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

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

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

INTRODUCTION

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

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

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

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

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

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

METHODS

Study area

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

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

Data collection

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

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

Analysis of the diet

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

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

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

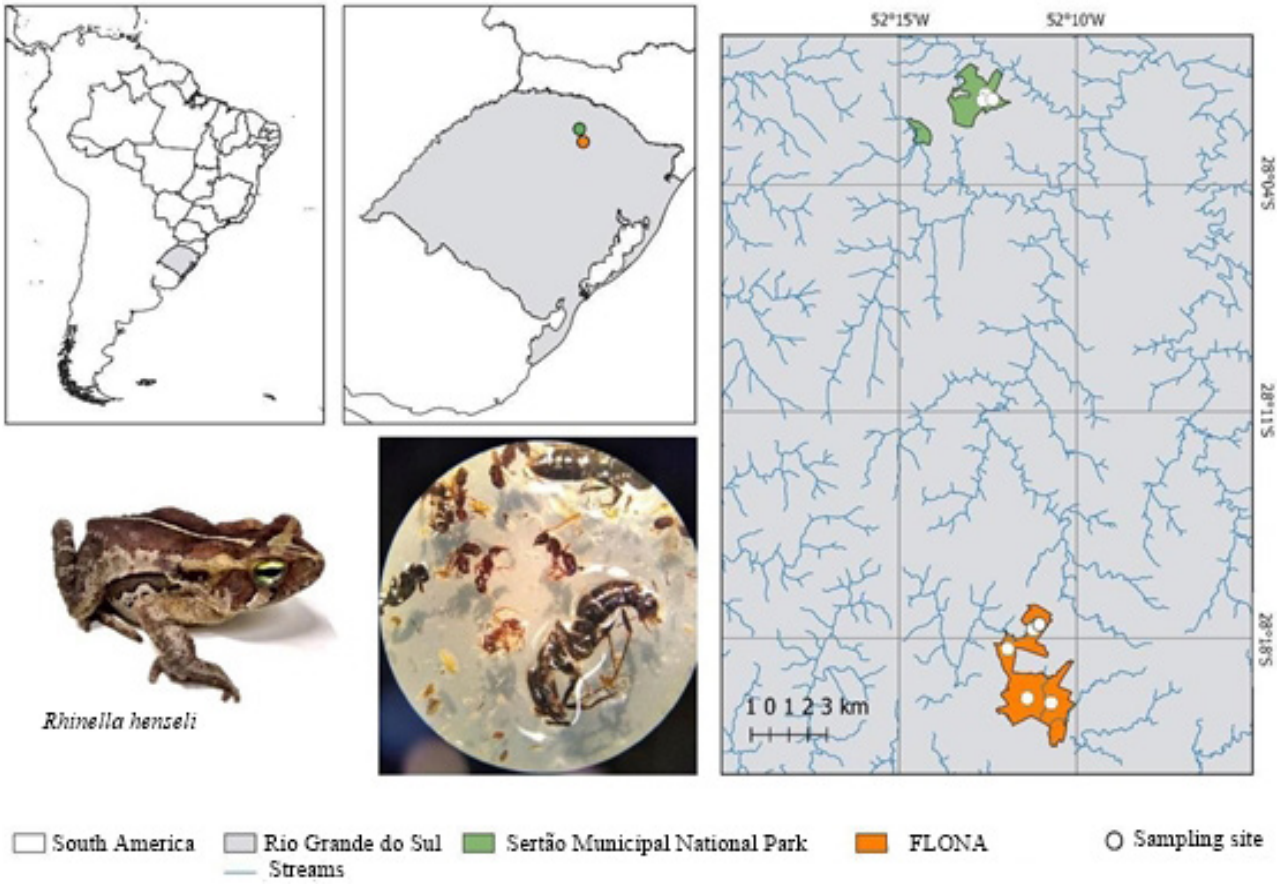

Figure 1. Location of the *R. henseli* (Anura: Bufonidae) populations sampled in the Atlantic Forest of southern Brazil and Sertão Municipal Natural Parkand Passo Fundo National Forest (FLONA Passo Fundo) in Rio Grande do Sul state, Brazil.

