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

Rebecca D. Tarvin[1,*], Jeffrey L. Coleman[2,3], David A. Donoso[4,5], Mileidy Betancourth-Cundar[6], Karem López-Hervas[7], Kimberly S. Gleason[8], J. Ryan Sanders[8], Jacqueline M. Smith[8], Santiago R. Ron[9], Juan C. Santos[10], Brian E. Sedio[2,3], David C. Cannatella[2,*], and Richard Fitch[8,*]

Affiliations

[1] Museum of Vertebrate Zoology and Department of Integrative Biology, University of California, Berkeley, Berkeley, CA 94720 USA

[2] Department of Integrative Biology and Biodiversity Collections, University of Texas at Austin, Austin, TX

[3] Smithsonian Tropical Research Institute, Apartado 0843–03092, Balboa, Ancón, Republic of Panama

[4] Grupo de Investigación en Ecología Evolutiva en los Trópicos (EETROP), Universidad de las Américas, Quito, Ecuador

[5] Ecological Networks Lab, Technische Universität Darmstadt, Darmstadt, Germany

[6] Departamento de Ciencias Biológicas, Universidad de los Andes, Bogotá, Colombia, 111711

[7] Max Planck Institute for Evolutionary Biology, Plön, Germany 24306

[8] Department of Chemistry and Physics, Indiana State University, Terre Haute, IN 47809, USA

[9] Museo de Zoología, Escuela de Biología, Facultad de Ciencias Exactas y Naturales, Pontificia Universidad Católica del Ecuador, Quito, Ecuador

[10] Department of Biological Sciences, St John's University, NY, USA 11439

*Corresponding authors: rdtarvin@berkeley.edu, catfish@utexas.edu, Richard.Fitch@indstate.edu

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

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 putatively aposematic (high alkaloid content and conspicuous coloration) and putatively undefended (low or zero alkaloid content and usually lacking conspicuous coloration) categories.

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

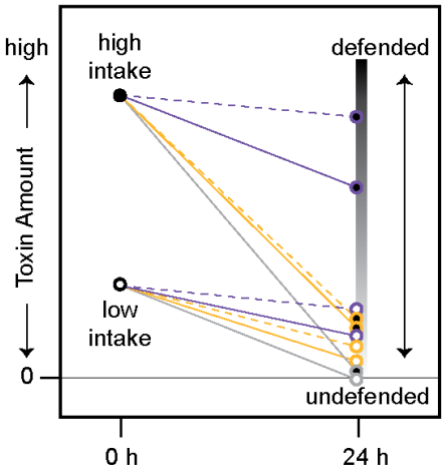

Figure 1. Major processes involved in the transition from the undefended to defended phenotype: 1) toxin intake, here visualized with two discrete points representing low and high rates; 2) toxin elimination rate (Elim. Rate), e.g., via toxin metabolism; 3) toxin sequestration rate (Seq. Rate), i.e., 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 line); 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 line); 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 line). 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 line). 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 line).

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 toxicity.

