snake was handled by the veterinarian carrying out the swab (DM) with new disposable nitrile or latex gloves and all the equipment eventually used (hooks, forceps, scale) was disinfected with 95% denatured ethanol or 5% sodium hypochlorite solution. The DNA has been extracted by placing each swab (with or without tissue) in a 1,5

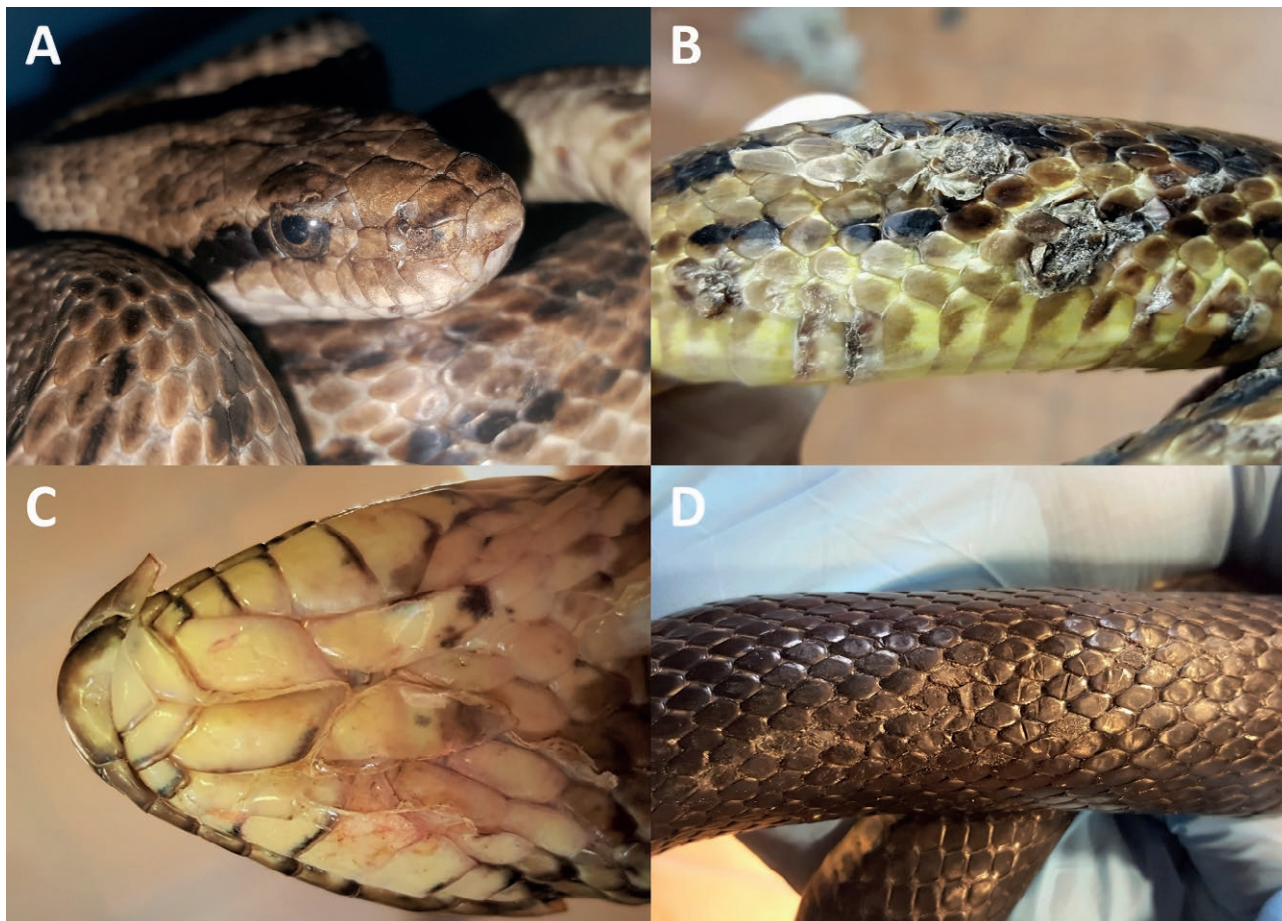


Fig. 1. Representative lesions of individuals grouped in category “1” (presence of signs consistent with ophidiomycosis): (A) EQ21, *Elaphe quatuorlineata* showing erosions of rostral, right internasal, nasal, preocular, ocular and supraocular scales and displaced dorsal scales; (B) EQ46, *Elaphe quatuorlineata* exhibiting multifocal swollen, eroded and hyperaemic (dorsal and ventral) scales associated with crusts and dysecdysis; (C) HV28, *Hierophis viridiflavus* with hyperaemia, erythematous skin, and retained exuvium in the gular region; (D) HV04, *Hierophis viridiflavus* showing wrinkled, depressed and crusty dorsal scales.

ml safe-seal tube with one aliquot (500 μ l) of lysis buffer (0.1 M Tris-HCl, pH 8.5, 0.05% Tween 20, 0.24 mg/ml proteinase K), then placed in a heat block first at 60 $^{\circ}$ C for 1 hour and after 95 $^{\circ}$ C for 15 minutes. Afterward, 10 μ l of each lysate were diluted in 90 μ l of nuclease free water. A broad-range standard panfungal PCR (amplifying the D1-D2 region of the large subunit [LSU - 28S] of the ribosomal RNA [rRNA] gene complex - Borman et al., 2006) was performed following Frankinos et al. (2017). Moreover, two different set of primers described by Bohuski and colleagues (2015), targeting specific regions of *Oo* genome - ITS2 (internal transcribed spacer region 2) and IGS (intergenic spacer region) within the rRNA gene - were employed for conventional PCR assays (Origgi et al., 2022). Each conventional (qualitative) PCR assay has been run in 30 μ l amplification mixture composed of 3 μ l of PCR Buffer, 0.6 μ l dNTP mix (10 mM

each), 0.75 μ l of each primer (100 mM), 0.3 μ l of Taq Polymerase, 2.5 μ l of DNA template (diluted lysates), 3.8 μ l of $MgCl_2$ (25 mM), and 18.3 μ l of water (Solys Biodyne, Luzerna Chem, Lucerne, CH). The reactions were carried out as follows: initial denaturation (95 $^{\circ}$ C for 3 min) followed by 35 cycles including 30 s at 95 $^{\circ}$ C (denaturation), 30 s at 52 $^{\circ}$ C for ITS and 50 $^{\circ}$ C for IGS (annealing), 30 s at 72 $^{\circ}$ C (elongation). A final extension at 72 $^{\circ}$ C for 10 min followed. Lastly, 5 μ l of the PCR product were resolved on a 2% agarose gel by electrophoresis and visualized under UV light.

A total of 129 snakes (adults, subadults and juveniles) were brought by *serpari* and examined by the scientific committee. Ophidians belonged to the following species: *Elaphe quatuorlineata* (n = 82), *Hierophis viridiflavus* (n = 31); *Zamenis longissimus* (n = 15), *Natrix helvetica* (n = 1). Twenty-three snakes out of 129 (17.8%)

Table 1. Individuals categorised as clinically affected (category “1”) and sampled at Cocullo’s festival in 2019. For each snake the table shows the identification code, species, sex and age class, snout-vent length (SVL), weight, type of collected sample, lesion scores (type, location, number, coverage) and the relative Infection Severity Score (ISS), and the description of gross signs. M: adult male, F: adult female, SAF: subadult female, S: dry swab, T: tissue. BCS: body condition score.

