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SHORT COMMUNICATION

Defensive alkaloid variation and palatability in sympatric poison frogs

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Abstract

Chemical defense in poison frogs derives from lipophilic alkaloids sequestered from dietary arthropods. Alkaloid composition varies extensively among individuals, populations, and species. Numerous causes of intraspecific variation have been identified, but the causes of interspecific variation are less clear, with both intrinsic (e.g., mechanism of sequestration) and extrinsic (e.g., arthropod availability) explanations being possible. Sympatric species afford a unique opportunity to investigate the causes and consequences of interspecific variation in natural populations, since they are potentially exposed to the same arthropod prey and predators. We used gas chromatography–mass spectrometry to identify alkaloids from 36 individuals of six species and three genera of dendrobatid poison frogs (*Adelphobates, Ameerega*, and *Ranitomeya*) collected in three Amazonian localities. We then compared alkaloid composition, richness, and quantity among sympatric species and analyzed the variation in alkaloid composition among con- and heterospecific populations at the two nearest localities. We also performed arthropod palatability experiments to investigate the biological significance of differences in alkaloids among sympatric species. Sympatric species differed in alkaloid composition, richness, and quantity, and conspecific individuals from different localities shared more alkaloids than heterospecific individuals from the same locality, strongly suggesting that variation is due to intrinsic causes. All analyzed alkaloid secretions were unpalatable, but palatability scores did not differ for most sympatric species, despite significant differences in alkaloid composition, richness, and quantity. Our results provide insights into the causes and consequences of interspecific variation in alkaloid profiles, but additional data are required to identify specific intrinsic causes and predator responses.

Keywords Allopatry · Chemical Defense · Dart-poison frogs · Dendrobatidae · Sympatry

Introduction

Chemically defended animals can acquire defensive compounds through several mechanisms, including endogenous biosynthesis and exogenous acquisition via symbiosis or dietary sequestration (Daly et al. 1994a, b; Mebs 2001; Saporito et al. 2009). Frogs employ a vast arsenal of defensive chemicals, most of which are endogenous, including peptides, proteins, steroid bufadienolides, biogenic amines, and volatile organic compounds (e.g., Daly 1995; Erspamer 1994; Mailho-Fontana et al. 2018; Gonzalez et al. 2021). However, frogs of five families, referred to as poison frogs, have independently evolved the ability to sequester lipophilic alkaloids from their arthropod diet (Rodríguez et al. 2010; Saporito et al. 2012).

One of the most salient features of poison frog chemical defense is the extensive variation in alkaloid composition among individuals, populations, and species. The causes and

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ecological consequences of this variation must be understood to identify the selective pressures driving the evolution of this chemical defense system. Most efforts to study the causes of alkaloid composition have focused on variation within species, with several variables being proposed to explain intraspecific variation, including arthropod availability (Daly et al. 1994b; McGugan et al. 2016; Moskowitz et al. 2022; Prates et al. 2019), geographic location (Saporito et al. 2006, 2007; Daly et al. 2007, 2008a; Sague et al. 2023), season and/or year (Saporito et al. 2006, 2007; Daly et al. 2007, 2008a; Basham et al. 2020), life stage (Daly et al. 2002; Brooks et al. 2023), individual age (Jeckel et al. 2015a), body size (Saporito et al. 2010; Stynoski et al. 2014; Jeckel et al. 2015a), and sex (Saporito et al. 2010).

Although the causes of intraspecific variation are far from fully understood, the causes of interspecific variation are even less clear. Presumably, intrinsic differences in sequestration ability or efficiency are responsible for much of the variation among species and clades. For example, Davison et al. (2021) found that *Dendrobates auratus* does not sequester 2,6-disubstituted piperidines, even though more than 20 alkaloids of that class have been detected in at least 15 other species of poison frogs (Daly et al. 1987, 1993, 2003, 2009; Edwards et al. 1988; Myers et al. 1995; Jeckel et al. 2019), and Waters et al. (2023) reported interspecific variation in the efficiency of epibatidine sequestration (see also Daly et al. 2003; Mebs et al. 2014). Little is known about the mechanism(s) of alkaloid sequestration, but Sánchez et al. (2019) reported a consistent pattern of up-regulation of genes related to muscle and mitochondrial processes in poison frogs following alkaloid consumption. Similarly, Caty et al. (2019) reported transcript and protein abundance patterns suggesting the involvement of small molecule transport proteins in alkaloid bioaccumulation, and Alvarez-Buylla et al. (2023) suggested a carrier plasma globulin could be responsible for alkaloid transport.

Additionally, spatial variation in the availability of arthropod sources also appears to be responsible for alkaloid variation among allopatric poison frogs. For example, although wild-caught *Oophaga lehmanni* differ from close relatives in lacking histrionicotoxins (Myers and Daly 1976), captive-reared individuals accumulate them when provided in the diet (Garraffo et al. 2001). Given that there are no apparent differences in prey selectivity between *O. lehmanni* and other species of *Oophaga* (Toft 1995), this finding suggests that histrionicotoxin-containing ants (Jones et al. 2012) are absent or rare within the distribution of *O. lehmanni* (Garraffo et al. 2001). Similarly, epibatidine is exceedingly rare in natural populations of poison frogs, being known from only two species of *Epipedobates* and one species of *Ameerega* (Spande et al. 1992; Daly et al. 2000, 2005; Tarvin et al. 2017). Nevertheless, species of *Dendrobates*, *Phyllobates*, and *Ranitomeya* also sequester epibatidine when administered orally (Waters et al. 2023), suggesting a geographically restricted distribution of epibatidine-containing arthropods rather than a phylogenetically restricted (and homoplastic) ability to sequester that alkaloid.

