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Dietary Alkaloid Sequestration in a Poison Frog: An Experimental Test of Alkaloid Uptake in *Melanophryniscus stelzneri* (Bufonidae)

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Abstract Several lineages of brightly colored anurans independently evolved the ability to secrete alkaloid-containing defensive chemicals from granular glands in the skin. These species, collectively referred to as 'poison frogs,' form a polyphyletic assemblage that includes some species of Dendrobatidae, Mantellidae, Myobatrachidae, Bufonidae, and Eleutherodactylidae. The ability to sequester alkaloids from dietary arthropods has been demonstrated experimentally in most poison frog lineages but not in bufonid or eleutherodactylid poison frogs. As with other poison frogs, species of the genus *Melanophryniscus* (Bufonidae) consume large numbers of mites and ants, suggesting they might also sequester defensive alkaloids from dietary sources. To test this hypothesis, fruit flies dusted with alkaloid/nutritional supplement powder were fed to individual *Melanophryniscus stelzneri* in two experiments. In the first experiment, the alkaloids 5,8-disubstituted indolizidine **235B'** and decahydroquinoline were administered to three individuals for 104 days. In the second experiment, the alkaloids 3,5-disubstituted indolizidine **239Q** and decahydroquinoline were given to three frogs for 153 days. Control frogs were fed fruit flies dusted only with nutritional supplement. Gas chromatography/mass spectrometry analyses revealed that skin secretions of all experimental frogs contained alkaloids, whereas those of all control frogs lacked alkaloids. Uptake of decahydroquinoline was greater than uptake of 5,8-disubstituted indolizidine, and uptake of 3,5-disubstituted indolizidine was greater than uptake of decahydroquinoline, suggesting greater uptake efficiency of certain alkaloids. Frogs in the second experiment accumulated a greater amount of alkaloid, which corresponds to the longer duration and greater number of alkaloid-dusted fruit flies that were consumed. These findings provide the first experimental evidence that bufonid poison frogs sequester alkaloid-based defenses from dietary sources.

Keywords Arthropods · Anura · Chemical defense · Diet · Feeding experiment

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Introduction

Amphibians are protected by an exocrine defense system composed of cutaneous poison glands (Toledo and Jared 1995)—specialized cells that secrete a variety of defensive chemicals, defined as substances produced that reduce the risk of bodily harm by another organism (Berenbaum 1995). The defensive chemicals that occur in amphibians include alkaloids, biogenic amines, bufadienolides, peptides and

proteins, steroids, and volatiles (Daly et al. 2005; Pukala et al. 2006) that are believed to function as important components of the innate immune system in defending against pathogens and parasites (Conlon 2011; MacFoy et al. 2005; Rivas et al. 2009) and also are involved in complex antipredator mechanisms (Brodie et al. 1991).

Approximately 150 species of brightly colored, primarily diurnal anurans are capable of secreting defensive chemicals that contain lipophilic alkaloids. These species form a polyphyletic assemblage referred to as 'poison frogs' and are distributed among seven lineages of the families Bufonidae (*Melanophryniscus*), Dendrobatidae (independendtly derived in *Ameerega*, *Epipedobates*, and Dendrobatinae), Eleutherodactylidae (part of the *Eleutherodactylus limbatus* group; Rodríguez et al. 2011, 2012), Mantellidae (*Mantella*), and Myobatrachidae (*Pseudophryne*) (for a recent review see Saporito et al. 2012). Over 900 lipophilic alkaloids of 28 structural classes have been detected in the skin of poison frogs (Garraffo et al. 2012; Saporito et al. 2012). Experiments have demonstrated that lipophilic alkaloids are sequestered from dietary sources in dendrobatid, mantellid, and myobatrachid poison frogs (Daly et al. 1994a, b, 1997, 2003; Smith et al. 2002), but experimental evidence is lacking for bufonids and eleutherodactylids.