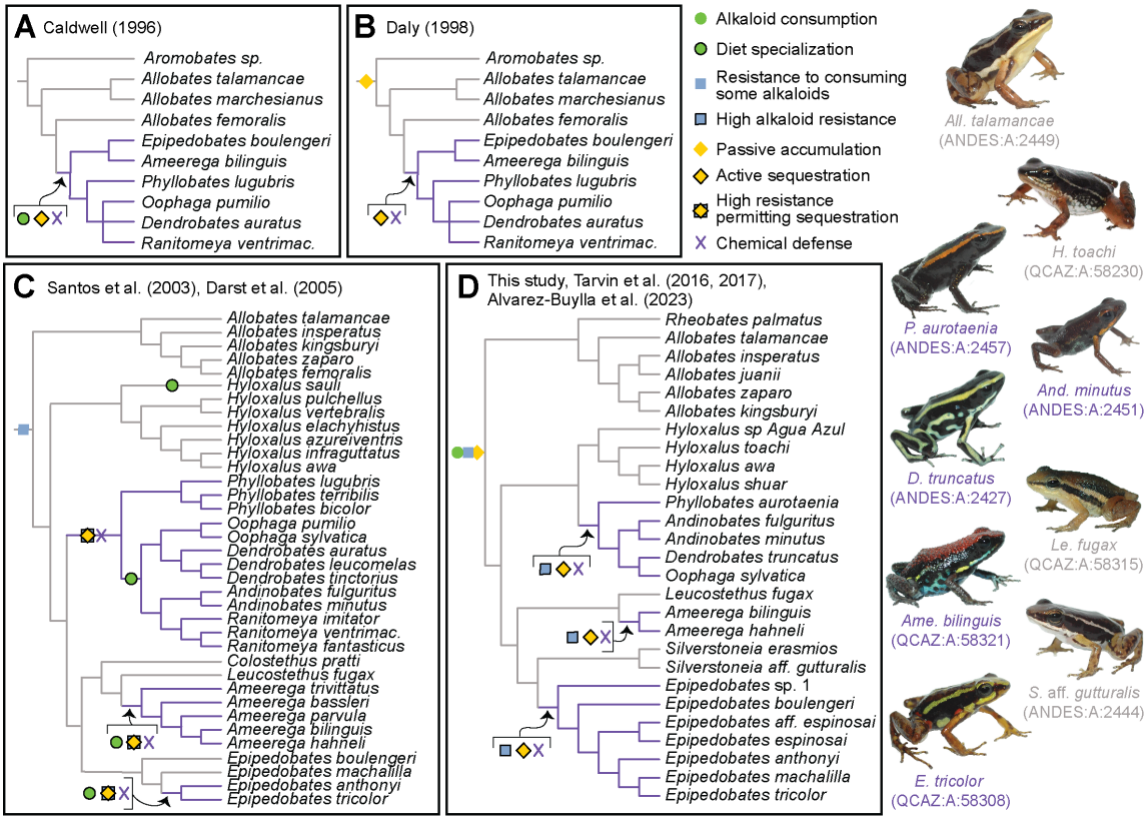

Figure 2. Evolutionary models of toxin sequestration in Dendrobatidae have changed over time. A) When several species of aposematic dendrobatids (purple lines) were found to have narrower dietary niches than undefended dendrobatids and other frogs [10,15,16], researchers hypothesized that diet specialization 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 aposematic dendrobatids (here denoted as active sequestration). **C)** A phylogenetic analysis of Dendrobatidae revealed that aposematism and diet specialization evolved independently several times [9]. The new 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 toxic clades and in some non-toxic species [20,21]. Some toxic 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].

(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, 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). 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 12 of 13 undefended species included in our study, although often with less diversity and relatively lower quantities than in aposematic lineages (Fig. 3, Table 1, Table S2, Table S3). We find substantially higher diversities of alkaloids in aposematic dendrobatid species than previously reported [5,42,44–46], and expand 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. The presumed chemical defense phenotype for poison frogs is given according to Santos and Cannatella [4]. Purple rows highlight toxic species.

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*	aposematic	1	21.97	21.97	133	133.0
Dendrobatidae	Colostethinae	*Ameerega hahneli*	aposematic	4	20.21 – 22.29	21.68	85 – 140	128.5
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
Dendrobatidae	Colostethinae	*Epipedobates* aff. *espinosai*	aposematic	2	18.44 – 20.20	19.32	83 – 131	107.0
Dendrobatidae	Colostethinae	*Epipedobates anthonyi*	aposematic	1	20.54	20.54	127	127.0
Dendrobatidae	Colostethinae	*Epipedobates boulengeri*	aposematic	2	18.87 – 19.39	19.13	77 – 94	85.5
Dendrobatidae	Colostethinae	*Epipedobates* sp. 1	aposematic	2	19.49 – 19.68	19.59	99 – 105	102.5
Dendrobatidae	Colostethinae	*Epipedobates espinosai*	aposematic	2	18.82 – 21.33	20.08	85 – 146	115.5
Dendrobatidae	Colostethinae	*Epipedobates machalilla*	aposematic	2	12.98 – 15.67	14.32	8 – 38	23.0
Dendrobatidae	Colostethinae	*Epipedobates tricolor*	aposematic	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*	aposematic	4	17.72 – 21.08	18.88	48 – 118	67.5

Dendrobatidae	Dendrobatinae	*Dendrobates truncatus*	aposematic	3	20.05 – 23.95	20.42	111 – 172	115.0
Dendrobatidae	Dendrobatinae	*Oophaga sylvatica*	aposematic	5	22.86 – 24.85	23.76	152 – 189	175.0
Dendrobatidae	Dendrobatinae	*Andinobates fulguritus*	aposematic	2	20.09 – 20.51	20.30	80 – 85	82.5
Dendrobatidae	Dendrobatinae	*Andinobates minutus*	aposematic	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
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 aposematic 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 aposematic 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 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 aposematic 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 *Adelphobates galactonotus* [12]) may thus be conserved in dendrobatids, or, non-exclusively, arthropod sources of the alkaloid class (likely myrmicine ants [54]) are widespread.