Unravelling the contributions of intrinsic (physiological alkaloid sequestration ability) and extrinsic (environmental alkaloid availability) causes is a major challenge to understanding interspecific variation in alkaloid composition. Controlled laboratory experiments that administer known quantities of specific alkaloids provide the most direct means of testing the ability to sequester alkaloids, but they entail captive breeding to produce alkaloid-free poison frogs and alkaloids to administer to the frogs. Given the cost and difficulty of maintaining and breeding poison frogs in captivity, as well as the fact that more than 1200 alkaloids representing 28 classes have been discovered in poison frogs (Hovey et al. 2018; Basham et al. 2020), only a few of which are available either commercially or from academic laboratories, it is not feasible to test them all experimentally.

Sympatric poison frog species afford an alternative means of distinguishing between intrinsic and extrinsic factors, given that sympatric frogs co-occur with the same arthropods. For example, Myers et al. (1995) compared sympatric *Oophaga granulifera* and *O. pumilio* from the Atlantic versant of Costa Rica and found that they differed in 42% of their alkaloids. Later, Mebs et al. (2014) compared *Phyllobates lugubris* and *P. vittatus* in Costa Rica with sympatric *Dendrobates auratus*, *O. granulifera*, and *O. pumilio* and found that the *Phyllobates* species contained only a small subset of the alkaloids present in the other species. Similarly, for mantellid species, Daly et al. (2008a) found that alkaloid composition differed between two sympatric species of Madagascan poison frogs (*Mantella baroni* and *M. madagascariensis*).

In addition to understanding the causes of alkaloid variation in poison frogs, the ecological consequences of this variation must also be understood to clarify its biological significance and identify the selective pressures driving the evolution of this system of chemical defense. To estimate their antipredator function, frog alkaloids have typically been injected into laboratory mice to determine minimum lethal doses (LD_{50}) or behavioral responses (e.g., Daly and Myers 1967; Darst et al. 2006; Maan and Cummings 2012). However, given that alkaloid defenses evolved in relation to naturally occurring predators, which do not include rodents, and alkaloids are externally contacted and ingested by potential predators, not injected into them, the relevance of those studies to understanding the ecology and evolution of poison frog chemical defense is questionable (Weldon 2017; Bolton et al. 2017; Saporito and Grant 2018; Lawrence et al. 2023).

Palatability assays provide an alternative method of testing the antipredator function of poison frog alkaloids. Given that birds are believed to be the main predator driving the evolution of visual aposematism in poison frogs, Lawrence et al.

(2019, 2023) performed alkaloid palatability assays with wild-caught Blue Tits (*Cyanistes caeruleus*) to investigate the link between amount and composition of skin alkaloids and predator response. They observed a significant aversive response, with different responses to the alkaloids from different populations of the poison frog *Dendrobates tinctorius*.

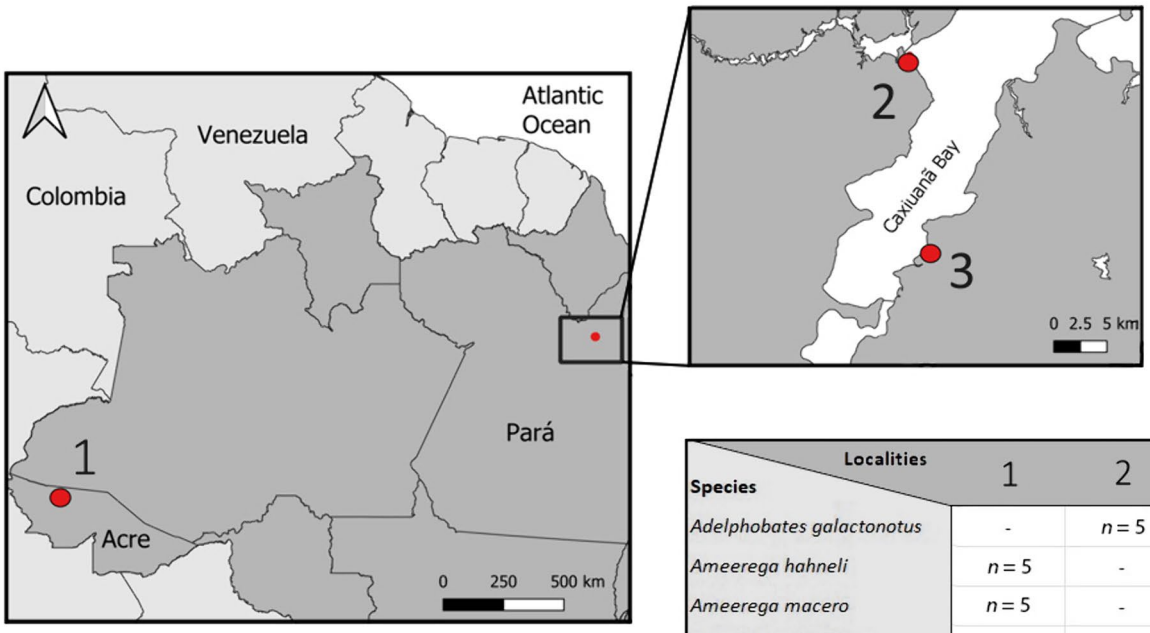
Arthropods are also known to predate frogs (including poison frogs; e.g., Nyffeler and Altig 2020), and arthropod predators that predominantly use chemoreception in prey detection are expected to be especially sensitive to variation in chemical defenses. Murray et al. (2016) found that the banana spider *Cupiennius coccineus* and bullet ant *Paraponera clavata*, both of which are natural frog predators, readily consumed the non-alkaloid containing rain frog *Craugastor bransfordii* but consistently rejected alkaloid-containing *Oophaga pumilio* following attack (see also Fritz et al. 1981; Szelistowski et al. 1985; Gray et al. 2010). Similarly, palatability assays have been used to test the antipredator function of individual poison frog secretions. Bolton et al. (2017) and Brooks et al. (2023) performed palatability assays using the Neotropical ant *Ectatomma ruidum*. Similarly, given that the fruit fly *Drosophila melanogaster* is commonly used as a model to study arthropod taste perception and specifically to understand arthropod perception of alkaloids, Bolton et al. (2017) and Jeckel et al. (2019) ran palatability assays using that species. To date, palatability assays have not been used to test the antipredator function of sympatric poison frogs.

