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Passive accumulation of alkaloids in non-toxic frogs challenges paradigms of the origins of acquired chemical defenses

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Abstract

Understanding the origins of novel, complex phenotypes is a major goal in evolutionary biology. Poison frogs of the family Dendrobatidae have evolved the novel ability to acquire alkaloids from their diet for chemical defense at least three times. However, taxon sampling for alkaloids has been biased towards colorful species, without similar attention paid to inconspicuous ones that are often assumed to be undefended. As a result, our understanding of how chemical defense evolved in this group is incomplete. Here we provide new data showing that, in contrast to previous studies, species from each undefended poison frog clade have measurable yet low amounts of alkaloids. We confirm that undefended dendrobatids regularly consume mites and ants, which are known sources of alkaloids. Further, we confirm the presence of alkaloids in two putatively non-toxic frogs from other families. Our data suggest the existence of a phenotypic intermediate between toxin consumption and sequestration—passive accumulation—that differs from active sequestration in that it involves no derived forms of transport and storage mechanisms yet results in low levels of toxin accumulation. We discuss the concept of passive accumulation and its potential role in the origin of chemical defenses in poison frogs and other toxin-sequestering organisms.

Keywords: toxin sequestration, toxin resistance, bioaccumulation, novelty, adaptive landscape, toxicokinetics

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1. Introduction

Complex phenotypes can evolve by leveraging phenotypic plasticity in existing traits with concerted change in developmental modules [1]. However, the evolutionary trajectory that animals take to traverse an adaptive landscape from one phenotype to another may be difficult to reconstruct given that they often must cross or avoid adaptive valleys, which include phenotypes that are not always readily observed in populations. Nevertheless, phenotype diversity can help us unravel origins of novel traits and reveal the physiological trade-offs associated with their evolutionary trajectory.

Acquired chemical defenses, or the ability to sequester and use chemicals from the environment against predators or parasites, is one complex phenotype whose evolutionary history has proved difficult to characterize [2,3]. How is it that animals transition from consuming to sequestering toxins? The following phases are likely to occur: 1) consistent exposure to a toxic compound; 2) prior existence or evolution of some resistance to the toxin; 3) change in the elimination rate of the compound that may lead to its prolonged retention, hereafter "passive accumulation" (see Fig. 1); 4) co-option of molecular pathways to transport and store the compound in a specific location, hereafter "active sequestration", which may in turn select for enhanced resistance. Note that while we focus on the processes underlying toxin resistance and sequestration, other phenotypes and selection pressures such as conspicuous coloration or predators may influence these patterns [4]. In the following text we use the terms alkaloid and toxin interchangeably, although the toxicity of each compound is not always known or very straightforward [5]. Similarly, for simplicity we broadly bin species into defended (high alkaloid content) and non-toxic or undefended (low or zero alkaloid content) categories.

	Process			
Accumulation Phenotype	Elim. Rate	Seq. Rate	Acc. Rate	Line Type
No accumulation	+++	0	0	—
Passive accumulation	++	0	+	—
	+++	+	+	- - -
Active sequestration	++	+++	++	—
	+	+++	+++	- - -

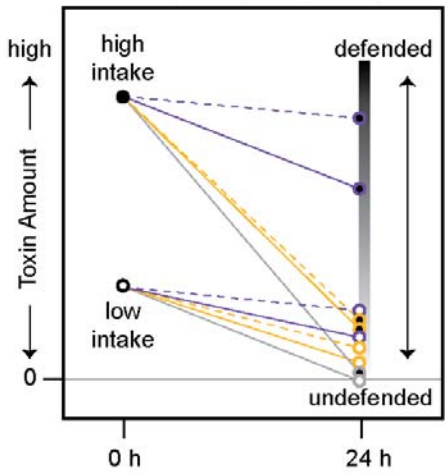

Figure 1. Major processes involved in the transition from the undefended to defended phenotype: 1) toxin intake, here visualized with two discrete points representing low and high rates; 2) toxin elimination rate (Elim. Rate), e.g., via toxin metabolism; 3) toxin sequestration rate (Seq. Rate), i.e., the active transport of toxins for storage in a specific location such as the skin; and 4) toxin accumulation rate (Acc. Rate), or the rate at which toxins are accumulated in the animal. Defense phenotypes are ultimately a result of how these processes interact over time, here arbitrarily from 0 h, immediately after toxin ingestion, to 24 h following ingestion. Although toxin intake influences the total possible amount of toxin accumulation, it cannot fully explain the defensive phenotype. We hypothesize that the “no accumulation” phenotype is characterized by the absence of any ability to sequester toxins in combination with a high rate of elimination, resulting in 0 toxin accumulation (solid grey lines); this phenotype is a likely ancestral state for many animals. In contrast, we hypothesize that “passive accumulation” is characterized by lower elimination rates than the no accumulation phenotype, leading to a low amount of toxin accumulation (solid yellow lines); however, some mechanisms of toxin transport could also exist, in which case a low sequestration rate could result in a passive accumulation phenotype when elimination rate is high (dashed yellow lines). We hypothesize that the “active sequestration” phenotype evolves from an intermediate passive accumulation phenotype through the addition of novel sequestration mechanisms that result in high sequestration rates (solid purple lines). However, elimination rates could still modulate the amount of toxins ultimately accumulated, with lower elimination rates resulting in a higher proportion of toxin accumulation overall (dashed purple lines).

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We use new data from poison frogs (Anura: Dendrobatidae) to shed some light on this complex transition. Approximately 100 of the ~340 dendrobatid poison frog species [6] fall into three conspicuously colored and alkaloid-sequestering (aposematic) clades: *Ameerega, Epipedobates,* and Dendrobatinae; the other ~240 species compose several other primarily inconspicuously colored clades that for the most part have been assumed to lack alkaloid defenses: i.e., all Aromobatinae (e.g., *Allobates, Rheobates,* and *Aromobates*), *Colostethus, Silverstoneia, Leucostethus,* and all Hyloxalinae (*Hyloxalus*) (Fig. 2). According to the phylogenetic placement of these traits, poison frogs have evolved sequestration of lipophilic alkaloids from consumed arthropods at least three times [7], making them an ideal group to study complex phenotypic transitions. Much of the research on poison frogs has focused on changes in diet (toxin intake) in the origins of chemical defenses [8–11] without much focus on rate of toxin elimination versus accumulation (Fig. 2; but see [12,13]). However, rates of intake, sequestration, and elimination all shape the ability of an animal to accumulate a compound (Fig. 1). Thus, characterizing the metabolism and sequestration of alkaloids in defended and undefended dendrobatid lineages is essential to understand the origins of chemical defense [14]. We propose that changes in toxin metabolism through selection on mechanisms of toxin resistance likely play a major role in the evolution of acquired chemical defenses.