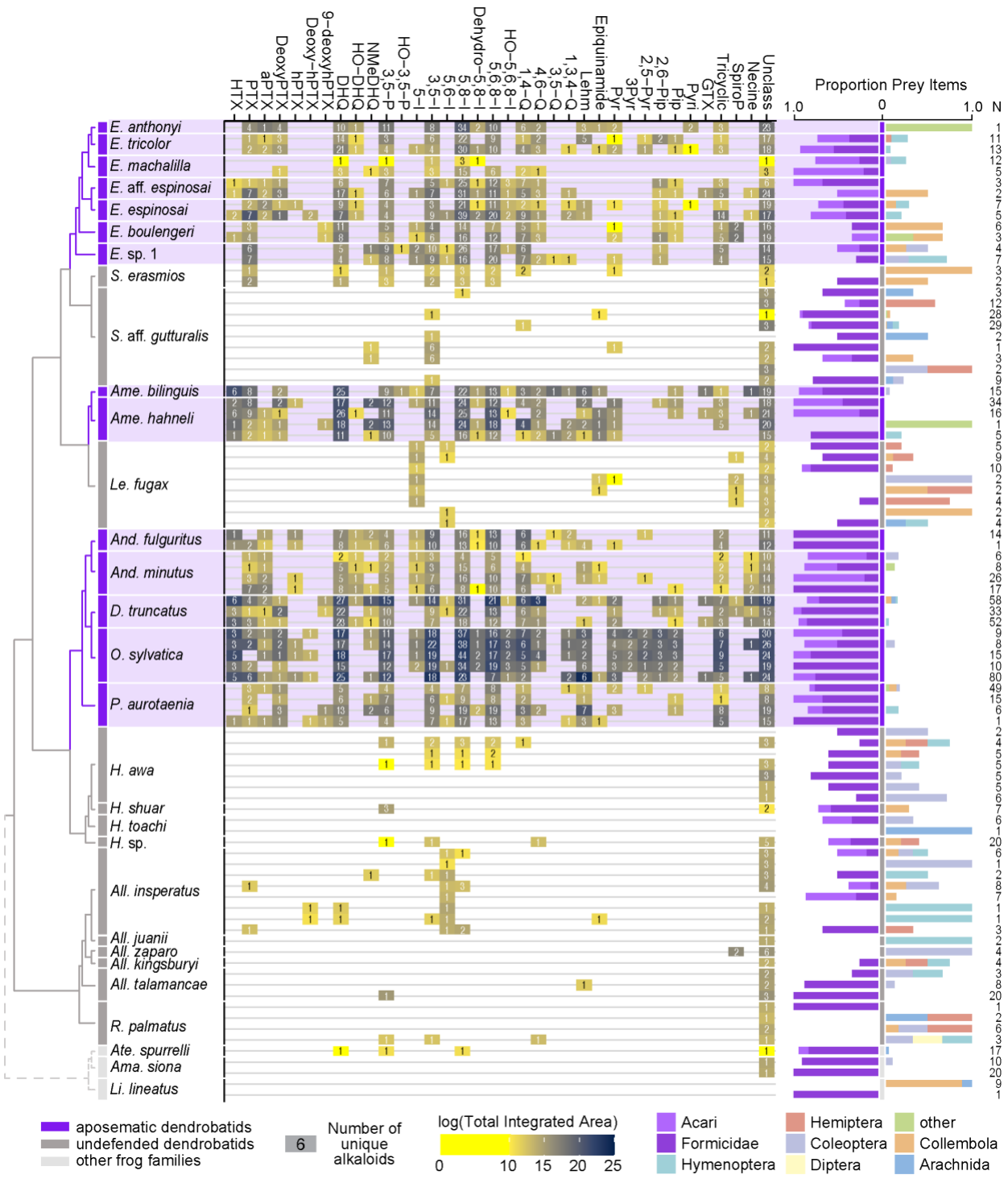

Figure 3. From left to right: an ultrametric tree showing phylogenetic relationships inferred previously [59] among sampled species with the three aposematic poison frog clades highlighted in purple, the undefended clades in dark gray, and non-dendrobatids in light gray (Bufonidae: *Amazophrynella siona* 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 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 [50,60] 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* [55] 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 [56]. 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 [57]. 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 [58]. 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 [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

