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Sequestered and Synthesized Chemical Defenses in the Poison Frog *Melanophryniscus moreirae*

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Abstract Bufonid poison frogs of the genus *Melanophryniscus* contain alkaloid-based chemical defenses that are derived from a diet of alkaloid-containing arthropods. In addition to dietary alkaloids, bufadienolide-like compounds and indolealkylamines have been identified in certain species of *Melanophryniscus*. Our study reports, for the first time, the co-occurrence of large quantities of both alkaloids sequestered from the diet and an endogenously biosynthesized indolalkylamine in skin secretions from individual specimens of *Melanophryniscus moreirae* from Brazil. GC/MS analysis of 55 individuals of *M. moreirae* revealed 37 dietary alkaloids and the biosynthesized indolealkylamine bufotenine. On average, pumiliotoxin **267C**, bufotenine, and allopumilitoxin **323B** collectively represent ca. 90 % of the defensive chemicals present in an individual. The quantity of defensive chemicals differed between sexes, with males possessing significantly less dietary alkaloid and bufotenine than females. Most of the dietary alkaloids have structures with branched-chains, indicating they are likely derived from oribatid mites. The ratio of bufotenine:alkaloid quantity decreased with increasing quantities of dietary alkaloids, suggesting that *M. moreirae* might regulate bufotenine synthesis in relation to sequestration of dietary alkaloids.

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Introduction

Chemical defenses are widespread among amphibians and represent complex adaptations that protect against predators, microbes, and parasites (Conlon 2011a, b; Mina et al. 2015; Savitzky et al. 2012; Toledo and Jared 1995). Skin secretions of amphibians contain a broad diversity of defensive chemicals that include biogenic amines, peptides, proteins, bufadienolides, tetrodotoxins, and lipophilic alkaloids (Daly 1998; Daly et al. 2005; Saporito et al. 2012). Amphibians produce most of these defensive chemicals, but multiple lineages of poison frogs, including some bufonids (*Melanophryniscus*), dendrobatids (*Epipedobates*, *Ameerega*, and Dendrobatinae), mantellids (*Mantella*), and myobatrachids (*Pseudophryne*), sequester alkaloid defenses from dietary arthropods (Hantak et al. 2013; Saporito et al. 2009, 2012). It also is likely that the alkaloids present in certain species of Eleutherodactylidae (*Eleutherodactylus limbatus* group) from Cuba result from dietary sequestration (Rodríguez et al. 2010).

Although dietary alkaloids generally are considered the main defensive compounds in poison frogs, the presence of both sequestered and endogenously biosynthesized defensive chemicals has been reported in a few species. Myobatrachids (*Pseudophryne* spp.) sequester pumiliotoxin alkaloids (PTXs) from a natural diet of arthropods, and also synthesize unique pseudophrynamine alkaloids (Smith et al. 2002). Sequestration of large quantities of PTXs appears to inhibit the synthesis of pseudophrynamines, suggesting that *Pseudophryne* can regulate the production of their

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manufactured defenses in response to the availability of sequestered defenses (Smith et al. 2002). The biosynthesized indolealkylamine, 5-hydroxytryptamine, also has been reported in the skin of *Pseudophryne* (Erspamer 1994). Similarly, in addition to sequestered alkaloids, small amounts of the biosynthesized peptide carnosine and trace levels of bufadienolide-like compounds have been reported in certain dendrobatids (Daly et al. 1987, Table 1 and references therein). Small quantities of bufadienolide-like compounds also have been identified in skin extracts of certain bufonids in the genus *Melanophryniscus* (Daly et al. 2008; Flier et al. 1980; but see Mebs et al. 2007a), as well as a number of indolealkylamines, including 5-hydroxytryptamine, N-methyl 5-hydroxytryptamine, bufotenine, and dehydrobufotenine (Cei et al. 1968; Daly et al. 1987; Erspamer 1994; Maciel et al. 2003; Mebs et al. 2007a) and the phenol hydroquinone (Mebs et al. 2005, 2007b).

