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ORIGINAL ARTICLE

Geographically separated orange and blue populations of the Amazonian poison frog *Adelphobates galactonotus* (Anura, Dendrobatidae) do not differ in alkaloid composition or palatability

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Abstract
As is typical of chemically defended animals, poison frogs present high variability in their alkaloid-based defenses. Previous studies have shown that geographically separated color morphs of *Oophaga* and *Dendrobates* species differ in both alkaloid composition and arthropod palatability. Here, we tested the generality of that finding by studying the alkaloid composition and palatability of geographically separated blue and orange morphs of the splash-backed poison frog, *Adelphobates galactonotus*. We identified and quantified the alkaloid composition of each individual frog using gas chromatography–mass spectrometry and evaluated the palatability of individual secretions to arthropods conducting feeding trials with *Drosophila melanogaster*. Despite their conspicuous differences in color and separation on opposite sides of a large aquatic barrier, the two morphs did not differ in alkaloid composition or palatability. This result shows that both color morphs are equally chemically protected and suggests that the color variation is not driven by predator selection.

Keywords Aposematism · Chemical defense · GC–MS · Polychromatism · Polytypism

Introduction

Poison frogs have evolved the ability to sequester defensive alkaloids from dietary arthropods (Saporito et al. 2009, 2012). As is typical of chemically defended animals (Speed et al. 2012), the alkaloid-based defenses of poison frog skin secretions are highly variable within species and even among individuals of the same population (e.g. Daly et al. 2008; Jeckel et al. 2015a). Alkaloid variation is related to multiple factors, including genetic or epigenetic differences in uptake (Daly et al. 2003; Hantak et al. 2013), availability of alkaloid-containing prey (Daly et al. 1994), size and abundance of granular glands (Saporito et al. 2010a), age (Jeckel et al. 2015b), sex (Saporito et al. 2010b), season (Saporito et al. 2006), habitat type (Andriamaharavo et al. 2010), and geographic location (e.g. Saporito et al. 2006, 2007a; Daly et al. 2008).

Alkaloid variation has also been associated with variation in skin coloration in poison frogs. Most poison frog species present gaudy, presumably aposematic coloration, and chromatic polytypism is common in this group (e.g. Silverstone 1975; Myers and Daly 1976; Brusa et al. 2013; Hoogmoed and Ávila-Pires 2012; Patrick and Sasa 2009; Noonan and Comeault 2008). Variation in alkaloid composition among polytypic populations has been studied most extensively in *Oophaga pumilio*, a dendrobatid poison frog distributed in lowland rainforests of the Caribbean slope in southern Nicaragua, Costa Rica, and northwestern Panama (Frost 2019). Throughout most of its range, populations of *O. pumilio* are similar in color, but the insular populations of the Bocas del Toro Archipelago, Panama are characterized by highly localized chromatic polytypism. Populations located on different islands differ wildly in coloration (Maan and Cummings 2012), alkaloid profiles (Saporito et al. 2006), toxicity (Daly and Myers 1967; Maan and Cummings 2012), and

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palatability (Bolton et al. 2017). Similar findings have also been reported for other polytypic species of *Oophaga*, such as *O. histrionica* (Myers and Daly 1976) and *O. granulifera* (Wang 2011).

Although *Oophaga* is the most well-studied polytypic genus of poison frog, chromatic polytypism also occurs in several other poison frog lineages and is especially common among the toothless dendrobatines (Dendrobatini; Grant et al. 2017) of the ADO clade, composed of *Adelphobates*, *Dendrobates*, and *Oophaga* (Grant 2019). Lawrence et al. (2019) recently found that two morphs of the chromatically polytypic species *D. tinctorius* differ in both alkaloid composition and palatability, thereby matching previous findings in *Oophaga*. However, no studies have examined polytypic species of *Adelphobates*. As such, to test the generality of findings in *Oophaga* and *Dendrobates* in this clade, we investigated the defensive alkaloids of two geographic color morphs of the splash-backed poison frog, *Adelphobates galactonotus*, a chromatically polytypic species distributed south of the Amazon River in Brazil (Hoogmoed and Ávila-Pires 2012). Specifically, to determine if geographically separated color morphs differ in skin alkaloid composition and palatability, we compared the alkaloid composition and arthropod palatability of secretions from the blue morph, known exclusively from the eastern side of Caxiuanã Bay, and the widespread orange morph, collected on the western side of the bay.