Among bufonids, *Melanophryniscus* is the only genus known to possess lipophilic alkaloids (Daly et al. 1984). *Melanophryniscus* includes 27 species of small (<40 mm), primarily diurnal toads (Peloso et al. 2012; Santos and Grant 2011), putatively aposematic toads with red or orange ventral coloration that can be displayed by performing the unken reflex (Fernández 1926), a defensive behavior in which an individual arches the vertebral column, elevates the head and limbs, and exposes palmar and plantar surfaces (Riemer 1958; Stebbins 1951). Approximately 170 alkaloids in 15 structural classes have been detected in 9 species of *Melanophryniscus* (Garraffo et al. 2012; Grant et al. 2012), the most common of which are 5,8-disubstituted indolizidines, 5,6,8-trisubstituted indolizidines, pumiliotoxins, tricyclics, and decahydroquinolines (Daly et al. 2005; Saporito et al. 2012). As in other poison frogs, the diet of *Melanophryniscus* includes large numbers of mites and ants (Bonansea and Vaira 2007; Daly et al. 2008; Garraffo et al. 2012; Quiroga et al., 2011), which are the primary dietary source of lipophilic alkaloids in dendrobatids and mantellids (Saporito et al. 2009). It is, therefore, likely that poison frogs of the genus *Melanophryniscus* also uptake alkaloids through their diet, but it has yet to be demonstrated experimentally.

In the present study, we tested the hypothesis that poison frogs of the genus *Melanophryniscus* sequester lipophilic alkaloids through dietary uptake by adding three distinct alkaloids (from three different alkaloids classes) to the diet of captive bred individuals of *M. stelzneri*. These experiments also allowed us to evaluate the relative uptake efficacy of the three alkaloid classes and examine the relationship between duration of alkaloid consumption and quantity of alkaloid sequestered.

Methods and Materials

Experimental Organisms Fifteen juvenile *M. stelzneri* were obtained from a captive breeding program at the Nashville Zoo, Nashville, Tennessee on 7 October 2011 and raised to maturity in terraria in the Department of Biology, John Carroll University before feeding experiments commenced. Frogs were housed in individual glass enclosures that contained damp paper towel and a plastic object to perch on. Each glass enclosure was covered with a glass lid to enclose both frogs and fruit flies (see below). To ensure complete isolation between control and experimental groups, the glass enclosures containing the control and experimental frogs were maintained inside separate 44 L glass terraria, each of which was covered with a glass lid to maintain constant temperature (23 °C) and humidity (97 %). Both terraria were misted with deionized (DI) water once per week, and individual glass enclosures were cleaned and misted with DI water every 3 d. Both 44 L terraria contained a shallow layer of rocks to allow water to drain. The research was performed in accordance with the John Carroll University IACUC protocol number 1101.

Frog feeding occurred and was closely monitored daily. Wingless fruit flies were raised on standard *Drosophila* medium (Carolina Biological Supply Co. Burlington, NC, USA). The fruit flies fed to the control frogs were dusted with Nekton-Rep nutritional supplement powder (Nekton, Clearwater, FL, USA). The flies fed to the experimental frogs were dusted with a 1 % alkaloid/nutritional supplement mixture. Fruit flies were dusted by placing them in a glass enclosure containing either the alkaloid mixture or pure nutritional supplement, gently shaking to ensure that all flies were thoroughly dusted, and then transferring flies individually with forceps to the corresponding frog enclosures. Different feeding containers and forceps were used for control and experimental fruit flies. The number of fruit flies added to each enclosure was recorded (5–15 fruit flies per frog daily), along with the number of flies that remained the next day. The three classes of alkaloids utilized in this study, 5,8-disubstituted indolizidine, 3,5-disubsituted indolizidine and decahydroquinoline, are common in *Melanophryniscus* poison frogs (Grant et al. 2012), including *M. stelzneri* (Daly et al. 2007; Garraffo et al. 1993). The following two experiments were performed:

Feeding Experiment One: DHQ and 5,8-I. This feeding experiment included 3 experimental and 2 control frogs and was conducted over 104 day from 18 February to 31 May 2012. In this experiment, 3 mg of synthetic 5,8-disubstituted indolizidine (5,8-I) **235B'** (Naoki Toyooka, University of Toyama, Japan) and 22.6 mg of synthetic decahydroquinoline (DHQ) (Acros Organics, New Jersey, USA) were combined and thoroughly mixed with 2.5 g of nutritional supplement to create a 1 % alkaloid/nutritional supplement mixture composed of a DHQ:3,5-I ratio of approximately 7.5:1.