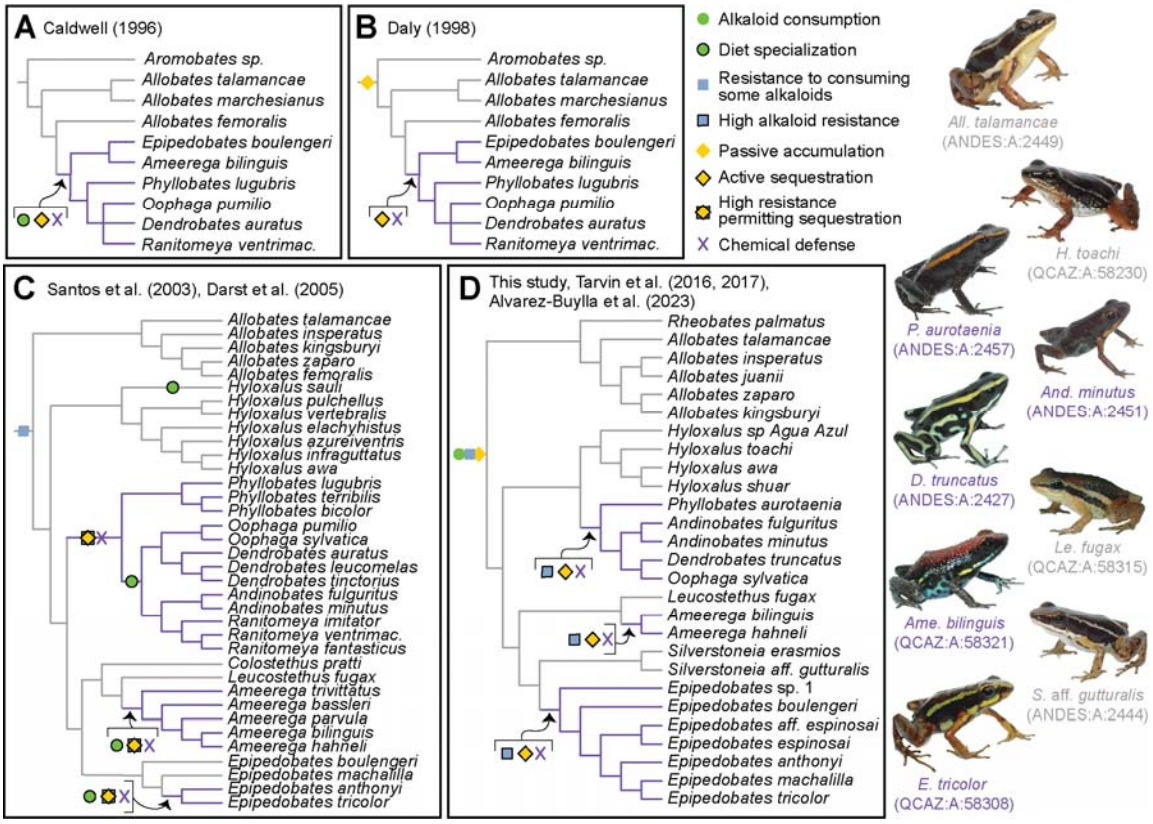

Figure 2. Evolutionary models of toxin sequestration in Dendrobatidae have changed over time. A) When several species of aposematic dendrobatids (purple lines) were found to have narrower dietary niches than undefended dendrobatids and other frogs [10,15,16], researchers hypothesized that diet specialization may have driven the radiation of aposematic dendrobatids [17]. **B)** Chemists hypothesized that aposematic dendrobatids sequester dietary alkaloids via an alkaloid uptake system [11]. Daly [18] postulated that an alkaloid uptake system was present in the ancestor of Dendrobatidae (here denoted as passive accumulation) and that it is "overexpressed" in defended dendrobatids (here denoted as active sequestration). **C)** A phylogenetic analysis of Dendrobatidae revealed that chemical defense and diet specialization evolved independently several times [9]. The new

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information helped generate the diet-toxicity hypothesis, which posits that shifts from a generalist to a specialist diet drove the multiple origins of alkaloid uptake through enhanced resistance and/or more efficient sequestration systems [4,8]. **D)** Here we propose a combination of these hypotheses, i.e., that passive accumulation, alkaloid consumption, and some level of alkaloid resistance was present in an early dendrobatid lineage; enhanced resistance and active sequestration mechanisms then arose later, resulting in the chemical defense phenotype. This hypothesis places less emphasis on dietary changes and more strongly emphasizes novel molecular mechanisms (e.g., binding proteins and target-site insensitivity [19–21]). Phylogenies in each subpanel highlight how increasing resolution impacted our understanding of phenotypic diversification in Dendrobatidae. All images of frogs were taken by RDT.

2. Results and Discussion

(a) Phases 1 and 2: Consistent exposure to toxins may select for resistance and sequestration

Many animals occasionally or frequently consume toxins, and a multitude have evolved toxin resistance. Some invertebrate pests resist pesticides [22–25], many insect herbivores resist plant toxins [26,27], some predators resist toxic prey [28], many animals resist environmental pollutants [29], and toxic organisms usually evolve resistance to their own defenses ("autoresistance") [3,30].

The general mechanisms of toxin resistance are toxin avoidance, toxin metabolism, and target modification [31]. If an animal does not or cannot *avoid* toxin exposure, it will need to *survive* exposure using toxin metabolism or target modification mechanisms such as biotransformation, elimination, alternative targets, and target-site resistance (see [31] for more details). Toxin metabolism, also known as toxicokinetics [32], is a set of mechanisms based on detoxification pathways than may provide toxin resistance. These pathways are common to all animals and were likely used by the ancestors of most if not all animals that eventually evolved toxin sequestration (Fig. 1).

The type of toxin resistance present in an animal may eventually affect that animal's ability or propensity to sequester toxins. For example, animals that possess target-site resistance may be more likely to evolve toxin sequestration than animals that avoid toxins [31]. Although one might expect that toxin metabolism may also prevent toxin sequestration, the ability to metabolize toxins can in some cases augment toxin defenses [33], increase the toxicity of a compound (e.g., pumiliotoxin to allopumiliotoxin in the poison frogs *Adelphobates galactonotus*, *A. castaneoticus*, *Dendrobates auratus*, and *D. tinctorius* [34,35]), or result in some amount of passive accumulation through increased toxin exposure [33,36]. In general, toxin-sequestering animals often have specialized mechanisms of toxin resistance when compared to non-toxic relatives [31]. For example, three amino acid replacements in the ATPα protein evolved in association with cardenolide sequestration in Danainae butterflies [36,37] and predatory fireflies that sequester lucibufagins have ATPα gene duplications that enhance lucibufagin resistance [38].

In dendrobatids, mechanisms of toxin resistance are still understudied [39]. Target-site resistance to some alkaloids appears to have evolved in several defended clades and in some undefended species [20,21]. Some defended species also appear to have alternative target mechanisms including binding proteins like alpha-binding globulin [19] and saxiphillin [40] that might prevent alkaloids from accessing their molecular targets. Accumulation of alkaloids in skin glands could help to prevent alkaloids from reaching their targets. Although direct evidence is lacking, some poison frogs may biotransform alkaloids into less toxic forms until they can be eliminated from the body, e.g., using cytochrome p450s [41].

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(b) Phases 3 and 4: Passive accumulation and active sequestration in poison frogs

Although the inconspicuously colored clades of poison frogs have long been considered to lack chemical defenses (i.e., they are undefended), many species have not been comprehensively assessed. By reviewing existing data from inconspicuous poison frog species, we found that of the 245 inconspicuous species described to date [6], only 30 have been assessed for toxicity, and sometimes using methods that would not necessarily detect lipophilic alkaloids (Table S1). Available data suggest that at least nine of these species might have alkaloids: *Allobates femoralis*, *Al. kingsburyi*, *Al. zaparo*, *Hyloxalus maculosus*, *H. nexipus, H. vertebralis, Leucostethus fugax, Paruwrobates erythromos,* and *Silverstoneia punctiventris* [4,8,42,43]. However, evolutionary studies have not fully incorporated these data (Fig. 1, Table S1, and see below).

We tested for possible alkaloid presence in a broad selection of inconspicuously colored poison frog lineages using GCMS. In total, we surveyed 89 animals representing 30 species of Neotropical frogs including 27 dendrobatid poison frogs and representatives from most of the major undefended clades in Dendrobatidae (Table 1). We also performed a highly sensitive, untargeted analysis (UHPLC-HESI-MS/MS) of *S. flotator* and non-dendrobatid *Eleutherodactylus cystignathoides* (Anura: Eleutherodactylidae) in which alkaloid diversities and types, but not quantities, were assessed. As far as we are aware, we provide alkaloid data for the first time for seven undefended species (*Rheobates palmatus, Allobates juanii, H. shuar, H.* sp. Agual Azul*, H. toachi, S.* aff. *gutturalis*, and *S. erasmios*) and one defended species (*Epipedobates* sp. 1). Overall, we detected alkaloids in skins from 13 of 14 undefended species included in our study, although often with less diversity and relatively lower quantities than in defended lineages (Fig. 3, Table 1, Table S2, Table S3). The ubiquity of low alkaloid levels in non-toxic dendrobatid lineages (Aromobatinae, Hyloxalinae, some species of Colostethinae) contrasts with the mixed or opposing evidence from previous analyses (Table S1).