Id	Species	Sex and age	SVL (cm)	Weight (g)	Sample type	Lesion			ISS (Infection Severity Score)	Gross signs description
						type score	location score	number score		
EQ21	<i>Elaphe quatuorlineata</i>	SAF	107	306	S	3	3	3	12	Crusty erosions of rostral, right internasal, nasal, preocular, ocular and supraocular scales. Crusty, dry, dusty, dislocated and (sometimes) eroded scales multifocally along the dorsum. Brownish and dusty crust of the tail (> 3x2cm).
HV04	<i>Hierophis viridiflavus</i>	M	89	264	S	2	1	3	9	Desquamations with dusty aspect. Concave, wrinkled or crusty dorsal scales. Traumatic and crusty lesions of ventral scales.
EQ24	<i>Elaphe quatuorlineata</i>	M	139	1086	S	2	2	3	9	Ectopic or crusty or dislocated dorsal and tail scales. Retained shed between supraocular, ocular and postocular scales. Focal discolorations. Low BCS.
HV11	<i>Hierophis viridiflavus</i>	M	95	260	S	2	2	2	8	Retained shed in the parietal and dorsal scales of head region. Crusty, dusty and/or raised dorsal scales. Light dehydration.
HV12	<i>Hierophis viridiflavus</i>	M	87	296	S	2	2	2	8	Coalescent desquamations of head and trunk scales. Concave or crusty dorsal scales.
EQ27	<i>Elaphe quatuorlineata</i>	F	142	854	S	2	2	2	9	Bilobed loose swelling (> 4x1,5cm - presumptively subcutaneous nodule) on the left lateral region of the trunk. Moderate number of ectopic and dislocated dorsal scales. Wrinkled scar between rostral and internasals scales.
ZL01	<i>Zamenis longissimus</i>	M	99	194	S	2	3	1	2	Tumefaction on the left side of the upper jaw. At buccal inspection, the mucosa was found hyperaemic, haemorrhagic and swelled.
ZL07	<i>Zamenis longissimus</i>	M	105	396	S	2	2	2	8	Desquamation with a slightly crusty and wrinkled aspect on the right loreal, preocular and supralabial scales. Dislocated, concave and ectopic dorsal scales.
HV15	<i>Hierophis viridiflavus</i>	M	96	320	S	1	2	2	7	Abrasion between rostral and internasals scales. Dislocated, ectopic, wrinkled or absent dorsal scales.
EQ39	<i>Elaphe quatuorlineata</i>	F	150	1000	S	2	2	2	7	Crusty lesion on right supraocular scale. Dislocated dorsal scale.
EQ40	<i>Elaphe quatuorlineata</i>	M	124	682	S	1	1	2	6	Focal discolorations. Concave dorsal scales.
EQ46	<i>Elaphe quatuorlineata</i>	F	112	380	S, T	3	1	3	10	Dysecdysis and retained moults in many locations. Tumefacted, eroded and hyperaemic (dorsal and ventral) scales. Several yellowish-brownish crusts lesions in the dorsal and ventral region of the trunk, multifocal pattern. Some swollen scales or crusts underlying nodular formations. Low BCS and muscular weakness.
EQ50	<i>Elaphe quatuorlineata</i>	M	116	444	S	1	2	2	7	Multifocal discolorations. Wrinkled dorsal scales. Irregular caudal edges of ventral scales.
EQ54	<i>Elaphe quatuorlineata</i>	F	138	966	S	2	2	2	9	Crusty lesions on dorsal scales. Concave and dislocated dorsal scales. Erythematous and hyperaemic ventral scales. Nodular formation on the tail (> 1x1cm).

Id	Species	Sex and age	SVL (cm)	Weight (g)	Sample type	Lesion			ISS (Infection Severity Score)	Gross signs description	
						type score	location score	number score			
EQ55	<i>Elaphe quatuorlineata</i>	M	117	556	S	2	1	3	3	9	Scattered dark scars. Dislocated, crusty, wrinkled or absent dorsal scales.
HV21	<i>Hierophis viridiflavus</i>	M	96	348	S	2	1	3	3	9	Brownish-yellowish moist fresh crusts dorsally and ventrally on the tail. Concave, crusty or absent dorsal scales.
HV23	<i>Hierophis viridiflavus</i>	M	93	288	S	3	1	2	2	8	Dislocated and concave dorsal scales. Patches of discolorations. Tumefaction (> 1x1 cm) on the left side of tail (adjacent erythematous and erosive ventral scales).
HV28	<i>Hierophis viridiflavus</i>	M	103	342	S, T	2	2	2	3	9	Dysecdysis. Retained exuvium at level of snout and chin. Hyperaemic and erythematous skin in the gular region. Desquamation and wrinkling of dorsal scales. Two nodular formations (0,5x0,5 cm) on the trunk. Irregular caudal edges of ventral scales.
ZL13	<i>Zamenis longissimus</i>	F	79	162	S, T	3	1	3	2	9	Crusty, raised or concave dorsal scales. Retained shed. Focal erosive fresh lesion on ventral scales. Multifocal crusts on caudal trunk and tail.
EQ58	<i>Elaphe quatuorlineata</i>	M	140	1040	S	2	3	3	2	10	Light multifocal discoloration. Crusty, dry or dislocated dorsal scales. Three crusty lesions on the ventral scales (one on the cloaca).
EQ62	<i>Elaphe quatuorlineata</i>	M	139	1088	S	3	1	2	3	9	Dry or dislocates dorsal scales. Swelling and hyperaemia or erosion and of ventral scales close to cloaca. One nodular formation at trunk level (1x1 cm), and one at tail level (0,5x0,5 cm).
EQ64	<i>Elaphe quatuorlineata</i>	M	134	790	S	2	1	2	2	7	Dislocated and ectopic dorsal scales. Desquamations. Crusty lesion of the tail.
EQ69	<i>Elaphe quatuorlineata</i>	M	136	858	S	2	1	2	2	7	Diffused dark dislocated and wrinkled dorsal scales. Three nodular formations on the trunk (< 1x1cm). Docked tail.

showed signs consistent with a fungal dermatitis and were assigned to the category “1” (apparently affected): *Elaphe quatuorlineata* (n = 13; 15.8%), *Hierophis viridiflavus* (n = 7; 22.6%); *Zamenis longissimus* (n = 3; 20%). Table 1 reports all the clinically affected ophidians and the macroscopic signs that allow ranking these individuals in category “1”, as well as each category calculated for counting the individual ISS. Among all the individuals, the ISS varied between 6 and 12, being 9 the median score. No influence of the species on the ISS was found ($\chi^2 = 11.49$, $P = 0.32$, $df = 10$). Cluster analysis of the ISSs – with and without normalization of the lesion coverage on the individual weight – did not reveal any particular trend or clustering (data not shown). A total of 23 swabs (and 3 tissues linked to one of them – Table 1) have been collected. After DNA extraction, for every sample (with or without tissue) a conventional PCR was carried out for each of the three targeted region of Oo (D1-D2, ITS2 and IGS regions – 23 samples x 3 reactions = 69 results). No product of consistent size was observed on agarose gel from each PCR electrophoresis (0/69). The number of category “1” individuals versus the number of category “0” individuals of each species did not differ statistically ($\chi^2 = 0.89$, $P = 0.64$, $df = 2$). The presence of clinical signs in adult *E. quatuorlineata* appeared to be associated with weight ($n_{cat1} = 12$, $x = 812.00 \pm 247.01$; $n_{cat0} = 63$, $x = 689.02 \pm 174.49$; t-test = 2.09 $P = 0.04$) but not with sex (Fisher test $P = 0.68$) and SVL ($n_{cat1} = 12$, $x = 131.42 \pm 10.67$; $n_{cat0} = 64$, $x = 127.88$; t-test = 0.98, $P = 0.33$). SVL and weight were correlated in both apparently clinically healthy ($r_{61} = 0.74$, $P < 0.01$) and clinically affected individuals ($r_{10} = 0.92$, $P < 0.01$). No females with clinical signs were observed in *H. viridiflavus* and, among males, the number of category “1” adults did not appear to be related to SVL ($n_{cat1} = 7$, $x = 94.14 \pm 5.24$; $n_{cat0} = 13$, $x = 91.65 \pm 6.14$; t-test = 0.91, $P = 0.38$). SVL and weight did not positively correlate in clinically affected ($r_5 = 0.62$, $P > 0.05$) while these morphometric parameters were correlated in unaffected individuals ($r_{11} = 0.82$, $P < 0.05$). Three adults of *Z. longissimus* (1 female and 2 males) out of 15 were grouped in category “1”. Oo genomic DNA was not detected in any of our samples (n= 23, observed prevalence 0%, Bayesian 95% credible intervals: 0.00-0.14).

