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The occurrence of defensive alkaloids in non-integumentary tissues of the Brazilian red-belly toad *Melanophryniscus simplex* (Bufonidae)

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Abstract The red-belly toads (*Melanophryniscus*) of southern South America secrete defensive alkaloids from dermal granular glands. To date, all information on *Melanophryniscus* alkaloids has been obtained by extraction from either skins or whole organisms; however, in other amphibians, tetrodotoxins, samandarines, and bufadienolides have been detected in both skin and other organs, which raise the possibility that lipophilic alkaloids may occur in non-integumentary tissues in *Melanophryniscus* as well. To test this hypothesis, we studied the distribution of alkaloids in the skin, skeletal muscle, liver, and mature oocytes of the red-belly toad *M. simplex* from three localities in southern Brazil. Gas chromatography and mass spectrometry of skin extracts from 11 individuals of *M. simplex* resulted in the detection of 47 alkaloids (including isomers), 9 unclassified and 38 from 12 known structural classes. Each alkaloid that was present in the skin of an individual was also present in the same relative proportion in that individual's skeletal muscle, liver, and oocytes. The most abundant and widely distributed alkaloids were the pumiliotoxins **251D**, **267C**, and **323A**, 5,8-disubstituted indolizidines **207A** and **223D**, 5,6,8-trisubstituted indolizidine **231B**, 3,5-disubstituted pyrrolizidines *cis*-**223B** and *cis*- and *trans*-**251K**, and izidine **211C**. We report the first record of piperidines in *Melanophryniscus*, bringing the total number of alkaloid classes detected in this genus to 16. Alkaloid composition differed significantly among the three study sites. The functional significance of defensive chemicals in non-integumentary tissues is unknown.

Keywords Anura · Amphibia · Chemical defense · Sequestration

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Introduction

Amphibians secrete a diverse arsenal of defensive chemicals from granular glands in the skin, including amines, alkaloids, bufadienolides, and peptides and proteins (Daly 1995). These secretions are believed to function as an important component of the innate immune system in defending against pathogens and parasites (Rivas et al. 2009; Conlon 2011) and are also involved in complex antipredator mechanisms (Brodie et al. 1991). All amphibians possess granular glands (Toledo and Jared 1995) and presumably secrete some sort of defensive chemical, but only approximately 150 species divided among eight lineages in

five anuran families secrete lipophilic alkaloids (Daly et al. 2005; Grant et al. 2006; Rodríguez et al. 2010), including Bufonidae (*Melanophryniscus*), Dendrobatidae (independently derived in *Epipedobates*, *Ameerega*, and Dendrobatinae), Eleutherodactylidae (*Eleutherodactylus*), Mantellidae (*Mantella*), and Myobatrachidae (*Pseudophryne*). Feeding experiments have demonstrated that dendrobatids, mantellids, and myobatrachids obtain lipophilic alkaloids by consuming alkaloid-containing arthropods, especially brachypiline mites and myrmicine ants (Saporito et al. 2009, 2011; Raspotnig et al. 2011). The bufonids and eleutherodactylids that secrete these alkaloids have diets that are rich in mites and ants and presumably also obtain their defensive alkaloids through sequestration (Saporito et al. 2009, 2011; Rodríguez et al. 2010).

Melanophryniscus includes 26 species in southeastern South America, extending from the Brazilian state of Minas Gerais to Uruguay (Frost 2011). Most of these small (<3.5-cm snout-vent length) toads are characterized by their diurnal activity (Santos and Grant 2011), and bright orange or red ventral coloration that they expose by arching the back and raising the limbs in the unken reflex (e.g., Fernández 1926; Fig. 1). Of the 26 known species, the defensive alkaloids of eight species have been studied, yielding 113 alkaloids (including isomers) of 15 structural classes and another 22 unclassified alkaloids (Table 1).

To date, all information on *Melanophryniscus* alkaloids has been obtained by extraction from either skins (Daly et al. 1984, 2007, 2008; Garraffo et al. 1993) or whole organisms (Mebs et al. 2005, 2007). However, the defensive chemical tetrodotoxin occurs in skin and other organs in several amphibians and other vertebrates (e.g. Noguchi and Arakawa 2008; Mebs et al. 2010), as do samandarine alkaloids in the salamandrid *Salamandra salamandra* (Mebs and Pogoda 2005) and bufadienolides in the bufonid toad *Rhinella marina* (Flier et al. 1980). Although these defensive chemicals have been shown or presumed to be synthesized by the organisms that contain them, it raises the possibility that lipophilic alkaloids of *Melanophryniscus*, which are derived from diet, may occur in non-integumentary tissues as well. To test this hypothesis, we studied the distribution of alkaloids in the skin, muscle, liver, and mature oocytes of the Brazilian red-belly toad *M. simplex* from three localities in southern Brazil.