In the present study, we tested whether alkaloid composition, richness, and quantity differ among sympatric dendrobatid poison frog species at three localities in two regions of the Amazon rainforest. We also analyzed the variation in alkaloid composition among con- and heterospecific populations at these localities. Finally, we tested how different alkaloid compositions would be perceived by arthropod predators by measuring the palatability of skin extracts from individual sympatric poison frogs.

Materials and methods

Sample collection

We collected poison frogs in two regions of the Brazilian Amazon rainforest (Fig. 1). In 2010, we collected five individuals each of *Ameerega hahneli, Am. macero, Am. trivittata*, and *Ranitomeya* cf. *cyanovittata* near Igarapé Esperança, within the protected area of Reserva Extrativista do Riozinho da Liberdade, municipality of Taruacá, Acre (Locality 1: 7°57′20.11″S, 72°4′35.41″W). Later, in January 2017, we collected individuals from two localities adjacent to Caxiuanã Bay, Pará. On the west side of the bay, inside the Caxiuanã National Forest protected area, municipality of Melgaço, we collected five individuals of *Adelphobates galactonotus* and three individuals of *R. amazonica*



Species \ Localities	1	2	3
Adelphobates galactonotus	-	*n* = 5	*n* = 5
Ameerega hahneli	*n* = 5	-	*n* = 3
Ameerega macero	*n* = 5	-	-
Ameerega trivittata	*n* = 5	-	-
Ranitomeya amazonica	-	*n* = 3	-
Ranitomeya cf. *cyanovittata*	*n* = 5	-	-

Fig. 1 Collection sites in the states of Acre and Pará, Brazil and the sample size of each species of poison frog

(Locality 2: 1°48′16.87″S, 51°26′45.31″W), and on the east side, in municipality of Portel, we collected five individuals of *Ad. galactonotus* and three individuals of *Am. hahneli* (Locality 3: 1°57′43″S, 51°25′09″W). All frogs were euthanized by either cooling followed by flash freezing in liquid nitrogen (Lillywhite et al. 2017) or pithing (McDiarmid 1994). Following euthanasia, whole skins were removed and stored in glass vials with Teflon-coated lids containing 1 mL 100% methanol. Data on *Ad. galactonotus* are from Jeckel et al. (2019).

Alkaloid extraction, identification, and quantification

We extracted alkaloids from skin samples using an acid–base extraction (Saporito et al. 2010). We added 100 µL of a nicotine solution (0.1 µg nicotine/µL methanol) to each of the 37 samples as an internal standard. Alkaloids were identified by comparing their retention times (Rt) via gas chromatography (GC) and mass spectral data with previously reported data on anuran alkaloids (Daly et al. 2005, 2007, 2008a, 2008b, 2009; Garraffo et al. 2012; Grant et al. 2012; Jeckel et al. 2015b; RAS unpubl. data). GC–MS analysis was performed on a Varian Saturn 2100 T ion trap MS coupled to Varian 3900GC with a 30 m 0.25 mm i.d Varian Factor Four VF-5 ms fused silica column. Gas chromatography was performed with a temperature program increasing from100 and 280 ℃ at a rate of 10 ℃ per minute, using helium as transport gas (1 mL/min). We considered as new isomers the alkaloids that shared the same mass spectral data with previously identified alkaloids but differed more than 0.15 min in Rt (Daly et al. 2005). We used a nicotine standard as a basis to quantify each alkaloid (Grant et al. 2012; Stynoski et al. 2014; Jeckel et al. 2015b, 2019; Crothers et al. 2016; Bolton et al. 2017; Basham et al. 2020; Alvarez-Buylla et al. Alvarez-Buylla et al. 2023; Davison et al. 2021). We analyzed samples in triplicate and used the average of the three measurements for statistical analysis. Prior to statistical analysis, alkaloid quantities (µg per frog skin) were standardized by dividing by wet frog skin mass (mg).

Palatability test

To test alkaloid palatability, we performed feeding trials with skin secretions from individuals of sympatric *Am. hahneli, Am. macero, Am. trivittata*, and *R.* cf. *cyanovittata* from Locality 1 using fruit flies (*Drosophila melanogaster*), which are commonly used as a model to study arthropod taste perception and specifically to assess alkaloid perception by arthropods (Devambez et al. 2013; Lee et al. 2015; Meunier et al. 2003; Sellier et al. 2011) and as a proxy for arthropod predators (Bolton et al. 2017; Jeckel et al. 2019). In this experiment, fruit flies were offered two sucrose solutions, one that contained alkaloid (treatment) and one that did not (control; Bolton et al. 2017). To distinguish between control and alkaloid-fed treatment inside the body of the fruit flies, we added blue and red food coloring to the treatment and control solutions, respectively, as previous studies have shown that *D. melanogaster* does not prefer either food coloring solution (Meunier et al. 2003; Sellier et al. 2011; Bolton et al. 2017).

Following Bolton et al. (2017), we made two stock solutions, one for the control solution and one for the treatment solution, each containing 20 mL of 20% sucrose/50% ethanol. For the control solution, we added 100 µL of red food coloring (Market Pantry®) to one stock solution. For the treatment solution, we added 50 µL of blue food coloring (Market Pantry®) to the other stock solution. A portion of the blue treatment solution was used to resuspend the naturally occurring alkaloids in each of the 20 frog skins, such that each treatment solution reflected the alkaloid defenses of an individual frog. In order to determine if alkaloid palatability in fruit flies is dose-dependent, we tested three concentrations: 0.625%, 1.25%, and 2.5% of the total alkaloid quantity in each individual skin. In total, we prepared four independent replicates assays for each one of the 20 skin samples, at each one of the three concentrations ($n = 12$ for each individual frog skin extract).