Materials and methods

Sample collection

We collected adult individuals of *A. galactonotus* in January 2017 in Pará state, Brazil (Fig. 1), including 5 (2 males, 3 females) of the orange morph collected on the western side of Caxiuanã Bay at a locality inside a protected area (Caxiaunã National Forest, 1°48′16.87″ S, 51°26′45.31″ W), and 5 (2 males, 3 females) of the blue morph collected on the eastern side of the bay, near riverside plantations (1°57′43″ S, 51°25′09″ W). We based our sample size on the results of previous studies that analyzed differences in alkaloid composition among populations (Saporito et al. 2006, 2007a; Andriamaharavo et al. 2010; Grant et al. 2012). To avoid interference of anesthetics commonly used to euthanize amphibians (Saporito and Grant 2018), we euthanized frogs

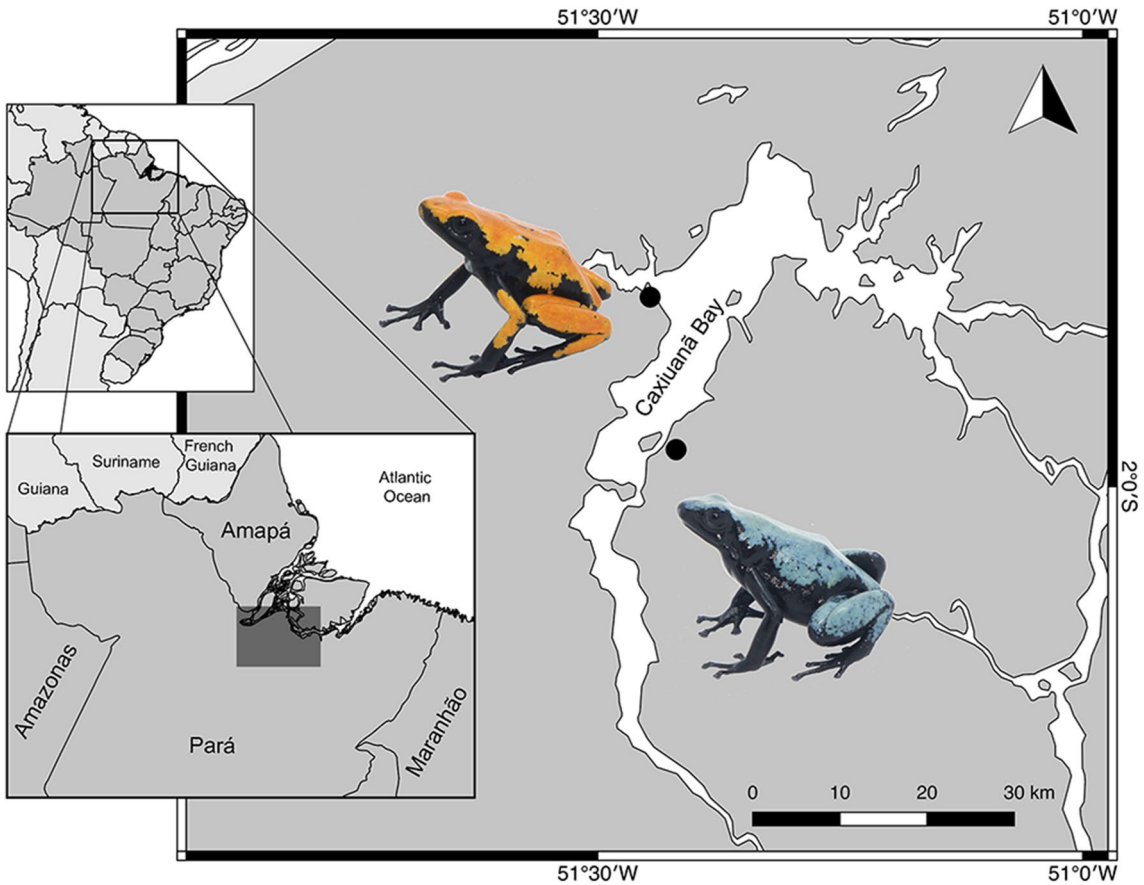

Fig. 1 Map of *Adelphobates galactonotus* collection localities around Caxiuanã Bay, in Pará, Brazil

by cooling followed by flash freezing in liquid nitrogen (e.g. Lillywhite et al. 2017). Following euthanasia, we removed and weighed the entire skin to 0.1 mg and examined gonads to confirm sex and maturity. We stored skins in individual 4-mL glass vials containing 100% methanol and sealed with Teflon-coated lids and deposited specimens in the amphibian collection of the Museum of Zoology of the University of São Paulo under voucher numbers MZUSP A158924–33.