Considering the increasing number of ophidiomycosis reports in Europe, a standardised monitoring for snake communities is warranted. To the best of our knowledge, this is the first systematic testing of a large sample size of a local snake community for the monitoring of *O. ophidiicola* in Italy. We investigated only individuals with signs consistent with a fungal dermatitis because the swabs coming from these ophidians are more likely

to result (true) positive compared to those showing no lesions (Hileman et al. 2018; Long et al. 2019). According to our data, none of the species studied shows an obvious high incidence of clinical signs compared to the other species and, within the same species, no particular trends emerged between clinically affected individuals versus clinically not affected ones and parameters as sex, SVL and weight. The used ISS was a helpful tool to characterize the severity of the infection of each individual, independently of the limitations associated with the lack of positive samples in our study. According to our experience, a normalization of the lesion coverage to the size of the animal is recommended (e.g., weight, surface – see Blanvillain et al., 2022). No evidence of the presence of Oo DNA was revealed by PCR. However, PCR negativity is consistent with either the actual absence of the target DNA sequence or its presence under the limit of detection. Furthermore, eventual inhibitors could also hamper the PCR results. Accordingly, we cannot rule out the occurrence of some false negative. Lastly, the snakes were sampled once, and repeated sampling of the same individuals was shown to significantly reduce the probability of a false-negative (Hileman et al. 2018). Hence, in order to detect eventual false negative and increase sensitivity such screening should be improved by performing multiple re-samplings (e.g., 3-5 swab applicators - Hileman et al. 2018; Marini et al., 2023). Also, the use of real-time (quantitative) PCRs instead of conventional (qualitative) ones would improve the detection of false negatives from swab samples (Allender et al., 2015a). This fungus might occur in all the temperate regions around the globe (Burbrink et al., 2017) and snake susceptibility may vary according to phylogenetic and ecological factors (Haynes et al., 2020). The actual natural history of the colonization of this fungus is still unclear. The possible introduction of the fungus into North America by pathogen pollution has been suggested (Ladner et al., 2022) along with evidenced of the presence of both the American (Switzerland) and the European clade (Italy) in the European continent for more than 60 years (Origgi et al., 2022). Therefore, it is essential to shed lights on the distribution of this fungus in European continent along with its associated (clade-specific). Accordingly, it is important to carry out screening in Italian territories, implementing what was started in Cocollo. On the other hand, the monitoring of the possible presence of Oo extends and enriches the health monitoring and conservation actions in place at Cocollo since 2010. In particular, to reduce the potential risk of disease and to ensure an excellent standard of handling and keeping of wild ophidians, a *vademecum* on snakes’ management in terraria has been published and delivered to the *Serpari*. Additionally, professional terraria have been allo-

cated to properly house the snakes every year, and dedicated exhibit with numerous environmental education and training activities concerning snakes are carried out for the thousands of tourists attending the ritual every year.

The annual monitoring of the ophidians involved in the Cocullo ritual will provide a great opportunity for collecting baseline data critical to assess the population health of the local snake community, which goes beyond the specific *Oo* screening, and which represents a paradigmatic example of how cultural traditions, citizen science and conservation may come all together.

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Assessment of fall season habitat and coverboard use by snakes in a restored tallgrass prairie community

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Abstract. We assessed habitat use and preference with respect to artificial coverboards for the snake community of a restored tallgrass prairie. Coverboards offer herpetofauna protection from predators and space to thermoregulate their body temperature. These covers also create microhabitats that differ from their surrounding habitat. We placed plywood and metal coverboards along a transect that crossed from prairie floodplain into upland prairie. Coverboards were checked over a three-week period during the fall season, during morning, afternoon, and dusk. Snake species were identified and counted, and ambient temperatures and humidity were checked under each coverboard. We found four snake species across the habitat gradient, common gartersnake (*Thamnophis sirtalis*), plains gartersnake (*T. radix*), Dekay's brownsnake (*Storeria dekayi*), and Western foxsnake (*Pantherophis ramspotti*). Species richness was greatest in the floodplain habitat and microhabitat associated with metal coverboards. The floodplain habitat was also the habitat predominantly used by common gartersnake and Dekay's brownsnake. Dekay's brownsnakes, furthermore, preferred utilizing metal coverboards over wood. The composition of snake species we observed suggests that the restoration efforts on this tallgrass prairie system have attracted some grassland snake species, but the possibility of a greater snake community remains. Our data suggest that using metal coverboards during the cooler active seasons, such as fall and spring, will increase capture success and more efficiently sample snake communities. Studies such as ours to better understand habitat and coverboard use will result in more efficient sampling of herpetofauna for conservation and monitoring efforts.

Keywords. *Thamnophis*, *Pantherophis*, *Storeria*, microhabitat use, thermal environment, humidity, artificial cover objects, snake ecology.

Prairie restoration is a common management strategy to increase the biodiversity of an ecosystem, restore native populations and communities, and store large amounts of available carbon (Jordon et al., 1988; Samson and Knopf, 1994; Anderson, 2009; Guiden et al., 2021). Restorations typically include the removal of agriculture plots, replanting native plant species, and relying on the "Field of Dreams" paradigm that if you build it species native to the area will come (Guiden et al., 2021). As such, this paradigm involves building a suitable habitat for organ-

isms in hopes they will find and stay in the restored area. Research shows prairie restorations can increase the abundance of animals, including herpetofauna such as snakes (King and Vanek, 2020). Although not readily observed because of their elusive nature, snakes can reach high abundance and greatly influence natural communities by influencing abundance and behavior of other species (Hisaw and Gloyd, 1926; Kotler et al., 1993; Sperry et al., 2008; Willson and Winne, 2018; King and Vanek, 2020). Accurately monitoring success of restoration efforts is dif-

difficult, however, because each restoration effort is unique, including the sampling techniques utilized.

Sampling herpetofauna in tallgrass environments can be difficult due to the cryptic nature of reptiles and amphibians (Fitch, 1987; Szaro et al., 1988). Visual sampling is a common method for sampling snake species (Foster, 2012), however, small, cryptic, or camouflaged species are often difficult to detect in areas of thick vegetation due to their slender bodies (Turner, 1977; Ward et al., 2017). One efficient form of sampling includes the use of coverboards constructed out of sheets of various heat conducting materials, such as metal, wood, rubber, or asphalt roofing (Fitch, 1987; Engelstoft and Ovaska, 2000). Coverboards can significantly increase detection of snakes compared to simple visual surveys (Halliday and Blouin-Demers, 2015). These covers provide attractive areas for snakes and other herpetofauna to seek refuge and an efficient way for scientists to observe, count, or capture snakes (Halliday and Blouin-Demers, 2015).