Fig. 1 The defensive, anti-predator posture of *Melanophryniscus simplex*. When threatened, individuals expose red or orange ventral coloration by arching the back and raising the limbs. Adult male, 26.4-mm snout-vent length, MCT 11922

Materials and methods

Frog collections

We collected 11 adult *Melanophryniscus simplex* from three localities in southern Brazil (Fig. 2): (1) Aratinga (São Francisco de Paula, RS; GPS coordinates 29°19′12.6″S, 50°12′13.3″W, ca. 775 m), 5 frogs (MCT 11922–26) collected 5 April 2008, (2) Gateados AC3 (Campo Belo do Sul, SC; GPS coordinates 27°59′42.19″S, 50°53′27.92″W), 3 frogs (UFRGS 5680 and two uncatalogued specimens) collected 16 July 2008, and (3) Gateados AD2 (Campo Belo do Sul, SC; GPS coordinates 28°1′30.14″S, 50°50′33.04″W), 3 frogs (UFRGS 5681–5682 and one uncatalogued specimen) collected 15 May 2008. The linear distance between the Aratinga and Gateados localities is approximately 156 km and between the two Gateados localities is approximately 6 km. We deposited specimens in the Coleção de Anfíbios e Répteis, Museu de Ciências e Tecnologia, Pontifícia Universidade Católica do Rio Grande do Sul (MCT) and the Coleção Herpetológica, Departamento de Zoologia, Universidade Federal do Rio Grande do Sul (UFRGS).

To determine the distribution of alkaloids in frogs, we removed whole skins, mature oocytes, and small sections of skeletal muscle (thigh) and liver from individual frogs. To prevent cross contamination we removed only a small section of skeletal muscle and liver and thoroughly cleaned instruments by wiping and soaking in methanol between organs and individuals. Due to equipment limitations, we could only obtain accurate mass measurements for whole skins. We stored samples of skin, skeletal muscle, liver, and oocytes separately in 100 % methanol in glass vials with teflon-lined caps.

General experimental procedures

We performed gas chromatography–mass spectrometry (GC–MS) analysis on a Varian Saturn 2100T ion trap MS instrument coupled to a Varian 3900 GC with a 30 m × 0.25 mm i.d. Varian Factor Four VF-5ms fused silica column. We achieved GC separation of alkaloids using a temperature program from 100 to 280 °C at a rate of 10 °C per minute with He as the carrier gas (1 mL/min). We analyzed each alkaloid fraction with both electron impact MS and chemical ionization MS with methanol as the CI reagent.

Table 1 Alkaloids detected previously in species of *Melanophryniscus*

Species	3,5-P	3,5-I	5,8-I	de-5,8-I	5,6,8-I	4,6-Q	1,4-Q	Izi	PTX	aPTX	hPTX	Do-hPTX	Spiro	DHQ	TRI	Unclass
M. atroluteus[a]									**237A**, **251D**, **265D**, **267C**, **307A**, **309A**, **323A**							
M. cupreuscapularis[b]		*5Z,9Z-* **223AB**, **239Q**	**259B(1)**			**275I**, **279H**	**259E**	**211C**	**277B**, **307F**(1), **307G**		**249F**(2)			**269AB**	**193C**, **205H**, **205I**, **235M**	**193K**, **207S**, **209R**, **223T**, **235S**, **293H**, **305H**
M. devincenzii[a]									**237A**, **251D**, **265D**, **267C**, **307A**, **309A**, **323A**							
M. klappenbachi[b]		**195B**, *5Z,9Z-* **223AB**, **239Q**	**207A**, **223D**, **259B**	**219G**, **249L**		**275I**			**251D**, **323A**		**249F**(1), **265N**			**223F**(1), **269AB**, **269B**, **271D**	**205I**	**207S**, **223EE**, **223T**, **235S**, **307J**
M. montevidensis[c, d, e]	*cis-***223H**, **237G**	*5E,9Z-* **195B**, *5Z,9E–* **195B**							**237A**, **251D**, **265D**, **267C**, **307B**, **309A**, **323A**		**319A**, **319B**, **321B**					
M. moreirae[f]									**267C**	**323B**						
M. rubriventris[e]		5Z,9Z- **223AB**	**261D**, **261D′**, **273C**		**195G**, **207C**, **221Q**			**191D**, **191E**, **205F**, **205G**, **207T**, **207T′**, **209N**, **221R**	**219G**, **237A**, **251D**, **275H**, **277B**, **277G**, **289C**, **291G**, **307A**, **307G**, **309A**, **319F**, **323A**	**267A**		**207O**			**193C**, **193C′**, **203B**, **205H**, **205H′**, **207U**, **221S**, **221W**, **237O** (2)	**183C**, **195L**, **209M** (2), **223T** (2), **267R**

