Journal of Chemical Ecology
https://doi.org/10.1007/s10886-023-01412-7

Linking Predator Responses to Alkaloid Variability in Poison Frogs

J. P. Lawrence[1,2] · Bibiana Rojas[3,4] · Annelise Blanchette[5,8] · Ralph A. Saporito[5] · Johanna Mappes[3,6] · Antoine Fouquet[7] · Brice P. Noonan[1]

Received: 21 September 2022 / Revised: 11 February 2023 / Accepted: 15 February 2023

Abstract

Many chemically-defended/aposematic species rely on diet for sequestering the toxins with which they defend themselves. This dietary acquisition can lead to variable chemical defenses across space, as the community composition of chemical sources is likely to vary across the range of (an aposematic) species. We characterized the alkaloid content of two populations of the Dyeing Poison Frog (*Dendrobates tinctorius*) in northeastern French Guiana. Additionally, we conducted unpalatability experiments with naive predators, Blue Tits (*Cyanistes caeruleus*), using whole-skin secretion cocktails to assess how a model predator would respond to the defense of individuals from each population. While there was some overlap between the two *D. tinctorius* populations in terms of alkaloid content, our analysis revealed that these two populations are markedly distinct in terms of overall alkaloid profiles. Predator responses to skin secretions differed between the populations. We identified 15 candidate alkaloids (including three previously undescribed) in seven classes that are correlated with predator response in one frog population. We describe alkaloid profile differences between populations for *D. tinctorius* and provide a novel method for assessing unpalatability of skin secretions and identifying which toxins may contribute to the predator response. In one population, our results suggest 15 alkaloids that are implicated in predator aversive response. This method is the first step in identifying the causal link between alkaloids and behavioral responses of predators, and thus makes sense of how varying alkaloid combinations are capable of eliciting consistent behavioral responses, and eventually driving evolutionary change in aposematic characters (or characteristics).

Keywords Unpalatability · *Dendrobates tinctorius* · Alkaloids · Birds · Aposematism

✉ J. P. Lawrence
JPLarry@gmail.com

[1] Department of Biology, University of Mississippi, University, MS 38677, USA

[2] Present Address: Lyman Briggs College, Michigan State University, East Lansing, MI 48825, USA

[3] Department of Biology and Environmental Science, University of Jyväskylä, P.O. Box 35, 40014 Jyväskylä, Finland

[4] Konrad Lorenz Institute of Ethology, Department of Interdisciplinary Life Sciences, University of Veterinary Medicine Vienna, Savoyenstraße 1, 1160 Vienna, Austria

[5] Department of Biology, John Carroll University, University Heights, OH 44118, USA

[6] Organismal and Evolutionary Biology Research Programme, Faculty of Biological and Environmental Sciences, Helsinki University, Helsinki, Finland

[7] Laboratoire Evolution et Diversité Biologique, UMR5174, Université Paul Sabatier, 31062 Toulouse Cedex 9, France

[8] Department of Ecology and Evolutionary Biology, Tulane University, New Orleans, LA 70118, USA

Introduction

Aposematism is a broadly-found defensive strategy in which prey species use warning signals to inform would-be predators of a secondary defense (Poulton 1890), often in the form of defensive chemicals. For a number of aposematic taxa (e.g., nudibranchs, butterflies, and dendrobatid frogs), defensive chemicals are sequestered from food sources rather than being synthesized *de novo* (Proksch 1994; Nishida 2002; Saporito et al. 2011), often resulting in interpopulation variation in defenses because food sources vary spatially and temporally (Saporito et al. 2006, 2007a; Daly et al. 2008; Prates et al. 2019). Consequences of this variation range from automimicry (within-population variation in the defense level of a prey species whose individuals have the same appearance; Brower et al. 1967; Speed et al. 2006) to polytypy (inter-population variation) in aposematic phenotypes (Siddiqi et al. 2004).

Published online: 01 March 2023

Qualitatively honest signaling occurs when a signal advertises the consequence of a secondary defense (Summers et al. 2015). If a signal is associated with a poor defense, predators will not strongly connect the signal with a defense (Rowland et al. 2007). Conversely, a poor signal that is over-defended may result in predators not strongly associating the signal with the defense (Lindström et al. 1999). Additionally, chemical defenses are often costly (Ojala et al. 2005; Sandre et al. 2007; Zvereva and Kozlov 2015; Burdfield-Steel et al. 2019). Therefore, having a well-matched signal should ensure that energy and resources are not wasted. While some studies suggested the occurrence of honest signaling among populations of aposematic species (e.g., see Vidal-Cordero et al. 2012; Maan and Cummings 2012), these conclusions have been based on analysis of the defense mechanism alone, not the effect this defense has on predators. Furthermore, this is not a universal trend, as ladybird beetles are known to exhibit a positive or negative correlation between signal and chemical defense depending on whether they are raised on low amounts of food or under resource abundance, respectively (Blount et al. 2012). Likewise, some species of poison frogs seem to display a tradeoff between secondary defense and signal (Darst and Cummings 2006; Wang 2011).