Alkaloid extract preparation

We isolated alkaloids from individual methanol extracts using an acid–base extraction following Saporito et al. (2010b) and Jeckel et al. (2015a). For each individual frog skin, we performed two extractions: one for alkaloid analysis and another for palatability assays. For the extractions used in alkaloid analyses, we added 100 µL of nicotine (10 µg nicotine/100 µL methanol) as an internal standard and resuspended the alkaloids in 100 µL of 100% methanol. For the extractions used in palatability assays, we resuspended the alkaloids in 100 µL of 20% sucrose/50% ethanol solution without adding nicotine.

Alkaloid identification and quantification

We identified alkaloids by comparing the observed mass spectrometry (MS) properties and gas-chromatography (GC) retention times (Rt) with those of previously reported anuran alkaloids (e.g. Daly et al. 2005). Most anuran alkaloids have been assigned code names that consist of a bold-face number corresponding to the nominal mass and a bold-face letter to distinguish alkaloids of the same nominal mass (Daly et al. 2005). We tentatively identified isomers of previously characterized alkaloids on the basis of their electron impact (EI) and chemical ionization (CI) mass spectral data and GC retention times. Following the methods of Garraffo et al. (2012), we considered alkaloids to be new isomers if they shared identical EI–MS data with a previously identified alkaloid but differed in Rt by at least 0.15 min (Daly et al. 2005). We analyzed each individual frog skin extract in three chromatographic replicates and determined the average quantity of defensive compounds by comparing the observed alkaloid peak areas to the peak area of the nicotine internal standard, using Varian MS Workstation v.6.9 SPI.

Palatability test

In addition to visually oriented vertebrate predators, chemically oriented arthropods also predate poison frogs (Fritz et al. 1981; Szelistowski 1985; Gray et al. 2010; Santos and Cannatella 2011; Stynoski et al. 2014a, b; Murray et al. 2016). *Drosophila melanogaster* is commonly used as a model to study arthropod taste perception and specifically to understand alkaloid perception by arthropods (Devambez et al. 2013; Lee et al. 2015; Meunier et al. 2003; Sellier et al. 2011), making it a suitable proxy to assess how arthropod predators might perceive variation in alkaloid defenses (Bolton et al. 2017).

To evaluate the palatability of *A. galactonotus* secretions to arthropods, we conducted feeding trials in which common fruit flies (*Drosophila melanogaster*) were allowed the option to feed on two different sucrose solutions (Bolton et al. 2017). In this assay, we added red food coloring to the control solutions (sucrose without alkaloids) and blue food coloring to the treatment solutions (sucrose with alkaloids) to distinguish between feeding preferences during trials. Previous studies have used *D. melanogaster* in multiple choice feeding trials and have demonstrated that fruit flies show no preference for different colored solutions (Meunier et al. 2003; Sellier et al. 2011; Bolton et al. 2017). Fruit fly abdomens are transparent, which enabled us to determine which colored solution they fed on or if they consumed a mixture of both colored solutions.

Following the procedures of Bolton et al. (2017), we made two stock solutions for use in the palatability assays, one for the control solution (no alkaloids) and one for the treatment solution (alkaloids). Each stock solution contained 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 alkaloid treatment solution, we added 50 µL of blue food coloring (Market Pantry®) to the other stock solution. We ran separate experiments for each of the 10 frog skins so that each treatment solution reflected an individual frog's naturally occurring alkaloid defenses. To determine if alkaloid palatability is dose-dependent, we tested three alkaloid concentrations for each individual frog, comprising, respectively, 2.5%, 1.25%, and 0.625% of the total quantity of the alkaloids present in each individual frog skin samples.

Each fruit fly palatability assay used 10 individual *D. melanogaster* (wingless, wild type, Carolina Science) that were 3–11 days old (average 5 days), grown on standard fruit fly media (Formula 4–24® Plain, Carolina Science), and starved for 24 h prior to the experiment. We placed these 10 fruit flies in a 9-cm Petri dish (Fisherbrand, 100 mm × 15 mm, sterile, Polystyrene) lined with filter paper dampened with deionized water (to provide moisture for the fruit flies) and containing 10 µL each of the control and treatment solutions on plastic cover slips (22 mm Fisherbrand® 2R Plastic Cover Slips). Following the methods of previous studies (Sellier et al. 2011; Devambez et al. 2013; Bolton et al. 2017), we allowed the fruit flies to feed on the solutions for 2 h in the dark, and then euthanized them by freezing.