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

337 2) Is the rate of toxin elimination faster in undefended lineages? Faster elimination would imply that
338 toxin metabolism impacts the defensive phenotype. Only a few studies have reviewed toxin metabolism
339 and elimination in dendrobatids, and none provided data for non-toxic species. Nevertheless, the
340 available data from aposematic species show species-level variation and plasticity in the metabolism and
341 elimination of alkaloids. *Epipedobates anthonyi, R. variabilis,* and *R. imitator* accumulate more than
342 twice as much ingested epibatidine compared to *P. vittatus* and *D. tinctorius* [68]. *Oophaga sylvatica* and
343 *D. tinctorius* upregulate detoxification genes such as cytochrome p450s upon alkaloid consumption
344 [35,41]. *Adelphobates galactonotus* sequesters the alkaloids HTX and DHQ less efficiently at higher
345 doses [12]. Some species metabolically alter the structure of alkaloids: *A. galactonotus*, *A. castaneoticus*,
346 *D. auratus*, and *D. tinctorius* can hydroxylate pumiliotoxin **251D** [34,35], making it more toxic (to mice);
347 *A. galactonotus* can also N-methylate DHQ [12]. These studies indicate that alkaloid elimination rate and
348 metabolism varies among aposematic species, but not enough information exists to infer much about
349 elimination rates in undefended lineages. Given that undefended lineages consume alkaloids yet show
350 much lower levels of alkaloids in the skin (Fig. 2), we hypothesize that their toxin elimination rates are
351 faster than in aposematic lineages (Fig. 1).

352 3) Are active sequestration mechanisms (Fig. 1) unique to chemically defended species, or can they also
353 be found in undefended ones? This would imply that the efficiency of sequestration mechanisms
354 impacts the defensive phenotype. Little is known regarding the mechanisms of toxin sequestration in
355 poison frogs or in other toxin-sequestering animals. An alkaloid-binding globulin was recently
356 characterized in the poison frog *O. sylvatica* [19]. While plasma assays demonstrated that the
357 aposematic species *O. sylvatica*, *E. tricolor*, and *D. tinctorius* can bind and sequester a PTX-like
358 photoprobe, plasma from the undefended *Allobates femoralis* showed no binding activity. In addition,
359 the evolutionarily distant mantellid species *Mantella aurantiaca*, which also sequesters alkaloids, did not
360 show binding activity. These data hint at variation in molecular mechanisms for alkaloid uptake across
361 lineages [19], which may be tuned to availability of specific alkaloids in each species' diet. One GCMS
362 analysis did not detect alkaloids in the skins of *Allobates talamancae* and *C. panamansis* after they
363 consumed fruit flies dusted with 5,8-disubstituted indolizidine **209B**, decahydroquinoline **195A**, and
364 histrionicotoxin **285C** for five weeks [11]. Other unpublished data suggest an inability by brightly colored

365 *H. azureiventris* to accumulate alkaloids (identities not reported) from fruit flies, though the frogs
366 apparently accumulated alkaloids dissolved in a methanol-saline solution [69]. Sparteine, a quinolizidine
367 structurally similar to epibatidine, was detected in *Allobates femoralis* skin after being fed sparteine-
368 dusted fruit flies for over a month [47].

