2022

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Funding information
Conselho Nacional de Desenvolvimento Científico e Tecnológico, Grant/Award Numbers: 305266/2019-5, 306823/2017-9, 309589/2017-7; Japan Society for the Promotion of Science Kakenhi, Grant/Award Number: JP19K05461; Fundação de Amparo à Pesquisa do Estado de São Paulo, Grant/Award Numbers: 2012/10000-5, 2016/09999-9, 2018/15425-0; Astellas Foundation for Research on Metabolic Disorders; Colleran-Weaver; JGC-S Scholarship Foundation; Kresge Foundation

Abstract

Sequestration of chemical defenses from dietary sources is dependent on the availability of compounds in the environment and the mechanism of sequestration. Previous experiments have shown that sequestration efficiency varies among alkaloids in poison frogs, but little is known about the underlying mechanism. The aim of this study was to quantify the extent to which alkaloid sequestration and modification are dependent on alkaloid availability and/or sequestration mechanism. To do this, we administered different doses of histrionicotoxin (HTX) **235A** and decahydroquinoline (DHQ) to captive-bred *Adelphobates galactonotus* and measured alkaloid quantity in muscle, kidney, liver, and feces. HTX **235A** and DHQ were detected in all organs, whereas only DHQ was present in trace amounts in feces. For both liver and skin, the quantity of alkaloid accumulated increased at higher doses for both alkaloids. Accumulation efficiency in the skin increased at higher doses for HTX **235A** but remained constant for DHQ. In contrast, the efficiency of HTX **235A** accumulation in the liver was inversely related to dose and a similar, albeit statistically nonsignificant, pattern was observed for DHQ. We identified and quantified the *N*-methylation of DHQ in *A. galactonotus*, which represents a previously unknown example of alkaloid modification in poison frogs. Our study suggests that variation in alkaloid composition among individuals and species can result from differences in sequestration efficiency related to the type and amount of alkaloids available in the environment.

KEYWORDS
Adelphobates galactonotus, biomodification, chemical defense, dendrobatidae, histrionicotoxin, lipophilicity

1 | INTRODUCTION

Numerous animal lineages have independently evolved the ability to sequester defensive chemicals from their diet—a process that involves the absorption, transport, and storage of extrinsic molecules for use in protection against predators and/or pathogens (Dussourd et al., 1989; Pasteels et al., 1983; Savitzky et al., 2012). Rather than sequestering a broad diversity of chemicals, most animals sequester a specific type or group of related chemicals, which is generally associated with dietary specialization. Well-known examples include monarch caterpillars that sequester cardenolides from milkweed (Brower et al., 1968), dorid nudibranchs that sequester terpenoids and terpene metabolites from marine sponges (Dean & Prinsep, 2017), and poison frogs that sequester lipophilic alkaloids from mites and ants (Saporito et al., 2012).

The degree of specificity can be remarkable. For example, flea beetles do not sequester all of the alkaloids present in the plants upon which they feed (Dobler et al., 2000), and chrysomelid leaf beetles sequester the alkaloids present in their hostplant more efficiently than those of other plants (Hartmann et al., 1997). Further, dietary sequestration can also involve enzymatic modifications, some of which act to increase uptake of defensive chemicals and/or convert sequestered chemicals into more or less toxic forms (Daly et al., 2003; Hartmann et al., 1990, 1997; Lindigkeit et al., 1997). The selectivity and modification of sequestered chemicals suggest the evolution of specific physiological mechanisms that are unique to particular lineages and types of chemicals.

Although sequestration of defensive chemicals appears to be rare among vertebrates (Savitzky et al., 2012), the ability to sequester lipophilic alkaloids from dietary arthropods and secrete them from dermal granular glands evolved at least four times within the neotropical poison frog family Dendrobatidae (Jones et al., 2012; Santos, 2012; Saporito et al., 2009, 2011, 2015; Grant et al., 2017). The quantity of a given alkaloid in skin secretions is highly variable, depending largely on environmental availability. For example, histrionicotoxin (HTX) alkaloids are absent from wild-caught *Oophaga lehmanni* but readily sequestered when administered in laboratory-based feeding experiments, suggesting that the arthropod source is either not sympatric with or not consumed by *O. lehmanni* (Garraffo et al., 2001; Jones et al., 2012). However, experimental studies have also revealed variation in the efficiency with which different species sequester certain alkaloids (Daly et al., 2000, 2003; Saporito et al., 2019). For example, laboratory-raised *Dendrobates auratus* readily sequester decahydroquinoline (DHQ), indolizidine, quinolizidine, and HTX alkaloids, but not pyrrolidine or piperidine alkaloids (Davison et al., 2021; Garraffo et al., 2001).