To quantify feeding preference, we used a dissecting microscope to examine the fruit flies and counted the individuals with red, blue, and purple (mixed) solutions in their

abdomens. From this count, we calculated a palatability index for each assay to determine the relative palatability of each alkaloid solution. The palatability index is a value that ranges from − 1 to + 1, where zero and positive values represent a palatable alkaloid solution and negative values indicate an unpalatable alkaloid solution relative to the control (Bolton et al. 2017). This index was calculated as followed: (# of blue fruit flies−# of red fruit flies − 0.5 * # of purple fruit flies)/(total # of fruit flies). We included each alkaloid extract from an individual frog in four independent replicate assays at each of the three concentrations ($n = 12$ for each individual frog skin extract).

Statistical analysis

We used non-metric multidimensional scaling (nMDS) to visualize and compare alkaloid composition (richness, type, and quantity of alkaloids) and one-way analysis of similarity (ANOSIM) to test for differences. Both nMDS and ANOSIM analyses were based on Bray–Curtis similarity matrices. We tested for differences in the quantity and richness of sequestered alkaloids between color morphs, sizes (SVL and mass), and sexes using Wilcoxon rank sum tests, and examined the relationship between alkaloid quantity and richness using linear regression. To test if frog alkaloids were considered unpalatable to fruit flies at each of the three concentrations, we performed one-tailed independent samples *t*-tests. Palatability index scores of zero or greater are considered palatable, and therefore average palatability indices for frogs were compared to a hypothesized mean of zero (Dyer et al. 2003; Bolton et al. 2017). To determine if there was a dose response in palatability among alkaloid concentrations, we used linear regression. To test for differences in alkaloid palatability, we performed an independent samples *t*-test. To examine the relationship between palatability and alkaloid quantity and alkaloid richness, we used linear regression. nMDS and ANOSIM were performed in PRIMER-E version 6, comparisons of alkaloid composition between morphs and sexes were performed using the statistical package R-3.6.0 (R Core Team 2019), and statistical analyses for the palatability assays were conducted using GraphPad Prism Software version 8.0.0 for Windows.

Results

Alkaloid composition

Alkaloid composition did not differ significantly between the two color morphs of *A. galactonotus* (alkaloid quantity [$W = 15$, $p = 0.69$], skin mass corrected alkaloid quantity [$W = 14$, $p = 0.84$], richness [$W = 14$, $p = 0.84$], and total composition analysis [Global $R = 0.12$, $p = 0.198$]; Fig. 2a–d). The total number and quantity of dietary alkaloids varied among individual skin extracts, including among individuals of the same population (Table 1). Females and males did not differ in size ($W = 18$, $p = 0.26$),