Alkaloid variation in dendrobatid poison frogs has been well-characterized in several species (Daly et al. 1987; Bolton et al. 2017). Over 1200 different types of diet-based alkaloids have been described from poison frogs (Daly et al. 2005; Hovey et al. 2018), defenses primarily derived from ants and mites (Saporito et al. 2003, 2007b; McGugan et al. 2016), and possibly some other invertebrates (i.e., millipedes and beetles; Saporito et al. 2007a). As the source of these defensive chemicals are arthropods (Darst et al. 2005), alkaloid profiles have been shown to vary among conspecific populations (Prates et al. 2019) and over time within populations (Saporito et al. 2006, 2007a). Characterizing the chemical profiles of secondary defenses in aposematic species is important for understanding how these vary among populations and how well these species are defended from predators. Even more important is to identify which chemicals are actually relevant for predator deterrence. Furthermore, because secondary defenses contribute to the efficacy of aposematic signaling, baseline data on alkaloid profiles can provide important insight into why aposematic signals vary among species and populations.

While alkaloid defenses have been the subject of scientific inquiry for decades, their relationship to aposematic signal variation is less understood. When examining the polytypy in the Strawberry Poison Frog (*Oophaga pumilio*) in Bocas del Toro, Panama, (Daly and Myers 1967) found no relationship between toxicity and color/pattern. Conversely, Maan and Cummings (2012) suggested that color/pattern of the poison frogs they studied were honest indicators of defense, particularly towards avian predators, and therefore there should be a strong relationship between chemical defense and conspicuousness of warning signals. This apparent contradiction may be the result of differences in methodological techniques. Daly and Myers (1967) assessed toxicity by determining LD50 (lethal dose for 50% of test subjects) of toxin extracts for mice from different populations, while Maan and Cummings (2012) examined the discomfort mice exhibited after injection (a method used in assessing toxicity of alkaloids; e.g., Darst et al. 2006; Amezquita et al. 2017; Protti-Sánchez et al. 2019). Evidence that these two methods of inferring defense provided by integumentary alkaloids are not complementary was provided by Bolton et al. (2017), who found divergent results for LD50 and discomfort for individual samples. While assays with mice may act as a proxy of toxicity, they may not be biologically relevant as these toxins are experienced by the predator in the oral cavity and digestive tract when capturing and consuming the prey species, rather than directly entering the bloodstream, musculature, and/or peritoneal cavity (Holen 2013; Weldon 2017; Saporito and Grant 2018). Importantly, however, the relationship between palatability (the direct means of predator interaction with chemical defenses and upon which they presumably base decisions) and toxicity appears to be unrelated (Bolton et al. 2017; but toxicity could be predator-dependent), nor is total alkaloid quantity predictive of palatability (Lawrence et al. 2019a). More importantly, not only are mice not relevant predators of poison frogs (and thus, not a selective agent behind the evolution of toxicity), but injections are an unrealistic method of assessing the biological function of defensive alkaloids, as anecdotal evidence on poison frog predation points to direct contact (e.g., mouthparts and antennae of ants; chelicerae and pedipalps of spiders; Murray et al. 2016) or ingestion (e.g., by birds; Master 1999) as the mechanism of exposure. Therefore, how the quantity and composition of alkaloids present in frog skins relate directly to predator responses presents a glaring knowledge gap in the evolutionary puzzle of signal honesty.

The Dyeing Poison Frog (*Dendrobates tinctorius*) is found throughout the Eastern Guiana Shield region (sensu Vacher et al. 2020) in northern South America. Throughout its range, it shows considerable color and pattern variation among (and sometimes within) populations (Noonan and Gaucher 2006; Rojas and Endler 2013; Lawrence et al. 2019a). *Dendrobates tinctorius* sequester alkaloids (Summers and Clough 2001; Santos et al. 2003), but only four populations have had their alkaloids characterized to date (Daly et al. 1987; Lawrence et al. 2019a). Here, we leverage the between-population warning signal

variability in this chemically-defended species to ask how variability in alkaloid defenses may relate to avoidance responses by model predators. First, if the skin alkaloids vary considerably among populations, it suggests that toxin content (or skin secretions) vary along with the environment and that individuals have relatively weak control over their toxic defenses. Second, if alkaloid content is different among populations, we expect predator responses to differ as well. If not, that would suggest weak (or relaxed) selection for alkaloid content - specifically alkaloids responsible for distastefulness. However, if the response by predators does differ between populations, is there a subset of alkaloids that would explain the response?