Table 1 continued

Species	3,5-P	3,5-I	5,8-I	de-5,8-I	5,6,8-I	4,6-Q	1,4-Q	Izi	PTX	aPTX	hPTX	Do-hPTX	Spiro	DHQ	TRI	Unclass
M. stelznerі[c, e]	*cis*-**223B**,	**195B**,	**193M**,	**219G**	**207C**,			**193I**,	**251D**				**236**	*cis*-**223F**,	**193A**,	**223W**,
	trans-	*5Z,9E*–	**207A**		**223X**,			**207S**						*trans*-	**193C**,	**231J**,
	223B,	**195B**,	**207A″**,		**235E**,									**223F**,	**235I**	**233K**,
	cis-	*5E,9Z*-	**259B**		**235E′**,									*cis*-**249D**,		**247L**,
	223H,	**195B**,			**237S**,									*trans*-		**247M**,
	cis-	*5Z,9Z*-			**251EE**									**249D**,		**249P** (2),
	251K,	**195B**,			(2)									*trans*-		**255F**,
	trans-	*5E,9E*–												**249E**,		**265BB**,
	251K	**195B**,												*cis*-**275B**		**267Y**
		5Z9Z-														
		223AB,														
		5E,9Z-														
		223AB														

The number of isomers detected is indicated in parentheses following the code designations. In some cases, prime symbols are used with alkaloid codes to indicate the specific isomer (see Daly et al. 2005)

Abbreviations for alkaloid classes: 3,5-P, 3,5-disubstituted pyrrolizidine; 3,5-I, 3,5-disubstituted indolizidine; 5,8-I, 5,8-disubstituted indolizidine; de-5,8-I, dehydro-5,8-disubstituted indolizidine; 5,6,8-I, 5,6,8-trisubstituted indolizidine; 4,6-Q, 4,6-disubstituted quinolizidine; 1,4-Q, 1,4-disubstituted quinolizidine; Izi, izidine; PTX, pumiliotoxin; aPTX, allopumiliotoxin; hPTX, homopumiliotoxin; Do-PTX, deoxyhomopumiliotoxin; Spiro, spiropyrrolizidine; DHQ, decahydroquinoline; TRI, tricyclic; Unclass, unclassified

[a] Mebs et al. (2007), [b] Daly et al. (2008), [c] Garraffo et al. (1993), [d] Mebs et al. (2005), [e] Daly et al. (2007), [f] Daly et al. (1984)

We prepared individual alkaloid fractions from methanol extracts of skin, skeletal muscle, liver, and oocytes for each specimen of *Melanophryniscus simplex*. For all samples from Gateados AC3 and Gateados AD2, we added 10 μg of nicotine ((-)-nicotine ≥99 %, Sigma-Aldrich, Milwaukee, Wisconsin) in a methanol solution (internal standard) and 50 μL of 1 N HCl to 1 mL of the original MeOH extract. We used the same procedure for the samples from Aratinga, except that we did not include the nicotine standard. We concentrated this combined MeOH extract with N_2 to 100 μL and then diluted it with 200 μL of water. We then extracted this solution 4 times, each time with 300 μL of hexane. We basified the HCl fraction with saturated $NaHCO_3$, followed by extraction 3 times, each time with 300 μL of ethyl acetate. We then dried the combined ethyl acetate fractions with anhydrous Na_2SO_4 and evaporated them to 100 μL.

We identified individual alkaloids by comparing the observed MS properties and GC retention times with those of previously reported anuran alkaloids (Daly et al. 2005). Anuran alkaloids have been assigned code names that consist of a bold-faced number corresponding to the nominal mass and a bold-faced letter to distinguish alkaloids of the same nominal mass (Daly et al. 2005). To determine the quantity of alkaloids in frog skins from Gateados AC3 and Gateados AD2, we compared the observed alkaloid peak area to the peak area of the nicotine internal standard, using a Varian MS Workstation v.6.9 SPI. We chose nicotine as an internal standard because its retention time is outside of the chromatographic range of most frog alkaloids.