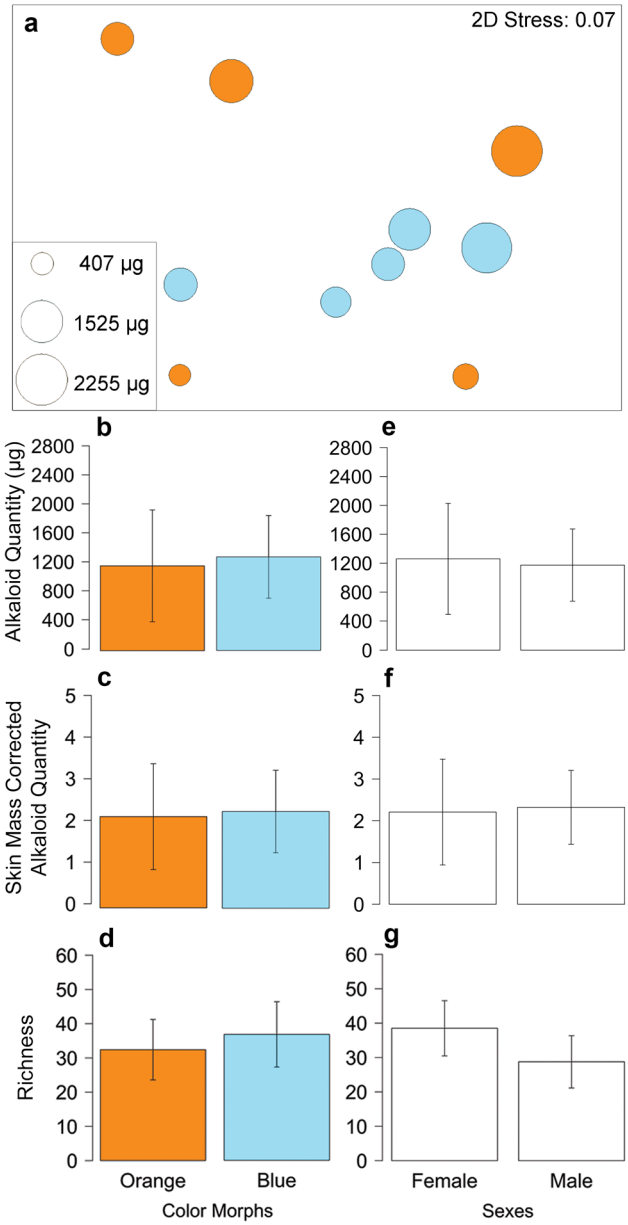

Fig. 2 Comparison of alkaloid composition between morphs and sexes of *Adelphobates galactonotus*. **a** nMDS plot of alkaloid composition between blue and orange morphs. Each circle represents an individual frog, and the distance between symbols represents the relative difference in alkaloid composition. The diameter of each circle is proportional to the quantity of alkaloids present in that frog (µg per frog skin). **b–d** Comparison of alkaloid quantity (µg), mass corrected alkaloid quantity, and alkaloid richness, respectively, between blue and orange morphs. **e–g** Comparison of alkaloid quantity (µg), mass corrected alkaloid quantity, and alkaloid richness, respectively, between males and females of both color morphs

Table 1 Summary of alkaloid variation in *Adelphobates galactonotus*

	Blue Morph	Orange Morph	Blue + Orange Morphs
Total quantity (mean µg per skin ± S.D.)	1285 ± 571 µg	1166 ± 772 µg	1225 ± 643 µg
Corrected quantity (mean µg per mg skin ± S.D.)	2 ± 1 µg	2 ± 1 µg	2 ± 1 µg
Richness (mean number of alkaloids per skin ± S.D.)	37 ± 10	32 ± 9	35 ± 9
Quantity range (µg per skin)	800–2180 µg	406–2255 µg	407–2255 µg
Richness range (per skin)	28–48	18–41	18–48

and there was no difference in alkaloid quantity ($W = 12$, $p = 1$), skin mass corrected alkaloid quantity ($W = 11$, $p = 0.91$), and richness ($W = 19$, $p = 0.17$; Fig. 2e–f). Total quantity and richness of alkaloids were not significantly related ($F_{1,8} = 4.5$, $R^2 = 0.4$, $p = 0.07$), even when corrected by wet skin mass ($F_{1,8} = 2.4$, $R^2 = 0.2$, $p = 0.16$).

We identified 89 alkaloids (including isomers) representing 16 structural classes (Table 2). Seven alkaloids are new, and we also identified several tentatively new isomers of previously characterized alkaloids. The MS data and Rt for all seven new alkaloids are shown in Supplementary material 1 and Rt for all of the new isomers are included in Supplementary material 2.

Overall, the most abundant alkaloid in *A. galactonotus* was histrionicotoxin (HTX) **259A** (348.8 ± 413.1 µg per skin), with 3 times the amount of the second most abundant alkaloid, HTX **261A** (103.6 ± 81.5 µg per skin). Both alkaloids were present in all individuals of both color morphs. Allopumiliotoxin (aPTX) **337D** and 5,6,8-indolozidine (5,6,8-I) **259C** were also present in all individuals of both populations, but in smaller amounts (26.1 ± 22.5 µg and 52.1 ± 43.7 µg per skin, respectively). 5,6,8-I **231B** and **249C**, aPTX **305A**, decahydroquinoline (DHQ) *trans*-**243A**, HTX **285B** and Izidine **211C** were present in all but one individual, and aPTX **323B** was present in all but two individuals.

Among the 89 alkaloids in total, 46 are shared between the two populations. Among the 26 alkaloids that are unique to the blue morph, 17 are present in only one individual, 6 are present in two individuals and 2 are present in three individuals. The only alkaloid present exclusively in all individuals of the blue morph is DHQ *5-epi-trans*-**243A**, which is an isomer of DHQ *trans*-**243A** found in all individuals of both morphs. In the orange morph, we found 15 unique alkaloids, 10 of which are present in only one individual, 3 in two individuals, and 2 in three individuals. All alkaloids present in each of the color morphs are listed in Table 2 (alkaloids and quantities for each individual are provided in Supplementary material 3). Although only 46 of the 89 alkaloids are shared among the two populations, the amount of the exclusive alkaloids in each color morph add up to only 7.2% of the total alkaloids found in the blue morph population and 3.2% of the total alkaloids in the orange morph population.