Each palatability assay used 10 *D. melanogaster* individuals that were 3–11 days old, grown on standard fruit fly media (Formula 4–24®Plain, Carolina Science), and starved for 24 h prior to the experiment. These fruit flies were then placed in a 9 cm Petri dish (Fisherbrand, 100 mm × 15 mm, sterile, Polystyrene) lined with filter paper dampened with deionized water and containing 10 µL of the control and treatment solution each on plastic cover slips (22 mm Fisherbrand® 2R Plastic Cover Slips). We allowed the fruit flies to feed for 2 h in the dark, at which time we euthanized them by freezing, following previous methods (Sellier et al. 2011; Devambez et al. 2013; Bolton et al. 2017; Jeckel et al. 2019). We then used a dissecting microscope to count the individuals with red, blue, or purple (mixed) abdomens. With these data, we calculated the palatability index for each assay, determined by: (blue fruit flies–red fruit flies–0.5 * purple fruit flies)/(total fruit flies). This index ranges from − 1 to + 1, with values equal to or greater than zero indicating palatable alkaloid solutions and negative values indicating unpalatable alkaloid solutions (Bolton et al. 2017).

Statistical analysis

All statistical analyses were performed in RStudio 2023.03.1 (RStudio Team 2023) using R 4.2.2 (R Core Team 2022). All plots were created using 'ggplot2' package (Wickham 2016). We used one-way analyses of similarity (ANOSIM) based on Bray–Curtis dissimilarity to compare alkaloid

composition among sympatric species and visualized variation using non-metric multidimensional scaling (nMDS) in 'vegan' package (Oksanen et al. 2022). Given the proximity of Localities 1 and 2, we also tested if the alkaloids detected on opposite sides of Caxiuanã Bay differed, and if frogs shared more or less alkaloids with conspecific frogs from different localities than they do with heterospecific frogs from the same locality. We created a matrix with individual frogs (rows) and the relative quantity of each identified alkaloid (columns; Supplementary material 1). Because our data are non-parametric, we used generalized linear models (GLMs) to compare alkaloid quantity (using gamma distribution because data are right skewed) and richness (using a negative binomial distribution because count data are over dispersed) among species and localities. The selected GLMs were used as input for post-hoc pairwise comparisons between sympatric species with Bonferroni correction using the 'emmeans' package (Lenth 2024).

We also used linear models to (1) test if alkaloids were palatable to fruit flies at each of the three concentrations and (2) compare differences in palatability among species. Alkaloids were considered palatable if palatability index scores were zero or greater, so palatability indices for all frogs were compared to a hypothesized mean of zero (Dyer et al. 2003; Bolton et al. 2017). Finally, we used GLMs to evaluate the relationship between alkaloid quantity, richness, and palatability. We reported statistical summaries as $\bar{x} \pm$ SE.

Results

Alkaloid composition

We identified 135 alkaloids, including isomers, representing 18 structural classes (Supplementary material 1). Histrionicotoxins (HTX), decahydroquinolines (DHQ), and 3,5-disubstituted indolizidines (3,5-I) were the only classes identified in all species, with HTXs and DHQs accounting for more than 50% of the total alkaloid quantity in all species (Fig. 2). The only alkaloids found in all species were HTX **239H**, HTX **259A**, DHQ *cis*-**243A**, and 3,5-I **223AB**. Epiquinamide (Epiqui), 4,6-disubstituted quinolizidine (4,6-Q) and pumiliotoxin (PTX) were exclusive to *Ad. galactonotus*, dehydro-5,8-disubstituted indolizidine (Dehydro-5,8-I) were exclusive to *Am. trivittata*, and *N*-methyl-decahydroquinoline was exclusive to *Am. macero*. Alkaloid richness and quantity were positively related ($\beta = 0.04$; $p = 0.05$). At Locality 1, *Am. trivittata* possessed the greatest alkaloid quantity (11.0 ± 1.0 µg/mg skin) and richness (37.8 ± 3.6 alkaloids/skin), followed by *Am. macero* (4.7 ± 1.6 µg/mg skin; 33.0 ± 4.3 alkaloids/skin), *R.* cf. *cyanovittata* (3.3 ± 0.8 µg/mg skin; 15.0 ± 1.9 alkaloids/skin), and *Am. hahneli* (1.6 ± 0.2 µg/mg skin; 13.2 ± 2.4 alkaloids/skin). At Locality 2, *Ad. galactonotus* had greater alkaloid quantity (2.3 ± 0.4 µg/mg skin) and richness (36.8 ± 4.3 alkaloids/skin) than *R. amazonica* (0.2 ± 0.03 µg/mg skin; 17.0 ± 2.1 alkaloids/skin). At Locality 3, *Ad. galactonotus* also had greater alkaloid quantity (2.2 ± 0.6 µg/mg skin) and richness (34.6 ± 2.8 alkaloids/skin) than *Am. hahneli* (0.4 ± 0.03 µg/mg skin; 24.7 ± 0.7 alkaloids/skin; Fig. 3).

Sympatry analyses

Our analyses revealed significant differences in alkaloid composition among sympatric species from Locality 1 ($R = 0.791$; $p = 0.001$), Locality 2 ($R = 0.949$; $p = 0.018$), and Locality 3 ($R = 1$; $p = 0.020$; Fig. 4). These differences were also observed in both alkaloid richness and quantity (Fig. 3; Supplementary material 2).

Samples from Localities 2 and 3 corroborated the conclusion by Jeckel et al. (2019) that alkaloid composition does not differ between frogs from opposite sides of the bay ($R = -0.0008$; $p = 0.370$), even though their analysis was restricted to *Ad. galactonotus* and ours also included *Am. hahneli* and *R. amazonica*. Among the 26 alkaloids reported by Jeckel et al. (2019) as unique to Locality 3, DHQ 5-*epi*-*trans*-**243A** was also found in one individual of *R. amazonica* at Locality 2. Likewise, among the 15 alkaloids reported by Jeckel et al. (2019) as unique to Locality 2, 5,6,8-I **249C** was also found in all but one specimen of *Am. hahneli* from Locality 3.