Feeding Experiment Two: DHQ and 3,5-I. The second feeding experiment included 3 experimental and 2 control frogs and was conducted over 153 day from 8 September 2012 to 8 February 2013. In this experiment, 9 mg of synthetic 3,5-disubsituted indolizidine (3,5-I) **239Q** (Naoki Toyooka, University of Toyama, Japan) and 16 mg of synthetic DHQ (Acros Organics, New Jersey, USA) were combined and thoroughly mixed with 2.5 g of nutritional supplement to create a 1 % alkaloid/nutritional supplement mixture composed of a DHQ:3,5-I ratio of approximately 2:1.

Upon conclusion of each feeding experiment, defensive skin secretions were harvested from each frog by using a transcutaneous amphibian stimulator (TAS; Grant and Land 2002) to apply a weak electric current to the skin, which caused the frogs to secrete the contents of their granular glands. The TAS treatment (Frequency: 50 Hz; Pulse width: 2 ms; Amplitude: 9 V) was standardized among frogs. The secretions were collected by gently wiping the surfaces of the frogs with Light-Duty Tissue Wipers (VWR International, West Chester, PA, USA) that were then placed into 1 ml of 100 % methanol in glass vials with Teflon-lined caps.

For each sample, 50 μl of 1 N HCl were added to 1 ml of the original MeOH extract. The combined MeOH extract was then concentrated with N_2 to 100 μl, followed by dilution with 200 μl of water. The resulting solution was extracted x 4, each time with 300 μl of hexane. Following extraction, the HCl fraction was basified with saturated $NaHCO_3$, followed by extraction x 3 with 300 μl portions of ethyl acetate. The combined organic fractions were dried with anhydrous Na_2SO_4, evaporated to dryness with N_2, and finally resuspended in 100 μl of 100 % methanol.

Gas chromatography/mass spectrometry (GC/MS) was performed for each sample on a Varian Saturn 2100 T ion trap MS instrument coupled to a Varian 3900 GC with a 30 m x 0.25 mm i.d. Varian Factor Four VF-5 ms fused silica column. To reach a GC separation of alkaloids, a temperature program was used from 100 to 280 °C at a rate of 10 °C per minute with helium as the carrier gas (1 ml/min). Each alkaloid fraction was analyzed with both electron impact MS and chemical ionization (CI) MS with methanol as the CI reagent.

Results

Feeding Experiment One: DHQ and 5,8-I Experimental frogs consumed an average of 873 alkaloid-dusted fruit flies. The skin secretions obtained from all three experimental frogs contained alkaloids, whereas no alkaloids were detected in those of the two controls (Fig. 1). The mass spectral data (not shown) illustrated that the skin alkaloids were identical to the dietary alkaloids. As expected based on the proportion of each alkaloid in the alkaloid/nutritional supplement mixture, in all specimens DHQ was more abundant than 5,8-I; however, the difference in relative abundance greatly exceeded the difference in the original proportions and varied considerably among the three individuals (DHQ:5,8-I=7.5:1 in the feeding mixture, 13.3–32.3:1 in frog skin secretions; Table 1).

Feeding Experiment Two: DHQ and 3,5-I Experimental frogs consumed an average of 1,596 alkaloid-dusted fruit flies. As in the first, shorter feeding experiment, the skin secretions of all three experimental frogs contained alkaloids, whereas no alkaloids were detected in the two controls (Fig. 2). The mass spectral data (not shown) demonstrated that the skin alkaloids were identical to the dietary alkaloids. Despite the greater proportion of DHQ in the feeding mixture, 3,5-I was much more abundant in the skin secretions (DHQ:3,5-I=1.8:1 in the feeding mixture, 1:2.1, 1:3, and 1:4.3 in frog skin secretions; Table 1).

Discussion

The results of our experiments provide conclusive evidence that the bufonid poison frog *Melanophryniscus stelzneri* has a similar (or identical) alkaloid uptake system to that of dendrobatid, mantellid, and myobatrachid poison frogs (Daly et al. 1994a, b, 1997, 2003; Smith et al. 2002). Skin secretions from individuals whose diets lacked lipophilic alkaloids did not possess detectable amounts of any alkaloid, whereas skin secretions from all individuals whose diets included lipophilic alkaloids possessed the same, unmodified alkaloids. Our findings support previous experiments that suggest a dietary source for most of the lipophilic alkaloids present in poison frog skin (reviewed in Saporito et al. 2009).