Methods and Materials

Field Collection We collected 18 (n[Matoury] = 10; n[Kaw] = 8) *Dendrobates tinctorius* in May-June 2013 and August 2014 from two populations in French Guiana: Matoury (4.89°N, 52.34 °W; 10 individuals) and Kaw Mountains (4.57°N, 52.21°W; 8 individuals). These individuals were used both for alkaloid analysis and associated behavioral analysis (see below). The dorsal color patterns of frogs from both populations consisted of colorful (white for Matoury, yellow for Kaw) stripes on a black background. For each encountered frog, we recorded individual variation (sex, snout-vent length). Next, frogs were euthanized by cervical transection and pithing in the field immediately after taking measurements. We skinned frogs and placed whole skins in 100% methanol in 4mL vials with PTFE caps.

Alkaloid Extraction We followed the protocol outlined by Saporito et al. (2010) to conduct acid-base fractionations of the methanol extracts. We took 1mL of the methanol extract and added it to a graduated conical vial along with 50µL of HCl and 100µL of an internal nicotine standard (L-Nicotine, 99+%, Acros Organics). Samples were then dried down to 100µL using a gentle flow of N_2 and then 200µL of DI H_2O was added. Following this, the sample was then extracted three times, each time with 300µL of hexane. The solution was then basified with $NaHCO_3$. The sample was then extracted three times, each time with 300µL of ethyl acetate and then dried with anhydrous $NaSO_4$, and evaporated to dryness under a gentle flow of N_2. The sample was reconstituted in 100µL of methanol for alkaloid analysis, referred to hereafter as the "methanol extract."

GC-MS Analysis Gas chromatography-mass spectrometry (GC-MS) was performed for each individual sample on a Varian Saturn 2100T ion trap MS instrument, which was coupled to a Varian 3900 GC with a 30 m x 0.25 mm inside diameter Varian Factor Four VF-5ms fused silica column. GC separation of alkaloids was achieved 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). Each alkaloid fraction was analyzed in triplicate with electron impact MS and once with chemical ionization (CI) MS with methanol as the ionizing reagent.

Individual alkaloids of *Dendrobates tinctorius* were identified based on comparison of mass spectral properties and GC retention times with those of previously reported alkaloids in dendrobatid frogs (Daly et al. 2005; Saporito, unpublished poison frog alkaloid library). Alkaloids in dendrobatid frogs have been assigned a series of code names that consist of a boldfaced number indicating the alkaloids' nominal mass, and a boldfaced letter to distinguish those alkaloids with the same nominal mass (Daly et al. 2005). Alkaloid quantities for each individual frog were calculated by comparing the average observed peak area of individual alkaloids to the average peak area of the nicotine standard from the triplicate EI-MS analyses using a Varian MS Workstation v.6.9 SPI. Only alkaloids that were present in quantities of ≥ 0.5 µg were included in the analyses (Bolton et al. 2017).

Unpalatability Assays We used data (Lawrence et al. 2019b) from a previously published study (Lawrence et al. 2019a, unpalatability assay A) to investigate the link between amount and composition of skin alkaloids and predator response. Briefly, the unpalatability assays consisted of the following methodology. We took 1mL of the methanol extract and evaporated it to dryness under a gentle stream of N_2 and then reconstituted the extract in 0.5mL ethanol to then be used in unpalatability trials with wild-caught Blue Tits (*Cyanistes caeruleus*). While Blue Tits are a palearctic species and thus would not encounter Neotropical frogs, predators of poison frogs driving the evolution of aposematism are assumed to be birds (Comeault and Noonan 2011; Chouteau and Angers 2011; Rojas et al. 2014; Paluh et al. 2014) and some anecdotal evidence seems to support this assumption (Master 1999; Alvarado et al. 2013). Other groups of animals are known predators of poison frogs as well (i.e., snakes, crustaceans, spiders, ants; see Murray et al. 2016, Rojas 2017) but it is unclear whether and how they may be driving color evolution in poison frogs. Bird taste systems are generally conserved across genera (Wang and Zhao 2015), which suggests that the response from Blue Tits to alkaloids will be similar to other birds. Notably, insectivorous and omnivorous birds, such as Blue Tits. show particular sensitivity to bitter tastes (Rowland, et al. 2015), such as alkaloids. The use of Blue Tits serves two additional advantages for this study: first, the use of sympatric species runs the risks that individuals have previously experienced these toxins and responses may be influenced

by prior experience. Thus, using this species allows us to use adult birds that are truly naive to these toxins; and second, blue tits have been used for multiple unpalatability assays and, thus, well established methods (see e.g., Rojas et al. 2017, 2019; Burdfield-Steel et al. 2018; Ottocento et al. 2022) and well-known responses to distasteful stimuli were both readily available.