Most alkaloids appear to be sequestered unchanged from their dietary source, but some dendrobatid poison frogs modify certain alkaloids following ingestion. For example, *Dendrobates* and *Adelphobates* species selectively hydroxylate dietary pumiliotoxin (+)-**251D** to allopumiliotoxin (+)-**267A**, whereas *Epipedobates* and *Phyllobates* species do not (Daly et al., 2003). Similarly, it also appears that the *N*-methyl DHQ class of alkaloids, which naturally occur in several species of *Ameerega* but not sympatric species of *Adelphobates*, result from the *N*-methylation of DHQ alkaloids derived from a diet of ants (Daly et al., 2009), although this hypothesis has not been tested experimentally.

The aim of this study was to quantify the extent to which alkaloid sequestration and modification are limited by either alkaloid availability or the sequestration mechanism itself (i.e., the organism's physiological ability to sequester alkaloids). To that end, we orally administered different, known doses of HTX **235A** and synthetic DHQ to captive-bred individuals of *Adelphobates galactonotus*, which enabled us to quantify variation in the amount of these alkaloids in different organs and feces and calculate the efficiency of accumulation (i.e., the percentage of the administered alkaloid detected in the frog), and the rate of conversion. Finally, because the absorption, movement across plasma membranes, metabolism, and excretion of ingested compounds are partly dependent on their lipophilicity (Lapins et al., 2018; Leo et al., 1971; Lipinski et al., 1997), we also modeled alkaloid lipophilicity as a possible explanation of our findings.

2 | MATERIALS AND METHODS

2.1 | Poison frogs

Adelphobates galactonotus (Steindachner, 1864) is a chromatically polytypic dendrobatid poison frog distributed south of the Amazon River in Brazil (Hoogmoed & Avila-Pires, 2012). It contains a broad diversity of dietary alkaloids (Daly et al., 2009; Jeckel et al., 2019) and hydroxylates dietary pumiliotoxin (+)-**251D** to form allopumiliotoxin (+)-**267A** (Daly et al., 2003). The *A. galactonotus* specimens used in this study were captive-bred at the Butantan Institute, São Paulo, Brazil. All frogs were 1-year-old subadult F1s bred by us from adults collected at Floresta Nacional de Caxiuanã, Pará, Brazil in January 2017. Before commencing experiments, all frogs were maintained in terraria with standard humidity (ca. 90%), temperature (ca. 23°C), and light cycle (12 h photoperiod; Lötters et al., 2007) and fed three times per week with fruit flies and crickets dusted with vitamin powder (NEKTON-Rep; Nekton Produkte). During the experiment, each frog was transferred to an individual plastic container (15 × 15 × 13 cm) with holes for ventilation and containing a wet paper towel substrate, water dish, and objects for concealment. The humidity, temperature, light cycle, and feeding regimen were as described above.

2.2 | Experimental design

2.2.1 | Alkaloids

We used two alkaloids in the experiment: HTX **235A** and DHQ (Figure 1a). A racemic mixture of HTX **235A** was synthesized as described by Matsumura et al. (2018, 2021), and *cis*- and *trans*-DHQ (Acros Organics) was purchased from Sigma-Aldrich.

2.2.2 | Alkaloid administration

Previous studies have combined known quantities of alkaloid with a vitamin powder that is then dusted onto fruit flies that are subsequently fed to frogs (e.g., Daly et al., 1994; O'Connell et al., 2021). This method has proved useful in testing several aspects of alkaloid sequestration, including selectivity (e.g., Daly et al., 1994), modification (e.g., Daly et al., 2003), and relative differences in uptake (e.g., Hantak et al., 2013; Saporito et al., 2019), but it does not allow quantitative assessment of sequestration because the amount of alkaloid on individual fruit flies is both unknown and variable (personal observation and e.g., O'Connell et al., 2021).