Coverboards of different types provide microhabitats of varying temperatures and humidities allowing individuals a choice regarding suitable areas for cover (Engelstoft and Ovaska, 2000). These microhabitats are impacted by the habitats in which they are placed and seasonality due to falling or rising temperatures. During the cooler months, for instance, snakes may utilize substrates or objects that absorb or retain heat to maintain their optimal body temperature more efficiently (Engelstoft and Ovaska, 2000). Individual preferences for specific microhabitats can arise through various needs associated with age, sex, shedding, food ingestion, circadian rhythms, and reproductive condition (Lilywhite, 1987). Sampling efficiency associated with coverboard type can therefore vary based on target species, habitat type, season, and interactions among these factors. These relationships mean that the coverboard types used can impact species-specific encounter rates during surveys and biodiversity assessments and can influence the herpetofaunal community composition detected (Grant et al., 1992; Engelstoft and Ovaska, 2000; Hampton, 2007).

Identifying habitat and microhabitat use of snake species in the tallgrass prairies of the Great Plains of North America has received relatively little attention, despite prairies being the largest vegetative community in North America (Samson and Knopf, 1994) and the Great Plains constituting one-third of the United States (Deitz, 2022). Understanding these preferences can be beneficial to conservation efforts and lead to more accurate and efficient sampling for specific species and communities. In this study, we used metal and plywood coverboards to assess snake habitat and coverboard preference in a restored tallgrass prairie system that includes a habitat

gradient from prairie floodplain to upland prairie. We specifically compared the preferences of the snakes for metal or wooden coverboards in association with habitat type, humidity, and ambient temperatures.

We sampled snakes within the Allwine Tract of Glacier Creek Preserve (41.19759N, -96.29893W), a 212 ha (525 acres) preserve that encompasses an entire sub watershed in eastern Nebraska, United States. The Allwine Tract (65 ha; 160 acres) was donated to the University of Nebraska at Omaha in 1959. In 1970, 57 ha (140 acres) of agricultural land within the Allwine Tract were seeded with five native prairie grass species and then over-seeded with a diverse mix of local native forbs in the following years. Between 2009 and 2019 an additional 147 ha (365 acres) were purchased and added to the preserve, including a mix of agriculture, wetlands, and woodlands. The reconstructed prairie is managed with a 3-year prescribed fire return interval that occurs in mid-spring, where no more than 2 of the 5 units are burned in one year. Additional details about the site can be found in Bragg et al. (2016), Dere et al. (2019), and Manning et al. (2022).

Data was collected during a 3-week period from September 17 to October 4, 2021, during three time blocks of 7:00-9:00, 14:00-16:00, and 18:00-20:00 CT (morning, afternoon, and dusk, respectively). Four data collection events occurred during each of the time blocks. We sampled 10 stations that were established in spring of 2018, reflecting 41 months since establishment. Sampling efficiency of artificial retreats can increase with time since establishment; however, studies have found that efficiencies reach asymptotic maximums within 12 months, well within our time frame (Grant et al., 1992; Croak et al., 2010). The stations ran along a north-south transect (800 m) that crossed Glacier Creek with 5 stations on the south slope and 5 stations on the north slope (104 m average distance between stations; min = 27 m, max = 145 m). Each station consisted of two artificial coverboards: a uniformly sized metal (corrugated, galvanized sheet metal) and plywood (12.2 mm or 1/2 inch thickness) coverboard each measuring approximately 122 x 122 cm (L x W) and placed approximately 1.5 m from each other. Therefore, there were 20 artificial retreats evenly divided between the two types of material. A Kestrel 5000 environmental meter (Nielsen-Kellerman Company) was used to collect relative humidity and temperature data.

During each sampling event each board was lifted, and the area underneath scanned to count number of individuals and identify snake species and life stage as either juvenile or adult. The Kestrel was then placed under the board to acclimate for 90 seconds. We recorded the relative humidity and temperature from the Kestrel and repeated this process for each of the stations

along the transect, whether or not snakes were present. For each station we also measured distance to creek using an aerial map in Arc Map GIS and recorded habitat type (prairie floodplain, floodplain-upland transition zone, and upland prairie). Because the transect ran across and perpendicular to the creek, the floodplain and transition zone were relatively narrow habitats with four coverboards (two stations) in each habitat and the remaining twelve coverboards (six stations) in the upland prairie.

We assessed snake species richness and encounters with respect to multiple covariates and factors such as time of day, date, coverboard type, habitat type, distance to creek, humidity, and temperature. Life stages of juvenile and adult were grouped for analyses because of low juvenile numbers. We employed generalized linear models (GLM) including covariates and factors as independent variables and species richness (a count of number of species) or species-specific encounters (a count of number of individual encounters) as the dependent variable. We tested dependent variables for normality and transformed data when necessary or ran models with the appropriate distribution, such as Poisson for count data or non-parametric tests. We also checked for relationships among our independent variables using analyses of variance (ANOVA), Pearson or Spearman correlations, or linear regressions where appropriate. When variables were related, we chose one to include in our initial model or used residuals from a regression of one variable on the other. Temperature and humidity were related, for instance, thus the residuals from a regression of humidity on temperature were used in the initial models. The date, or sequence of sampling days, did not influence species richness and was therefore not included in further models ($Wald \chi^2 = 0.423$, $P = 0.516$). Because of relationships between some of our independent variables (see results) our initial models included coverboard type, habitat type, and temperature. Interactions that were not significant were eliminated from final models. All statistical analyses were conducted with IBM SPSS for Windows, Version 29.0.

Habitat and coverboard type both influenced species richness and individual species encounters (Table 1, Fig. 1 and 2). More species were found in the prairie floodplain than in the transition zone or drier upland prairie (Fig. 1). We also found more species, almost double the number, underneath the metal coverboards than the wooden coverboards. Similar trends were observed at the individual level of species encounters (Fig. 2). We encountered significantly more common gartersnakes (*Thamnophis sirtalis*, $n = 27$) and Dekay's brownsnakes (*Storeria dekayi*, $n = 24$) in the prairie floodplain than the other habitats (Fig. 1) and more Dekay's brownsnakes under the metal coverboards (Fig. 2). We found too few

Table 1. Effects of habitat and coverboard type on snake species richness and encounters of individual species in a restored tallgrass prairie. Habitats consisted of prairie floodplain, floodplain to upland transition zone, and upland prairie. Coverboard types included plywood and corrugated sheet metal. Sampling occurred at Glacier Creek Preserve in Bennington, Nebraska, across 240 sampling occasions during the fall season. Bold text denotes statistical significance at the $\alpha = 0.05$ level based on generalized linear models.

Predictors	df	Species Richness		Common Gartersnake Encounters		Dekay's Brownsnake Encounters	
		Wald χ^2	P	Wald χ^2	P	Wald χ^2	P
Intercept	1	12.704	< 0.001	18.499	< 0.001	299.111	< 0.001
Coverboard Type	1	4.229	0.040	2.042	0.153	10.964	0.001
Habitat Type	2	24.077	< 0.001	16.865	< 0.001	35.427	< 0.001
Temperature	1	0.226	0.635	2.727	0.099	2.421	0.120

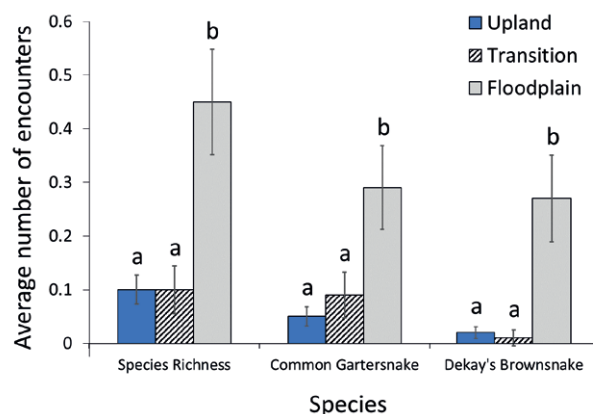


Fig. 1. Average species richness and number of encounters for each species (per coverboard) found under coverboards in each habitat type. Averages are estimated marginal means based on the generalized linear model for a Poisson distribution of count data. Associated count totals for Common Gartersnakes (*Thamnophis sirtalis*) and Dekay's Brownsnakes (*Storeria dekayi*) in the Upland = 8 and 4, Transition = 5 and 1, and Floodplain = 15 and 18, respectively. Upland translates to prairie upland, Transition to upland/floodplain transition zone, and Floodplain to prairie floodplain. A low occurrence of individuals precluded a species-specific analysis of Plains Gartersnakes (*Thamnophis radix*) and pattern detection in Western Foxsnakes (*Pantherophis ramspotti*). Different letters above bars denote significant differences between habitats ($\alpha = 0.05$). Error bars are ± 1 SE.

plains gartersnakes (*Thamnophis radix*, $n = 3$) to statistically examine their abundances across habitat and coverboard types and no significant patterns in Western foxsnakes (*Pantherophis ramspotti*, $n = 6$, GLM all $P > 0.4$). We caution direct interpretation of our encounters as relative abundances because we did not mark individuals, thus it is possible some were recounted, which could bias the count data.