Statistical analysis

We used non-metric multidimensional scaling to visualize patterns of alkaloid richness in *Melanophryniscus simplex* among the three localities. To assess the significance of the differences in alkaloid composition among localities we used a one-way analysis of similarity. For both analyses, we used Bray-Curtis dissimilarity matrices. We ran all multivariate statistical analyses using PRIMER-E version 6. Given the close proximity of Gateados AC3 and Gateados AD2, we report the total quantity of alkaloid per skin (mean ± standard error) and tested for differences between localities using a *t* test in SPSS version 17.0 for Mac (SPSS, Inc., Chicago, IL).

Results

GC–MS analyses of extracts from 11 individuals of *Melanophryniscus simplex* resulted in the detection of 47 alkaloids (including isomers), including 9 unclassified

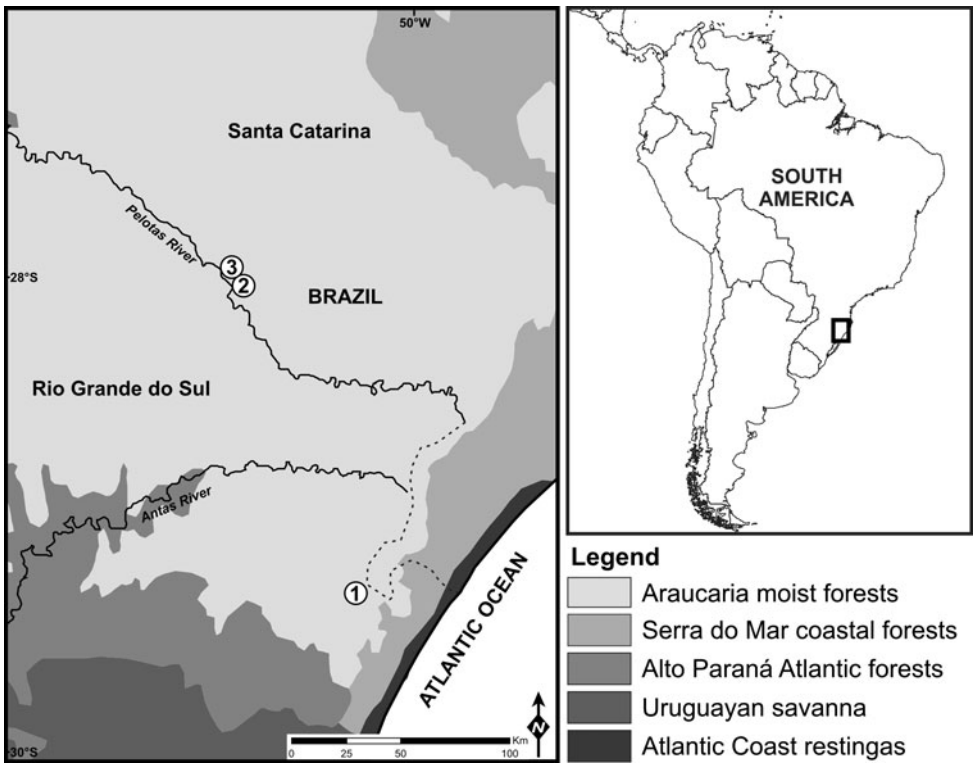

Fig. 2 Map of *Melanophryniscus simplex* collecting localities in southern Brazil. *1* Aratinga. *2* Gateados AD2. *3* Gateados AC3. Ecoregions follow Olson et al. (2001)

alkaloids and 38 alkaloids from 12 known structural classes (Table 2). Five alkaloids and two isomers are new (for chemical properties see Supplementary Information). The composition (type, number, and quantity) of alkaloids varied among individual frogs at each locality (see below); however, each alkaloid that was present in the skin of an individual was also present in that individual's skeletal muscle, liver, and, in one case, oocytes. Limitations in our ability to accurately measure tissue mass prevented us from quantifying the amount of alkaloids in muscle, liver, and oocyte samples; however, alkaloids are found in the same relative proportions in these tissues as in the skin (Fig. 3; Table 3).

The most abundant and widely distributed alkaloids detected in *Melanophryniscus simplex* were the pumiliotoxins (PTX) **251D**, **267C**, and **323A**, 5,8-disubstituted indolizidines (5,8-I) **207A** and **223D**, 5,6,8-trisubstituted indolizidine (5,6,8-I) **231B**, 3,5-disubstituted pyrrolizidines (3,5-P) *cis*-**223B** and *cis*- and *trans*-**251K**, and izidine **211C**. The PTXs, 5,8-Is, and 5,6,8-I all have branched carbon skeletons (Fig. 4) and are likely derived from a diet of oribatid mites, whereas the 3,5-Ps have unbranched carbon skeletons and are presumably obtained from dietary ants (Saporito et al. 2009, 2010). Although the chemical structure of izidine **211C** has not yet been determined, it was recently detected in an oribatid mite (Saporito et al. 2009, 2010).