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

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

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

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

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

Antimicrobial activity tests

We mixed the chemical secretions obtained from the toads

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

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

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

RESULTS

Diet of *Rhinella henseli*

We collected data on the diet of 82 toads, of which 66 were collected in Area A (16 during the cool season and 50 during the warm season) and 16 in Area B (warm season). We did not detect any significant variation in the composition of the diet of the toads captured by active searching or pitfall traps ($p = 0.07$), which allowed us to pool these data. The *R. henseli* stomachs contained six insect orders, including six families of the order Coleoptera and 15 genera of the

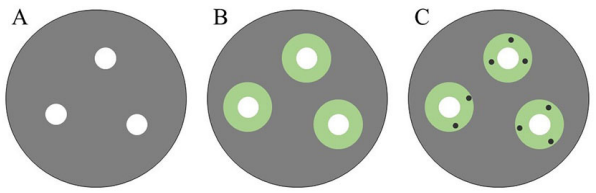

Figure 2. The figure demonstrates the hypothesised results for the agar plug diffusion method. Grey color: fungal and bacterial growth in the agar. White color: 50 µl wells containing the parotoid macroglands secretions. Green color: inhibition zones of microbial growth around the wells. **A)** No microbial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

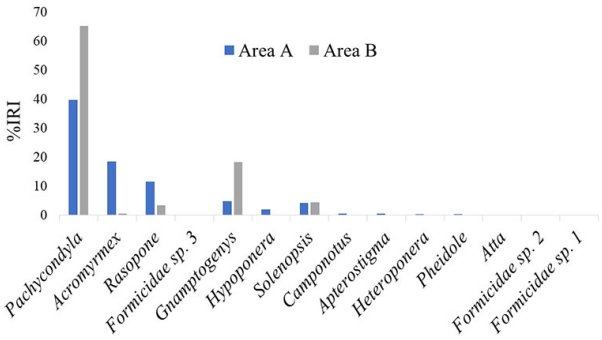

Figure 3. Percentage index of relative importance of ant genera consumed by *R. henseli* in two areas of Atlantic Forest in southern Brazil. This comparison only includes the data from the warm season. Note that in area B the genus *Pacycondylais* dominant, while in area A the values are better distributed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

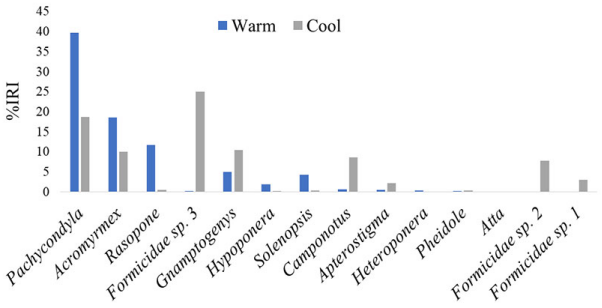

Figure 4. Percentage index of relative importance of ant genera consumed by *R. henseli* in two seasons in the Atlantic Forest of southern Brazil. This comparison only includes the data from the area A. Note that in the cold season, the most consumed genus becomes Formicidae sp. 3, including other prey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

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

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

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

hymenopteran family Formicidae (Table 1).