The Brazilian red belly toad *Melanophryniscus moreirae* has been reported to contain both sequestered alkaloids and the biosynthesized indolealkylamine bufotenine, albeit not in the same studies. Cei et al. (1968; see also Erspamer 1994) reported large amounts of bufotenine in the skin of 3,190 individuals of *M. moreriae*. Subsequently, Daly et al. (1984) reported PTX **267C** and allopumiliotoxin (aPTX) **323B** in the skin of 52 individuals, which likely were derived from a diet of oribatid mites (Saporito et al. 2007, 2009, 2011). The occurrence of both large quantities of biosynthesized indolealkylamines and dietary derived alkaloids in *M. moreirae* provides a rare opportunity to explore the relationship between synthesized and sequestered chemical defenses in a poison frog.

The main goals of the present study were to corroborate the co-occurrence of bufotenine and alkaloids in skins of *M. moreirae* from Serra da Mantiqueira, Brazil, and determine if a relationship exists between the quantities of these biosynthesized and sequestered chemical defenses (bufotenine and alkaloids, respectively). Dietary alkaloids are known to vary among individuals, between sexes, and over time (Saporito et al. 2012), so variation among individuals and between sexes in both the quantity of bufotenine and the composition (type, quantity, and number) of alkaloids was also examined in *M. moreirae*.

Methods and Materials

Sample Collection A total of 55 adult toads (40 males, 15 females) were collected in Itatiaia National Park (Serra da Mantiqueira, Rio de Janeiro, Brazil, GPS coordinates: 22°23′05.88″ S, 44°40′41.83″ W) on November 30, 2013. All toads were measured for snout-to-vent length (nearest 0.1 mm), sexed, and weighed (nearest 0.1 mg). The entire skin was removed from each individual toad. Skin samples were stored in individual 4 ml glass vials with Teflon-coated lids, containing 100 % methanol (hereafter, referred to as methanol extracts). Specimens are deposited in the amphibian collection of the Museu de Zoologia da Universidade de São Paulo under

Table 1 Alkaloids identified in *Melanophryniscus moreriae* arranged by structural class

Structural Class	5,8-I	5,6,8-I	PTX	aPTX	hPTX	Tri	Unclass
1	**225D**	**225L**	**251D**	**293K**	**281K**	**261J**	**237W**
2	**241K**(1)	**277E**(1)[a]	**253F**	**305A**(1)[f]		**265S**(1)[h]	**251GG**
3	**297G**	**279F**(3)[b]	**265D**	**323B**(2)[g]			**281R**
4		**281H**[c]	**267C**(2)[d]	**337D**			**283H**
5		**295G**	**295F**[e]				
6		**297H**	**323A**				

Alkaloids that are **underlined** represent new alkaloids that have not been previously described. The mass spectral data and retention times for each alkaloid are in Figure 1 of the *Supplemental Information*

The number of isomers detected for each alkaloid is indicated in parentheses. The retention times for these tentatively new isomers are in Table 1 of the *Supplemental Information*

[a] Two new isomers of 5,6,8-I **277E** were identified

[b] Two new isomers of 5,6,8-I **279F** were identified

[c] One new isomer of 5,6,8-I **281H** was identified

[d] One new isomer of PTX **267C** was identified

[e] One new isomer of PTX **295F** was identified

[f] One new isomer of aPTX **305A** was identified

[g] One new isomer of aPTX **323B** were identified

[h] One new isomer of Tri **265S** was identified

Abbreviations for alkaloid structural classes are as follows: 5,8-I (5,8-disubstituted indolizidine); 5,6,8-I (5,6,8-trisubstituted indolizidine); PTX (pumiliotoxin); aPTX (allopumiliotoxin); hPTX (homopumiliotoxin); Tri (tricyclic); Unclass (unclassified as to structure)

voucher numbers MZUSP 154089–154091, 154093–154106, 154109–154146.