The secretions of all studied species of *Melanophryniscus* contain lipophilic alkaloids (Daly et al. 1984, 2007, 2008; Garraffo et al. 1993, 2012; Grant et al. 2012; Mebs et al.

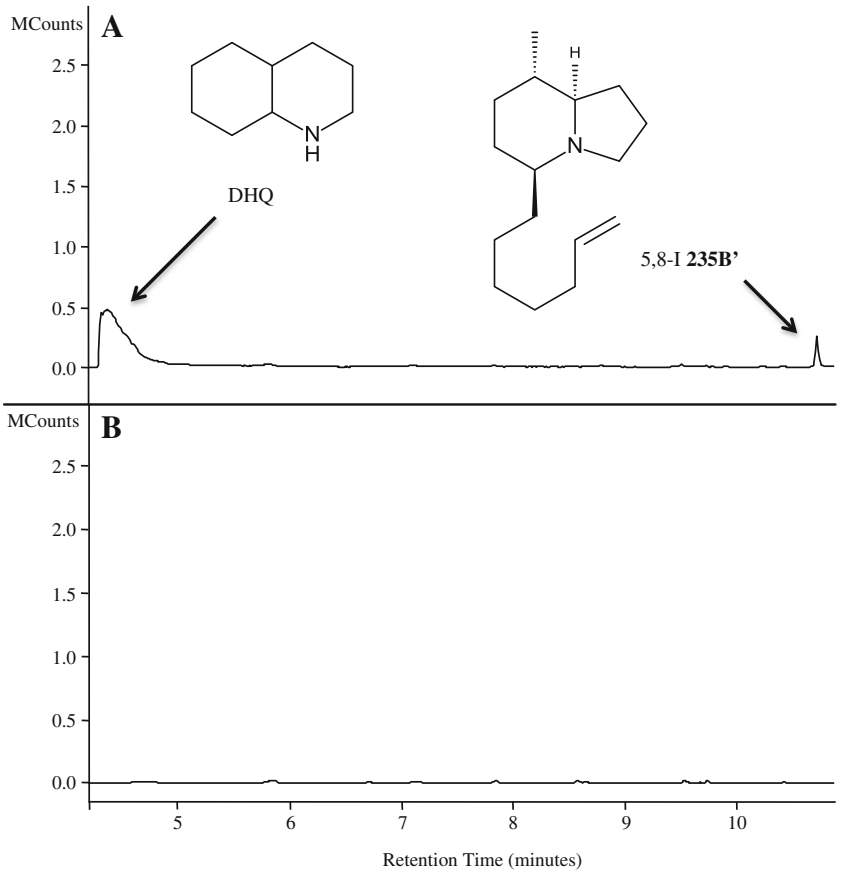

Fig. 1 Gas chromatograms illustrating: **a** uptake of 5,8-I **235B'** and decahydroquinoline (DHQ) by experimental *Melanophryniscus,* and **b** no alkaloids present in control *Melanophryniscus.* Small peaks are not alkaloids

2005, 2007), and it is reasonable to assume that those species also obtain alkaloids through dietary uptake. Certain groups of mites and ants appear to be the primary sources of alkaloids in poison frogs (Raspotnig et al. 2011; Saporito et al. 2009, 2011, 2012), and studies of the stomach contents of *M. cupreuscapularis, M. klappenbachi, M. montevidensis, M. stelzneri,* and *M. rubriventris* have found large numbers of ants and mites (for review of previous studies see Daly et al. 2008; see also Bonansea and Vaira 2007; Garraffo et al. 2012; Quiroga et al., 2011).