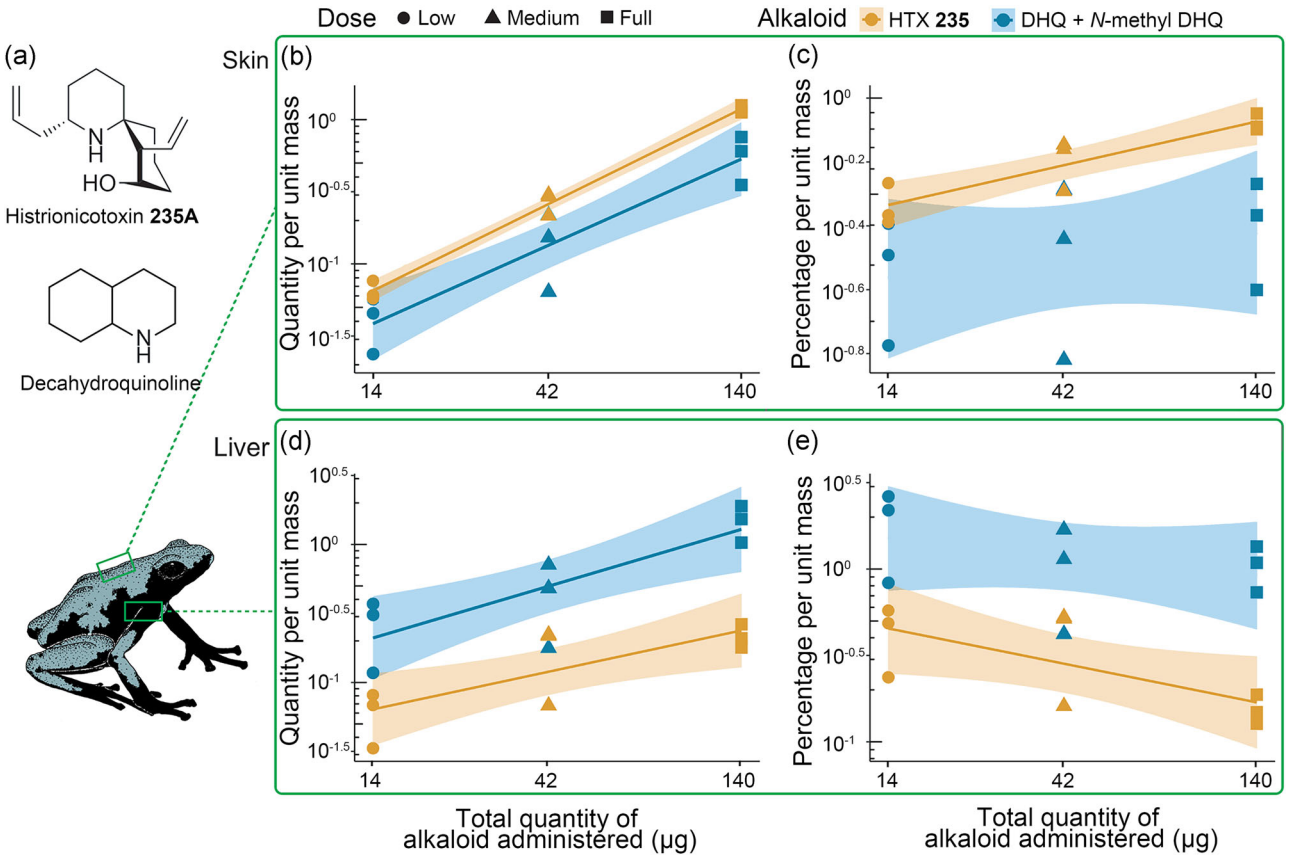

FIGURE 1 Dose response and efficiency of accumulation of histrionicotoxin (HTX) **235A** and decahydroquinoline (DHQ + *N*-methyl DHQ). (a) Chemical structures of HTX **235A** and DHQ used in the experiments. Dose response accumulation of both alkaloids in (b) skin and (d) liver. Accumulation efficiency of both alkaloids in (c) skin and (e) liver. Each symbol represents an individual of *Adelphobates galactonotus* for each experimental group ($n = 3$). Quantity and percentage of alkaloid detected per unit mass were $\log_{10}$ transformed for the simple regression. Regression lines are only included for results that were statistically significant

Instead, we orally administered 5 µl of a known quantity of alkaloid (see below) in a 50% ethanol solution using a micropipette. We gently opened the mouth of each frog with a disposable plastic pipette, allowing the tip of the micropipette to be inserted into the oral cavity of the frog before delivering the alkaloid solution. Alkaloids found in poison frogs are lipophilic; for this reason, we used the 50% ethanol solution to dissolve the alkaloids used in this study. To ensure the frogs would not be negatively affected by the 50% ethanol solution, we conducted a 2-week pilot study with six adult, lab-raised *Dendrobates auratus* (Girard, 1853) in which we monitored loss of appetite, emaciation, lethargy, and/or uncoordinated movements as potential signs of intoxication or disturbance in metabolism (Lötters et al., 2007). None of these symptoms were observed in the pilot study, but we also monitored experimental animals for the same symptoms throughout the course of the study. During the 21-day experiments (14 days of alkaloid administration + 7 days of latency), we fed the frogs three times per week with fruit flies and crickets dusted with vitamin powder.

Studies of vertebrate herbivores have shown that certain toxins can be removed through the feces without modification by gut mucosal metabolism (Patey et al., 2020; Sorensen et al., 2004; Thacker et al., 2012). Therefore, we collected all feces during the experiment to determine if alkaloids were present. Previous amphibian pharmacokinetics studies have reported a half-life of 1.2–48 h for administered drugs to be cleared from the circulatory system (D'Agostino et al., 2007; Fox & Russell, 1987; Guénette et al., 2007, 2008; Howard et al., 2010; Lalonde-Robert et al., 2012; Rifkin et al., 2017). Assuming that circulating alkaloids from our feeding experiments act similarly, and to ensure that unsequestered alkaloids had cleared the frog's systems before chemical analysis, we euthanized frogs 7 days after the last alkaloid treatment.