Despite the lack of significant differences in alkaloid composition on opposite sides of Caxiuanã Bay (Localities 2 and 3), we observed large differences in the number of alkaloids shared between pairs of species, with allopatric conspecific pairs possessing a significantly higher mean number of shared alkaloids ($\bar{x} = 11.5$) than sympatric heterospecific pairs ($\bar{x} = 6.9$; $p < 0.001$). At Locality 2, sympatric *Ad. galactonotus* and *R. amazonica* shared only 18 of 76 alkaloids (24%), and at Locality 3, sympatric *Ad. galactonotus* and *Am. hahneli* shared only 20 of 84 alkaloids (24%). In contrast, the two populations of *Ad. galactonotus* from opposite sides of Caxiuanã Bay (Localities 2 and 3) shared 46 of 89 alkaloids (52%; Jeckel et al. 2019).

Palatability

A dose response was present for alkaloid palatability across all three concentrations ($p < 0.0001$ for all comparisons), with the highest dose concentration being the least palatable and the lowest concentration the most palatable (Fig. 5).

Given that all alkaloid concentrations were unpalatable, we used the intermediate concentration of 1.25% for species comparisons. We found strong evidence for differences in palatability among species ($R^2 = 0.57$; $p < 0.001$), with pairwise

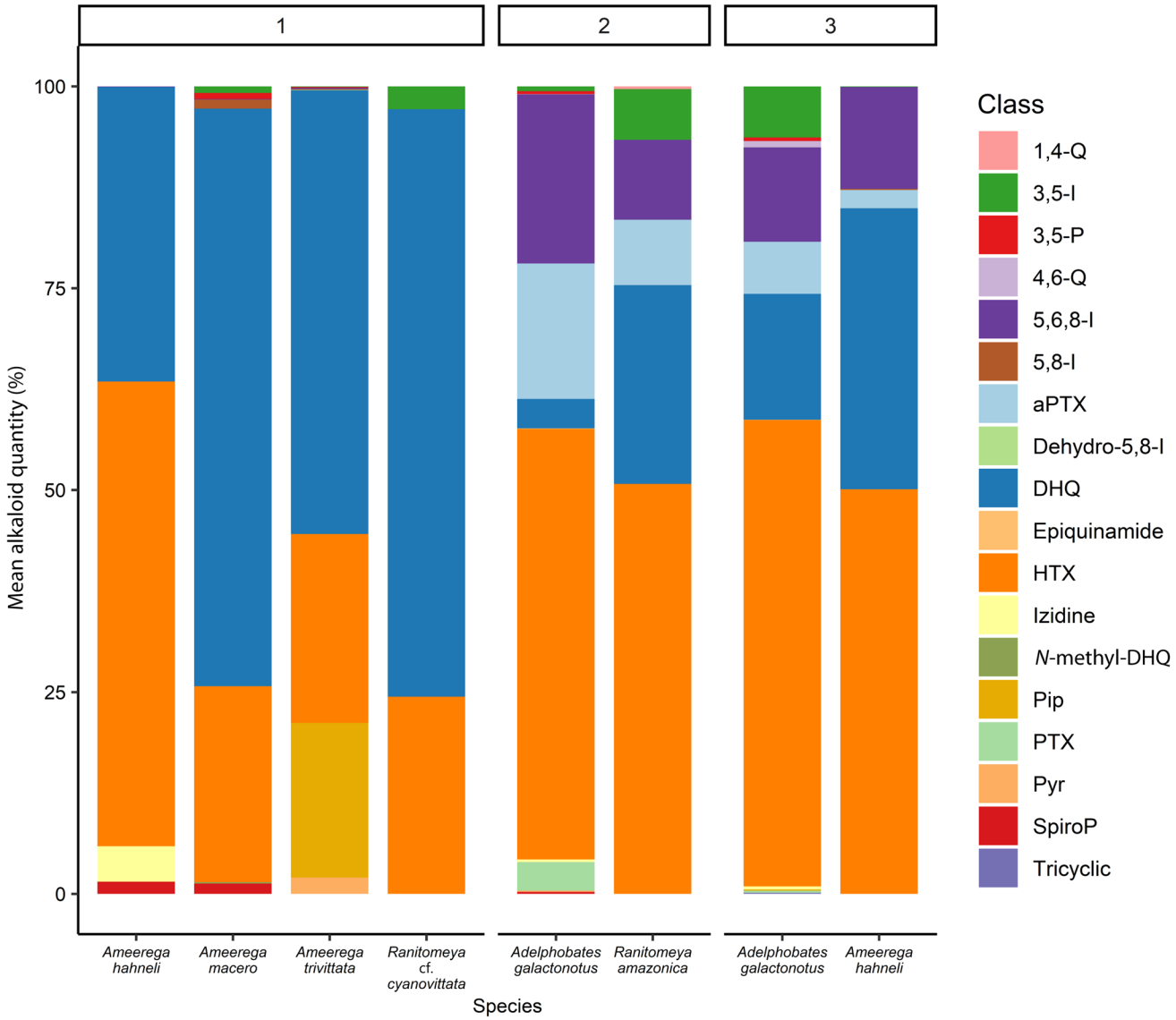


Fig. 2 Relative quantities of all alkaloid classes. Species are separated by locality (1, 2 and 3). Alkaloid class abbreviations: 1,4-disubstituted quinolizidine (1,4-Q); 3,5-disubstituted indolizidine (3,5-I); 3,5-disubstituted pyrrolizidine (3,5-P); 4,6-disubstituted quinolizidine (4,6-Q); 5,6,8-trisubstituted indolizidine (5,6,8-I); 5,8-disubstituted indolizidine (5,8-I); allopumiliotoxin (aPTX); dehydro-5,8-disubstituted indolizidine (dehydro-5,8-I); decahydroquinoline (DHQ); histrionicotoxin (HTX); *N*-methyl-decahydroquinoline (*N*-methyl-DHQ); piperidine (Pip); Pumiliotoxin (PTX); Pyrrolizidine (Pyr) and Spiropyrrolizidine (SpiroP)