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

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

Parotoid macrogland chemical composition

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

Antimicrobial activity of parotoid secretions

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

DISCUSSION

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

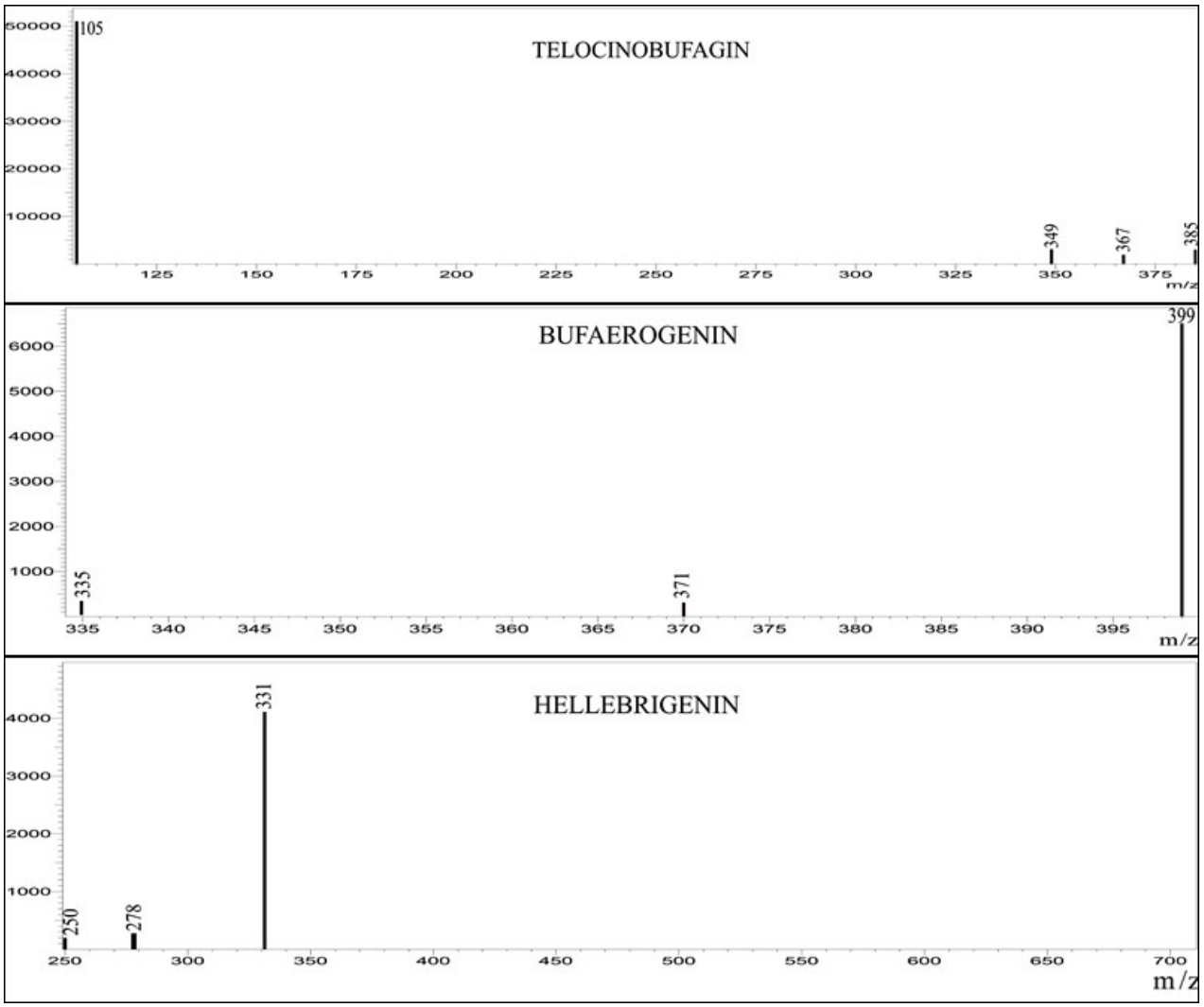

Figure 5. Compounds tentatively identified from precursor ions and fragment ions (M+H)+. Telocinobufagin and Hellebrigenin are recovered in the literature as compounds with activity against pathogens.

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

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

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

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

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

*x = present;-- = absent.

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

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

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

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

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

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

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

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

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

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Authors' contribution

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

Ethical statement

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

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Conflicts of interest

The authors declare having no competing interests.

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