Previous studies have reported sequestered alkaloids in several organs in addition to the skin (Grant et al., 2012; Stynoski et al., 2014). As such, following euthanasia by cooling followed by flash freezing in liquid nitrogen (Lillywhite et al., 2017; Shine et al., 2015), we collected the entire skin, liver, kidney, and the muscles from one thigh. All organs and feces were stored at room temperature in 1 ml of 100% methanol in a 4-ml glass vial with Teflon-lined lids until preparation for alkaloid analysis. Skin and liver samples were dried in a vacuum oven at 60°C for 24 h and then weighed. Muscle samples were not dry weighed.

2.2.3 | Alkaloid dosage

To ensure that the amounts of alkaloid administered in our experiments were biologically realistic, we estimated daily alkaloid consumption in nature. On the basis of stomach contents (Supporting Information 1), we estimated a daily rate of ant consumption of 61 ants per day. The estimated rate corresponds to a mean alkaloid intake of 18.2 μg/day. We corrected for the difference on average body mass between three wild-caught adult *A. galactonotus* (4.3 ± 0.5 g) and the subadults used in our study (2.3 ± 0.4 g), resulting in a full daily dosage of 9.8 μg of alkaloid, which we rounded to 10.0 μg to facilitate preparation of alkaloid solutions.

From the 20 individuals available for the study, we randomly assigned 2 individuals to the control group and the remaining 18 individuals to one of three experimental groups for each alkaloid (i.e., three individuals per experimental group) and administered one of three alkaloid doses to each experimental group every day for 14 days. We determined sample size ($N = 3$) on the basis of frog and alkaloid availability, as well as results of previous studies that examined alkaloid sequestration in laboratory-based feeding experiments (e.g., Hantak et al., 2013; O'Connell et al., 2021). The first experimental group received a low dose (10% of the full dose = 1.0 μg), the second received a medium dose (30% of the full dose = 3.0 μg), and the third received the full dose (100% = 10.0 μg). Each dose was diluted in 5 μl of 50% ethanol. Each individual in the experimental group was administered 5 μl doses of only one alkaloid (DHQ or HTX **235A**) in a 50% ethanol solution. Individuals in the control group received 5 μl doses of 50% ethanol without alkaloid.

2.3 | DHQ methylation methods

To confirm the presence of *N*-methylated DHQ in skin extracts, we compared mass and infrared spectral properties of synthetic *N*-methyl DHQ to the supposed *N*-methyl DHQ from the skin extracts (Supporting Information 2). We also compared spectral properties to previously identified *N*-methyl DHQs obtained from other dendrobatid frogs (Daly et al., 2005, 2009).

2.4 | Gas chromatography-mass spectrometry analysis

We isolated alkaloids from individual methanol extracts using an acid–base extraction and nicotine internal standard (Jeckel et al., 2015; Saporito et al., 2010) (Supporting Information 3).

2.5 | Lipophilicity analysis

The two most common measures of lipophilicity are log*P*, which is the log of the partition coefficient of a neutral (or nonionized) molecule between two immiscible solvents (e.g., octanol:water), and log*D*, which is the distribution coefficient, which considers both the ionized and non-ionized forms of a molecule at a specific pH. Given that frog alkaloids are ionizable and subject to changes in pH in the digestive tract of frogs, we calculated and compared the predicted log*D* for HTX **235A**, DHQ, *N*-methyl DHQ using the log*D* function in MarvinSketch (version 20.13) and plotted the results with GraphPad Prism (version 8.4.2). For comparison to a common, naturally found DHQ, which also represents the parent member of the DHQ class of frog alkaloids (Daly et al., 2005), we also included DHQ **195A** in the log*D* analysis.

2.6 | Statistical analysis

Before statistical analysis, we used the dry mass of all tissue samples to calculate the quantity of alkaloids detected per unit mass. We then transformed the data to $\log_{10}$ for analysis. To test for differences in accumulation efficiency among experimental groups of the same alkaloid and between alkaloids, we performed simple regression analyses. We performed an analysis of covariance (ANCOVA) to evaluate differences in the slope of accumulation curves between alkaloids and then utilized a Tukey posthoc to test for differences in adjusted means between alkaloids. Statistical analyses and graphical representations (package ggplot2; Wickham, 2016) were performed using R-3.6.0 (R Core Team, 2019).

3 | RESULTS

3.1 | HTX 235A and DHQ dose–response accumulation and efficiency

None of the frogs exhibited signs of intoxication. We detected both HTX **235A** and DHQ in the skin, liver, muscle, and kidney of all individuals of *A. galactonotus*. The alkaloids in the skin and liver were quantifiable; however, we were only able to reliably quantify the alkaloids in the muscle extracts from the "full" dose group (Table 1) due to the small amount in the muscle and kidney samples. We report the remaining muscle and all kidney extracts as containing trace amounts (<100 ng). Feces contained detectable amounts of alkaloid exclusively in the "full" dose group of DHQ, but in trace amounts (<100 ng). No alkaloid was detected in the control frogs.