Table 1 Relative amounts of alkaloids secreted by individual *Melanophryniscus,* expressed as ratios

Experiment 1	
	DHQ:5,8-I **235B'**
1 % alkaloid mixture	7.5:1
Individual frog	
1	32.3:1
2	13:1
3	24:1
Experiment 2	
	DHQ:3,5-I **239Q**
1 % alkaloid mixture	2:1
Individual frog	
1	1:2
2	1:3
3	1:4

In both experiments, the relative amounts of each alkaloid in skin secretions differed greatly from those of the feeding mixtures. Uptake of decahydroquinoline was approximately 2–4 times greater than uptake of the 5,8-disubstituted indolizidine. Decahydroquinolines in wild-caught frogs are straight-chained alkaloids that appear to be derived from myrmicine ants (Jones et al. 1999; Saporito et al. 2009), whereas 5,8-disubstituted indolizidines are branched-chained izidines found mostly in oribatid mites (Saporito et al. 2007, 2011). Uptake of the 3,5-disubstituted indolizidine, a straight-chained alkaloid found in both mites and ants, was approximately 2–4 times greater than uptake of decahydroquinoline. As such, although our results are limited, they suggest that *M. stelzneri* accumulates straight-chained alkaloids more efficiently than branched alkaloids. Studies of wild-caught specimens of *Melanophryniscus stelzneri* have reported 47 alkaloids (including isomers), of which 19 are branched (including the izidines **193I** and **207S**, for which tentative structures were proposed by Daly et al. 2007), 18 are straight-chained, and 10 are unclassified (Daly et al. 2007; Garraffo et al. 1993; Grant et al. 2012). Both branched and

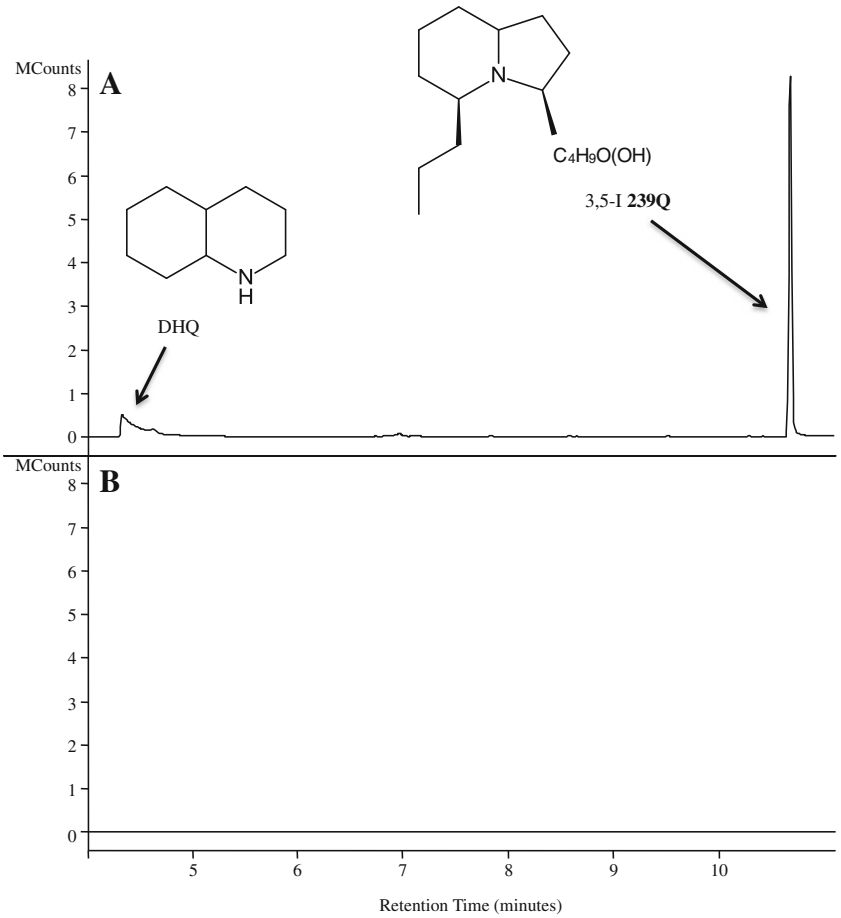

Fig. 2 Gas chromatograms illustrating: **a** uptake of 3,5-I **239Q** and decahydroquinoline (DHQ) by experimental *Melanophryniscus,* and **b** no alkaloids present in control *Melanophryniscus*

straight-chained alkaloids have been reported as major alkaloids in *M. stelzneri* (>50 μg of alkaloid per 100 mg of skin; Daly et al. 2007).