The birds used in this study were caught in November and December 2017 at Konnevesi Research Station (Central Finland) from feeding sites using peanuts as bait (e.g., Rojas et al. 2017; Burdfield-Steel et al. 2019). Birds were housed individually in plywood cages with a daily light regime of 11 h:13 h (light:dark), fed on sunflower seeds and peanuts, and provided with fresh water ad libitum. Each bird was weighed before and after the experiment, and ringed before being released to the same place of capture. For the behavioral assays, each bird was transferred to an experimental plywood box (50 × 60 × 45 cm) which contained a perch, a little bowl with water and a moving hatch attached to a visual barrier (see below for details; Nokelainen et al. 2012; Rojas et al. 2017; Ottocento et al. 2022).

We used a protocol developed by (Rojas et al. 2017) that is designed to test the chemical defense efficacy where the target compound is offered to a predator in a novel context. Prior to experiments, Blue Tits were trained to eat oat flakes in their home boxes. We tested a total of 25 birds, eight with extracts from the Kaw population, 10 with extracts from the Matoury population, and seven controls with just ethanol. Each of two oats were soaked with 15 µl of the extract of one frog skin and left for 24 h at room temperature to ensure that all ethanol had evaporated. Two other oats were soaked each with 15 µl of pure ethanol that were used at the beginning and end of the experiment with each bird. Each bird went through four trials. The first trial consisted of a control oat which needed to be consumed entirely by the bird before the experiment could be initiated. Following this, two consecutive extract treatments each consisted of a single oat with extract. The final trial involved the second control oat which was offered to ensure that the birds were not refusing to eat the oats coated with extract out of satiation or lack of motivation to eat in general. Birds in the control treatment received oats soaked with pure ethanol for all four trials in order to compare directly the response of birds to oats containing frogs' extracts vs. oats with ethanol only.

Each oat was presented on a hatch that had a visual barrier, which allowed us to identify the exact moment at which the oat was seen which determined the actual beginning of each of the two experimental trials where birds were exposed to toxins. We recorded the percentage of the oat eaten as an analog for how distasteful the oat was: 100% when the whole oat flake was eaten, 50% when half was eaten, 0% when the oat was left untouched. When less than a whole oat but more than half of the oat was eaten, we assigned 75%, and when some of the oat was eaten, but not as much as half of it, we assigned 25%. We also recorded the number of times each bird wiped its beak against a surface, a well-known response to distasteful food (Skelhorn and Rowe 2009; Rojas et al. 2019). Birds were watched for a 2-min period after they finished eating the oats, or for a maximum of 5 min in the cases in which the oat was not fully eaten, to make sure that any delayed response to the oat taste was not going to be missed. This assay was done between November and December 2017.

Data Analysis As individuals and populations can be quite variable in alkaloid content, it is unlikely that the entire suite of alkaloids is contributing to the behavioral response of predators. In order to identify the most likely alkaloid candidates driving predator response, we performed an exploratory factor analysis using the quantities of each type of alkaloid. Factor analyses group independent variables into loadings that explain population variation in these variables. In this analysis, alkaloids were grouped at random into smaller loadings, which were then used to explore whether these groups, collectively, could be explanatory for predator response. Following this, we performed a multiple linear regression to identify which loadings are explanatory for variation in behavioral response. For this analysis, we used the natural variation assay (Assay A in Lawrence et al. 2019a, b) for behavioral responses to alkaloids. We performed this analysis for each population to determine what, if any, alkaloids are important for behavioral response to the frogs' chemical defenses. By using this exploratory factor analysis and subsequent multiple linear regression, we can narrow down alkaloids from the complex profiles found in these frogs. While this analysis will not determine what alkaloids in these loadings is driving response (i.e., it is possible that a loading may have nonsignificant alkaloids paired with significant alkaloids), this analysis functions to allow a stepping stone for future research to determine what alkaloids are directly responsible for behavioral responses. All analyses were conducted with R (R 2016) using the packages *psych* and *GPArotation*.

Results

Results regarding unpalatability assays were previously reported in Lawrence et al. 2019a, b. Briefly, despite individuals from the Matoury population having higher amounts of alkaloids, we found a stronger aversive response by birds to the skin extracts of the Kaw population than to controls. Namely, birds wiped their beak more often and took longer to consume the offered oats when these were soaked in the

skin extracts of frogs from the Kaw population (i.e., frogs from the Kaw population are more unpalatable); we detected no differences in predator aversive response between controls and the skin extracts from the Matoury population. Here, we present a new analysis linking these behavioral responses (namely, oat consumption) to alkaloid variability in these two populations.

The Matoury and Kaw populations showed similar diversity, though different composition, of alkaloids (49 in 11 classes and 46 in 12 classes, respectively; Table 1). Fifteen alkaloids were found in both populations, albeit in different quantities (Fig. 1). Only the Matoury population had significant loadings which implicated 15 alkaloids with behavioral response (Table 1). Of these, two (**251T** and **259 C**) averaged to be major alkaloids (quantity greater than 50 µg), one (**249 C**) was minor (5–50 µg), and the rest were trace (< 5 µg). Five of these fifteen were only found in a single individual.