In the skin, alkaloid quantification showed a dose response accumulation of both alkaloids, with greater amounts of administered alkaloid resulting in more alkaloid accumulating in the skin (DHQ: $F_{1,7} = 47.34$, $p < 0.0001$, $r^2 = 0.87$; HTX **235A**: $F_{1,7} = 717.8$, $p < 0.0001$, $r^2 = 0.99$, Figure 1b). Even though the accumulation slopes did not differ between alkaloids (ANCOVA: $F_{1,14} = 0.48$, $p = 0.5$), greater quantities of HTX **235A** were accumulated at all doses relative to DHQ (Tukey: $p = 0.001$; Figure 1b). Additionally, HTX **235A** accumulation efficiency increased at higher concentrations ($F_{1,7} = 30.86$, $p = 0.001$, $r^2 = 0.82$), whereas DHQ efficiency remained constant ($F_{1,7} = 0.73$, $p = 0.42$, $r^2 = 0.09$; Figure 1c and Table 1).

TABLE 1 Total amount and percentage of alkaloids detected in the organs of *Adelphobates galactonotus* per experimental group (Histrionicotoxin [HTX] **235A** and decahydroquinoline [DHQ])

		Skin (μg)	Skin (%)	Liver (μg)	Liver (%)	Muscle sample (μg)	Muscle sample (%)	Kidney (μg)	Kidney (%)
DHQ	Full	25.5 ± 4.0	18.2 ± 2.9	10.2 ± 3.2	7.3 ± 2.3	1.5 ± 0.2	1.1 ± 0.1	Trace	Trace
	Medium	7.1 ± 1.8	16.9 ± 4.2	3.9 ± 1.2	9.4 ± 2.9	Trace	Trace	Trace	Trace
	Low	2.0 ± 0.2	14.4 ± 1.2	2.1 ± 0.2	15.0 ± 1.5	Trace	Trace	Trace	Trace
HTX **235A**	Full	67.4 ± 13.8	48.2 ± 9.9	2.0 ± 0.4	1.4 ± 0.3	0.6 ± 0.1	0.4 ± 0.1	Trace	Trace
	Medium	13.4 ± 0.8	31.9 ± 1.8	1.2 ± 0.4	2.8 ± 1.0	Trace	Trace	Trace	Trace
	Low	3.8 ± 0.4	27.5 ± 3.1	0.5 ± 0.1	3.5 ± 0.5	Trace	Trace	Trace	Trace

Note: Quantities <100 ng are shown as trace amounts. Mean ± SD.

Different relationships were found for the liver and muscle samples. The quantity of both alkaloids also increased at higher doses in the liver (DHQ: $F_{1,7} = 15.42$, $p = 0.006$, $r^2 = 0.69$; HTX **235A**: $F_{1,7} = 10.98$, $p = 0.01$, $r^2 = 0.61$; Figure 1d), but there was more DHQ than HTX **235A** (Figure 1d). Further, the percentage of alkaloid in the liver was inversely related to HTX **235A** dosage, with alkaloid retention being proportionally higher in the "low" dose group compared to the "full" dose group ($F_{1,7} = 6.16$, $p = 0.04$, $r^2 = 0.49$; Figure 1e). A similar relationship was observed for DHQ in the liver, suggesting this might be biologically important; however, it was not statistically significant ($F_{1,7} = 1.11$, $p = 0.33$, $r^2 = 0.14$). Although we were only able to quantify alkaloids in muscle for the "full" dose (see above), muscle also contained more DHQ than HTX **235A** (Table 1).

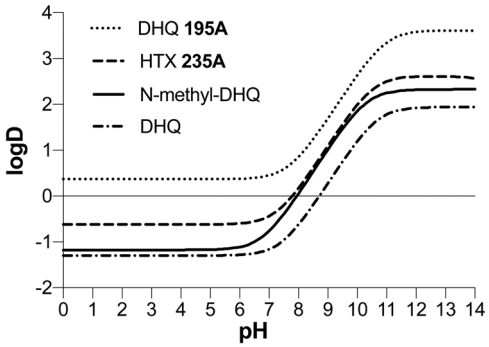

FIGURE 2 Predicted log*D* of decahydroquinoline (DHQ), *N*-methyl decahydroquinoline (*N*-methyl-DHQ), decahydroquinoline **195A** (DHQ **195A**), and histrionicotoxin **235A** (HTX **235A**) ranging from 0 to 14 pH

3.2 | *N*-methylation of DHQ

All frog skin extracts possessed unmodified HTX **235A**, but 31.5% ± 6.9% of the accumulated DHQ (5.1% ± 0.9% of the total ingested DHQ) and 28.4% ± 5.3% of the DHQ in the liver (3.0% ± 1.3% of the total ingested DHQ) was *N*-methylated (also see Supporting Information 2).