Palatability test

Frog alkaloids were significantly unpalatable to fruit flies at all three concentrations ($p \leq 0.001$ for all comparisons). There was no statistically significant dose response in palatability among concentrations for either morph (Orange: $F_{1,13} = 3.43$, $p = 0.087$; Blue: $F_{1,13} = 1.37$, $p = 0.264$); however, on average, the higher concentrations of alkaloids were more unpalatable (Fig. 3). Being conservative, the lowest concentration of 0.6% was used for all of the remaining analyses. There were no significant differences in palatability between the orange and blue populations of *A. galactonotus* ($t = 0.43$, $p = 0.681$; Fig. 4). There was no relationship between alkaloid palatability and alkaloid quantity for either morph (Orange: $F_{1,3} = 5.27$, $p = 0.106$, $R^2 = 0.637$; Blue: $F_{1,3} = 0.477$, $p = 0.539$, $R^2 = 0.137$; Fig. 5a) or alkaloid richness (Orange: $F_{1,3} = 0.763$, $p = 0.447$, $R^2 = 0.203$; Blue: $F_{1,3} = 1.43$, $p = 0.318$, $R^2 = 0.322$; Fig. 5b); however, there was a trend towards a decrease in palatability with an increase in alkaloid quantity and richness (Fig. 5).

Discussion

In the only previous study that examined the alkaloids of wild-caught *A. galactonotus*, Daly et al. (2009) analyzed a single specimen from Tucuruí (reported as "Tucurvi"), Pará. Although Daly et al. (2009) did not provide color information, only orange frogs are known from that region (Hoogmoed and Ávila-Pires 2012). Among the four alkaloids we observed in all individuals, Daly et al. (2009) also detected HTX **259A** (trace amount), HTX **261A** (minor constituent), and 5,6,8-I **259C** (major constituent); however, they did not detect any aPTX **337D**. Daly et al. (2009) also reported HTX **291A**, aPTX **253A** and **267A**, DHQ *trans*-**243A**, and 5,6,8-I **249C** as major constituents; in our results, HTX **291A** was present in both blue and orange populations, aPTX **253A** and **267A** were absent in both populations, and DHQ *trans*-**243A** and 5,6,8-I **249C** were present in both populations.

Table 2 Alkaloids identified in blue and orange morphs of *Adelphobates galactonotus*, arranged by structural class

Structural classes	3,5-I	3,5-P	4,6-Q	5,6,8-I	5,8-I	aPTX	Epiq	DHQ	HTX	Izidines	Pip	PTX	Tri	Unclass	New
Blue Morph															
	195B	*trans*-**223M**	**195C**	**209C**	**217A**	**293K**	**196**	*cis*-**195A**	**235A**	**211C**	**269C**	**277B**	**193C**	**227**	**209**
	5E, 9E **223AB**	*cis*-**223M**		**231B** (2)	**243C**	**305A** (3)		**195J**	**239H**			**323F**		**271B**	**213**
	5E, 9Z **223AB**	**249I**		*cis*-**249C** (2)		**323A**		*5-epi-trans*-**219A**	**259A** (2)						**245**
	5Z, 9E **223AB**	**251K** (2)		**259C**		**323B** (3)		*2-epi-cis*-**219A**	**261A** (3)						**251**
	5Z, 9Z **223AB**			**261B**		**337D**		*cis*-**219A**	**265E**						**265**
	223R			**263A** (2)		**341A**		*5-epi-trans*-**243A** (2)	**283A**						
	275C			**265U** (2)				*cis*-**243A**	**285B** (2)						
				273A				*trans*-**243A** (2)	**291A**						
				281M				**245Q**							
Structural classes	3,5-I	3,5-P	4,6-Q	5,6,8-I	aPTX	Epiq	DHQ	HTX	Izidines	PTX	Pyr	SpiroP	Unclass	New	
Orange Morph															
	5E, 9Z **223AB**	*trans*-**223M**	**195C**	**207C**	**293K**	**196**	*cis*-**195A**	**235A**	**211C**	**277B**	**211T**	**222**	**223DD**	**209**	
	5Z, 9E **223AB**	**251K** (2)		**231B** (3)	**305A** (4)		*cis*-**243A**	**239H**		**323A**		**236**		**232**	
	5Z, 9Z **223AB**			**233G**	**307C**		*trans*-**243A** (2)	**259A** (2)		**323F**				**245**	
				249C (2)	**323B** (4)		**245Q**	**261A** (3)						**293**	
				259C (2)	**337D**			**265E**							
				261B				**283A**							
				263A				**285B** (2)							
				263D				**291A**							
				265U											
				273A											
				281M											