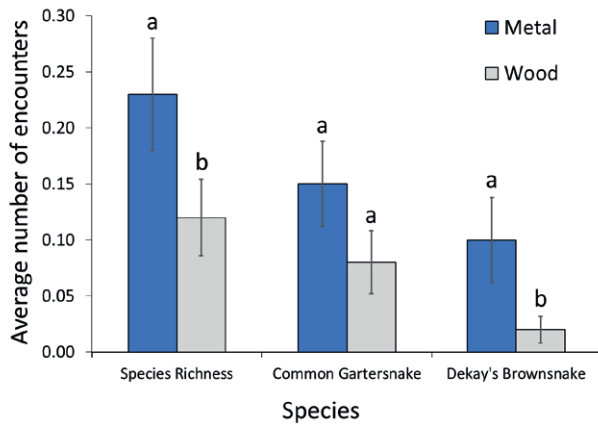


Fig. 2. Average species richness and number of encounters for each species (per coverboard) found under wood and metal coverboards. Averages are estimated marginal means based on the generalized linear model for a Poisson distribution of count data. Associated count totals for Common Gartersnakes (*Thamnophis sirtalis*) and Dekay's Brownsnakes (*Storeria dekayi*) found under wood = 10 and 4, and metal = 18 and 19, respectively. A low occurrence of individuals precluded a species-specific analysis of Plains Gartersnakes (*Thamnophis radix*) and pattern detection in Western Foxsnakes (*Pantherophis ramspotti*). Different letters above bars denote significant differences between treatments ($\alpha = 0.05$). Error bars are ± 1 SE.

Relative humidity under the coverboards was not influenced by coverboard type (as humidity residuals on temperature; $F_{1, 238} = 3.002$, $P = 0.084$), but it was influenced by habitat type (as humidity residuals on temperature; $F_{2, 237} = 8.961$, $P < 0.001$) and negatively related to temperature ($F_{1, 238} = 279.76$, $P < 0.001$, $R^2 = 0.54$). Temperature under the coverboards also was not influenced by coverboard type ($F_{1, 238} = 0.246$, $P = 0.620$), but was related to time of day ($F_{2, 237} = 207.78$, $P < 0.001$) with afternoons being the warmest period (Mean \pm SE for Morning = $17.3 \pm 0.5^\circ$ C, Afternoon = $30.7 \pm 0.5^\circ$ C, Dusk = $26.0 \pm 0.5^\circ$ C). The distance between coverboards and the creek was related to both the relative humidity underneath coverboards (residuals on temperature; Spearman's rho = -0.163 , $P = 0.012$) and to habitat type (Kruskal-Wallis H = 185.406, df = 2, $P < 0.001$).

The results of our study revealed habitat use and coverboard preference of the snake community in a restored tallgrass prairie system during the fall season. We found four snake species at Glacier Creek Preserve including the common gartersnake (*Thamnophis sirtalis*; Fig. 3A), plains gartersnake (*T. radix*), Western foxsnake (*Pantherophis ramspotti*; Fig. 3B), and Dekay's brownsnake (*Storeria dekayi*). We also observed one lizard species, the Northern prairie skink (*Plestiodon septentrionalis*). Snake species richness was greatest in the prairie floodplain as were snake encounter rates in general (Table 1,

Fig. 1). The greater overall snake encounters were a result of the significantly higher average number of individuals among common gartersnakes and Dekay's brownsnakes in the floodplain compared to the transition areas and upland prairie (Fig. 1). More snakes may have occupied the prairie floodplain because the proximity of a known hibernaculum used for overwintering (TJC, personal observation; Fig. 3). Because this study occurred in the fall, snakes may have been moving from the upland areas toward their hibernacula in preparation for winter brumation (McAllister, 2018; Bridger and Geluso, 2021).

Using both metal and plywood coverboards provided snakes with an opportunity to choose specific microhabitats. A greater number of common gartersnakes and Dekay's brownsnakes chose to settle under metal coverboards than wood, which is consistent with other studies for *Thamnophis* (Engelstoft and Ovaska, 2000, Hampton, 2007) and *Storeria* species (Halliday and Blouin-Demers, 2015). Snakes often seek materials with higher heat conductivity especially in cooler seasons (Hoyer, 1974; Fitch, 1987; Barker and Hobson, 1996; Engelstoft and Ovaska, 2000). Common gartersnakes in British Columbia, for instance, preferred metal and asphalt coverboards over wood during fall and spring (Engelstoft and Ovaska, 2000). Coverboards may be used less often generally during summer (e.g., mid-August) because associated microhabitat temperatures often rise above 40° C, which is above critical thermal maximum for most reptile species (Engelstoft and Ovaska, 2000; Angilletta, 2009). One might hypothesize that wooden coverboards would be used more during warmer summer months because they maintain both higher humidities and more stable temperatures compared to metal (Grant et al., 1992), often providing thermal environments similar to ambient conditions (Engelstoft and Ovaska, 2000). In other Nebraska grasslands reptile preference for wooden coverboards has been observed during the warmer months (Brown and Geluso 2022; approximately 370 km southwest of Glacier Creek Preserve). However, plywood coverboards were not used by reptiles in the British Columbia community more often in the summer compared to spring and fall (Engelstoft and Ovaska, 2000). Coverboard use may similarly vary with other herpetofauna such as amphibians, where some studies have found more amphibian species under wood coverboards than metal (Grant et al., 1992), and others have found no difference in amphibian abundance under metal versus wood coverboards (Hampton, 2007). During the lower fall season temperatures, the preference of snakes at Glacier Creek Preserve for metal coverboards likely occurred because these spaces heated up more quickly allowing snakes to more efficiently attain optimal body temperature for various behaviors and bod-



Fig. 3. Map of Glacier Creek Preserve, a tall grass prairie restoration site located in Bennington, Nebraska, United States in the heart of the Great Plains ecosystem of North America. The inset highlights the placement of coverboard stations along the habitat gradient from floodplain to upland prairie. The floodplain is depicted by the lighter beige strip of vegetation (75-300 m) covering both sides of Glacier Creek. The upland prairie is depicted by the darker tan areas of vegetation moving both north and south of the floodplain, up the slopes (200-600 m). The transition zone consists of the narrow strip (20-30 m) where the floodplain habitat (light beige vegetation) transitions into the upland prairie (darker tan vegetation).

ily functions such as foraging and digestion (Grant et al., 1992; Lillywhite, 1987).