comparisons indicating significant differences between *Am. hahneli* and *Am. trivittata* ($t = 4.57$, $p < 0.01$) and *A. trivittata* and *Ranitomeya* cf. *cyanovittata* ($t = -4.24$, $p < 0.01$); none of the other comparisons were significant (Supplementary material 3). Also, we observed a negative correlation between palatability and alkaloid richness ($R^2 = 0.53$; $p < 0.001$) and quantity ($R^2 = 0.51$; $p < 0.001$). For example, the species with the highest alkaloid quantity and richness, *Am. trivittata*, was also the least palatable (Palatability Index = -0.962 ± 0.121), and the species with the lowest quantity and richness, *Am. hahneli*, was the most palatable (Palatability Index = -0.338 ± 0.07).

Discussion

Early research on poison frogs assumed their alkaloid-based defenses were acquired through endogenous biosynthesis (for historical account, see Saporito et al. 2009). However, following the discovery that poison frogs sequester their defensive alkaloids from dietary arthropods (Daly et al. 1994a, b), evidence has mounted that the presence and quantity of a given alkaloid in a frog's skin depends on both the frog's intrinsic ability to sequester that alkaloid and the extrinsic availability of that alkaloid in dietary arthropods.

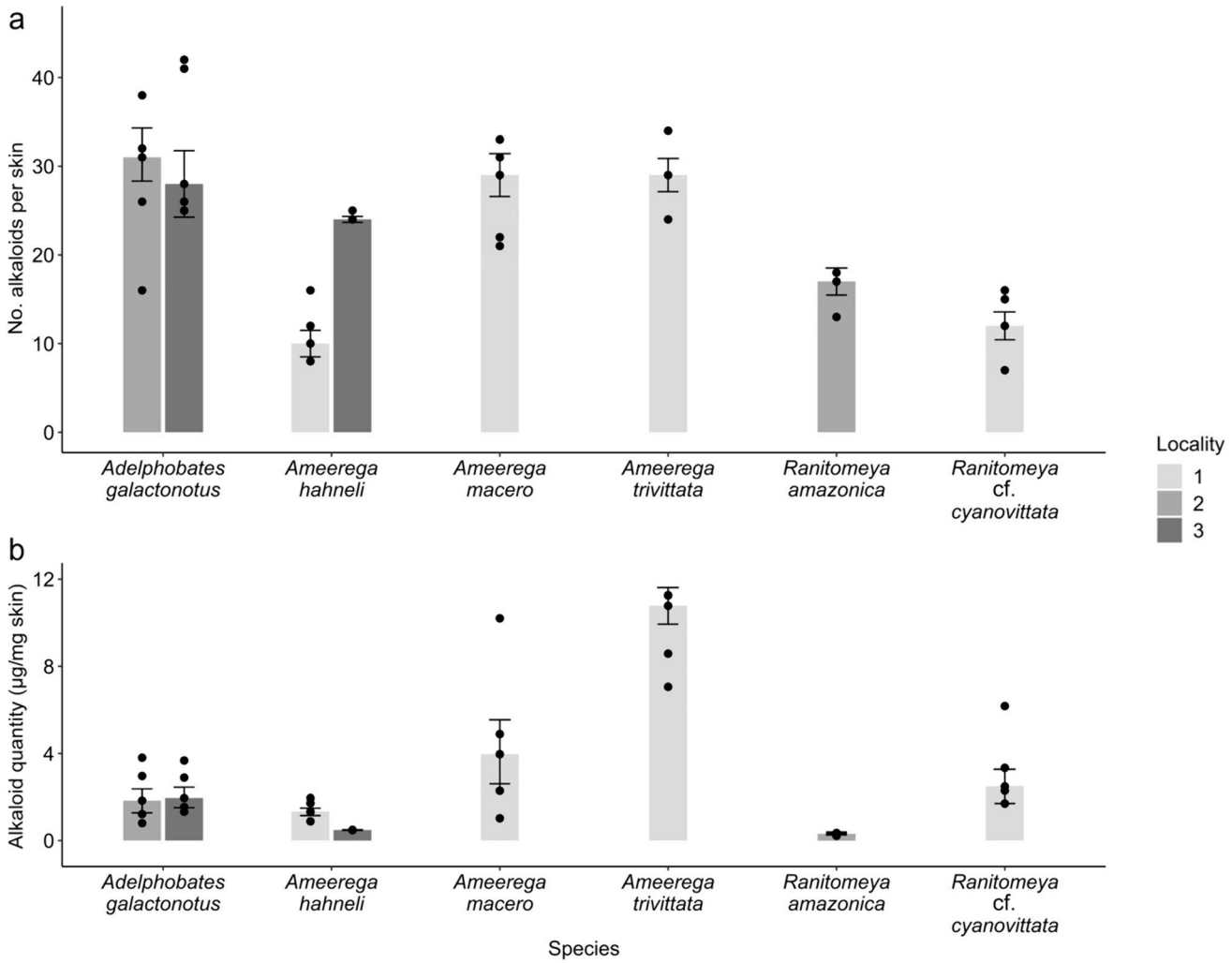


Fig. 3 Differences in **a** alkaloid richness (number of alkaloids per skin) and **b** relative alkaloid quantity among sympatric poison frogs. For results of statistical comparisons see Supplementary material 2

Teasing apart intrinsic and extrinsic causes is essential to explaining the high amount of variation among individuals, populations, and species but is especially challenging when studying natural populations due to the many variables that must be considered.