Chemical Analyses Alkaloids and bufotenine were isolated from individual methanol extracts using an acid-base extraction (following Saporito et al. 2006). In brief, 10 μg of nicotine ((-)-nicotine≥99 %, Sigma-Aldrich) in a methanol solution (internal standard) and 50 μl of 1 N HCl were added to 1 ml of the original methanol extract. This combined methanol extract was concentrated with nitrogen gas to 100 μl and then diluted with 200 μl of deionized water. This solution was extracted four times, each time with 300 μl of hexane. The aqueous layer was then treated with saturated $NaHCO_3$, followed by extraction 3 times, each time with 300 μl of ethyl acetate. The combined ethyl acetate fractions were dried with anhydrous Na_2SO_4, evaporated to dryness, and then reconstituted with methanol to 100 μl.

Gas mass spectrometry/mass spectrometry (GC/MS) analysis was performed on a Varian Saturn 2100 T ion trap MS instrument coupled to a Varian 3900 GC with a 30 m 0.25 mm i.d. Varian Factor Four VF-5 ms fused silica column. GC separation was achieved by using a temperature program from 100 to 280 °C at a rate of 10 °C per min with helium as the carrier gas (1 ml/min). Alkaloid/bufotenine fractions were analyzed with both electron impact MS (EI-MS) and chemical ionization MS (CI-MS) with methanol as the CI reagent. Vapor phase Fourier-transform infrared spectral data (GC-FTIR) were obtained using an Hewlett-Packard model 5890 gas chromatograph, with an Agilent J&W DB-5 capillary column (30 m, 0.25 mm i.d., 0.25 μm), using the same temperature program as above, coupled with a model 5965B (IRD) narrow band (4000–750 cm^{-1}) infrared detector.

Individual alkaloids were identified by comparison of the observed MS properties (and FTIR properties for PTX **251D** and bufotenine), and GC retention times (R_t) with those of previously reported anuran alkaloids (Daly et al. 2005). Most 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). Identification of pumiliotoxin **251D** and bufotenine was based on comparisons to reference standards of each compound (PTX **251D**: Daly et al. 2003; **Bufotenine**: bufotenine solution, B-022, Cerilliant, Sigma-Aldrich). Isomers of previously characterized alkaloids were tentatively identified based on comparisons of EI mass spectral data and GC retention times. Following the methods of Garraffo et al. (2012), alkaloids were considered new isomers if they shared identical EI-MS data to a previously identified alkaloid, but differed in their R_t ±0.15 min from the R_t previously reported (Daly et al. 2005). The only exception to this was a tentative new isomer of aPTX **305A**, which differed by only 0.07 min from the previously identified aPTX (see Supplementary Information).

Individual frog skin extracts were analyzed in three chromatographic replicates, and the average quantity of defensive compounds was determined by comparing the observed alkaloid peak areas to the peak area of the nicotine internal standard, using a Varian MS Workstation v.6.9 SPI. It is noted, however, that our quantification using nicotine should be considered 'semi-quantitative'. The response of the MS detector is expected to differ for individual alkaloids, and ideally, a unique standard for each alkaloid should be used for absolute quantification; however, standards are not available for most alkaloids, especially new alkaloids, and, therefore, this was not possible.

Statistical Analysis Non-metric multidimensional scaling (nMDS) was used to visualize and compare alkaloid composition (number, type, and quantity of alkaloids), and a one-way analysis of similarity (ANOSIM) was used to test for statistical differences between males and females. nMDS and ANOSIM were based on Bray-Curtis similarity matrices, and these analyses were performed using PRIMER-E version 5. Differences in the quantity of sequestered alkaloids and manufactured bufotenine between males and females were examined using independent samples *t*-tests. The quantity of alkaloids and bufotenine were corrected for wet skin mass, and analyses for both the corrected and uncorrected quantities are reported. Linear regression was used to determine if the (1) quantity of alkaloids is related to the number of alkaloids, (2) quantity of alkaloids is related to the snout–vent length (SVL) and wet skin mass, and (3) number of alkaloids is related to the SVL and mass of males and females. Linear regression also was used to examine differences in the ratio of bufotenine:alkaloid quantity as a function of the total quantity of alkaloid per frog. In order to meet the assumptions of normality and homoscedasticity, these data were log_{10}-transformed. All parametric statistical analyses were performed using the statistical package R-3.0.1 (R R Core Team 2013). All error is reported as±1 S.E.