Alkaloid richness differed significantly among the three study sites (Fig. 5; Global $R = 0.99$; $P \leq 0.001$). Alkaloid quantity per skin was more variable among specimens from Gateados AC3 (490–2524 μg, $\bar{x} = 1{,}577 \pm 591$ μg) than those from Gateados AD2 (200–492 μg, $\bar{x} = 374 \pm 89$ μg). Due to small number of samples and large variance of the Gateados AC3 sample, the difference in the mean quantity of alkaloid per skin at Gateados AC3 and Gateados AD2 was not significant ($t_4 = 2.01$; $P = 0.110$).

Discussion

Lipophilic alkaloids are anatomically widespread in *Melanophryniscus simplex*. Although the quantity of alkaloids appears to be greatest in skin, where defensive chemicals are secreted from granular glands, all of the alkaloids found in the skin of a given toad also occur in the same relative proportions in muscle, liver, and oocytes of that toad (Fig. 3; Table 3). Sequestered alkaloids are accumulated over the lifetime of an organism from multiple dietary sources that vary in both space and time. The amount of alkaloid in non-integumentary tissue and the fact that the alkaloids occur in the same relative proportions as in the skin suggests that they are not simply in the process of being transported to the granular glands but instead accumulate in those organs as well.

This is the first record of lipophilic alkaloids in organs other than the skin in amphibians. Few data have been published on the anatomical distribution of these defensive chemicals, so it is unclear if their occurrence in multiple

Table 2 Occurrence of alkaloids in skin extracts of *Melanophryniscus simplex* and their likely dietary arthropod source

Alkaloids	Dietary Source
Pumiliotoxins	
237A, **251D**, **265G**, **267C**, **277B**, **305I**, **323A**[a]	Mites
Homopumiliotoxins	
265N	Mites
5,8-Disubstituted indolizidines	
207A, **221A**[b], **209B**, **219L**, **223D**, **237D**, **239G**, **239G** [c], **267CC**, **271G**	Mites
5,6,8-Trisubstituted indolizidines	
197H, **223X**, **231B**, **235E**, **263A**	Mites
1,4-Disubstituted quinolizidines	
231A	Mites
3,5-Disubstituted indolizidines	
5Z,9Z-**223AB**, **223R**	Ants
3,5-Disubstituted pyrrolizidines	
cis-**223B**, *trans*-**223B**, **239Y**, *cis*-**251K**, *trans*-**251K**	Ants
4,6-Disubstituted quinolizidines	
275I	Ants
Piperidines	
223K, **225I**	Ants
Izidines	
211C	Ants
Spiropyrrolizidines	
236, **252B**	Millipedes/Mites
Tricyclics	
193C	Beetles/Mites
Unclassified	
223DD, **235S**, **235X**, **235FF**, **249P**, **253M**, **267Y**, **305J**, **307N**	Unknown[d]

Additional alkaloids present in trace amounts were detected, but data were not sufficient to assign a code number and letter. New alkaloids and new isomers of previously known alkaloids are underlined

[a] A dimethylsilanate derivative of **323A** (MW 379) generally accompanied the presence of PTX **323A**

[b] New diastereomer of a previously reported alkaloid 5,8-I **221A**; see Supplemental Information (Table 1) for chemical properties

[c] New diastereomer of a previously reported alkaloid 5,8-I **239G**; see Supplemental Information (Table 1) for chemical properties

[d] Some unclassified alkaloids have recently been identified in oribatid mites (Saporito et al. 2009)

organs is common or limited to a subset of lineages or species. Daly et al. (1994:660) reported that no alkaloids were detected in "muscle or internal organs" of wild-caught specimens of the dendrobatid *Dendrobates auratus* or in liver or muscle of captive specimens of the same species fed decahydroquinoline **195A** for 2 months; however, recent experiments indicate that at least some dendrobatids also possess detectable amounts of alkaloids in muscle and liver tissue (Saporito, Donnelly, Garraffo, Spande, pers. comm.). Similarly, tetrodotoxins (TTX) occur in skin, egg, ovary, muscle, liver, and blood in amphibians and other vertebrates (Noguchi and Arakawa 2008; Mebs et al. 2010), samandarine and samandarone occur in skin, liver, testes, and ovaries in the salamandrid *Salamandra salamandra terrestris* (Mebs and Pogoda 2005), and bufadienolides circulate in the plasma of the bufonid *Rhinella marina* (Flier et al. 1980). Sequestered batrachotoxins occur in the feathers, skin, heart, skeletal muscle, and liver of birds of the genus *Pitohui* (Dumbacher et al. 2009).