In an effort to reduce variables and isolate possible causes of alkaloid variation, most studies of naturally occurring poison frogs have focused on intraspecific variation. For example, Jeckel et al. (2015a) controlled for phylogenetic, spatial, and temporal variables by studying individuals of a single species collected at the same time and locality, finding that alkaloid richness depends on individual age—probably because older frogs have consumed a greater diversity of arthropod prey over their lifetime than younger frogs (i.e., an extrinsic cause)—whereas alkaloid quantity depends on individual size, with larger frogs having larger and more abundant poison glands for alkaloid storage (i.e., an intrinsic cause). Given that sympatric poison frog species co-occur with the same arthropod prey, studies of heterospecific poison frogs control for spatial variation by focusing on frogs from a single locality. Our finding that defensive alkaloids vary among sympatric Amazonian poison frog species is strongly suggestive of intrinsic causes. This conclusion is further strengthened by our finding that the alkaloid profiles of allopatric conspecific frogs are more similar to each other than those of sympatric heterospecific frogs and is consistent with previous studies of sympatric poison frogs in Central America (Myers et al. 1995; Mebs et al. 2014) and Madagascar (Daly et al. 2008a).

Although our results are suggestive of intrinsic causes, additional data are required to determine which specific intrinsic causes explain the observed variation. For example, the species we studied vary considerably in size, with *Am. trivittata* being both largest (37–55 mm snout–vent length [SVL]; Zapata-Hernández and Herrera-Lopera

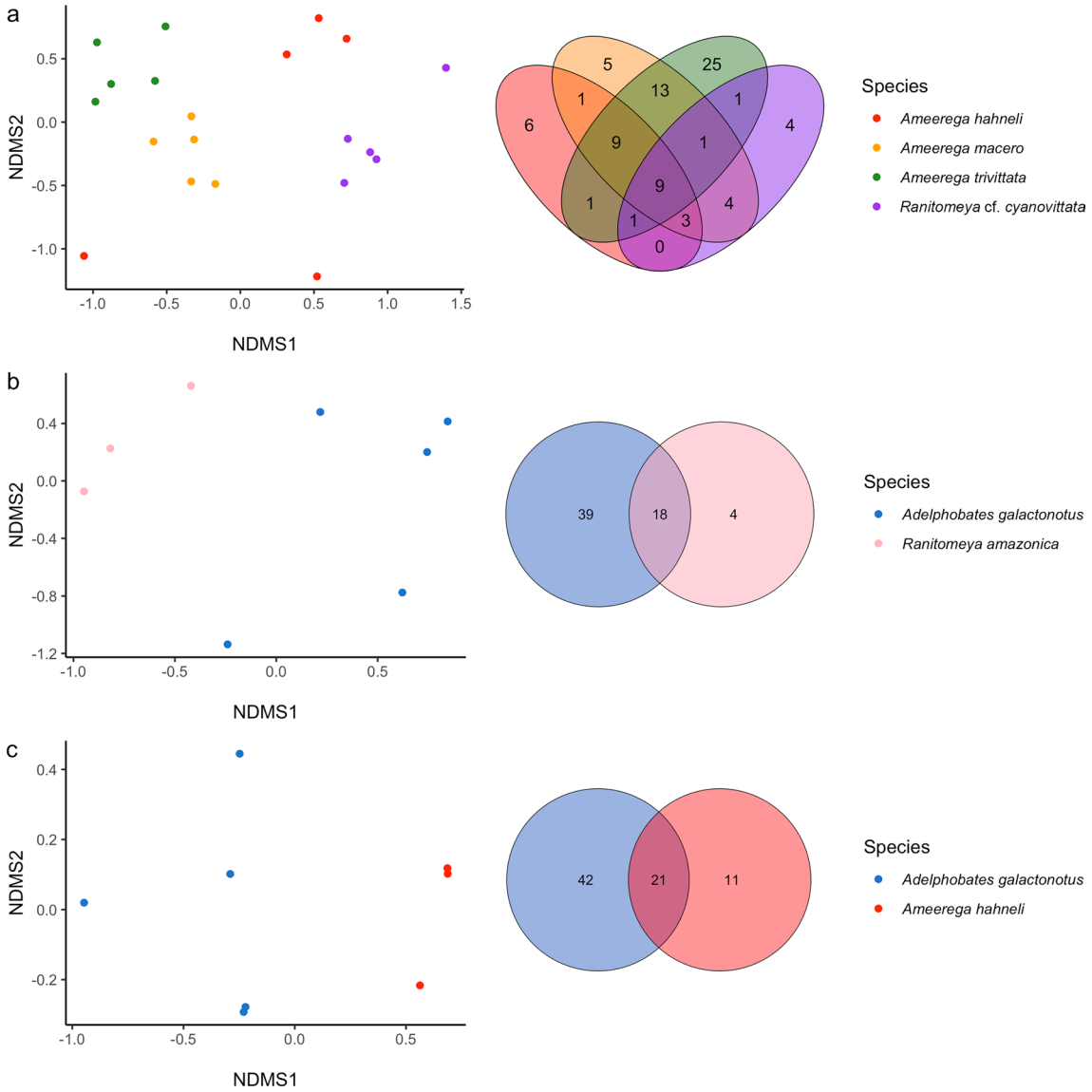


Fig. 4 nMDS plots using Bray–Curtis dissimilarity for alkaloid composition and Venn diagrams showing number of shared alkaloids among sympatric species at **a** Locality 1, **b** Locality 2, and **c** Locality 3

2021) and having the greatest alkaloid richness and quantity, whereas the two species of *Ranitomeya* were smallest (15–19 mm SVL; Perez-Peña et al. 2010; Brown et al. 2011) and had considerably lower alkaloid richness and quantity. Nevertheless, the relationship between species size and alkaloid richness and quantity is not straightforward. For example, the two species of *Ranitomeya* are approximately the same size but differed greatly in alkaloid richness and quantity, and *R.* cf. *cyanovitatta* possessed a greater quantity of alkaloid per mg skin than the much larger (30.5–42 mm SVL; Silverstone 1975; Hoogmoed et al. 2012) *Ad. galactonotus*. Clearly, larger samples and additional data (e.g., diet, age, size) are required to determine which intrinsic causes (e.g., variation in microhabitat, feeding behavior, physiological mechanism of sequestration) explain this variation.