We detected 49 different alkaloids representing 11 structural classes (Table 1; Fig. 1) from frogs in the Matoury population. Twelve of the 49 alkaloids (24.4%) were only detected once in the Matoury samples (i.e., found in one individual). Of the 49 alkaloids, 14 (two 3,5-disubstituted indolizidines (3,5-I) [**223AB** and **275 C**], four 5,6,8-trisubstituted indolizidines (5,6,8-I) [**231B**, **251T**, **259 C**, and **267R**], two 3,5-disubstituted pyrrolizidines (3,5-P) [**209Q** and **251 K**], one histrionicotoxin (HTX) [**235 A**], two decahydroquinolines (DHQ) [**219** and **243 A**], one 1,4-disubstituted quinolizidines (1,4-Q) [**231 A** and **249 N**], and two new alkaloids of molecular weight **247** and **275** were found in most individuals. The new alkaloids could not be assigned a structural class and will be further characterized.

The factor analysis revealed five loadings that explained 76% of the variation among alkaloid profiles in the Matoury population. A subsequent multiple linear regression (multiple $R^2 = 0.97$, $F_{5,4} = 34.07$, $p = 0.002$) revealed two loadings that significantly deviated from the null when examining the proportion of oats eaten by blue tits. Loading 1 (t = -11.47, $p = 0.0003$), which explains 18% of the population variation, contained the alkaloids Tri (tricyclic) **205B**, Tri **207GH**, spiropyrrolizidine (Spiro) **236**, cyclopentaquinazoline (CPQ) **245 A**, 5,6,8-I **265 L**, and allopumiliotoxin (aPTX) **339 A**. Loading 3 (t = -3.113, $p = 0.0350$), which explains 17% of the population variation, contained the alkaloids new **245**, new **247**, 5,6,8-I **249 C**, Unclassified (Unclass) **249 N**, 5,6,8-I **251T**, 5,6,8-I **259 C**, 5,6,8-I **261B**, 5,6,8-I **263 A**, and DHQ **269B**.

We detected 46 different alkaloids representing 12 different structural classes from frogs in the Kaw population (Table 1; Fig. 1). Notably, of the eight Kaw individuals examined, one individual showed approximately ten times the quantity of alkaloids as compared to other individuals in the population and was thus considered an outlier and removed from analysis. Of the 46 different alkaloids, eighteen were found in single individuals (39.1%) as opposed to being found repeatedly in the population. Eleven alkaloids are represented in a majority (> 50%) of individuals in this population including one 3,5-I (**223AB** and **275 C**), one 5,6,8-I (**231B**), DHQs (**195 J**, **219 A**, and **221D**), one 1,4-Q (**231 A**), one Spiro (**236**), one Unclass (**235BB**), one new piperidine (PIP) of molecular weight **213**, and one new alkaloid of molecular weight **247**. Both the new piperidine and alkaloid of molecular weight 247 (also present in Matoury population), will be further characterized.

The factor analysis revealed four loadings that explained 70% of the variation observed in toxin profiles in the Kaw population. After factor analysis, however, multiple linear regression revealed no differences among loadings when examining either proportion of oats eaten (multiple $R^2 = 0.07$, $F_{4,3} = 0.05$, $p = 0.99$).

Discussion

Dendrobatid frogs are characterized by their impressive alkaloid diversity used as secondary defenses combined with their conspicuous color signals. Whether this diversity is necessary for effective defenses or a subset of important alkaloids primarily drives predator responses has not been previously tested. We report the diversity of alkaloids from two populations of *D. tinctorius* (sampled punctually so as to avoid long-term temporal change in alkaloids; Saporito et al. 2007a) and infer which alkaloids may be correlated with predator behavior. These populations show similar richness in alkaloids (numbers and structural classes), but the composition of the alkaloid cocktails is different between the two populations (Lawrence et al. 2019a). Only three alkaloids are well-represented in both populations (**223AB**, **231B**, and **219 A**). Mites are known sources for both **223AB** and **231B** (Saporito et al. 2007b; McGugan et al. 2016) and, while no source of **219 A** has been identified, as it is a decahydroquinoline, the source for that alkaloid is likely ants (Saporito et al. 2007a). These overlapping and common alkaloids suggest that some prey may be common to both populations, which is not surprising as the two populations are less than 50 km from one another. Matoury shows a large diversity of 5,6,8-trisubstituted indolizidines, six of which (**249 C**, **251T**, **259 C**, **261B**, **263 A**, and **265 L**) are implicated in predator aversive response. Allopumiliotoxins and tricyclics were found only in Matoury while dehydro-5,8-indolizidines, 4,6-quinolizidines, and piperidines were found only in Kaw. These classes are known to come from both ants and mites (Saporito et al. 2007b) which, given the population specificity of these alkaloid classes, suggests differential availability of these sources between the two populations.