Our GCMS assessment revealed substantially higher diversities of alkaloids in defended dendrobatid species than previously reported [5,42,44–46], and expands knowledge on major classes of alkaloids within genera. Because chemical standards for most poison frog alkaloids do not exist, it is not possible to provide absolute quantification of alkaloids. Reported values are in units of integrated area, which do not directly correspond to alkaloid quantity because of differences in ion yield. Nevertheless, qualitative comparisons of integrated areas can provide insight into how species differ in degrees of magnitude.

Table 1. Range and median of alkaloid quantity (estimated by the sum of integrated areas) and alkaloid diversity (number of different compounds) by species from the GCMS assessment. The presumed chemical defense phenotype for poison frogs is given according to Santos and Cannatella [4]. Purple rows highlight defe species. *Data are from UHPLC-HESI-MS/MS, which does not provide quantitative data.

Family	Subfamily	Species	Phenotype	Sample Size (frogs)	Log (Total Integrated Area)		Alkaloid Number	
					Range	Median	Range	Median
Dendrobatidae	Aromobatinae	*Rheobates palmatus*	undefended	4	13.07 – 14.24	14.00	1 – 4	1.5
Dendrobatidae	Aromobatinae	*Allobates insperatus*	undefended	8	13.47 – 15.44	14.99	1 – 9	5.0
Dendrobatidae	Aromobatinae	*Allobates juanii*	undefended	1	14.10	14.10	1	1.0
Dendrobatidae	Aromobatinae	*Allobates kingsburyi*	undefended	1	13.63	13.63	2	2.0
Dendrobatidae	Aromobatinae	*Allobates talamancae*	undefended	3	14.89 – 16.27	15.09	2 – 4	3.0
Dendrobatidae	Aromobatinae	*Allobates zaparo*	undefended	1	16.78	16.78	8	8.0
Dendrobatidae	Colostethinae	*Leucostethus fugax*	undefended	8	12.57 – 15.33	14.00	3 – 8	4.5
Dendrobatidae	Colostethinae	*Ameerega bilinguis*	defended	1	21.97	21.97	133	133.0
Dendrobatidae	Colostethinae	*Ameerega hahneli*	defended	4	20.21 – 22.29	21.68	85 – 140	128.5
Dendrobatidae	Colostethinae	*Silverstoneia flotator**	undefended	12	NA	NA	57 – 68	62
Dendrobatidae	Colostethinae	*Silverstoneia* aff. *gutturalis*	undefended	9	11.80 – 17.33	15.40	1 – 10	3.0
Dendrobatidae	Colostethinae	*Silverstoneia erasmios*	undefended	2	14.70 – 16.11	15.41	15 – 15	15.0

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Dendrobatidae	Colostethinae	*Epipedobates* aff. *espinosai*	defended	2	18.44 – 20.20	19.32	83 – 131	107.0
Dendrobatidae	Colostethinae	*Epipedobates anthonyi*	defended	1	20.54	20.54	127	127.0
Dendrobatidae	Colostethinae	*Epipedobates boulengeri*	defended	2	18.87 – 19.39	19.13	77 – 94	85.5
Dendrobatidae	Colostethinae	*Epipedobates* sp. 1	defended	2	19.49 – 19.68	19.59	99 – 105	102.5
Dendrobatidae	Colostethinae	*Epipedobates espinosai*	defended	2	18.82 – 21.33	20.08	85 – 146	115.5
Dendrobatidae	Colostethinae	*Epipedobates machalilla*	defended	2	12.98 – 15.67	14.32	8 – 38	23.0
Dendrobatidae	Colostethinae	*Epipedobates tricolor*	defended	2	18.36 – 19.07	18.72	91 – 114	102.5
Dendrobatidae	Hyloxalinae	*Hyloxalus awa*	undefended	7	0.00 – 16.05	13.58	0 – 12	3.0
Dendrobatidae	Hyloxalinae	*Hyloxalus shuar*	undefended	1	14.92	14.92	5	5.0
Dendrobatidae	Hyloxalinae	*Hyloxalus* sp. Agua Azul	undefended	1	14.30	14.30	8	8.0
Dendrobatidae	Hyloxalinae	*Hyloxalus toachi*	undefended	2	0.00 – 0.00	0.00	0 – 0	0.0
Dendrobatidae	Dendrobatinae	*Phyllobates aurotaenia*	defended	4	17.72 – 21.08	18.88	48 – 118	67.5
Dendrobatidae	Dendrobatinae	*Dendrobates truncatus*	defended	3	20.05 – 23.95	20.42	111 – 172	115.0
Dendrobatidae	Dendrobatinae	*Oophaga sylvatica*	defended	5	22.86 – 24.85	23.76	152 – 189	175.0
Dendrobatidae	Dendrobatinae	*Andinobates fulguritus*	defended	2	20.09 – 20.51	20.30	80 – 85	82.5
Dendrobatidae	Dendrobatinae	*Andinobates minutus*	defended	4	16.57 – 18.77	18.07	34 – 80	66.0
Bufonidae		*Amazophrynella siona*	NA	2	14.12 – 14.40	14.26	1 – 1	1.0
Bufonidae		*Atelopus* aff. *spurrelli*	NA	1	11.58	11.58	4	4.0
Eleutherodactylidae		*Eleutherodactylus cystignathoides**	NA	3	NA	NA	62 – 66	63
Leptodactylidae	Leptodactylinae	*Lithodytes lineatus*	NA	2	0.00 – 0.00	0.00	0 – 0	0.0

For Aromobatinae, we surveyed the undefended genera *Rheobates* and *Allobates.* Alkaloids were detected in all four *R. palmatus* individuals sampled, with one individual having at least four classes of compounds represented (4,6-disubstituted quinolizidines, 3,5-disubstituted indolizidines, 3,5-disubstituted pyrrolizidines, and unclassified). We found that five species of *Allobates* all had detectable levels of alkaloids. *Allobates insperatus* had a relatively high level of alkaloid diversity, with at least eighteen alkaloids from nine classes detected, and at least one class found in each of the eight sampled individuals. In contrast, only one unclassified alkaloid was identified in a single individual of *Allobates juanii* while two were found in one individual of *Allobates kingsburyi*. At least two alkaloids were identified in each of the three sampled individuals of *Allobates talamancae* (including the lehmizidine **277A** and five new alkaloids). Eight alkaloids were identified in the single surveyed *Allobates zaparo* individual (including the spiropyrrolizidines **222-1** and **222-2** as well as six unclassified alkaloids). Prior assessments using thin-layer chromatography suggested the presence of alkaloids in three *Al. kingsburyi* [4], but none in twelve *Al. insperatus* [8]. Four studies (Table S1) failed to identify any alkaloids in *Allobates talamancae. Allobates zaparo* was shown to possibly have trace alkaloids, although the interpretation of these data was absence of alkaloids [8]. There are no known defended species from this subfamily, although we note conflicting evidence on the presence of alkaloids in *Allobates femoralis* [42,47–49] (Table S1).