All the skin secretions we tested were unpalatable, suggesting that they serve to deter arthropod predators. As found in previous studies (Bolton et al. 2017; Jeckel et al. 2019; but see Lawrence et al. 2019), secretions of greater alkaloid richness and quantity were least palatable. It is possible that species for which chemical defenses are less unpalatable rely more on mechanisms of predator avoidance than antipredator chemical defenses (Brodie et al. 1991). However, despite exhibiting different alkaloid profiles, the secretions of most of the sympatric poison frogs we tested did not differ in palatability. Variation in palatability is an adaptative trait if predators are able to

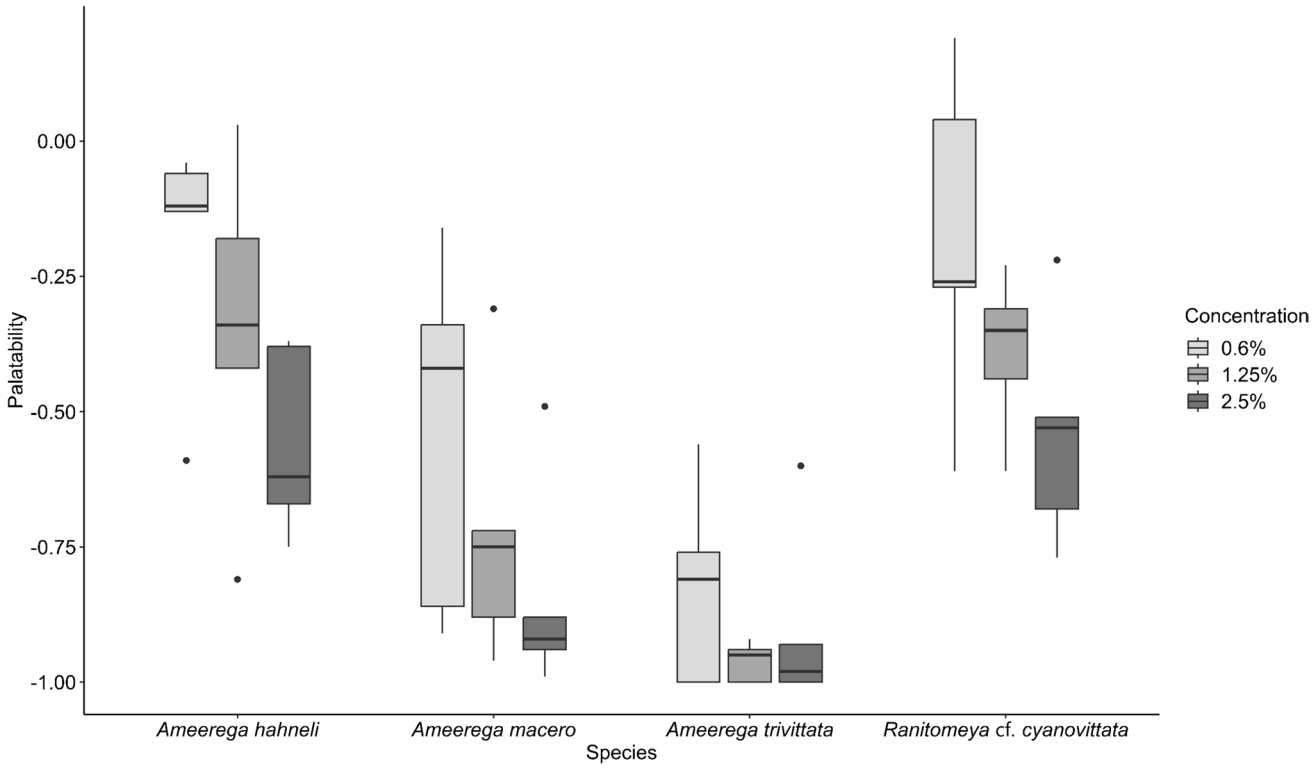


Fig. 5 Mean palatability scores at three alkaloid concentrations for each species collected at Locality 1. Values equal to or greater than zero are indicate palatability; negative values indicate unpalatability

distinguish and respond to such variation (Speed et al. 2012; Ottocento et al. 2023), so lack of differences in palatability could indicate a lack of selection via predation. Alternatively, it is likely that different predators respond differently to particular alkaloids, and it is possible that chemically oriented vertebrate predators (e.g., snakes) might perceive differences in palatability that arthropods do not. Most reports of poison frog predation are based on anecdotal observations (e.g., Summers 1999; Gray and Christy 2000; Alvarado et al. 2013; Lenger et al. 2014; Nyffeler and Altig 2020), and additional information is required about natural predators and their responses to specific alkaloids.

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Author contribution AMJ, JAP, RAS, and TG contributed to study conception and design. AMJ, PSB, and TG collected specimens. AMJ, JAP, and RAS performed chemical analyses. RAS and SK performed palatability tests. DYMN ad JAP performed statistical analyses. JAP wrote the first draft of the manuscript, which was then modified and approved by all authors.

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Data availability All data supporting the findings of this study are included within the paper and its Supplementary materials, including Supplementary material 1. Alklaloid richness and quantity and skin mass for the 36 individual poison frogs examined in the present study; Supplementary material 2. Differences in alkaloid richness and quantity among sympatric poison frogs, estimated with pairwise comparisons after Bonferroni correction using GLMs; and Supplementary material 3. Pairwise differences in palatability between sympatric poison frogs in Locality 1.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Permits Collection permits were issued by the Brazilian Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio 12178-2, 54640-1, 13173-2), and authorization to export samples from Brazil to

the USA was issued by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA 17BR025049/DF).

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