Results

GC/MS analysis of 55 individual *Melanophryniscus moreirae* skin extracts resulted in the detection of 37 dietary alkaloids (including isomers), representing seven different structural classes (Table 1). In addition, the samples contained the indolealkylamine bufotenine. We did not detect the phenol hydroquinone in any samples. Five of the dietary alkaloids present in our samples have been reported in other species of *Melanophryniscus*, including allopumiliotoxin (aPTX) **323B** and the pumiliotoxins (PTX) **251D**, **265D**, **267C**, and **323A** (Table 1). Eleven alkaloids are new and have not been detected previously in poison frogs. A number of tentative new isomers of previously characterized alkaloids were identified

(Table 1). The mass spectral data and retention times for all 11 new alkaloids are available in Figure 1 *Supplementary Information*, and the retention times for all of the new isomers are included in Table 1 *Supplementary Information*.

Overall, the three most abundant dietary alkaloids were PTX **267C** (average quantity in 55 samples=279±32 μg per skin), aPTX **323B** (37±6 μg per skin), and tricyclic (Tri) **265S** (16±2 μg per skin) (see Fig. 1 for alkaloid structures), representing 89 % of the total dietary alkaloid quantity and 71 % of the total mass of chemical defenses (dietary alkaloids+bufotenine) in *M. moreirae*. PTX **267C** was present in all individuals, whereas aPTX **323B** and TRI **265S** were present in all but two individuals. PTX **265D** was present in all but three individuals, and PTX **323A**, aPTX **337D**, and an isomer of PTX **267C** were present in ca. 70 % of individuals. aPTX **323B** (isomer), aPTX **305A**, and 5,8-disubstituted indolizidine (5,8-I) **297G** were present in ca. 30 % of all individuals examined. The remaining 28 dietary alkaloids were detected in only a few (1–15) individuals and in variable quantities (0.5–42 μg per skin).

The total number and quantity of dietary alkaloids differ among individual skin extracts. Individuals possessed 5–17 alkaloids (**mean**: 10±1 per skin) and 37–1382 μg of alkaloids (**mean**: 369±35 μg per skin). There is no linear relationship between the total number and quantity of dietary alkaloids among individuals ($F_{1,53}$=0.539, P=0.466). There is no linear relationship between the number of dietary alkaloids and wet skin mass or SVL (**skin mass**: $F_{1,53}$=0.003, P=0.958; **SVL**: $F_{1,53}$=0.071, P=0.791); however, there is a linear relationship between the quantity of dietary alkaloids and wet skin mass ($F_{1,53}$=17.41, P<0.001) and SVL ($F_{1,53}$=15.19, P<0.001).

Females are larger than males in SVL (t_{53}=10.05, P<0.001) and wet skin mass (t_{53}=8.56, P<0.001). Dietary alkaloid composition (Global R=0.098, P=0.071; Fig. 2) and the total number of dietary alkaloids did not differ significantly between males and females (t_{53}=0.491, P=0.625; **mean number males**: 10±1 per skin; **mean number females**: 10±1 per skin). Although females had significantly larger quantities of dietary alkaloids compared to males (t_{53}=4.28, P<0.001; **mean QTY males**: 289±32 μg per skin; **mean QTY females**: 582±72 μg per skin), when corrected for wet skin mass, the total quantity of dietary alkaloids did not differ between males and females (t_{53}=1.83, P=0.073; **mean QTY males**: 2±0.2 μg per skin wet mass; **mean QTY females**: 2±0.3 μg per skin wet mass).