Within Colostethinae, we surveyed individuals from two undefended clades, *Leucostethus* and *Silverstoneia*, and from two defended clades, *Epipedobates* and *Ameerega*. From *Leucostethus fugax*, we identified a total of twelve 5-substituted indolizidine, 5,6-disubstituted indolizidine, pyrrolidine, spiropyrrolizidine, and unclassified alkaloids (**196A**, **225C**, **222-1**, **222-2**, and eight new alkaloids), with three to eight unique compounds detected in each of the eight sampled individuals. Our data are consistent with prior thin-layer chromatography data showing that *Leucostethus fugax* tested positive for skin compounds [4], though prior interpretation of these data were different (Table S1). We also surveyed two species of *Silverstoneia*. We found alkaloids in all nine *S.* aff. *gutturalis*, with a total of fourteen alkaloids identified across seven classes (**196A**, **223I**, **233A**, **235B**, **237U**, three isomers of **239AB**, two isomers of **239CD**, and four new alkaloids). In just two individuals of *S. erasmios,* we detected a total of 26 alkaloids, including some pumiliotoxins (**325B**, **323B**) and pyrrolizidines (**225C**). While *S. erasmios* and *S.* aff. *gutturalis* had not been surveyed for alkaloids previously, a study detected

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13 alkaloids in *S. punctiventris* [43]. In addition, we conducted a highly sensitive, untargeted analysis (UHPLC-HESI-MS/MS) of *S. flotator*, which revealed that alkaloids were present in all 12 sampled individuals (>99% alkaloid pathway probability; Table S6). At this probability level, we found 67 alkaloids including one quinolizidine, two pyridines, and an analog of epibatidine (Tables S5 and S6).

In terms of the defended clades of Colostethinae that we sampled, most of the individual skins of *Epipedobates* and *Ameerega* contained dozens to more than one hundred unique alkaloids (see Table S3 for full details). For *Ameerega*, we surveyed 5 individuals representing 2 species, all of which had integrated areas that were more than 75,000x compared to individuals of its sister clade, *Leucostethus fugax* (Table 1). Similarly, alkaloid diversity was 10–20x greater in *Ameerega* than in *Leucostethus.* Histrionicotoxins and decahydroquinolines were considered previously to be the dominant alkaloid classes in genus *Ameerega* [50]; here we also found high levels of indolizidines (Fig. 3). Patterns for *Epipedobates* as compared to sister genus *Silverstoneia* were similar, although less extreme. We surveyed 13 individuals representing 7 species in *Epipedobates* and identified at least 370 alkaloids, which contrasts with studies using a less sensitive method (thin-layer chromatography) that found mixed evidence for the presence of alkaloids in *E.* aff. *espinosai* (then referred to as *E. boulengeri*) and *E. machalilla* [4,8]. However, the quantity and diversity of alkaloids in *E. machalilla* was substantially lower than in other *Epipedobates* species, occurring at levels similar to *Silverstoneia* spp. (Table 1, Fig. 3). Except for *E. machalilla*, each *Epipedobates* species had about 10x higher quantities and diversities of alkaloids compared to members of *Silverstoneia.* We found trace levels of epibatidine in *Epipedobates anthonyi* but not in other *Epipedobates* species. Epibatidine and its analogs have also been detected in *E. espinosai, Ameerega silverstonei*, *S. flotator* [51], this study], and *Ameerega petersi* or a closely related, undescribed species (reported as *Dendrobates pictus* from Loreto, Peru in [42], but see taxonomic revision by [52]).

Within Hyloxaline, a generally undefended clade, we surveyed four species of *Hyloxalus*, three of which had detectable levels of alkaloids. We identified seventeen different alkaloids in *H. awa* (**197D**, **197H**, **199B**, **217B**, **221P**, **223AB**, **231A**, **231C**, **247E**, and eight previously undescribed alkaloids), with the seven sampled individuals having zero to twelve alkaloids each. We detected five alkaloids in a single individual of *H. shuar* (**197D**, **199B**, **237G**, and two isomers of **239K**) and eight alkaloids in a single individual of *H.* sp. Agua Azul (**195C**, **197D**, **199B**, **251K**, and four new alkaloids). Our detection of low levels of alkaloids in *H. awa* are consistent with the observations that avian predators consume *H. awa* [53]. No alkaloids were detected in two individuals of *H. toachi*, the only undefended species from which we failed to detect alkaloids.

According to the most recent phylogenetic reconstructions [7], the sister clade to Hyloxalinae is Dendrobatinae. Dendrobatinae contains exclusively (or near exclusively) toxic species. From this subfamily, we surveyed 18 individuals representing 5 species. We identified a total of 187 unique alkaloids from four *Phyllobates aurotaenia*, 316 alkaloids from five *Oophaga sylvatica*, and 213 alkaloids from three *Dendrobates truncatus*. These three species are all relatively large poison frogs (snout-vent lengths 20–35 mm; Table S2), which may in part explain their high alkaloid diversities and quantities. In *Andinobates minutus* and *Andinobates fulguritus*, which are members of the same subfamily but are much smaller in size (11–15 mm; Table S2), we detected 129 and 109 alkaloids, respectively. Three of the *Andinobates minutus* individuals were juveniles. The total alkaloid quantities (integrated areas) in *D. truncatus* and *O. sylvatica* were comparable to those of *Ameerega* but were higher than quantities detected in *Epipedobates*. We also report for the first time, to the best of our knowledge, the occurrence of N-Methyldecahydroquinolines outside the genus *Ameerega* (in *E.* aff. *espinosai*, *E.* sp. 1, *S.* aff. *gutturalis*, *Andinobates minutus*, *Andinobates fulguritus*, *D. truncatus*, *O. sylvatica*, *P. aurotaenia*, and *Allobates insperatus*) [50]. The ability to N-methylate DHQ (demonstrated experimentally in

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Adelphobates galactonotus [12]) may thus be conserved in dendrobatids, or, non-exclusively, arthropod sources of the alkaloid class (likely myrmicine ants [54]) are widespread.