Table 1 Alkaloid variation seen in the Matoury and Kaw populations

		Matoury Population											Kaw Population							
		M1	M2	M3	M4	M5	M6	M7	M8	M9	M10		K1	K2	K3	K4	K5	K6	K7	
	Sex	F	F	M	M	M	M	F	M	M	F		F	F	F	M	F	M	F	
Structural Class	Alkaloid											Matoury Average								Kaw Average
3,5-I	**195B**																	o		o
	223AB	†	†	†	o	†	*	*	†	*	o	†	†	†	†	*	†	†	*	†
	275C			o	†	o	†	o	†		†	o	†	†		†	†		†	†
5,6,8-I	**193G**															o				o
	195D						o					o		o						o
	205A																	o		o
	207C																o			o
	219N								o			o								
	223A																o			o
												o								o
	225K						†	†									o		o	
	231B	*	†	*	†	†	*	*	†	†	†	*	o	†	o	†	†	†	†	†
	233G			o				o		o		o								
	235E								o	o	o	o								
	237C												o	o						o
	245G									o		o								
	249C	†		†	o			†				†								
	249BB				†	o			†	†	o	†								
	251T	*	*	*	o	*	*	*	*	*	*	*								
	259C	*	†	*	†	†	†		†	†	†	*								
	261B	†		†								o								
	263A	o		o								o								
	265L	o		†								o								
	265U	o		o						o		o								
	265L								†		†	o								
	267R				o	o	o	o	o	o		o								
3,5-P	**209Q**			†			†	†	o	o	o	o					o			o
	223B								†			o	o			o				o
	251K	†	†	†	o	o	*	*	†	†	*	†	o	o					†	†
HTX	**235A**	†	*		o	†	†	*	†	†	†	†	*	*			*			*
	238A													†						o
	245A			†								o								
	259A						†	†	†	†		o								
	261A															o		o	o	o
	283A												†							o
	285A												†							o
	285C													†						o
	291A							†			†	o								
DHQ	**195A**													o						o
	195J												†	†		o		†	o	†
	219A	o	o	†			†	o		o	o	†	†	†	†	*	†	*	*	†
	221D														o	o	o	o	†	o
	223F													o						o
	243A	*	*	†	†	†	*	*	†	*	†	*	†					o	†	o
	245Q												o							o
	269B	o	o									o								
1,4-Q	**231A**	†	o	o			†		†	o		o	o	o	o	o	o	o		o
	235U										o	o								
aPTX	**305A**					†	†					o								
	339A			†								o								
5,8-I	**243C**						o					o								
	237D																o		o	o
Spiro	**236**		o	†				†				o		†	†	†		†	o	†
4,6-Q	**195C**												†	o			o			o
	275I												†						†	o
Dehydro-5,8-I	**265T**																†			o
Tri	**205B**			o								o								
	205E						o					o								
	207G			o								o								
Unclass	**209G**																	o		o
	209M						o					o								
	227													o					o	o
	235BB														*	†		†	†	†
	247M												o							o
	249N	†	o	o	o	o	o	o	o	o		o								
Pip	**"213"**														o	o	o	†		o
New	**"171"**													o						o
	"193"		o									o								
	"207"		†									o								
	"209"		o									o		†						o
	"217"		†									o								
	"223"												o	o						o
	"229"												o	o						o
	"233"														†			†	o	o
	"235"															o			†	o
	"237"																			
	"245"	o										o								
	"247a"	o		o			o	o		o	o	o								
	"247b"														†	†	†	†	†	†
	"253"		o	o								o		o						o
	"275"		o	o	o	†	†	†	o	o	o	o								
Total		17	18	25	13	13	22	19	19	20	17	49	19	22	10	15	16	16	18	46

Alkaloids with gray background were found to significantly impact predator behavior in unpalatability experiments. Alkaloids are divided into the following structural classes: 3,5-disubstituted indolizidines (3,5-I), 5,6,8-trisubstituted indolizidines (5,6,8-I), 3,5-disubstituted pyrrolizidines (3,5-P), histrionicotoxins (HTX), decahydroquinolines (DHQ), 1,4-disubstituted quinolizidines (1,4-Q), allopumiliotoxins (aPTX), 5,8-disubstituted indolizidines (5,8-I), spiropyrrolizidine (Spiro), 4,6-disubstituted quinolizidines (4,6-Q), dehyrdo-5,8-disubstituted indolizidines (Dehydro-5,8-I), tricyclics (Tri), unclassified alkaloids (Unclass), piperidine (Pip). The piperidine and fifteen (New) alkaloids have not previously been described but are in quotes as further characterization is needed in order to be documented as new alkaloids. Major alkaloids (*) are found in quantities greater than 50 μg, minor alkaloids (†) are found in quantities between 5 and 50 μg, and trace alkaloids (o) are found in quantities less than 5 μg.