Alkaloids present in both morphs are underlined

The number of isomers detected for each alkaloid is indicated in parentheses. The retention times for these tentatively new isomers are in Supplementary material 2. Abbreviations for alkaloid structural classes are as follows: 3,5-I (3,5-disubstituted indolizidine); 3,5-P (3,5-disubstituted pyrrolizidine); 4,6-Q (4,6-disubstituted quinolizidine); 5,6,8-I (5,6,8-trisubstituted indolizidine); 5,8-I (5,8-disubstituted indolizidine); aPTX (allopumiliotoxin); Epiq (Epiquinamide); DHQ (2,5-disubstituted decahydroquinoline); HTX (histrionicotoxin); Pip (2,6-disubstituted piperidine); PTX (pumiliotoxin); Pyr (2,5-disubstituted pyrrolidine); SpiroP (Spiropyrrolizidine); Tri (tricyclic); Unclass (unclassified as to structure); New (New alkaloids - Supplementary material 1)

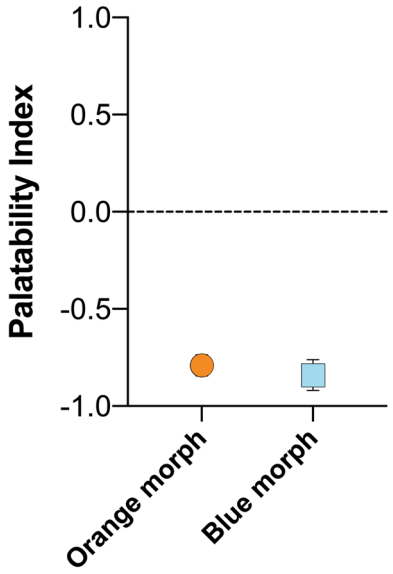

Fig. 3 Mean palatability (±1 S.E.) of orange and blue morphs of *Adelphobates galactonotus*. The dotted line represents the point at which the solution of alkaloids is considered palatable

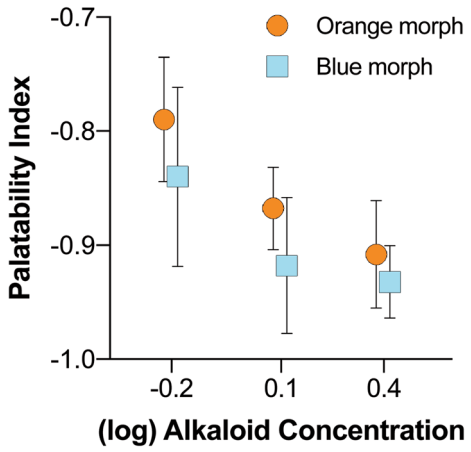

Fig. 4 Dose response of mean palatability (±1 S.E.) for each of the three alkaloid concentrations tested between orange and blue morphs of *Adelphobates galactonotus*. Each data point is offset ± 0.02 units for clearer visualization of data

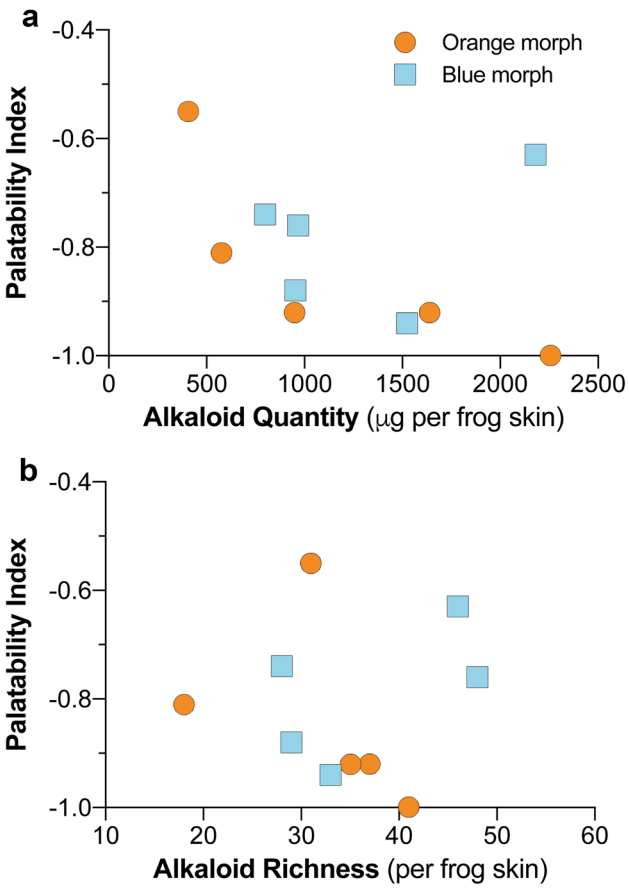

Fig. 5 The relationship between palatability and **a** alkaloid quantity or **b** alkaloid richness for orange and blue morphs of *Adelphobates galactonotus*