Relationships among our environmental variables have ecological implications for habitat and coverboard use by snakes. Our final model only contained coverboard type, habitat type, and temperature as independent variables and temperature was the only variable that did not directly influence species encounters or richness. Interestingly, coverboard type was not directly related to either humidity or temperature in our study, which contrasts with other studies (Engelstoft and Ovaska, 2000; Grant et al., 1992). This is a case where the statistical significance may not match the biological significance as we found increased snake presence under metal coverboards even though we did not observe significantly greater temperatures or relative humidity. The relatively mild fall season climate likely diminished environmental effects of coverboard type that may be more significant during

late spring and summer seasons (Engelstoft and Ovaska, 2000). It may also be a dissociation of measured temperatures at time of capture versus biologically significant temperatures during snake movement and microhabitat choice. Our analyses of relationships among environmental variables determined that temperature was related to time of day (afternoons the warmest) and habitat type was related to humidity and distance from the creek. Neither relative humidity nor temperature were significant factors in our models of coverboard selection, but our temperature and humidity data were collected at the time of sampling. It is possible that temperature and humidity influence habitat and coverboard use most when the snake first moves to the space, which may occur within a specific window during the day, although we did not observe an effect of time of day on species presence or absence. We do not know when during the day snake activity and coverboard choice occurred, but future stud-

ies examining daily temperature cycles along with snake activity and microhabitat preferences could shed light on this relationship. Habitat type, humidity, and distance from creek were all related, with humidity decreasing with distance from creek as habitat changed from the prairie floodplain and transitioned to the upland prairie. The prairie floodplain had the greatest species richness, suggesting that greater humidity and creek proximity could attract more snake species. Whether it was the humidity itself or proximity to the creek, however, we cannot discern.

Because our study was limited to three weeks of a single climatic season, we caution against any definitive conclusion regarding the overall snake community. However, comparing this prairie snake community with assessments of other prairie snake communities within a day's drive suggests that the Glacier Creek restoration effort at least partially reflects the "Field of Dreams" paradigm. The Nachusa Grassland is a tallgrass prairie system in the State of Illinois, 600 km to the east of our restoration site. An extensive survey of the Nachusa Grassland system detected a snake community nearly identical to ours, including the common gartersnake (*Thamnophis sirtalis*), plains gartersnake (*Thamnophis radix*), and Dekay's brownsnake (*Storeria dekayi*), in addition to the Eastern foxsnake (*Pantherophis vulpinus*) rather than our Western foxsnake (*Pantherophis ramspotti*; King and Vanek, 2020). The Konza Prairie is another well surveyed tallgrass prairie in the State of Kansas, approximately 500 km south of our site with a reported snake community of ten species (Wilgers and Horne, 2006). The Konza Prairie snake community overlapped with two of our species, the common gartersnake and Dekay's brownsnake, but contained eight more species, four of which are known to occur in Douglas County, Nebraska, where Glacier Creek Preserve resides, the Gophersnake (*Pituophis catenifer*), Eastern Racer (*Coluber constrictor*), Lined Snake (*Tropidoclonion lineatum*), and Ring-necked Snake (*Diadophis punctatus*; Fogell, 2010). Together, these comparisons show two snake species common to all three prairies and the possibility of four more species inhabiting Glacier Creek Preserve.

Our collective knowledge about maintaining, restoring, and monitoring prairie communities is of global importance because grasslands cover one-third of the world's land area (Nunez, 2019). Our study is an important first step in the assessment of restoration success with regard to the reptile community of this tallgrass prairie ecosystem in the Great Plains. Our study determined a baseline estimate of the current reptile community (four snake and one lizard species) and the relative efficacy of coverboard type (metal coverboards created a

preferred microclimate) in the tallgrass prairie ecosystem. Our data suggest that fall surveys should incorporate metal coverboards and focus on the prairie floodplain if the goal is to assess snake diversity. Using an array of different coverboard types may be beneficial across seasons because of the different microclimates that they create along with different preferences exhibited by the different species, life stages, or physiological state of individual snakes (Halliday and Blouin-Demers, 2015). Although the snake community we recorded was similar to one comparable system, it was different than another. Further studies in this system, such as a long-term monitoring program over multiple years and seasons will provide further insight into the restored snake community such as seasonal influence on habitat and coverboard use, and possibly the season in which sampling should be conducted for highest detection rates.

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Revisiting the polyploidy in the genus *Odontophrynus* (Anura: Odontophrynidae)

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Abstract. The genus *Odontophrynus*, composed of ten species, is found in practically the entire south of South America. *Odontophrynus americanus* was the first vertebrate registered to present natural polyploidy, considering that most individuals have $2N = 4x = 44$ chromosomes, although having $2N = 2x = 22$ chromosomes is considered the ancestral condition for all genera of the family Odontophrynidae. The present study aimed to analyze the karyotype of *O. americanus*, providing a detailed and comparative description of conventional chromosomal markers, with focus on a possible diploidization process operating in this polyploid genome. The individuals were collected in a fragment of Atlantic Forest in the south-central region of Paraná State, Brazil. The analyzed individuals presented the tetraploid pattern, with banded chromosomes. The C-banding showed heterochromatic regions restricted to centromeres and telomeres. Among homologous chromosomes of the same quartet, small differences were observed in morphology, possibly the result of differentiation after the polyploidization event. Finally, the 45S rDNA (Nucleolar Organizer Regions) was mapped in the short arm of quartet 11, showing the nucleolus organizing regions active in the four homologous chromosomes. This genome, although structurally polyploid, may be undergoing a process of diploidization, by becoming functionally equivalent to a diploid genome, via chromosomal rearrangements, epigenetic mechanisms, and/or repetitive DNA dynamics.

Keywords. Amphibian, diploidization, heterochromatin, rDNA.

According to Frost (2023), the family Odontophrynidae currently contains 55 species distributed in three genera *Macrogenioglottus* Carvalho, 1946, *Odontophrynus* Reinhardt and Lütken, 1862, and *Proceratophrys* Miranda-Ribeiro, 1920. Earlier phylogenies validate the monophyly of the family, as well as that *Macrogenioglottus* and *Odontophrynus* are sister taxa (Pyron and Wiens, 2011; Feng et al., 2017). The genus *Odontophrynus* is composed of eleven species widely distributed in southern and eastern South America. *Odontophrynus americanus* (Duméril and Bibron, 1841), a small fossorial anuran with no apparent sexual dimorphism (Quiroga et al., 2015), has the greatest distribution, its range extends to central and

southern Argentina, southern Paraguay, southern Brazil, and Uruguay (Frost, 2023).

Odontophrynus americanus was the first case of natural polyploidy found in vertebrates (Beçak et al., 1966). The *Odontophrynus americanus* species group is a complex of morphologically indistinguishable diploid and tetraploid species. It includes currently four diploid species: *O. cordobae* Martino and Sinsch, 2002, *O. juquinha* Rocha, Sena, Pezzuti, Leite, Svartman, Rosset, Baldo, and Garcia, 2017, *O. lavillai* Cei, 1985 and *O. maisuma* Rosset, 2008 with $2N = 2x = 22$ chromosomes, and one widely distributed tetraploid species (*O. americanus*) with $2N = 4x = 44$ chromosomes (Beçak et al., 1966; Ruiz et

al., 1981; Martino and Sinsch, 2002; Rosset et al., 2006; Rosset, 2008). Martino et al. (2019) established the existence of cryptic diversity and overestimation of species richness by combining molecular, morphological, and bioacoustic data. Populations known as *O. americanus* comprise at least three species.

Polyploidy plays an important role in speciation and evolution in anurans, with about 50 polyploid species described in several families (Bogart, 1980; Mable et al., 2011; Evans et al., 2012; Schmid et al., 2015). Polyploids originate by autopolyploidization (intraspecific whole-genome duplication) or allopolyploidization (associated with interspecific hybridization). Thus, individuals with an autotetraploid genome can originate by fusion of unreduced (i.e., diploids) gametes, or by suppression of the first mitotic division in fertilized eggs (Schmid et al., 2015). In recently evolved autopolyploids, the homologous chromosomes of a quartet are expected to exhibit identical chromosome banding patterns in somatic metaphases, leading to the multivalent formation during the first meiotic division. On the other hand, in an allopolyploid genome, if there are differences among the karyotypes of the parental species, the banding techniques or the genomic *in situ* hybridization (GISH) allow chromosomes from parental species to be distinguished (Schwarzacher et al., 1989), which will form bivalent configurations in meiosis (Schmid et al., 2015).