Boldface notation denotes the nominal mass of the alkaloid

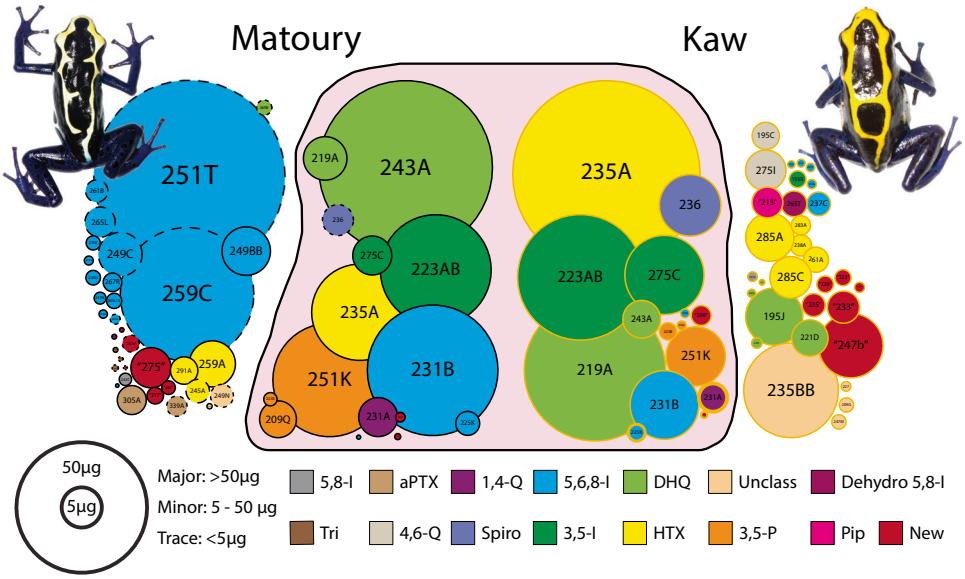

Fig. 1 Distribution of alkaloids between the two populations. Alkaloids common to both populations of *D. tinctorius* are surrounded by the pink shape. Text in the circles represent the type of alkaloid. Size of circles represents relative proportions of the population average of the alkaloid. Circles with dashed lines denote alkaloids that were implicated in predator response (See Table 1 for complete list). Color of the circle represents the structural class in which the alkaloid is found. Alkaloids are divided into the following structural classes: 3,5-disubstituted indolizidines (3,5-I), 5,6,8-trisubstituted indolizidines (5,6,8-I), 3,5-disubstituted pyrrolizidines (3,5-P), histrionicotoxins (HTX), decahydroquinolines (DHQ), 1,4-disubstituted quinolizidines (1,4-Q), allopumiliotoxins (aPTX), 5,8-disubstituted indolizidines (5,8-I), spiropyrrolizidine (Spiro), 4,6-disubstituted quinolizidines (4,6-Q), dehyrdo-5,8-disubstituted indolizidines

With over 1200 alkaloid toxins known from poison frogs (Daly et al. 2005; Hovey et al. 2018) from all over the world, it is likely that palatability varies widely across populations and species. Whole toxin profile examination provides insight into this chemical diversity however, it lacks the ability to identify the specific alkaloid components of the diverse toxin cocktail that elicit aversion. Our unpalatability assay offers a new perspective on the linkage between chemical defense and predator response (Lawrence et al. 2019a), which together drive the evolution of aposematic phenotypes. Coupling predator responses with individual alkaloid profiles offers the opportunity to isolate which individual alkaloids or combinations of alkaloids influence predator aversive responses. Given the large variety of alkaloids present in toxin profiles of *D. tinctorius* and the small sample size of this study, we acknowledge that the interpretation of our results is limited. However, our study does provide a novel approach for future studies to tease out the contributions of individual alkaloids in driving predator response. Chemical identification alone does not explain how these defensive compounds function in antipredator behavior, and likewise, predator assays do not address the question why predators respond as they do. By using this comprehensive approach of both chemical identification and predator assay, we are able to tease apart the function of defensive compounds.

The proximate causes of predator behavior remain unknown and unstudied, with prior studies focusing on the effects of alkaloid defenses on predators (e.g., Daly and Myers 1967; Maan and Cummings 2012; Bolton et al. 2017) or identification of alkaloids (e.g., Daly et al. 1987; Saporito et al. 2006; McGugan et al. 2016). Given the variability of alkaloids within and among populations, a subset of alkaloids that may be common to most individuals in a population may be responsible for predator responses. Consistency in predator response, despite varied alkaloid profiles, would be important for aposematism to evolve. In this study, we identify 15 alkaloids implicated in predator response for only the Matoury population. Interestingly, 14 of the 15 alkaloids are unique to this population, with only the spiropyrrolizidine **236** being common to both. Six of these fifteen alkaloids are 5,6,8-trisubstituted indolizidines. This alkaloid class has the highest representation, both in terms of quantity and diversity, of the fifteen implicated alkaloids, perhaps suggesting the importance of this class in eliciting aversive responses in avian predators. The lack of significant loadings in the Kaw population is a puzzling result given the strong avoidance of skin extracts seen for this population (Lawrence et al. 2019a, b). We suspect that this may be a function of the low sample size for this population. Alternatively, perhaps there are more aversive alkaloids in Kaw defenses and our analysis was unable to distinguish between loadings. As 39.1% of the alkaloids in the Kaw population are only represented once,

it is plausible that this is not enough to determine whether these singletons are responsible for predator response. Our approach narrows this large variation into a subset of alkaloids for future studies, allowing for investigations of the proximate causes of predator behavior.