3.3 | Lipophilicity

The predicted log*D* indicates that DHQ **195A** is most lipophilic, followed by HTX **235A**, *N*-methyl DHQ, and DHQ (Figure 2).

4 | DISCUSSION

Our experiments demonstrated that total quantities of accumulated HTX **235A** and DHQ increase with higher doses. However, efficiency of accumulation differs among alkaloids and organs analyzed, suggesting that the quantity of alkaloid in a frog is limited by both the environmental availability of dietary sources and the mechanism of sequestration, in conjunction with other intrinsic factors. For example, Stynoski et al. (2014), and Jeckel et al. (2015) found that larger individuals of the dendrobatid poison frog *Oophaga pumilio* and the bufonid poison frog *Melanophryniscus moreirae*, respectively, had greater quantities of alkaloid than smaller individuals, which they explained as owing to the greater size and abundance of granular glands for alkaloid storage in larger individuals.

Accumulation in the skin was more efficient at higher doses for HTX **235A** but remained constant at all doses for DHQ. In contrast, the efficiency of HTX **235A** accumulation in the liver was inversely related to dose, and a similar, albeit statistically nonsignificant, pattern was observed for DHQ. Although seemingly contradictory, these results may provide an important insight into the mechanism of alkaloid sequestration in poison frogs. Dietary uptake of alkaloids likely involves passage through the liver before accumulation in the skin (Jeckel et al., 2020; O'Connell et al., 2021), which has been demonstrated for the dietary uptake of tetrodotoxin in pufferfish (Kono et al., 2008). Our experiments suggest the existence of an as-yet-unidentified mechanism associated with the liver that may be triggered (or increased) following ingestion of larger amounts of alkaloids, possibly resulting in quicker transport from the liver to skin glands. Kowalski et al. (2020) provided evidence of dose-dependent expression of ABCB transporters for diet-derived cardenolide-defenses in leaf beetles, and a similar process could exist in poison frogs. Although this hypothesis appears to contradict the proposal of

Caty et al. (2019) that expression of saxiphilin (a presumed alkaloid transporter) might be upregulated when alkaloids are scarce in the blood to increase the capture and transport of low quantities of alkaloid, additional biochemical and regulatory experiments are needed to better understand sequestration and how it is regulated.

The biological role of alkaloids in nonintegumentary tissues is unclear. Savitzky et al. (2012; p. 145) defined sequestration as "the evolved retention within tissues of specific compounds, not normally retained in the ancestors of the taxon in question, which confers a selective advantage through one or more particular functions." The accumulation of alkaloids in the dermal granular glands clearly meets that definition (Conlon, 2011; Mina et al., 2015; Savitzky et al., 2012; Toledo & Jared, 1995), as does the accumulation of alkaloids in nutritive oocytes and tadpoles in the dendrobatids *Oophaga granulifera*, *O. pumilio*, and *O. sylvatica* (Fischer et al., 2019; Saporito et al., 2019; Stynoski et al., 2014). Although it is unlikely that the comparatively meager accumulation of alkaloids in liver and muscle significantly enhances antipredator defenses beyond the more formidable integumentary chemical defense, it is conceivable that it confers a selective advantage by defending against pathogens and/or parasites inside the body (cf., Robert et al., 2017; but see Santos et al., 2018), thereby qualifying as true sequestration. Nevertheless, until that hypothesis is tested, it is preferable to refer to this occurrence agnostically as accumulation.

Our study revealed significant differences in accumulation efficiency in the skin between HTX **235A** and DHQ, independent of dosage, in *A. galactonotus*. Different relative sequestration efficiencies among alkaloids have been shown previously (Daly et al., 1994, 2003; Hantak et al., 2013), but this is the first time these differences have been quantified. Although the difference between alkaloids was clear, with, on average, twice as much HTX **235A** accumulated than DHQ, we also observed differences among individuals. Our findings suggest that differences in uptake efficiency are an important factor in explaining the variation in alkaloid composition among individuals, populations, and species of poison frogs.

The differences in accumulation between HTX **235A** and DHQ could also be the result of differences in lipophilicity between these two alkaloids. The absorption, movement across plasma membranes, metabolism, and excretion of compounds (including alkaloids) are partly dependent on lipophilicity (Lapins et al., 2018; Leo et al., 1971; Lipinski et al., 1997). Our results suggest that HTX **235A** is more lipophilic than DHQ (Figure 2), which might explain its increased accumulation. Although the precise location of uptake in the frog digestive system is unknown, the pH can vary from 6.5 in the esophagus to 4.5 in the stomach and 7.5 in the small intestine (Yang et al., 2019). Furthermore, in a fasting frog, the pH of the stomach can be as low as 3 (Takeuchi et al., 1983). Although these differences will affect lipophilicity, HTX **235A** was consistently more lipophilic throughout the pH range in our models. Interestingly, the predicted lipophilicity of *N*-methyl DHQ was greater than that of DHQ and was almost identical to that of HTX **235A** at pH greater than 6.