In this study, the structure of polyploid karyotype *O. americanus* from a southern Brazilian population is described and subjected to comparative analysis in order to add new data regarding the speculated species complex. Additionally, the data are placed in an evolutionary context, thus contributing to a better understanding of the evolutionary scenario concerning ploidy levels in this group.

Cytogenetic analyses were carried out on six juveniles of *O. americanus* collected in União da Vitória, Paraná

State, Brazil (26°13'48"S and 51°05'09"W). Chromosome preparations were performed directly from bone marrow, according to Baldissera et al. (1993). Briefly, the animals received intraperitoneal injection of aqueous solution of colchicine (0.01 ml/g body weight) 1% per 6 h, and then subjected to deep sedation euthanasia by dermal absorption of Lidocaine 5% pomade, following the recommendations of the Ethical Committee in Animal Use from Universidade Estadual do Paraná.

Conventional staining was performed using 5% Giemsa in sodium-phosphate buffer (pH 7.0, for 10 min). Detection of the constitutive heterochromatin was accomplished according to Sumner (1972). Silver staining technique (Ag-NOR detection) was carried out according to Howell and Black (1980). The mitotic metaphases were analyzed under a Carl Zeiss Axiolab A1 microscope equipped with the software Zen Lite and a Zeiss AxioCam ICc1 camera with a resolution of 1.4 megapixels (Carl Zeiss, Oberkochen, Germany). Chromosomes were classified based on the centromeric index according to Green and Sessions (1991) and were arranged in decreasing size.

The specimens of *O. americanus* showed a karyotype of $2N = 4x = 44$ chromosomes, distributed in eight metacentric quartets (1, 5–11) and three submetacentric quartets (2–4), thus presenting a fundamental number (FN) = 88 (Fig. 1). There was no variation among the specimens karyotyped. Exclusively between homologous chromosomes of quartets 2, 3, and 4, small differences were observed in terms of chromosomal morphology, which often made it difficult to organize these quartets. The centromeric indexes were established confirming the morphology discrepancies between homologs of the same quartet (Fig. 1). According to the relative size of the chromosomes, the species has a karyotype with four different sizes of chromosomes: one large quartet (1),

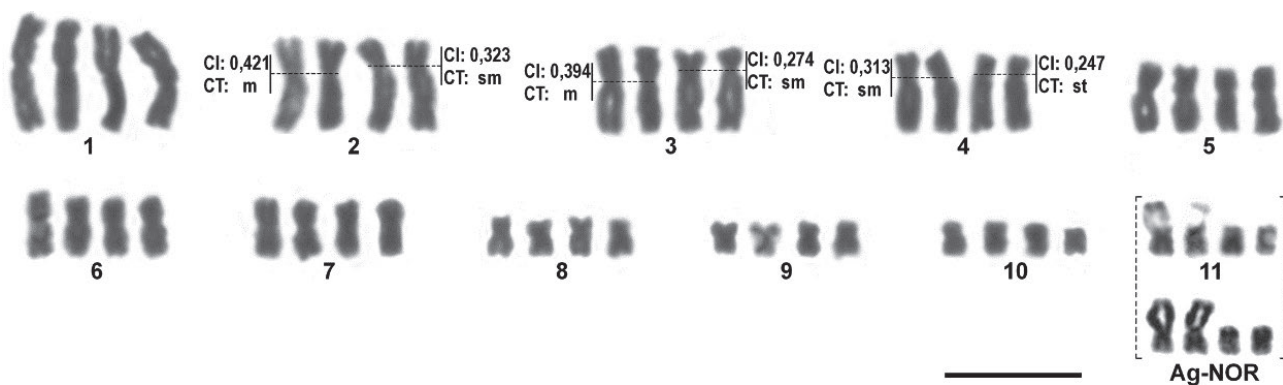


Fig 1. Giemsa-stained karyotype of *O. americanus*. Highlighted the Ag-NORs site localized on the quartet 11. CI: centromeric index; CT: chromosome type; m: metacentric; sm: submetacentric; st: subtelocentric. Bar = 10 μ m.

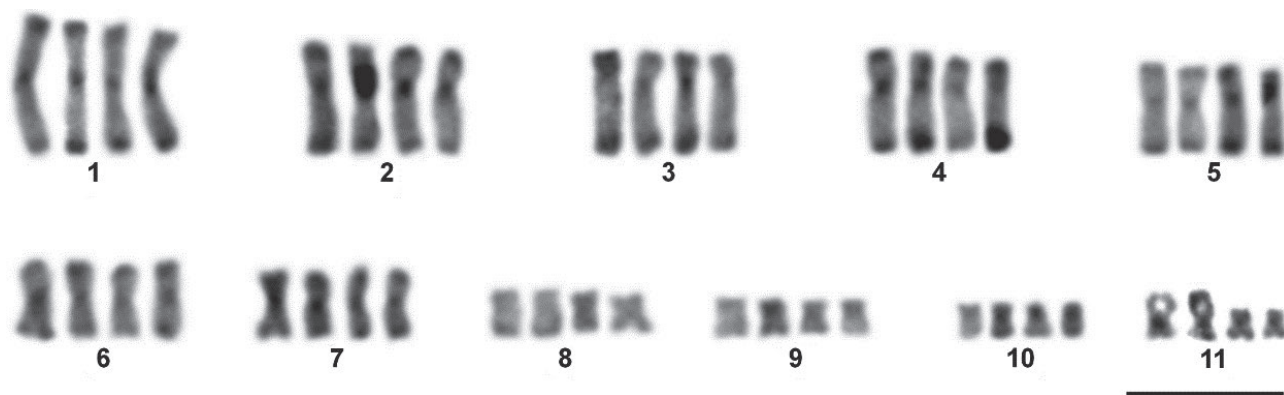


Fig 2. C-banding karyotype of *O. americanus*. Bar = 10 μ m.

three medium quartets (2–4), four small (5–8), and three very small (9–11).

Nucleolus Organizer Regions (NORs) were observed on the short arm of quartet 11. Such regions are coincident with secondary constrictions (Fig. 1). A NOR size heteromorphism between homologous chromosomes of the quartet was frequently observed.

The C-banding showed the presence of constitutive heterochromatin in the centromeric and telomeric regions of almost all quartets (absence of centromeric bands in quartets 8 and 9), and coincident with Ag-NOR staining (quartet 11) (Fig. 2).

The family Odontophrynidae was first established as a tribe within the (then) huge family *Leptodactylidae* (Lynch, 1971). The karyotype with $2N = 2x = 22$ chromosomes is considered the ancestral condition, given its high frequency in all three genera. This characteristic karyotype is believed to have arisen from the differentiation of the primitive chromosome number of $2N = 26$ chromosomes present in the family *Leptodactylidae*, followed by centric fusions (Bogart, 1973).

Karyotype descriptions of the genus *Odontophrynus* reveal so far a very similar and conserved karyotype, which is composed exclusively of biarmed chromosomes, reflecting in fundamental numbers always twice the $2N$, with some constant pairs in morphology between the species (Table 1). These small variations are a consequence of chromosomal rearrangements that only modify the chromosome morphology, such as pericentric inversions, although the centromere repositioning, which alters the chromosome morphology without any accompanying chromosomal rearrangements (Rocchi et al., 2012), could be an alternative pathway leading to chromosomal remodeling.