The functional significance of defensive chemicals in organs other than the skin is unclear. Williams et al. (2004) suggested that some populations of the non-venomous garter snake *Thamnophis sirtalis* might employ TTX accumulated in the liver following consumption of TTX-containing salamanders as an anti-predator mechanism. Also, the proliferation of defensive chemicals in other organs might help prevent or suppress infection by non-skin penetrating parasites, such as ingested platyhelminths and nematodes. The occurrence of alkaloids in oocytes raises the possibility that mothers might transmit defensive chemicals to offspring, as occurs in the salamander *Taricha granulosa.* In that species, the TTX in eggs appears to be maternally derived (Hanifin et al. 2003). However, although ovaries of *Salamandra salamandra terrestris* were found to contain samandarine alkaloids, intrauterine larvae lacked any trace of alkaloids (Mebs and Pogoda 2005). Mebs et al. (2007) found that post-ovipositional eggs and larvae of *Melanophryniscus devincenzii* lacked alkaloids, and further study is required to determine if

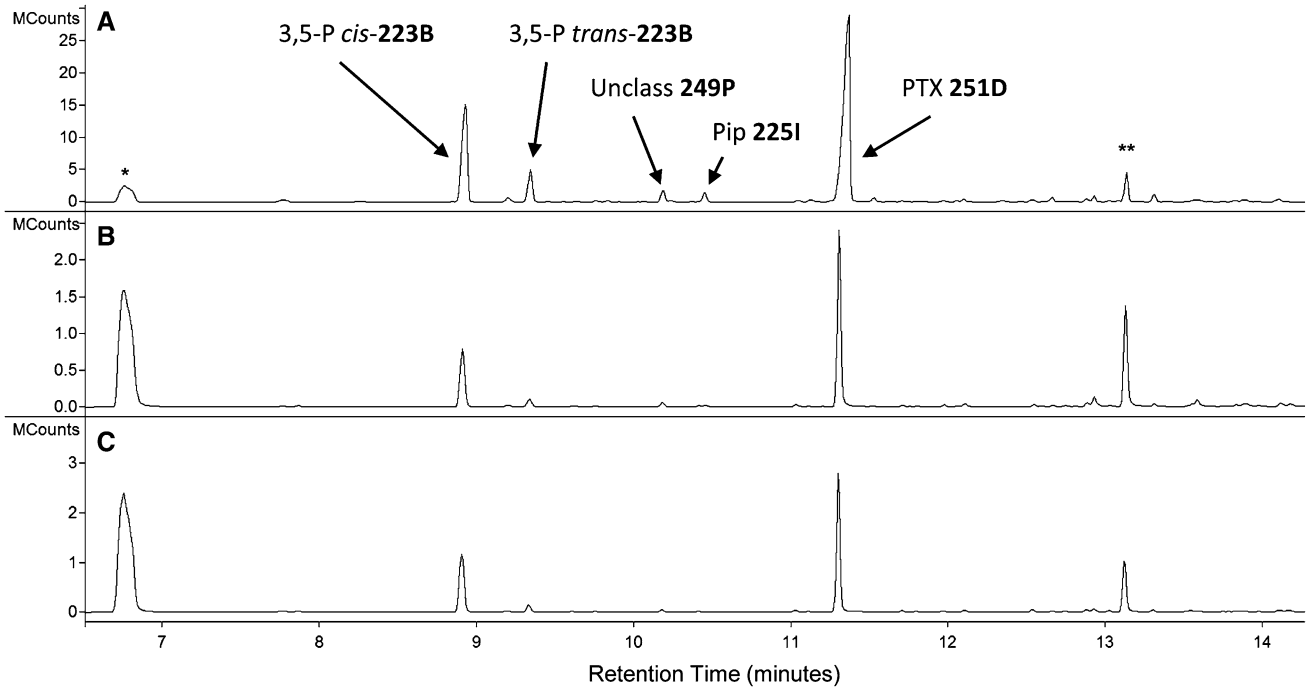

Fig. 3 Chromatograms illustrating the major alkaloids present in different organs of *Melanophryniscus* simplex from Gateados AD2 (individual 2; see Table 3). All alkaloids are present in each tissue type, and the relative proportion of alkaloids is similar among all tissue types. **a** Skin, **b** muscle, and **c** liver. The *asterisk* (*) indicates the nicotine standard. The *double asterisk* (**) indicates a peak that is not an alkaloid

post-ovipositional eggs and larvae of *M. simplex* possess alkaloids or if alkaloids are lost following oviposition.