Bufotenine was the second most abundant defensive compound in terms of quantity (average quantity in 55 samples=103±11 μg per skin), representing on average 22 % of the total mass of chemical defenses in *M. moreirae*. Bufotenine was detected in all individuals, albeit in variable quantities (4–395 μg per skin). Although females contained larger quantities of bufotenine compared to males (t_{53}=3.45, P<0.001; **mean QTY male**: 83±8 μg per skin; **mean QTY female**: 157±29 μg per skin), when corrected for wet skin mass, there is no statistical difference in bufotenine quantity between males and females (t_{53}=1.30, P=0.200; **mean QTY male**: 0.5±0.04 μg per skin wet mass; **QTY female**: 0.6±0.1 μg per skin wet mass). There is a positive linear relationship between bufotenine quantity and wet skin mass ($F_{1,53}$=15.98, P<0.001) and SVL ($F_{1,53}$=14.30, P<0.001). The ratio of bufotenine:alkaloid quantity decreased with increases in total alkaloid quantity ($F_{1,53}$=44.75, P<0.001, r^2=0.46; Fig. 3). When analyzed separately, the ratio of bufotenine:alkaloid quantity decreased with increases in total alkaloid quantity in males ($F_{1,38}$=49.00, P<0.001, r^2=0.56; Fig. 3), but there was no relationship in females ($F_{1,13}$=0.88, P=0.370; Fig. 3).

The rank order of defensive chemicals by quantity varied extensively among individuals, but the three most abundant chemicals were consistently PTX **267C**, bufotenine, and aPTX **323B** (Table 1 *Supplementary Information*). The most abundant chemical was PTX **267C** in 45 individuals (82 %), followed by bufotenine in 8 (14 %) and aPTX **323B** in 2 (4 %). When PTX **267C** was most abundant, it was 1.0–13.6 (3.5±0.4) times more abundant than the next most abundant chemical and comprised 38–79 % (61.2±0.02 %) of the total quantity of defensive chemicals; when bufotenine was most abundant, it was 1.1–4.5 (1.9±0.4) times more abundant and comprised 36–71 % (49.2±0.04 %) of the total quantity; and when aPTX **323B** was most abundant, it was 1.7–2.1 (1.9±0.2) times more abundant and comprised 34–43 % (38.0±0.04 %) of the total quantity. The second most abundant chemical was bufotenine in 39 individuals (71 %), followed by aPTX **323B** in 12 (22 %), and PTX **267C** in 4 (7 %). The third most abundant chemical was TRI **265S** in 22 individuals (40 %), followed by aPTX **323B** in 18 (33 %), PTX **267C** in 5 (9 %), bufotenine in 4 (7 %), PTX **265D** in 3 (5 %), and PTX **323A**, aPTX **323B**, and aPTX **337D** in a single individual (2 %) each.

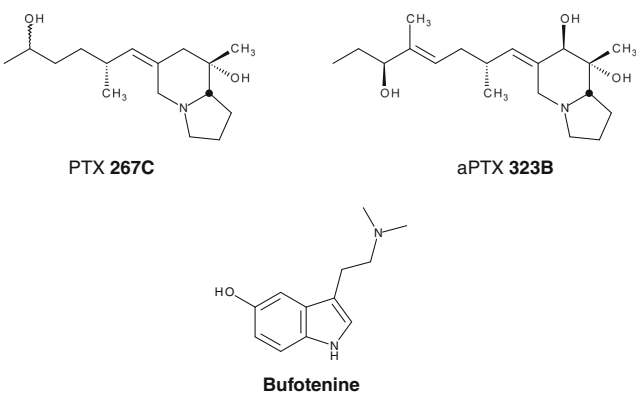

Fig. 1 Chemical structures of the three most abundant defensive chemicals in *Melanophryniscus moreirae*