A special interest has been devoted to the study of the occurrence of diploid ($2N = 2x = 22$) and tetraploid ($2N = 4x = 44$) constitutions in the *O. americanus* spe-

cies group (see Table 1). In this sense, several studies have indicated that it could consist of a complex of species (Rosset et al., 2006; Lanzone et al., 2008; Cianciarullo et al., 2019; Martino et al., 2019) and thus, the *O. americanus* listed with 22 chromosomes are expected to probably be other distinct species.

The difficulty in organizing some quartets (i.e., 2–4) in conventional staining, due to small differences in the position of centromeres, may represent a prognosis for an incipient process of diploidization, as observed in other populations (Ruiz et al., 1981; Schmid et al., 1985). A structural heterogeneity must be created between homologous of quartets in the polyploid karyotype, which can originate even from small rearrangements such as pericentric inversions (Ohno, 1970; Ohno, 1974). Therefore, the differences within the quartets in question can be interpreted as post-polyploid events, indicating a diploidization process operating in this polyploid genome (Ohno, 1970; Schmid et al., 1985; Beçak, 2014).

The variation of NORs location in species of *Odontophrynus* is the result of translocations (Beçak and Beçak, 1974) and/or transposable elements-mediated transpositions events (Gray, 2000; Mandrioli, 2000), which switched these ribosomal genes to other pairs promoting karyotype diversification. The karyotype with NORs on pair 11 is considered as the plesiomorphic condition, found in diploid species from three species groups of *Odontophrynus*, as well as in most individuals studied from tetraploid populations of *O. americanus* (see Table 1).

A size heteromorphism between homologous was frequently observed. The presence of NOR-associated heterochromatin demonstrated that this heteromorphism between homologous of quartet 11 comprises both functional and structural aspects. This condition may have facilitated breaks and transpositions of rRNA genes to other sites in different species and populations of *Odontophrynus* (Wiley et al. 1989; Carvalho et al. 2014).

Table 1. Summary of the chromosome findings of the species of *Odontophrynus*: diploid number (2N), centromeric heterochromatin (©), telomeric heterochromatin (Ⓣ), interstitial heterochromatin (Ⓜ), Nucleolus Organizer Region (NOR), short arm (p), long arm (q), fundamental number (FN), *Artificial hybrid.

Species group	Species	Locality	2N	Ploidy level	C-banding	NOR	FN	Reference	
<i>O. americanus</i>	<i>O. americanus</i>	Brazil	22	2x	-	4p	44	Ruiz et al. (1981)	
		Argentina	22	2x	©ⓉⓂ	4p	44	Ruiz et al. (1981)	
		Brazil	22	2x	©ⓉⓂ	4p, 11p	44	Ruiz et al. (1981)	
		Argentina	33	3x	-	-	66	Grenat et al. (2018)	
		Brazil	44	4x	-	11p	88	Beçak et al. (1966)	
		Argentina	44	4x	-	11q	88	Bogart (1967)	
		Uruguay	44	4x	-	4p	88	Ruiz et al. (1981)	
		Argentina	44	4x	-	-	88	Grenat et al. (2018)	
		Brazil	44	4x	©ⓉⓂ	11p	88	Ruiz et al. (1981)	
		Uruguay	44	4x	©ⓉⓂ	4p, 11p	88	Ruiz et al. (1981)	
		Argentina	44	4x	©ⓉⓂ	11p	88	Schmid et al. (1985)	
		Brazil	44	4x	©Ⓣ	11p	88	Present study	
		Uruguay	66*	6x	-	11p	132	Ruiz et al. (1981)	
		<i>O. cordobae</i>	Argentina	22	2x	-	11-	44	Martino and Sinsch (2002)
				22	2x	-	4p	44	Salas and Martino (2007)
<i>O. juquinha</i>	Brazil	22	2x	-	4p	44	Rocha et al. (2017)		
		22	2x	-	4p	44	Rosset et al. (2006)		
<i>O. lavillai</i>	Argentina	22	2x	-	4p	44	Rosset et al. (2006)		
		22	2x	-	4p	44	Rosset (2008)		
<i>O. maisuma</i>	Uruguay	22	2x	-	4p	44	Rosset (2008)		
		22	2x	©Ⓜ	4p	44	Borteiro et al. (2010)		
<i>O. reigi</i>	Argentina, Brazil, Paraguay	22	2x	-	4p	44	Rosset et al. (2021)		
		22	2x	©ⓉⓂ	11p	44	Ruiz and Beçak (1976)		
<i>O. cultripes</i>	Brazil	22	2x	©ⓉⓂ	8p	44	Ruiz et al. (1981)		
		22	2x	©ⓉⓂ	9q, 11p	44	Ruiz et al. (1981)		
<i>O. occidentalis</i>	<i>O. occidentalis</i>	Argentina	22	2x	©ⓉⓂ	9q, 11p	44	Ruiz et al. (1981)	

Heteromorphic NORs could also be related to differences in genetic activity (Amaro-Ghilardi et al., 2008). In fact, in polyploids, while the number of 45S rDNA citrons is proportional to the degree of ploidy, gene expression may be equivalent to a diploid genome (Schmidtke and Engel, 1976). Epigenetic mechanisms are responsible for modulating gene expression through chemical modifications of histones, via methylation, acetylation, and/or phosphorylation (Furey and Sethupathy, 2013). Equalization of gene activity between 2x and 4x species could be at the transcriptional level, probably by rDNA methylation (Hashimshony et al., 2003). Indeed, Ruiz and Brison (1989) found high levels of methylation of ribosomal genes in tetraploid genomes of *O. americanus*. It has been validated by Cianciarullo et al. (2000), who found only 25–30% more ribosomes in *O. americanus* tetraploid than do 2N cells. Therefore, polyploid genomes may become functionally diploid throughout evolution (Schmid et al., 2015).

The presence of constitutive heterochromatin on centromeric and telomeric regions is an expected pattern in *Odontophrynus*. The eventual variation involves the additional presence of interstitial bands that characterize

some species/populations (see Table 1). The variation in the distribution pattern of constitutive heterochromatin is generally associated with the dynamics of different classes of repetitive DNA. Heterochromatin is normally rich in repetitive sequences, which can have important functions in speciation and/or adaptation, as they are less subject to selective pressures, favoring the accumulation of differences throughout the evolutionary process (Martins, 2007; Böhne et al., 2008).

In conclusion, the intra- and interpopulation chromosomal variability in *Odontophrynus* is a consequence of its wide geographic distribution throughout South America. Regarding polyploidy within the group, its origin via autopolyploidization seems to be the most accepted, mainly due to the presence of multivalents at meiosis (Beçak et al., 1966; Schmid et al., 1985; Lanzone et al., 2008). However, multivalent formation can also be observed in some allopolyploids, because the structure of chromosomes from different species (i.e., homeologous) can be sufficiently conserved to permit multivalent associations. Autopolyploids, on the other hand, might also have mechanisms that prevent multivalent con-

figuration and thus form bivalents (Gregory and Mable, 2005). Therefore, distinguishing between auto- and allopolyploidization is difficult, since the scenario possibly involves a combination of both mechanisms. The disjunct tetraploid populations are closely associated with several diploid species, which suggests that polyploidy has multiple origins, with putative older lineages accumulating more chromosomal changes within the homologous quartets. The evidence suggests that the benefits of polyploidization are stabilized by epigenetic mechanisms, small structural rearrangements, and repetitive DNA dynamics, which lead the tetraploid genomes to become functionally diploid (diploidization). Given this scenario, the analysis throughout the chromosomal mapping of repetitive elements represents a crucial tool for clarifying the dynamic processes concerned with the karyotype diversification in *Odontophrynus* species, especially in this group with the uncertain taxonomic assignment.

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