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

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

383 **(c) Predictions arising from the passive accumulation hypothesis**

384 Data from this and other studies indicate that nearly all dendrobatids consume alkaloid-containing prey
385 and species vary in their ability to clear alkaloids. Some species appear to lack specific transport and
386 storage mechanisms for consumed alkaloids ("active sequestration"), yet they have detectable levels of
387 alkaloids in their skin; we refer to this phenotype as "passive accumulation" and suggest that it is an
388 evolutionary intermediate between toxin consumption (with no sequestration) and sequestration (Fig.
389 1). We predict that the ancestral state of poison frogs (and potentially other clades with alkaloid-
390 sequestering species, such as *Melanophryniscus* and *Eleutherodactylus*) is alkaloid consumption and low
391 levels of alkaloid resistance, accompanied by passive alkaloid accumulation. Interestingly, we also
392 detected small amounts of alkaloids in two species of bufonid toads and one eleutherodactylid (but not
393 in a leptodactylid), suggesting that passive accumulation may be present in an even older ancestor.
394 Importantly, our concept of passive accumulation requires no major evolutionary innovations, only the
395 tweaking of metabolic efficiency and/or toxin intake, along with the ability to survive consuming certain
396 toxins (Fig. 2). Passive accumulation would also be expected to result in the diffusion of alkaloids across
397 many tissues, rather than concentration of alkaloids within a specific tissue. Desorption electrospray
398 ionization mass spectrometry imaging data indicate that alkaloids diffuse across various tissues in the
399 aposematic dendrobatid *Dendrobates tinctorius* immediately following intake, possibly an evolutionary
400 trace of the low elimination rates that may have initial evolved in an ancestor with the passive
401 accumulation phenotype [13].

402 Toxin resistance is associated with toxin sequestration in dendrobatid poison frogs [20,21]. Although
403 available data supports the presence of target-site resistance in some but not all poison frogs, we
404 anticipate that some alkaloid resistance evolved in the ancestor of Dendrobatidae or in an even older
405 ancestor, but is yet to be described [3,8] (Fig. 1D). Such resistance may be difficult to characterize using
406 the comparative method if it involves mutations of small effect [70] or pleiotropic processes. Regardless,
407 it is clear that all or nearly all dendrobatid poison frogs consume some amount of alkaloid-containing
408 arthropods [4,8,10] (Fig. 3) and they do not appear to suffer substantially from doing so (e.g. [35,47,68]).
409 While little to no adaptation appears necessary to passively accumulate lipophilic alkaloids, additional

410 adaptive changes are likely necessary to more efficiently clear or accumulate alkaloids. New research is
411 beginning to identify major molecular players involved in this process [19].

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

426 In sum, for toxin-resistant organisms, there is little cost to accumulating a toxin, yet there may be
427 benefits in doing so. If toxin accumulation is both low-cost and beneficial, then slow toxin elimination
428 rates could evolve quite readily, resulting in passive accumulation. Two recent studies support the idea
429 that some toxin resistance permits toxin intake and results in passive accumulation. In one, nicotine-
430 resistant *Drosophila melanogaster* fruit flies that were fed nicotine accumulated measurable amounts of
431 the toxin in their bodies, more than nicotine-sensitive flies [33]. In another study, ouabain-resistant *D.*
432 *melanogaster* flies that were fed ouabain accumulated measurable amounts of ouabain in their bodies,
433 more than ouabain-sensitive flies [36]. In a more extreme scenario, cardenolide defense in milkweed
434 butterflies may not rely on any active forms of toxin transport or storage, but rather is simply a result of
435 a high rate of toxin intake relative to toxin clearance [76]. Two cardenolide-resistant species (*Danaus*
436 *plexippus* and *D. gilippus*) accumulate the cardenolides in the midgut and store the compounds in the
437 hemolymph as well as integument and adhering tissues. In contrast, a susceptible, non-sequestering
438 species (*Euploea core*) appears to degrade and clear cardenolides. In another case, the aphid *Athalia*
439 *rosae* shows constant turnover of its glucosinolate toxins, suggesting that they cannot effectively store
440 glucosinolates, yet their metabolic clearing is inefficient enough that they still maintain a high level of
441 toxins in the hemolymph [77]. It is conceivable, then, that in some cases, accumulation of defensive
442 chemicals results from a mechanism that enables high net toxin intake, followed by passive entry into
443 the bloodstream and long-term storage in tissues.

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

451 **(d) Limitations**

452 Our study presents a novel alkaloid dataset for dendrobatid frogs and some relatives, yet it is limited in
453 the following ways. For some species we only sampled one or two individuals, which may paint an
454 incomplete picture of toxin diversity and quantity in the group. Poison frogs vary substantially over time

455 and seasons in their alkaloid profiles [78], yet we did not conduct serial sampling. Standards are
456 unavailable for most frog alkaloids and thus we could not measure absolute quantity. Relative
457 quantitation was performed based on integration of the extracted ion chromatogram of the base peak
458 for each alkaloid for maximum sensitivity and selectivity. The nature of these data mean that qualitative
459 comparisons may be meaningful but quantitative comparisons across alkaloid structures could be
460 misleading, especially given our small sample sizes for some species. Therefore, we refrained from
461 conducting additional quantitative analyses of integrated area data. Finally, batrachotoxin and
462 tetrodotoxin are too heavy to study using GCMS; we cannot exclude the possibility that they occur in the
463 sampled species.