Previous experiments with other poison frog lineages also identified variation in the uptake efficiency of different alkaloids. In experiments with the dendrobatid *Dendrobates auratus*, Daly et al. (1994b: 662) reported that "the ratio of alkaloids in the dusting powder was usually quite similar to the ratio of alkaloids subsequently found in the frog skin after feeding mixtures of decahydroquinolines, pyrrolidines, indolizidines, quinolizidines and histrionicotoxins, suggesting a rather non-specific uptake for lipophilic alkaloids." However, they also noted that the 2,5-disubstituted pyrrolidines and 2,6-disubstituted piperidines were not accumulated, revealing that sequestration is not indiscriminate. Daly et al. (1994b) also reported that the dendrobatoids *Allobates talamancae* and *Colostethus panamansis* (as *C. talamancae* and *C. inguinalis*, respectively; see Grant 2004; Grant et al. 2006) did not accumulate dietary alkaloids, confirming the lack of alkaloids in studies of wild-caught specimens. In experiments with the mantellid *Mantella viridis*, Daly et al. (1997) reported that an allopumiliotoxin, a synthetic 2,5-dimethyl-cis-decahydroquinoline, and two 5,8-disubstituted indolizidines were readily accumulated, but that only a small amount of the isodihydrohistrionicotoxin and none of the piperidine could be detected in skin secretions. Experiments with the myobatrachid *Pseudophryne semimarmorata* illustrated sequestration (and reduction/hydroxylation) of dietary pumiliotoxin **307A**, but not a histrionicotoxin, a 3,5-disubstituted indolizidine, or a decahydroquinoline (Smith et al. 2002). Similarly, experiments with species of the dendrobatid genera *Dendrobates* and *Adelphobates* demonstrated stereoselective hydroxylation of dietary pumiliotoxin (+)-**251D** to a more toxic allopumiliotoxin (+)-**267A** (Daly et al. 2003).

Although all experimental specimens of *Melanophryniscus stelzneri* exhibited the same pattern of alkaloid uptake, there appear to be differences in the relative uptake efficiency among individuals. Despite the different durations and classes of alkaloids used in the two experiments, in both experiments the relative amounts of the two alkaloids varied among individuals from 2–4X. Evidence is lacking to explain the individual differences in relative uptake efficiency, but the fact that all individuals in each experiment consumed the same ratio of alkaloids suggests that it is not

due to differences in feeding behavior or environmental availability.

The experimental frogs from the second experiment accumulated more alkaloids than the frogs from first experiment. Given that both experiments used 1 % alkaloid/nutritional supplement mixture and employed the same feeding procedures, this difference appears to be due to the greater duration of the second experiment, which was more than 1.5 months longer than the first experiment. The longer period of time allowed the frogs to consume a greater number of dusted fruit flies, which provided the alkaloids. It also is possible that some of this variation could be due to unequal dusting of fruit flies, with flies from the shorter experiment containing less alkaloid than flies of the second experiment. Likewise, it could be due to different levels of secretion during each of the experiments. Nevertheless, our finding that frogs that consume alkaloids for a longer period of time possess more alkaloid in their skin secretions than frogs that consume alkaloids over a shorter period of time is consistent with studies of dendrobatid poison frogs (Daly et al. 1994b), and it is unlikely that in all of these cases differential dusting and/or secretion would correlate with feeding experiment duration.

Although experimental evidence demonstrates conclusively that the lipophilic alkaloids in the defensive skin secretions of bufonid, dendrobatid, mantellid, and myobatrachid poison frogs are sequestered through dietary uptake, the molecular mechanisms involved in alkaloid sequestration are poorly known. To date, the mechanism of resistance in poison frogs has been investigated only in *Phyllobates terribilis*, in which resistance to batrachotoxin seems to be due to a modification of the regulatory site controlling voltage-dependent sodium channel activation and permeability, thus preventing binding by batrachotoxin (Daly et al. 1980; Wang and Wang 1999; Wang et al. 2006). However, poison frog alkaloids have diverse mechanisms of activity (Daly et al. 1999, 2005), and Grant et al. (2012) showed that lipophilic alkaloids are not confined to skin glands, as previously believed, but are instead anatomically widespread, suggesting that physiological resistance and uptake must have evolved in tandem. Moreover, the molecular mechanisms of alkaloid uptake, including capture, transport, and accumulation in dermal granular glands, are entirely unknown. Future feeding experiments will undoubtedly provide additional insight and further understanding of the mechanisms responsible for alkaloid sequestration in poison frogs.

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