We did not perform statistical comparisons of the Tucuruí population with the blue and orange populations we studied because only one individual from that population has been analyzed. However, given that the orange morph has a broad distribution and additional morphs exist (Hoogmoed and Ávila-Pires 2012), future studies should compare multiple populations to test the generality of our findings in this species.

Alkaloid composition does not differ between the orange and blue populations of *A. galactonotus*, despite their conspicuously different coloration and geographic separation on opposite sides of a significant aquatic barrier. This result contrasts with those of previous studies of other species of poison frog that found significant differences (Saporito et al. 2006, 2007a; Daly et al. 2008; Grant et al. 2012; McGugan et al. 2016; Lawrence et al. 2019). The two main reasons for the lack of significant differences are: 1) the high amount of shared alkaloids between the populations, including the two most abundant alkaloids (HTX **259A** and HTX **261A**), and 2) the low amounts and occurrence of unshared alkaloids. The difference between results found in *A. galactonotus* and other species could be due to lack of variation in alkaloid-containing arthropods on either side of Caxiuanã Bay and variation among localities of other species. For example, in a study of *Oophaga sylvatica*, McGugan et al. (2016) attributed differences among populations to differences in arthropod availability. Unfortunately, data on arthropod availability are lacking for our study sites. Alternatively, the difference could be due to genetically or epigenetically determined alkaloid uptake being the same in orange and blue morphs of *A. galactonotus* but differing

among populations of other poison frog species. The physiological and genetic aspects of alkaloid sequestration are not understood, but experiments suggest that they play a role in the variation of alkaloid composition (Daly et al. 1994, 2003; Hantak et al. 2013).

The unpalatability of alkaloid defenses in *A. galactonotus* is consistent with previous studies of alkaloid palatability in poison frogs (Schulte et al. 2017; Bolton et al. 2017; Lawrence et al. 2019). Given the lack of differences in alkaloid composition between blue and orange morphs, the lack of differences between the two color morphs in palatability is expected. Previous studies have found that conspicuousness of dorsal skin coloration in poison frogs is an honest indicator of alkaloid presence for visually oriented predators (Stuckert et al. 2014, 2018), and native predators are able to recognize and avoid aposematic coloration (e.g. Saporito et al. 2007b; Noonan and Comeault 2008). However, variation in visual cues and alkaloid levels is not necessarily correlated (Daly and Myers 1967; Wang 2011; Stuckert et al. 2014, 2018; Crothers et al. 2016; Bolton et al. 2017) and do not predict differences in predation risk (Hegna et al. 2011; Stuckert et al. 2014). In *A. galactonotus*, the orange morph is brighter than the blue morph (Rojas et al. 2015); however, the lack of differences in alkaloid composition and palatability illustrate that this difference in brightness and hue is not a qualitative indicator of toxicity in this species.

The lack of differences in the defensive chemicals and palatability of the orange and blue morphs of *A. galactonotus* suggests that the color polytypism in this species is not related to predation. Indeed, our results are consistent with previous findings that the frequency of attacks by vertebrates on paraffin models representing these two color morphs does not differ in either population (Rojas et al. 2015). Similarly, accumulation of dietary carotenoids has been shown to be unrelated to defensive chemicals in other poison frogs (e.g. Crothers et al. 2016), and captive breeding experiments with *A. galactonotus* have shown that orange and blue morphs are not diet dependent (AMJ and TG, unpublished data). Instead, we suggest that the different color morphs in *A. galactonotus* might be related to female mate preference. Nothing is known about mate preferences in this species; however, females of *O. pumilio* prefer males of the same color morph (Maan and Cummings 2008) through parental imprinting (Yang et al. 2019). If female *A. galactonotus* also imprint on parental coloration and prefer males of the same color morph, then assortative mating could be the main driver of the distinctive color polytypism in *A. galactonotus*.

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Author contribution AMJ, RAS, and TG contributed to the study conception and design. Specimens were collected by AMJ and TG. Chemical analysis was performed by AMJ and RAS. Palatability tests were performed by SK and RAS. The first draft of the manuscript was written by AMJ and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

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

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