464

465 3. Conclusion

466 The large-scale evolutionary transition from consuming to sequestering toxins has occurred in a plethora
467 of invertebrates [79] and vertebrates [30]. Here we provide new evidence showing that undefended
468 poison frogs and frogs in closely related families (Bufonidae, Eleutherodactylidae) contain measurable
469 amounts of alkaloids. We confirm that they consume some amount of toxic arthropod prey. We propose
470 that passive accumulation of consumed alkaloids is an ancestral state in the group, and possibly in
471 related taxa, and that selection acted on the efficiency of toxin elimination and sequestration to result in
472 chemical defense. Future studies of the kinetics of alkaloids in different tissues of both aposematic and
473 undefended poison frogs will provide further insight into these putative intermediate evolutionary
474 steps.

475

476 4. Methods

477 (a) Field collection

478 In the case of *Silverstoneia flotator* and *Eleutherodactylus cystignathoides*, animals were collected and
479 euthanized with benzocaine in 2022 in Gamboa, Panama (9.136, -79.723) and in 2024 in Austin, Texas,
480 USA (30.285, -97.736). Dorsal and ventral skins were removed and placed separately in ~1-mL MeOH in
481 1-dram glass vials for UHPLC-HESI-MS/MS analyses (see below). For all other species, animals were
482 collected in 2014 and euthanized with an overdose of lidocaine. Whole skins were removed and placed
483 in ~1-mL MeOH in glass vials with PTFE-lined caps. Stomachs of all species were removed and placed in
484 95% ethanol.

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

499 *flotator*).

500 **(b) Alkaloid identification and quantification**

501 For samples from Ecuador and Colombia, a 100-µL aliquot of the MeOH was sampled from each vial and
502 transferred to a 200-µL limited volume insert and analyzed directly by GC-MS. The system used was a
503 Thermo AS-3000 autosampler interfaced to a Trace GC Ultra interfaced to a iTQ 1100 ion trap mass
504 spectrometer autotuned with FC-43 (PFTBA) operating in positive ion mode. AS conditions were as
505 follows: 2 pre-wash cycles of 5 µL MeOH, then 3 plunger strokes and withdrawal of 1.00 µL sample with
506 1-µL air gap, injection with no pre- or post-injection dwell followed by 3 post wash cycles of 5 µL MeOH.
507 GC conditions were as follows: splitless injection, splitless time 1.00 min with surge (200 kPa for 0.70
508 min, to sharpen early peaks), split flow 50 mL/min; injector temperature 250C, oven temperature
509 program 100C for one minute, then ramped at 10C/min to 280C and held 10 min; transfer line
510 temperature 300C. MS conditions were as follows: for electron ionization (EI), collection mode profile, 1
511 microscan, 25 µsec max ion time, range 35–650 µ, source temperature 250 C, solvent delay 3.00 min,
512 source voltage 70 eV; for chemical ionization (CI), reagent gas NH3 (1.8 mL/min). Samples for CI were
513 run in ddMS2 mode (3 precursor ions) with 1 microscan, 50 ms max ion time, 0.450 µ precursor width
514 and dynamic exclusion duration 0.2 min.

515 EI spectra were compared with published data [51,60,80] to identify class and likely ID. A set of known
516 standards was run to give accurate retention times across the range of alkaloids and normalized to
517 literature data using linear regression. Sample retention times were then normalized, and molecular
518 weights were obtained from CI MS1 spectra. These were then directly compared to archival Daly GC-MS
519 data where possible. CI MS2 spectra were also used where possible to confirm functional groups such as
520 alcohols by loss of water, etc. Kovats retention indexes (semi-standard nonpolar) are also provided
521 based on retention times and published indexes for background silicone impurities. Accuracy of index
522 assignments were confirmed based on fatty acid methyl esters from skin lipids present in extracts.
523 Epibatidine coelutes with the lipid methyl palmitoleate and the latter caused a number of false positives
524 in the GC-MS data. We thus reviewed LC-HRMS data at the known elution time relative to a known
525 standard. Epibatidine was only found in one sample in trace quantities and is marked as such.