The alkaloids found in *Melanophryniscus simplex* disrupt ion-channel activity or neurotransmitter-receptor binding in nerve and muscle cell (Daly et al. 1999). Although autotoxicity might be prevented or reduced by compartmentalizing toxins in dermal granular glands (Daly et al. 1980; Saporito et al. 2011), the occurrence of alkaloids in non-integumentary tissues suggests that physiological resistance evolved in these toads. Physiological resistance to batrachotoxin in the dendrobatid *Phyllobates terribilis* appears to be due to modification of the regulatory site controlling sodium-channel activation and permeability, thus preventing binding by batrachotoxin (Daly et al. 1980). Similarly, physiological resistance to TTX evolved multiple times in garter snakes (*Thamnophis*) through specific mutations in an important functional region of a TTX-sensitive sodium-channel gene ($tsNa_v1.4$) that alters the channel pore and significantly reduces TTX binding affinity (Geffeney et al. 2005; Geffeney and Ruben 2006; Feldman et al. 2009). It is likely that physiological resistance in *M. simplex* arose through similar modifications.

Previous studies of *Melanophryniscus* detected approximately 140 alkaloids (including isomers) in eight species based on extractions of multiple individuals and localities (Table 1). We detected 47 alkaloids in 11 specimens of *M. simplex* from three localities. Over half of these alkaloids are unknown in other bufonids, including seven novel alkaloids and isomers and 17 previously known alkaloids that had not been detected in *Melanophryniscus*. The new discoveries include the first record of 2,6-disubstituted piperidines (Pip) in *Melanophryniscus*, bringing the number of alkaloids classes in *Melanophryniscus* to 16. The two piperidines we detected, Pip **223K** and **225I**, were previously known only in Dendrobatidae (Daly et al. 2005) and are probably obtained from ants (Saporito et al. 2009, 2010). Among species of *Melanophryniscus*, a spiropyrrolizidine (Spiro) was previously known only in *M. stelzneri*; we detected the same alkaloid, Spiro **236**, and also Spiro **252B**, which was previously known only in *Mantella* from Madagascar and *Pseudophryne* from Australia (Daly et al. 2005). Spiro **236** is probably obtained from dietary mites, but it also occurs in certain millipedes (Saporito et al. 2009, 2010); Spiro **252B** has not been detected in any arthropods. Likewise, 1,4-quinolizidines (1,4-Q) were previously known only in *M. cupreuscapularis*, in which 1,4-Q **259E** was reported by Daly et al. (2008). We did not detect that alkaloid but instead identified the mite alkaloid (Saporito et al. 2009, 2010) 1,4-Q **231A**, previously known in dendrobatids and *Mantella* (Daly et al. 2005).

Our observation of variation in alkaloid composition among individuals and populations of *Melanophryniscus simplex* is consistent with previous findings for other

Table 3 Relative proportion of the five most abundant alkaloids in tissue types among individuals of *Melanophryniscus simplex* from Gateados AC3 and AD2

Individual	Major alkaloids	Location		
		Skin	Muscle	Liver
Gateados AC3				
1	PTX **251D**	0.41	0.41	0.38
	PTX **237A**	0.20	0.19	0.20
	3,5-P *trans*-**251K**	0.20	0.16	0.14
	3,5-P *cis*-**223B**	0.11	0.12	0.15
	3,5-I **223AB** (*5Z,9Z*)	0.07	0.10	0.14
2	PTX **251D**	0.37	0.32	0.33
	3,5-P *cis*-**223B**	0.28	0.34	0.33
	3,5-P *trans*-**251K**	0.22	0.18	0.17
	5,8-I **223D**	0.10	0.13	0.13
	5,8-I **207A**	0.03	0.04	0.05
3	PTX **251D**	0.64	0.63	0.74
	3,5-P *trans*-**251K**	0.20	0.27	0.12
	3,5-P *cis*-**223B**	0.10	0.06	0.07
	3,5-I **223AB** (*5Z,9Z*)	0.04	0.03	0.04
	5,8-I **207A**	0.02	0.01	0.02
Gateados AD2				
1	PTX **251D**	0.64	0.70	0.61
	Spiro **252B**	0.23	0.19	0.30
	Izi **211C**	0.06	0.07	0.07
	5,6,8-I **235E**	0.04	0.02	0.01
	3,5-P *trans*-**251K**	0.03	0.02	0.02
2	PTX **251D**	0.61	0.65	0.61
	3,5-P *cis*-**223B**	0.27	0.29	0.34
	3,5-P *trans*-**223B**	0.07	0.04	0.04
	Unclass **249P**	0.03	0.02	0.01
	Pip **225I**	0.02	0.01	0.00
3	3,5-P *cis*-**223B**	0.40	0.46	0.42
	PTX 251D	0.38	0.37	0.25
	3,5-P *trans*-**251K**	0.09	0.09	0.22
	3,5-P *cis*-**251K**	0.09	0.03	0.03
	5,6,8-I **197H**	0.04	0.06	0.07