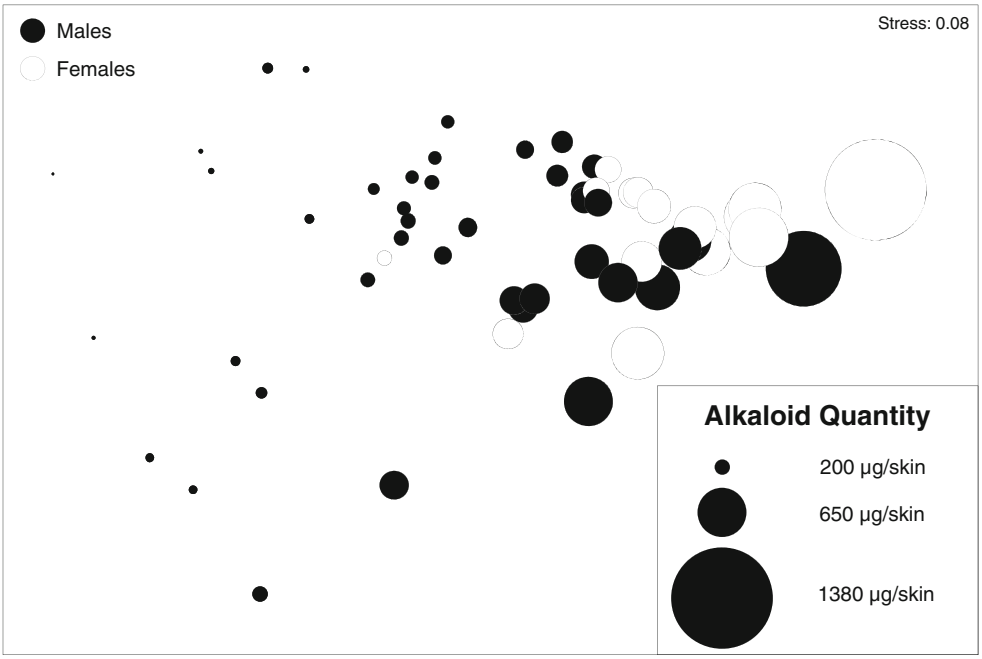

Fig. 2 nMDS plot of alkaloid composition between males and females of *Melanophryniscus moreirae. Each circle* represents an individual male or female frog, and the distance between symbols represents the difference in alkaloid composition. The diameter of each circle is proportional to the quantity of alkaloids present in that frog (µg per frog skin)

Discussion

Our study reports, for the first time, the co-occurrence of large quantities of both sequestered dietary alkaloids and the biosynthesized indolealkylamine bufotenine in the same skin secretions of *M. moreirae*. The defensive chemicals of nine of the 26 species of *Melanophryniscus* (Frost 2014) have been examined (including the present study), revealing approximately 200 dietary alkaloids organized into 15 different structural classes (Daly et al. 2007; Garraffo et al. 2012; Grant et al.