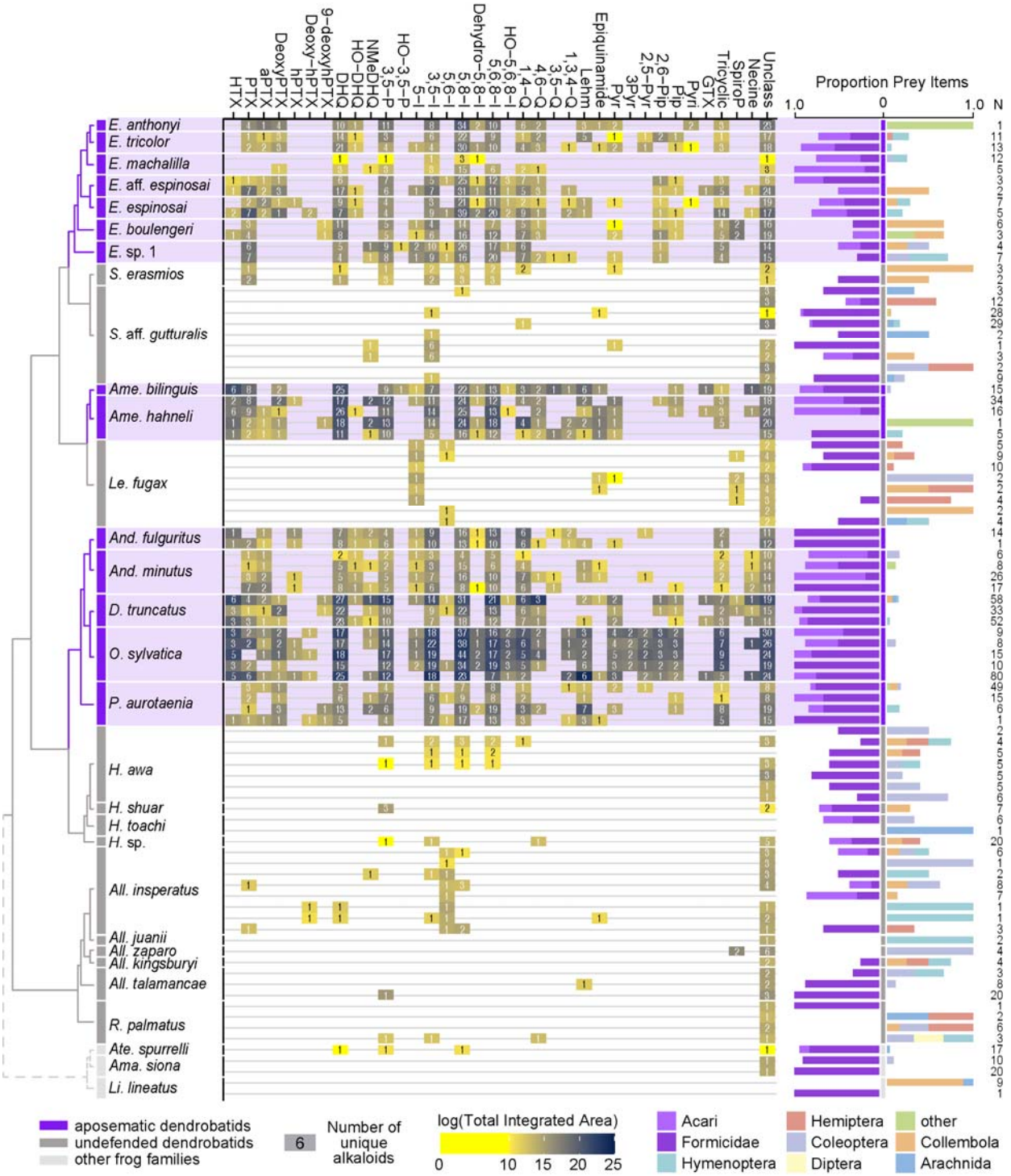

Figure 3. From left to right: an ultrametric tree showing phylogenetic relationships inferred previously [55] among sampled species with the three defended poison frog clades highlighted in purple, the undefended clades in dark gray, and non-dendrobatids in light gray (Bufonidae: *Amazophrynella siona* and *Atelopus* aff. *spurrelli*; Leptodactylidae: *Lithodytes lineatus*). Tile color indicates the log of the total quantity of alkaloids in each class as measured by the sum of integrated areas of alkaloids of that class from GCMS data per individual. The number in

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each tile indicates the number of alkaloids (including isomers) detected in each individual for each class. On the right are prey items recovered from the stomach of each individual, colored by arthropod group and scaled to 1 (total number of prey identified are shown under N). Note the large proportion of ants (Formicidae, dark purple) and mites (Acari, light purple) in many of the individuals compared to other prey types. See Table S3 for alkaloid-level data and Table S4 for raw diet data. Poison frog genera names are abbreviated as follows: *All.*, *Allobates*; *Ame.*, *Ameerega*; *And.*, *Andinobates*; *D.*, *Dendrobates*; *E.*, *Epipedobates*; *H.*, *Hyloxalus*; *Le.*, *Leucostethus*; *O.*, *Oophaga*; *P.*, *Phyllobates*; *R.*, *Rheobates*; *S.*, *Silverstoneia*; Alkaloid class abbreviations are based on [50,56] and are as follows: HTX, histrionicotoxins; PTX, pumiliotoxins; PTXB, Pumiliotoxin B; aPTX, allopumiliotoxins; DeoxyPTX, deoxypumiliotoxins; hPTX, homopumiliotoxins; deoxy-hPTX, deoxy-homopumiliotoxins; DHQ, decahydroquinolines; NMeDHQ, N-Methyldecahydroquinolines; HO-DHQ, hydroxy-decahydroquinolines; 3,5-P, 3,5-disubstituted pyrrolizidines; HO-3,5-P, hydroxy-3,5-disubstituted pyrrolizidines; 5-I, 5-substituted indolizidines; 3,5-I, 3,5-disubstituted indolizidines; 5,6-I, 5,6-disubstituted indolizidines; 5,8-I, 5,8-disubstituted indolizidines; Dehydro-5,8-I, Dehydro-5,8-Indolizidines; 5,6,8-I, 5,6,8-trisubstituted indolizidines; HO-5,6,8-I, Hydroxy-5,6,8-trisubstituted indolizidines; 1,4-Q, 1,4-disubstituted quinolizidines; 4,6-Q, 4,6-disubstituted quinolizidines; 3,5-Q, 3,5-disubstituted quinolizidines; 1,3,4-Q, 1,3,4-trisubstituted quinolizidines; Lehm, lehmizidines; Epiquinamide, epiquinamide; 2-Pyr, 2-substituted pyrrolidine; 3-Pyr, 3-substituted pyrrolidine; 2,5-Pyr, 2,5-disubstituted pyrrolidines; Pyr, pyrrolizidine of indeterminate substitution; 2,6-Pip, 2,6-disubstituted piperidines; Pip, other piperidines; Pyri, pyridines (including epibatidine); GTX, gephyrotoxins; Tricyclic, coccinelline-like tricyclics; SpiroP, spiropyrrolizidines; Necine, unspecified necine base; Unclass, unclassified alkaloids without known structures.

Outside of Dendrobatidae, we detected a new unclassified alkaloid, New159, in each of two *Amazophrynella siona* (Bufonidae) and four alkaloids in one individual of *Atelopus* aff. *spurrelli* (Anura: Bufonidae; 3,5-disubstituted pyrrolizidine **237R**-1, decahydroquinoline **243A**-3, 5,8-disubstituted indolizidine **251B**-2, and an unclassified alkaloid, New267-2). To the best of our knowledge, the detection of a decahydroquinoline and a 3,5-disubstituted pyrrolizidine in a bufonid frog other than *Melanophryniscus* [57] is novel and may provide useful context for understanding the evolution of chemical defense in the family. We detected no alkaloids in two *Lithodytes lineatus* (Leptodactylidae) individuals, which is surprising because *Lithodytes lineatus* has been hypothesized to be a Müllerian mimic of poison frogs, though the composition of its chemical defenses may be primarily proteinaceous [58]. These findings are also interesting in light of the fact that *Lithodytes lineatus* live and breed in ant colonies using chemical signals that provide camouflage [59]. In addition, we identified 55 alkaloids in 3 sampled individuals of the frog *Eleutherodactylus cystignathoides* (Eleutherodactylidae), 40 of which were likely identical to compounds identified in *S. flotator* according to our analyses (Tables S5, S6). A few other species of *Eleutherodactylus* frogs from Cuba are also known to have alkaloids [60]. Thus, these patterns suggest that some alkaloids may be widespread byproducts of frog metabolism or that passive accumulation may occur more generally in frogs. Our data do not allow us to discriminate between these possibilities.

Dietary data from these same specimens point to the ubiquity of mites and ants in dendrobatid diets, and possibly more generally in other leaf-litter dwelling frogs (Fig. 3; see below). This finding in concert with the detection of low levels of alkaloids in the lineages that putatively lack chemical defenses leads us to hypothesize that dietary shifts are not sufficient to explain the presence or absence of the chemical defense phenotype within Dendrobatidae or possibly in other families (Bufonidae, Eleutherodactylidae). The total amount of alkaloids accumulated is a result of multiple rates including toxin intake, toxin elimination, and toxin sequestration (Fig. 1) – not just intake alone. Answers to the following questions would help further evaluate the relative roles of diet versus sequestration mechanisms in the evolution of toxin sequestration in frogs.