Although the function of *N*-methylation is not known, it could be related to the movement, metabolism, or excretion of DHQs. Further, DHQ serves as the backbone structure for the 2,5-disubstituted DHQs present in wild-caught frogs; however, DHQ alone lacks the substitutions in the C-2 and C-5 positions that are common to most DHQs found naturally in dendrobatids. Although the importance of these substitutions for alkaloid uptake is not known, a comparison of the DHQ backbone to DHQ **195A** (a common DHQ present in frogs and containing the C2 and C5 substitutions) shows that the latter has greater lipophilicity (Figure 2). Additional studies are needed to determine the role of lipophilicity in alkaloid uptake and the degree to which our predicted (i.e., modeled) results correspond to what occurs in these frogs.

Animals that are dependent on food for chemical protection usually possess mechanisms to prevent autointoxication, such as physiological mechanisms that regulate the quantity of compounds available in the body (e.g., induced mucosal and hepatic enzymatic detoxification; Gordon et al., 2000) or gastrointestinal motility that lowers the amount of toxin available for absorption (Camara, 1997). Many herbivores eliminate a portion of the ingested compounds as a strategy to avoid self-intoxication (Amidon et al., 1995; Brückmann et al., 2000; Ehmke et al., 1990; Sorensen et al., 2004). By analyzing the feces of the poison frogs, we aimed to test whether some portion of the alkaloids was eliminated before absorption; however, we detected either trace amounts or no alkaloids at all in the feces, suggesting the lack of elimination of unmodified alkaloids and that the undetected portion of the consumed alkaloid was metabolized for detoxification and elimination or storage.

Alkaloid-based defenses in dendrobatids appear largely to be the result of uptake and sequestration of chemically unchanged alkaloids from dietary arthropods (Saporito et al., 2009, 2012). To date, the only known exception involves species of *Dendrobates* and *Adelphobates* in which more than 70% of dietary pumiliotoxin (+)-**251D** is converted to allopumiliotoxin (+)-**267A** by an enantio- and stereoselective hydroxylase (Daly et al., 2003). Although the chemical and/or physiological function(s) of pumiliotoxin hydroxylation has not been studied, subcutaneous injection of aPTX **267A** into mice results in a five-fold increase in toxicity when compared to PTX **251D**, suggesting a possible defensive function (Daly et al., 2003). Herein, we provide experimental evidence that *A. galactonotus* is capable of an additional modification, specifically the *N*-methylation of synthetic DHQ. Approximately 31.5% of the accumulated dietary DHQ in the skin and 28.4% of that detected in the liver was *N*-methylated to *N*-methyl DHQ. Indeed, the high amounts of alkaloids detected in the liver could be due to the *N*-methylation process, but further analysis is needed to confirm this assumption.

Decahydroquinoline serves as the backbone structure of the more than 35 alkaloids in the 2,5-disubstituted DHQ class yet lacks the 2,5 substitutions that are common to most DHQs present in dendrobatids. Although the mechanism by which *N*-methylation occurs in *A. galactonotus* is unknown, it is likely to be enzyme mediated (similar to hydroxylation of PTX **251D**), possibly by an *N*-methyl transferase, an enzyme class that is common in animals (Weinshilboum et al., 1999); however, it is also possible that the presence of *N*-methylated DHQs in wild-caught frogs is due to the

consumption of *N*-methylated alkaloids and absence of a demethylation process. Future studies will need to examine the mechanism by which *N*-methylation occurs, as well as the potential chemical and physiological function(s) of *N*-methylation.