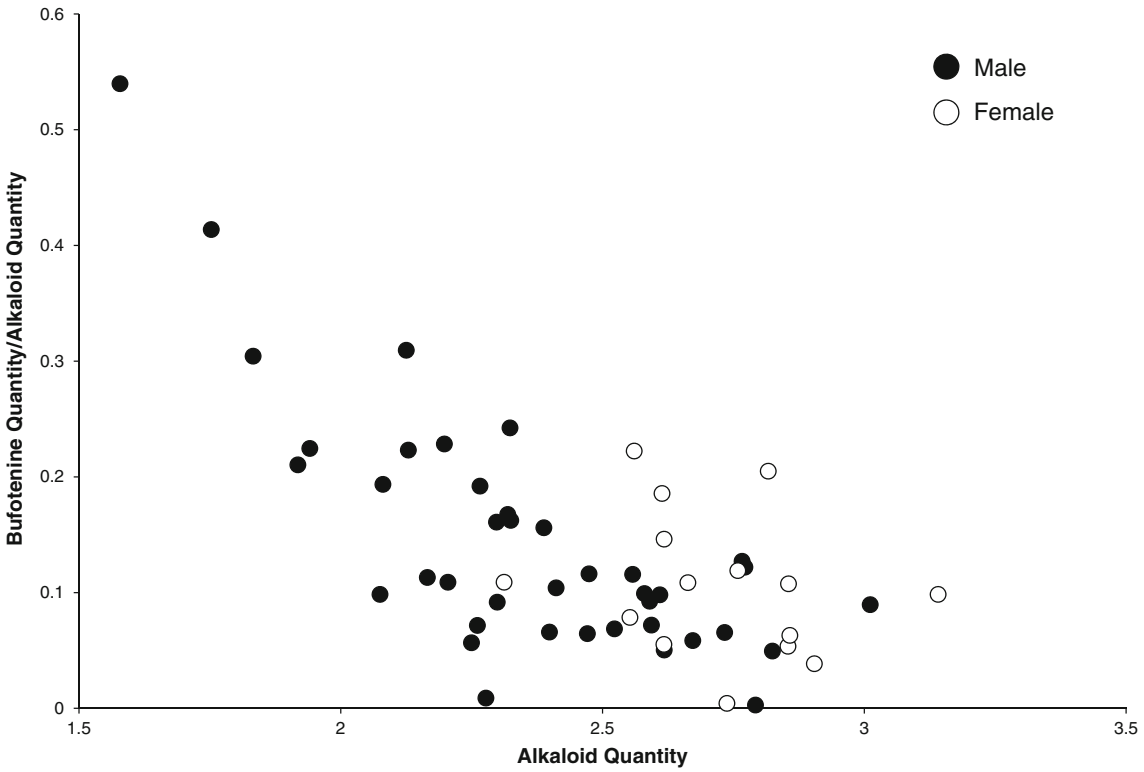

Fig. 3 Relationship between bufotenine quantity/alkaloid quantity (µg per frog skin) and the quantity of alkaloids (µg per frog skin) in males and females of *Melanophryniscus moreirae. Filled circles* indicate males and *open circles* indicate females. Graph axes are $\log_{10}$-scaled

2012). As in other poison frogs, the large variety of dietary alkaloids in bufonids appears to reflect a similar diversity of alkaloids in their arthropod prey (Saporito et al. 2009, 2012).

The three most abundant defensive chemicals in the skins of *M. moreirae* were pumiliotoxin (PTX) **267C**, bufotenine, and allopumiliotxon (aPTX) **323B** (Fig. 1), respectively, which collectively represent almost 90 % of the average quantity of defensive chemicals present in an individual toad. Bufotenine alone comprises>22 % of these chemicals, and ranks as one of the three most abundant chemicals in 93 % of toads, accounting for up to>70 % of the total quantity of defensive chemicals in individual toads. These findings suggest that bufotenine plays a prominent role in *M. moreirae* chemical defense. Some pumiliotoxins and allopumiliotoxins are known to be quite toxic, with LD50 values of ca. 2.5 mg/kg mouse (Daly et al. 2005). The specific activity of PTX **267C** is unknown, but it appears to be an effective defense against arthropods (Weldon et al. 2013). aPTX **323B** is a voltage-dependent sodium channel agonist, causing activation of sodium flux (Daly et al. 1990). Bufotenine is a toxic (LD50= 1.3 mg/kg mouse; Erspamer 1994), hallucinogenic (Ujváry 2014) indolealkylamine with antiviral activity (Vigerelli et al. 2014).

Pumiliotoxin and allopumiliotoxin alkaloids are derived from dietary mites (Saporito et al. 2007, 2009, 2011), and both PTX **267C** and aPTX **323B** are widespread among poison frogs, occurring in bufonids, dendrobatids, mantellids, and myobatrachids (Daly et al. 2005). In contrast, although the serotonin-derived bufotenine is synthesized by frogs and is widespread among amphibians (Daly et al. 1987; Erspamer 1994; Mebs et al. 2007a), it appears to be rare among poison frogs, in which it has been reported exclusively in *M. moreirae* (Cei et al. 1968; Erspamer 1994), *M. cambaraensis* (Maciel et al. 2003), and possibly *M. stelzneri* (small quantity tentatively reported by Cei et al. 1968, but not detected in subsequent studies; *e.g.*, Daly et al. 2007; Hantak et al. 2013).

Individuals of *M. moreirae* that had higher levels of alkaloids also contained more bufotenine, and variation in both bufotenine and the most abundant alkaloids was similar. Interestingly, however, the ratio of bufotenine:alkaloid quantity decreased with increasing quantities of dietary alkaloids (Fig. 3), suggesting that these toads might regulate bufotenine production in relation to the total quantity of sequestered dietary alkaloids. A similar possibility was suggested by Smith et al. (2002), who proposed that high levels of dietary pumiliotoxins in *Pseudophryne* spp. (myobatrachid poison frogs) might turn off biosynthesis of pseudophrynamine alkaloids. It also is possible that bufotenine production is merely correlated with, but is not regulated in response to, alkaloid uptake, and further investigation is required to test the causal relationship between acquired and biosynthesized chemical defenses in poison frogs, including *M. moreirae*.