1) Is total alkaloid intake lower in undefended lineages? If so, this would imply that behavioral or environmental changes affect diet and impact the defensive phenotype. Several of the lipophilic alkaloids found in dendrobatid frogs have been traced to arthropod sources, specifically mites [61], ants

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[62], and beetles [63], but the amount and diversity of alkaloids in each arthropod prey type is generally unknown. Shifts in diet content towards a higher proportion of ants and mites have been hypothesized to play an important role in the origin of chemical defense in poison frogs [4,8]. We quantified gut contents for the same individuals that we analyzed by GCMS and found that both undefended and defended species consume a large proportion of ants and mites (Fig. 3; Table S4). Although the defended clades tend to consume proportionally more ants and mites, as found in other studies, the undefended lineages do consume a high proportion of ants and mites. Other data support this general pattern: ants and mites constituted up to 51% and 60% of the stomach contents of the undefended species *Allobates talamancae* [64] and *H. sauli* [8], respectively. Ants and mites compose nearly 50% of the arthropods (36 and 10%, respectively) found in the *S. flotator* stomachs we analyzed (Table S4). Sympatric populations of the undefended *H. awa* and defended *E. espinosai* (formerly *E. darwinwallacei* [65]) are both diet specialized, with the former consuming mostly ants and beetles and the latter consuming mostly mites and springtails [66]. In a lab experiment, the defended species *D. tinctorius* was shown to prefer fruit fly larvae over ants when given the choice [67], suggesting that even in defended species, consumption of possible alkaloid-containing prey is not necessarily a preference. One study found that *O. sylvatica* alkaloid quantity is inversely correlated with ant and mite stomach contents; however, this species consumed more mites and ants than sympatric *H. infraguttatus* [46]. Although in many cases defended species consume more mites and ants than undefended species, the undefended lineages clearly consume toxic prey items, and some of the consumed alkaloids reach the skin (Fig. 3). In sum, the available data do not strongly support that changes in diet alone are sufficient to explain differences in alkaloid skin quantities.

2) Is the rate of toxin elimination faster in undefended lineages? Faster elimination would imply that toxin metabolism impacts the defensive phenotype. Only a few studies have reviewed toxin metabolism and elimination in dendrobatids, and none provided data for non-toxic species. Nevertheless, the available data from defended species show species-level variation and plasticity in the metabolism and elimination of alkaloids. *Epipedobates anthonyi, R. variabilis,* and *R. imitator* accumulate more than twice as much ingested epibatidine compared to *P. vittatus* and *D. tinctorius* [68]. *Oophaga sylvatica* and *D. tinctorius* upregulate detoxification genes such as cytochrome p450s upon alkaloid consumption [35,41]. *Adelphobates galactonotus* sequesters the alkaloids HTX and DHQ less efficiently at higher doses [12]. Some species metabolically alter the structure of alkaloids: *A. galactonotus*, *A. castaneoticus*, *D. auratus*, and *D. tinctorius* can hydroxylate pumiliotoxin **251D** [34,35], making it more toxic (to mice); *A. galactonotus* can also N-methylate DHQ [12]. These studies indicate that alkaloid elimination rate and metabolism varies among defended species, but not enough information exists to infer much about elimination rates in undefended lineages. Given that undefended lineages consume alkaloids yet show much lower levels of alkaloids in the skin (Fig. 2), we hypothesize that their toxin elimination rates are faster than in defended lineages (Fig. 1).

3) Are active sequestration mechanisms (Fig. 1) unique to chemically defended species, or can they also be found in undefended ones? This would imply that the presence and efficiency of sequestration mechanisms impact the defensive phenotype. Little is known regarding the mechanisms of toxin sequestration in poison frogs or in other toxin-sequestering animals. An alkaloid-binding globulin was recently characterized in the poison frog *O. sylvatica* [19]. While plasma assays demonstrated that the defended species *O. sylvatica*, *E. tricolor*, and *D. tinctorius* can bind and sequester a PTX-like photoprobe, plasma from the undefended *Allobates femoralis* showed no binding activity. In addition, the evolutionarily distant mantellid species *Mantella aurantiaca*, which also sequesters alkaloids, did not show binding activity. These data hint at variation in molecular mechanisms for alkaloid uptake across lineages [19], which may be tuned to availability of specific alkaloids in each species' diet. One GCMS analysis did not detect alkaloids in the skins of *Allobates talamancae* and *C. panamansis* after they

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consumed fruit flies dusted with 5,8-disubstituted indolizidine **209B**, decahydroquinoline **195A**, and histrionicotoxin **285C** for five weeks [11]. Other unpublished data suggest an inability by brightly colored *H. azureiventris* to accumulate alkaloids (identities not reported) from fruit flies, though the frogs apparently accumulated alkaloids dissolved in a methanol-saline solution [69]. Sparteine, a quinolizidine structurally similar to epibatidine, was detected in *Allobates femoralis* skin after being fed sparteine-dusted fruit flies for over a month [47].

Additional data on potential uptake mechanisms in dendrobatids exist for benzocaine, a synthetic lipophilic compound that is used for anesthesia and euthanasia in amphibians and has a structure similar to poison frog alkaloids. Benzocaine is readily taken up orally to the skin in the defended poison frog *D. auratus*, the non-toxic ranid frog (*Lithobates clamitans*), and the alkaloid-sequestering bufonid toad *Melanophryniscus moreirae* [48]. Although the same amount of benzocaine was injected into each frog, twice as much benzocaine was recovered from *D. auratus* than *L. clamitans* and three times as much was recovered from *M. moreirae* (see their Fig. 2), suggesting that lipophilic compound uptake occurs without specialized mechanisms of sequestration in *L. clamitans* (e.g., possibly "passive sequestration") but that *D. auratus* and *M. moreirae* likely have distinct active sequestration mechanisms that result in much higher levels of benzocaine accumulation.

Although more data will be necessary to evaluate phylogenetic patterns of active sequestration mechanisms, these data suggest that active sequestration mechanisms might be absent in undefended lineages, and that sequestering species differ substantially in their ability to actively transport and store specific compounds (Fig. 1).

(c) Predictions arising from the passive accumulation hypothesis

Data from this and other studies indicate that nearly all dendrobatids consume alkaloid-containing prey and species vary in their ability to clear alkaloids. Some species appear to lack specific transport and storage mechanisms for consumed alkaloids ("active sequestration"), yet they have detectable levels of alkaloids in their skin; we refer to this phenotype as "passive accumulation" and suggest that it is an evolutionary intermediate between toxin consumption (with no sequestration) and sequestration (Fig. 1). We predict that the ancestral state of poison frogs (and potentially other clades with alkaloid-sequestering species, such as *Melanophryniscus* and *Eleutherodactylus*) is alkaloid consumption and low levels of alkaloid resistance, accompanied by passive alkaloid accumulation. Interestingly, we also detected small amounts of alkaloids in two species of bufonid toads and one eleutherodactylid (but not in a leptodactylid), suggesting that passive accumulation may be present in an even older ancestor. Our concept of passive accumulation requires no major evolutionary innovations, only the tweaking of metabolic efficiency and/or toxin intake, along with the ability to survive consuming certain toxins (Fig. 1). Passive accumulation would also be expected to result in the diffusion of alkaloids across many tissues, rather than concentration of alkaloids within a specific tissue. Desorption electrospray ionization mass spectrometry imaging data indicate that alkaloids diffuse across various tissues in the defended dendrobatid *Dendrobates tinctorius* immediately following intake, possibly an evolutionary trace of the low elimination rates that may have initially evolved in an ancestor with the passive accumulation phenotype [13].

Alkaloid resistance is associated with alkaloid sequestration in dendrobatid poison frogs [20,21]. Although available data supports the presence of target-site resistance in some but not all poison frogs, we anticipate that some alkaloid resistance evolved in the ancestor of Dendrobatidae or in an even older ancestor, but is yet to be described [3,8] (Fig. 1D). Such resistance may be difficult to characterize using the comparative method if it involves mutations of small effect [70] or pleiotropic processes. Regardless, it is clear that all or nearly all dendrobatid poison frogs consume some amount of alkaloid-

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containing arthropods (Fig. 3) and do not appear to suffer substantially from doing so as it is part of their regular diet [4,8,10]. While little to no adaptation appears necessary to passively accumulate lipophilic alkaloids, additional adaptive changes are likely necessary to more efficiently clear or accumulate alkaloids. New research is beginning to identify major molecular players involved in this process [19].