As *Dendrobates tinctorius* is highly polytypic throughout its distribution (Noonan and Gaucher 2006; Wollenberg et al. 2008; Lawrence et al. 2019a), future research should focus on further characterizing alkaloid diversity among populations and across time. Given that 16 of the 81 (19.7%) alkaloids described here have not previously been described in the literature, we speculate that a large number of undescribed alkaloids are present in other populations of *D. tinctorius*. Further, our research represents the first study that seeks to understand the drivers of predator response. Future research should expand upon this to determine if there is a subset of toxins that are primarily responsible for predator response. Doing so will give predictive power to future alkaloid characterization studies in how predators will respond to toxins. Importantly, while our study is not able to tease apart which alkaloids may be driving response, our approach narrows down diverse alkaloid profiles into potentially functional alkaloids important for predator responses.

Aposematism is a complex interplay between a warning signal (i.e., coloration) and a secondary defense mechanism (e.g., toxins). While a large amount of research has focused on the warning signal and the psychology of learned avoidance, considerably less research has focused on understanding intraspecific variation in secondary defense and its consequences for predator response, despite being more common than previously thought (Speed et al. 2012). By examining which toxins may be important in driving predator response, we can now begin to predict how predators will respond to highly varied secondary defenses both within and among populations of aposematic prey. Importantly, this study highlights how aposematism may evolve under conditions where secondary defense may appear highly variable. In the case of these two populations, while individual profiles are highly variable within and between populations, there is an overlap among individuals in the Matoury population of alkaloids that are implicated in predator response. This suggests that while there may be considerable noise in alkaloid profiles, there are core alkaloids that are not as variable among individuals. These core alkaloids may provide the consistency in protection necessary for aposematic warning signals to be maintained. Without consistent secondary defenses, which are at risk for those with a dietary origin, aposematism may break down when predators are unable to associate, at least qualitatively, conspicuous signals with predictable defenses. Identifying these core alkaloids (and thus reducing the noise of alkaloid variation among individuals) will allow us to better address concepts such as automimicry and honest signaling that are hypothesized drivers of diversification in aposematic signals (Speed et al. 2006, 2010; Maan and Cummings 2012). Diversity within and among populations likely varies over space and time. It is this variation that provides fertile ground for further exploration of aposematic color evolution as it likely evolves under specific conditions of alkaloid availability and predator community.

Acknowledgements We highly value equity, diversity, and inclusion in science (EDI) and thus took into account EDI best practice. Our team includes researchers from (4) different countries (United States, Colombia, Finland, and France), and diverse backgrounds, and career stages, all of which significantly contributed to the fulfillment and quality of our study. The data used in this study can be found in Lawrence, et al. 2019b. Blue tit experiments were conducted under the Central Finland Centre for Economic Development, Transport, and Environment and license from the National Animal Experiment Board (ESAVI/9114/04.10.07/2014), and the Central Finland Regional Environment Centre (VARELY/294/2015), respectively. Animals were exported under CITES permit FR1397300015-E. This study was supported in part by an Investissement d'Avenir Grant (CEBA ANR-10-LABX-25-01) of the Agence Nationale de la Recherche; the American Society of Ichthyologists and Herpetologists' Gaige Award (to JPL); and a Society for the Study of Amphibians and Reptiles' grant in Herpetology (to JPL). BR and JM were funded by the Finnish Centre of Excellence in Biological Interactions (Project 28466, to JM.). BR also acknowledges funding from the Academy of Finland (Academy Research Fellowship, No. 318404).

Author Contributions All authors contributed to the study conception and design. Material collection, data preparation, and analysis were performed by J.P.L., B.R., J.M., A.B., and R.S. Samples were collected by A.F. and B.R. J.P.L. and B.N. wrote the first draft of the manuscript, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding This study benefited from an "Investissement d'Avenir" grant managed by the Agence Nationale de la Recherche (CEBA, ref. ANR-10- LABX-25-01. B.R. and J.M were funded by the Finnish Centre of Excellence in Biological Interactions (Project 28466, to J.M.). B.R. is supported by funding from the Academy of Finland (Academy Research Fellowship No. 318404).

Data Availability Data are available at Lawrence et al. 2019b.

Declarations

Competing Interests The authors declare no competing interests.

Conflict of Interest The authors declare no conflicts of interest in relation to this study.

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