The quantity of dietary alkaloids and bufotenine increased with skin mass and SVL. The greater quantity of dietary alkaloids in larger individuals is probably related to individual age. Anurans possess indeterminate growth, and among conspecifics, larger frogs usually are older than smaller frogs (*e.g.*, Monnet and Cherry 2002). Dietary alkaloids in poison frogs accumulate over an individual's lifetime, and, therefore, older individuals usually will have consumed more alkaloids than younger individuals. Although the relationship between age, size, and alkaloid quantity has not been tested among adults, in the dendrobatid *Oophaga pumilio*, larger tadpoles possess more alkaloids than younger, smaller ones and adults possess more alkaloids than juveniles (Stynoski et al. 2014). The increase in amount of bufotenine with toad size suggests that larger and presumably older individuals either produce or retain more of this indolealkylamine than smaller, younger individuals.

The composition of dietary alkaloids did not differ significantly between sexes. In contrast, the quantity of defensive chemicals did, with males possessing significantly less dietary alkaloid and bufotenine than females; however, females were larger than males, and size-corrected values were not significantly different. Previous studies have reported mixed findings on sex-related differences in dietary alkaloid defenses. The clearest evidence was found in the dendrobatid *Oophaga pumilio*, which exhibits differences in composition, in number, and quantity of alkaloids that are not attributable to differences in size (Saporito et al. 2010). Among species of *Melanophryniscus*, Daly et al. (2007) examined two breeding pairs of *M. stelzneri* and found that nine alkaloids present in trace amounts were restricted to one sex. Garraffo et al. (2012) did not detect sex-related differences in *M. rubriventris*, but the sample included only a single female. Overall alkaloid composition did not differ significantly between males and females of the mantellid poison frog *Mantella bernhardi*, although certain alkaloids were more common in one sex (Daly et al. 2008). Sex-related differences in quantity of bufotenine have not been studied previously.

In addition to PTX **267C** and aPTX **323B**, which occurred in all 55 individuals and comprised the principal dietary alkaloids, we detected 35 additional alkaloids (Table 1), almost all of which are branched chain compounds and likely are derived from oribatid mites (Saporito et al. 2007, 2011). There was no relationship between the number and quantity of alkaloids detected, which reflects the fact that chemical defenses of *M. moreirae* are dominated by a few alkaloids that are present in large quantities. In their study of 52 toads collected 35 years ago at the same locality, Daly et al. (1984) found only PTX **267C** and aPTX **323B**. The composition of dietary alkaloids is known to vary among frogs in the same population over time, presumably in relation to variation in arthropod availability (Daly et al. 2007; Saporito et al. 2006, 2007), which might explain our different results; however, it

also is likely that improvements in instrument sensitivity allowed us to detect alkaloids that were overlooked previously.

The causes and consequences of the extensive individual variation observed in the alkaloid composition of poison frog skin are poorly understood. Studies have shown that intraspecific alkaloid variation can be explained by frog sex and geographic and temporal variation in arthropod availability (reviewed by Saporito et al. 2012), but other potential causes, such as post-metamorphic age, fine-scale alkaloid abundance in different microhabitats, and genetic differences in alkaloid uptake capacity, remain to be studied. We found that the ratio of bufotenine:alkaloid quantity decreased with increasing quantities of dietary alkaloids, suggesting that *M. moreirae* might regulate bufotenine synthesis in relation to sequestration of dietary alkaloids. In order to understand the biological significance of our observations, it must be determined if a regulatory mechanism indeed exists or if variation in the ratio of bufotenine:alkaloid quantity is due to some other underlying cause (*e.g.*, individual age). The effectiveness of bufotenine and dietary alkaloids in defending against predators, parasites, and pathogens also must be evaluated.

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