Passive accumulation of toxins is not a novel concept, as it has been discussed previously in terms of self-medication [71,72] and bioaccumulation (e.g., of environmental pollutants [32]), and it is also conceptually analogous to some medical treatments in humans (e.g., chemotherapy). Any organism that consumes something toxic might simultaneously suffer from toxin exposure yet benefit from the compound's effect on disease, infection, parasites, or predators. For example, in the presence of parasitoids, *Drosophila suzukii* flies preferentially lay their eggs on the insecticide atropine, which protects them from being parasitized but prolongs development [73]. Mechanisms that likely underlie passive accumulation may also be analogous to key organismal functions [74]. For example, humans accumulate vitamin E in the liver [75] and use a transfer protein abundant in liver cells to shuttle the vitamin into the plasma where it becomes bioavailable [76]. The transition from passive accumulation to active sequestration in poison frogs may also rely on overexpression of genes whose encoded proteins bind to and transport alkaloids [41] (Fig. 1B). Alternatively, because most poison-frog alkaloids are fat-soluble, the passive diffusion of alkaloids, perhaps using fat-storage mechanisms, could have evolved with few changes to the ancestral physiological machinery.

In sum, for toxin-resistant organisms, there is little cost to accumulating a toxin, yet there may be benefits in doing so. If toxin accumulation is both low-cost and beneficial, then slow toxin elimination rates could evolve quite readily, resulting in passive accumulation. Two recent studies support the idea that some toxin resistance permits toxin intake and results in passive accumulation. In one, nicotine-resistant *Drosophila melanogaster* fruit flies that were fed nicotine accumulated measurable amounts of the toxin in their bodies, more than nicotine-sensitive flies [33]. In another study, ouabain-resistant *D. melanogaster* flies that were fed ouabain accumulated measurable amounts of ouabain in their bodies, more than ouabain-sensitive flies [36]. In a more extreme scenario, cardenolide defense in milkweed butterflies may not rely on any active forms of toxin transport or storage, but rather is simply a result of a high rate of toxin intake relative to toxin clearance [77]. Two cardenolide-resistant species (*Danaus plexippus* and *D. gilippus*) accumulate the cardenolides in the midgut and store the compounds in the hemolymph as well as integument and adhering tissues. In contrast, a susceptible, non-sequestering species (*Euploea core*) appears to degrade and clear cardenolides. In another case, the aphid *Athalia rosae* shows constant turnover of its glucosinolate toxins, suggesting that they cannot effectively store glucosinolates, yet their metabolic clearing is inefficient enough that they still maintain a high level of toxins in the hemolymph [78]. It is conceivable, then, that in some cases, accumulation of defensive chemicals results from a mechanism that enables high net toxin intake, followed by evasion of elimination mechanisms, passive entry into the bloodstream, and long-term storage in tissues.

Are these cases of active sequestration? Under our definition they are not, given that these species do not actively transport and store these compounds, as far as we know. Rather, these organisms merely fail to efficiently metabolize and eliminate these compounds, leading to their temporary diffusion in certain tissues that provides a transient benefit against parasites or predators. Evidence for this "passive accumulation" phenotype as an intermediate stage on the path towards toxin sequestration is scarce, but passive accumulation is a pervasive pattern in studies of ecological toxicology and may be more common in toxin-sequestering lineages than we currently know.

(d) Limitations

Our study presents a novel alkaloid dataset for dendrobatid frogs and some relatives, yet it is limited in

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the following ways. For some species we only sampled one or two individuals, which may paint an incomplete picture of toxin diversity and quantity in the group. Poison frogs vary substantially over time and seasons in their alkaloid profiles [79], yet we did not conduct serial sampling. Standards are unavailable for most frog alkaloids and thus we could not measure absolute quantity. Relative quantitation of GCMS data was performed based on integration of the extracted ion chromatogram of the base peak for each alkaloid for maximum sensitivity and selectivity. The nature of these data mean that qualitative comparisons may be meaningful but quantitative comparisons across alkaloid structures could be misleading, especially given our small sample sizes for some species. Finally, batrachotoxin and tetrodotoxin are too heavy to study using GCMS; we cannot exclude the possibility that they occur in the sampled species.

3. Conclusion

The large-scale evolutionary transition from consuming to sequestering toxins has occurred in a plethora of invertebrates [74] and vertebrates [30]. Here we provide new evidence showing that undefended poison frogs and frogs in closely related families (Bufonidae, Eleutherodactylidae) contain measurable amounts of alkaloids. We confirm that they consume some amount of toxic arthropod prey. We propose that passive accumulation of consumed alkaloids is an ancestral state in the group, and possibly in related taxa, and that selection acted on the efficiency of toxin elimination and sequestration to result in toxin accumulation and chemical defense. Future studies of the kinetics of alkaloids in different tissues of both defended and undefended poison frogs will provide further insight into these putative intermediate evolutionary steps.

4. Methods

(a) Field collection

In the case of *Silverstoneia flotator* and *Eleutherodactylus cystignathoides*, animals were collected and euthanized with benzocaine in 2022 in Gamboa, Panama (9.136, -79.723) and in 2024 in Austin, Texas, USA (30.285, -97.736). Dorsal and ventral skins were removed and placed separately in ~1-mL MeOH in 1-dram glass vials for UHPLC-HESI-MS/MS analyses (see below). For all other species, animals were collected in 2014 and euthanized with an overdose of lidocaine. Whole skins were removed and placed in ~1-mL MeOH in glass vials with PTFE-lined caps. Stomachs of all species were removed and placed in 95% ethanol.

Instruments and dissection surfaces were cleaned with 95% ethanol between dissections. Although contamination across samples is possible, it is unlikely to invalidate the identification of alkaloids in undefended species based on the following patterns. 1) At several sites, we only sampled undefended species, and these individuals were found to contain alkaloids (e.g., Las Brisas: *Rheobates palmatus*; El Valle: *Silverstoneia* aff. *gutturalis*; Santa Maria: *Hyloxalus* sp. Agua Azul); i.e. these cannot possibly have come from contamination by defended species. 2) In one site where we collected both undefended and defended species, the undefended species show no alkaloids (Lita: *H. toachi*); i.e., the preparation of both types does not imply cross-contamination of samples. 3) At two sites where the undefended species were prepared on a different day from the defended species (Valle Hermoso: *H. awa* and *E. boulengeri*; Canelos: *L. fugax* and *A. hahneli*) and could not have been cross-contaminated, the undefended species still show evidence of alkaloids. 4) All chromatograms in the sequence and integration data were inspected manually. Peaks with low areas or following samples with high areas and subject to carryover were excluded from further analysis. 5) Data from Panama collected by a

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different team using different methods also identify alkaloids in an undefended dendrobatid (*S. flotator*).

(b) Alkaloid identification and quantification

For samples from Ecuador and Colombia, a 100-µL aliquot of the MeOH was sampled from each vial and transferred to a 200-µL limited volume insert and analyzed directly by GC-MS. The system used was a Thermo AS-3000 autosampler interfaced to a Trace GC Ultra interfaced to a iTQ 1100 ion trap mass spectrometer autotuned with FC-43 (PFTBA) operating in positive ion mode. AS conditions were as follows: 2 pre-wash cycles of 5 µL MeOH, then 3 plunger strokes and withdrawal of 1.00 µL sample with 1-µL air gap, injection with no pre- or post-injection dwell followed by 3 post wash cycles of 5 µL MeOH. GC conditions were as follows: splitless injection, splitless time 1.00 min with surge (200 kPa for 0.70 min, to sharpen early peaks), split flow 50 mL/min; injector temperature 250C, oven temperature program 100C for one minute, then ramped at 10C/min to 280C and held 10 min; transfer line temperature 300C. MS conditions were as follows: for electron ionization (EI), collection mode profile, 1 microscan, 25 µsec max ion time, range 35–650 µ, source temperature 250 C, solvent delay 3.00 min, source voltage 70 eV; for chemical ionization (CI), reagent gas NH3 (1.8 mL/min). Samples for CI were run in ddMS2 mode (3 precursor ions) with 1 microscan, 50 ms max ion time, 0.450 µ precursor width and dynamic exclusion duration 0.2 min.