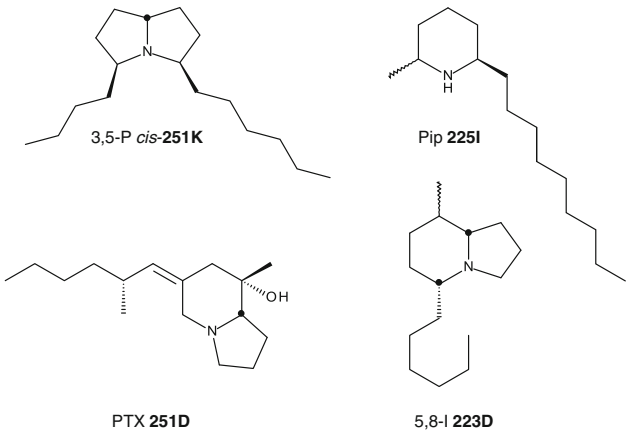

Fig. 4 Structures of four of the major alkaloids detected in *Melanophryniscus simplex*. The unbranched 3,5-disubstituted pyrrolizidine (3,5-P) *cis*-**251K** and piperidine (Pip) **225I** (the first record of this class detected in *Melanophryniscus*) are likely obtained from dietary ants, whereas the branched pumiliotoxin (PTX) **251D** and 5,8-disubstituted indolizidine (5,8-I) **223D** are probably obtained from dietary mites

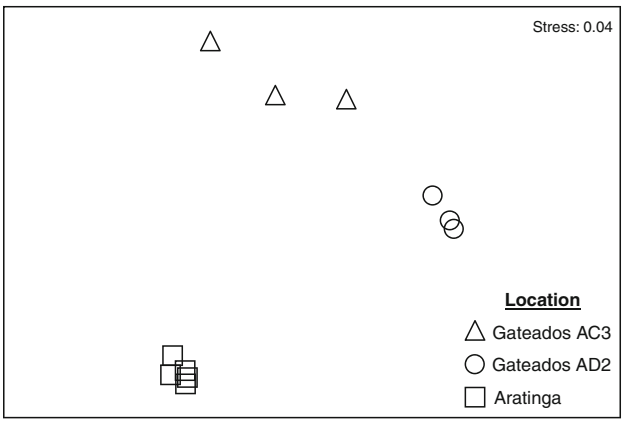

Fig. 5 Non-metric multidimensional scaling plot of alkaloid richness in *Melanophryniscus simplex* at three localities. Alkaloid richness is significantly different among locations (Global $R = 0.99$; $P \leq 0.001$). *Each symbol* represents an individual toad from one location. The distance between symbols corresponds to differences in alkaloid richness between frogs

alkaloid-sequestering anurans, including species of *Melanophyrniscus* (Mebs et al. 2005; Daly et al. 2007, 2008). The causes and consequences of alkaloid variation among species, populations, and individuals are not well understood. Differences among species are due, at least in part, to heritable differences in the ability to uptake different classes of alkaloids (Grant et al. 2006), although the extent that this holds true can be obscured by other causes of variation. The primary cause of intraspecific variation appears to be differences in the availability of alkaloid-containing arthropods and the kinds and amounts of alkaloids they contain (Saporito et al. 2009), which suggests that the three localities we studied differ markedly in their arthropod fauna. Alkaloid composition is known to vary ontogenetically (Myers et al. 1978; Daly et al. 2002) and sexually (Saporito et al. 2010), which could be due to intrinsic (e.g. developmental, genetic) differences but is more likely due to variation in habitat use and arthropod availability at finer scales than have been investigated to date.

We are unable to explain the difference in alkaloid quantity between specimens from Gateados AC3 and AD2 or the unexpectedly large variation in alkaloid quantity among specimens from Gateados AC3. Although not statistically significant, the quantities were markedly different, with the lowest quantity at Gateados AC3

(490 μg) being barely less than the greatest quantity of alkaloid at Gateados AD2 (492 μg). Moreover, the maximum quantity at Gateados AC3 (2,524 μg) was five times greater than the maximum quantity at AD3. Further data are required to understand the basis of variation in alkaloid composition in *Melanophryniscus simplex*.

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