Our experimental demonstration that *A. galactonotus* *N*-methylates dietary DHQ suggests that some, if not all, naturally occurring *N*-methyl DHQs are the result of *N*-methylation. *N*-methyl DHQs were originally discovered and characterized in several species of *Ameerega* (Daly et al., 2009) but have since been detected in some species of *Adelphobates*, *Oophaga*, *Dendrobates*, and *Ranitomeya*, including a small amount of the *N*-methyl DHQ **245Q** in wild-caught *A. galactonotus* (Hovey et al., 2018; Jeckel et al., 2019; Lawrence et al., 2019; Stuckert et al., 2014). *N*-methyl DHQs are best known in species of *Ameerega*, yet there appears to be variation in their presence, which might indicate differences in the ability to *N*-methylate. Among the examined *Ameerega* species, *A. macero*, *A. parvula*, *A. picta*, *A. trivittata*, and the formally undescribed *Ameerega* sp. "Porto-Walter1" (sister species of *A. macero*; Guillory et al., 2020; Grant et al., 2017; Twomey & Brown, 2008) possessed either one or both of the DHQs **219A** and **243A** and their *N*-methylated versions (*N*-methyl DHQs **223C** and **257A**; Daly et al., 2009). Individuals of *A. cainarachi* and *A. hahneli* varied in the amounts of *N*-methyl DHQs and corresponding DHQs, presenting lower amounts of *N*-methyl DHQs than DHQs, suggesting differences in the ability or extent of methylation in these species. Among the remaining *Ameerega*, *A. bassleri*, *A. bilinguis*, *A. petersi*, and *A. pulchripecta* contained DHQs without the corresponding *N*-methylated DHQs, suggesting that these species are unable to *N*-methylate, and *A. erythromos* and *A. silverstonei* did not contain DHQs or *N*-methyl DHQs. The biological and chemical significance of *N*-methylation, as well as the taxonomic distribution of *N*-methylation among dendrobatids is unknown.

We have shown that, although the accumulation of alkaloids in poison frogs is mainly limited by alkaloid availability in dietary items, accumulation efficiency can be dose-dependent and differ among classes of alkaloids. We have also provided experimental evidence for the biomodification of sequestered alkaloids by poison frogs, specifically the *N*-methylation of sequestered DHQ. Thus, in addition to previously known factors—including geographic location (Saporito et al., 2006; Saporito et al., 2007), season (Basham et al., 2020; Saporito et al., 2006), and age and life stage (Daly et al., 2002; Jeckel et al., 2015; Stynoski et al., 2014)—variation in accumulation efficiency in relation to both the type and amount of alkaloid also contributes causally to the high variation in alkaloid composition among poison frog individuals and species. We also raise the possibility that differences in alkaloid lipophilicity might affect sequestration efficiency. The role that physiological adaptations play in uptake and the ecological consequences of differences in sequestration and modification efficiency of dietary alkaloids remain to be tested.

ACKNOWLEDGMENTS

We thank J. P. Marcos, I. Cavalcanti, V. Seiko, and the staff of Structural Biology Lab, Instituto Butantan, for assistance in the maintenance of the frogs. We also thank M. Postal, M. Rada, R. Montesinos, J. P. Marcos, and M. Anganoy-Criollo for assistance during alkaloid administration. We thank M. Nichols for his assistance in maintaining the GC-MS. This study was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq Proc. 306823/2017-9 to Taran Grant, Proc. 305266/2019- 5 to Carlos Jared, and Proc. 309589/2017-7 to Marta M. Antoniazzi), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP Proc. 2012/10000-5 and 2018/15425-0 to Taran Grant and 2016/09999-9 to Adriana M. Jeckel), Kresge Foundation (Ralph A. Saporito [RAS]), Colleran-Weaver (RAS), Astellas Foundation for Research on Metabolic Disorders, JGC-S Scholarship Foundation (Nikki Saneyoshi Scholarship), and Japan Society for the Promotion of Science Kakenhi (grant number JP19K05461 to Kunihiro Matsumura, Keisuke Nishikawa, and Yoshiki Morimoto).

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Adriana M. Jeckel, Taran Grant, and Ralph A. Saporito conceived, designed, and coordinated the study. Adriana M. Jeckel coordinated the maintenance and reproduction of frogs in captivity, performed the alkaloid feeding experiments, collected and analyzed GC-MS data, performed statistical analyses, and drafted the manuscript. SKB performed the analysis and quantification of synthetic and natural N-methyl DHQ using GC-MS and GC-MS-FTIR, and critically revised the manuscript. KRW assisted with the collection and analysis of GC-MS data. Marta M. Antoniazzi and Carlos Jared coordinated the maintenance and reproduction of frogs in captivity and critically revised the manuscript. Kunihiro Matsumura, Keisuke Nishikawa, and Yoshiki Morimoto synthesized the HTX used in the feeding experiments and critically revised the manuscript. Taran Grant coordinated the maintenance and reproduction of frogs in captivity, participated in data analysis, and helped draft and revise the manuscript. Ralph A. Saporito conceived the method to orally administer alkaloids, conceived and performed the analysis of alkaloid lipophilicity, coordinated the alkaloid analysis on GC-MS and GC-MS-FTIR, participated in data analysis, and helped draft and revise the manuscript. All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Jeckel, A. M., Bolton, S. K., Waters, K. R., Antoniazzi, M. M., Jared, C., Matsumura, K., Nishikawa, K., Morimoto, Y., Grant, T., & Saporito, R. A. (2022). Dose-dependent alkaloid sequestration and *N*-methylation of decahydroquinoline in poison frogs. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, 337, 537–546. https://doi.org/10.1002/jez.2587