EI spectra were compared with published data [51,56,80] to identify class and likely ID. A set of known standards was run to give accurate retention times across the range of alkaloids and normalized to literature data using linear regression. Sample retention times were then normalized, and molecular weights were obtained from CI MS1 spectra. These were then directly compared to archival Daly GC-MS data where possible. CI MS2 spectra were also used where possible to confirm functional groups such as alcohols by loss of water, etc. Kovats retention indexes (semi-standard nonpolar) are also provided based on retention times and published indexes for background silicone impurities. Accuracy of index assignments were confirmed based on fatty acid methyl esters from skin lipids present in extracts. Epibatidine coelutes with the lipid methyl palmitoleate and the latter caused a number of false positives in the GC-MS data. We thus reviewed LC-HRMS data at the known elution time relative to a known standard. Epibatidine was only found in one sample in trace quantities and is marked as such.

Samples from Panama and Texas were extracted on separate occasions, then filtered and run in tandem with ultra-high-performance liquid-chromatography heated-electrospray-ionization tandem mass spectrometry (UHPLC-HESI-MS/MS), following an untargeted metabolomics profile, with conditions optimized specifically for retention and subsequent identification of alkaloids [81]. Briefly, for extraction, methanol was evaporated and skins were homogenized with stainless steel beads in a TissueLyser QIAGEN™ and resuspended in 1800 µL of extraction solvent (9:1 MeOH: pH 5 water). Samples were then extracted for 3 hr at 4˚C in a ThermoMixer (Eppendorf US, Enfield, CT, USA), followed by evaporation of the methanol component with a speedvac concentrator (Thermo Fisher Scientific, Waltham, MA, USA). Next, samples were freeze-dried with a lyophilizer overnight and resuspended in 500 µL extraction solvent. Resuspended samples were then filtered, diluted 1:7 in 100% MeOH, and analyzed using UHPLC-HESI-MS/MS on a Thermo Vanquish LC and QExactive quadrupole-orbitrap MS. Instrumental methods were identical to those described by [81]. A positive reference of 1 µg/µL ≥98% (±)-epibatidine dihydrochloride hydrate (Sigma-Aldrich, St. Louis, MO, USA) was included in the run.

Following UHPLC-HESI-MS/MS, chromatographic data were processed using MZmine 3 (v3.9.0) [82], applying a stringent MS1 noise threshold parameter >100000, as used by other workers (e.g., [81]). We did not use a gap filling algorithm, a step often used in analysis of chemically homogeneous datasets to backfill overlooked metabolite occurrences, so as to avoid the creation of false positive metabolite

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observations. MZmine 3 assigns chromatographic features to putative compounds based on molecular mass and retention time. MZmine 3 feature tables and MS2 data were then uploaded to the Global Natural Products Social Molecular Networking (GNPS) platform [83] for Feature-Based Molecular Networking [84]. We used SIRIUS [85] and CSI:FingerID [86] to infer molecular formulae and predict structures including the elements H, C, N, O, P, and S. CANOPUS was used to classify metabolites [87], following the ClassyFire [88] and NPClassifier molecular taxonomies [89]. Only compounds assigned to the alkaloid pathway with an NPClassifier pathway probability score >99% were retained in the feature table; epibatidine (the positive reference) was among the compounds recovered at this confidence level. This untargeted metabolomics approach yielded correct annotations for epibatidine at the levels of most specific class ("epibatidine analogues": ClassyFire) and class and superclass ("pyridine alkaloids" and "nicotinic alkaloids": NPClassifier). As expected, the compound was detected only in the positive reference sample.

(c) Diet identification

Stomach content was inspected under a stereoscope and all prey items identified to order (or family, in the case of Formicidae). Given the low sample sizes in many individuals, we did not conduct statistical comparisons of diet composition across species.

(d) Analyses

We summarized and plotted data from Ecuadorian and Colombian samples in R v4.3.1 [90] using the packages *ggplot2* [91], *cowplot* v1.1.1 [92], and *dplyr* v1.1.2 [93]. Samples from Panama and Texas were analyzed using a different instrument that has higher sensitivities to detect more diverse compounds but lower retention-time resolution, as well as untargeted analytical methods, reducing confidence in structural inferences. Therefore, data are not directly comparable, and they could not be included in Figure 3. Phylogenies were subsetted from [55] using *ape* v5.7.1 [94] and *phytools* v1.9.16 [95]. Any compounds known to co-elute with other compounds were unable to be identified, so we averaged their quantities across the co-eluting compounds. Corrections for mass were not included; we instead opted to provide data from full skins.

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Author contributions. RDT: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, visualization, writing-original draft, writing-review & editing; JLC: data curation, formal analysis, funding acquisition, investigation, resources, writing-original draft, writing-review & editing; SRR, MBC, KLH, JCS: investigation, resources, writing-review & editing; DD: data curation, investigation, resources, writing-review & editing; KSG, JMS, JRS: data curation, investigation, writing-review & editing; BES: formal analysis, funding acquisition, investigation, resources; DCC: funding acquisition, investigation, project administration, resources, supervision, writing-original draft, writing-review & editing; RWF: data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, visualization, writing-original draft, writing-review & editing. All authors gave final approval for publication and agreed to be held accountable for the work described herein.

Ethics. Collection was performed under permits (COL: Res. 1177 at Universidad de los Andes) and Contrato Marco Acceso a los Recursos Genéticos Nro. 005-14 IC-FAU-DNB/MA (Ecuador) as well as Ministerio de Ambiente Permiso de Colecta Científica No. ARBG-0038-2022 and Permiso de Transferencia de Material Genético y/o Biológico No. PA-01-ARG-096-2022 (Panama). The animal use protocols were approved by the University of Texas at Austin (IACUC AUP-2012-00032) and the Smithsonian Tropical Research Institute (SI-22017). Voucher specimens are deposited in the Museo de Zoología (QCAZ) de Pontificia Universidad Católica del Ecuador (PUCE) and the Museo de Historia Natural C.J. Marinkelle (ANDES) at the Universidad de los Andes in Bogotá, Colombia.

Use of Artificial Intelligence (AI) and AI-assisted technologies. No AI or AI-assisted technologies were used in the preparation of this manuscript.

Data accessibility. The datasets supporting this article have been uploaded as part of the supplementary material. GCMS and LCMS data are available at the Global Natural Product Social Molecular Networking (GNPS) (accession numbers pending). Other raw data are available here as supplementary tables.

Supplementary Information

Table S1. A summary of data available on alkaloid detection in “non-toxic” lineages of poison frogs.

Table S2. Collection localities, specimen numbers, size, sex, and summary of alkaloid quantities and diversity for each individual.

Table S3. Alkaloid-level data for every individual analyzed by GCMS.

Table S4. Stomach content data for every individual.

Table S5. List of the subset of classes and most specific classes of compounds in *Silverstoneia flotator* annotated as alkaloids (“Alkaloid Pathway” of NPClassifier) at >99% probability and data on presence/absence of each in *Eleutherodactylus cystignathoides*.

Table S6. Feature table with details about determined chemical properties, annotations, and presence/absence information for a) *Silverstoneia flotator* skin alkaloids, and b) identifying information for run numbers listed in Table S6a columns.

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