526 Samples from Panama and Texas were extracted on separate occasions, then filtered and run in tandem
527 with ultra-high-performance liquid-chromatography heated-electrospray-ionization tandem mass
528 spectrometry (UHPLC-HESI-MS/MS), following an untargeted metabolomics profile, with conditions
529 optimized specifically for retention and subsequent identification of alkaloids [81]. Briefly, for extraction,
530 methanol was evaporated and skins were homogenized with stainless steel beads in a TissueLyser
531 QIAGEN™ and resuspended in 1800 µL of extraction solvent (9:1 MeOH: pH 5 water). Samples were
532 then extracted for 3 hr at 4˚C in a ThermoMixer (Eppendorf US, Enfield, CT, USA), followed by
533 evaporation of the methanol component with a speedvac concentrator (Thermo Fisher Scientific,
534 Waltham, MA, USA). Next, samples were freeze-dried with a lyophilizer overnight and resuspended in
535 500 µL extraction solvent. Resuspended samples were then filtered, diluted 1:7 in 100% MeOH, and
536 analyzed using UHPLC-HESI-MS/MS on a Thermo Vanquish LC and QExactive quadrupole-orbitrap MS.
537 Instrumental methods were identical to those described by [81]. A positive reference of 1 µg/µL ≥98%
538 (±)-epibatidine dihydrochloride hydrate (Sigma-Aldrich, St. Louis, MO, USA) was included in the run.

539 Following UHPLC-HESI-MS/MS, chromatographic data were processed using MZmine 3 (v3.9.0) [82],
540 applying a stringent MS1 noise threshold parameter >100000, as used by other workers (e.g., [81]). We
541 did not use a gap filling algorithm, a step often used in analysis of chemically homogeneous datasets to
542 backfill overlooked metabolite occurrences, so as to avoid the creation of false positive metabolite
543 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 [59] 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.

Acknowledgements. We thank Fray Arriaga, Josué Collins (STRI, Panama), Cristian Florez-Pai (FELCA, Colombia), Valentina Gómez-Bahamón, Camilo Isaza (Cafam, Colombia), Roberto Márquez, Daniel Nastacuaz, Pablo Palacios-Rodriguez, Andrea Paz, Santiago Vega, and many others for their assistance in the field. We thank the communities of Laguna de Cube (Esmeraldas) and Laguna de San Pedro (Orellana) in Ecuador for their support and efforts towards conserving local ecosystems. We also thank Kameron T. Bell, Nicholas R. Andreasen, and Megan M. Reid for their help acquiring and processing GCMS data. Finally, JLC would like to thank Raineldo Urriola, Isis Ochoa, Lil Marie Camacho, Zurenayka Alain, Roberto Ibáñez, Roberto Cambra, Roberto Borrell, Rivieth De Liones, Félix Rodriguez, and Orelis Arosemena for their warmth, generosity, and logistical support during his time at STRI.

Funding. RDT was supported by an NIH MIRA (1R35GM150574), start-up funding from University of California Berkeley, and grants from the Society of Systematic Biologists, North Carolina Herpetological Society, Society for the Study of Reptiles and Amphibians, Chicago Herpetological Society, Texas Herpetological Society, the EEB Program at University of Texas at Austin, National Science Foundation Graduate Research Fellowship Program Graduate Research Opportunities Worldwide (in partnership with USAID), and a 2014 National Geographic Young Explorer Grant. Additional support to DCC, RWF, JLC, and RDT was provided by NSF DBI-1556967, and BES received support from Stengl-Wyer Endowment Grant SWG-22-01. RWF and students KSG, JMS, and JRS were supported by NSF DUE-0942345, NSF CHE-1531972, and NSF IOS-1556982.

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.

626 **References**

627 1. West-Eberhard MJ. 2003 *Developmental plasticity and evolution*. 1st edn. New York: Oxford
628 University Press.

629 2. Berenbaum MR. 1995 The chemistry of defense: theory and practice. *Proc. Natl. Acad. Sci. U. S. A.*
630 **92**, 2–8.

631 3. Santos JC, Tarvin RD, O'Connell LA. 2016 A review of chemical defense in poison frogs
632 (Dendrobatidae): ecology, pharmacokinetics, and autoresistance. In *Chemical Signals in Vertebrates*
633 *13* (eds BA Schulte, TE Goodwin, MH Ferkin), pp. 305–337. Switzerland: Springer International
634 Publishing.

635 4. Santos JC, Cannatella DC. 2011 Phenotypic integration emerges from aposematism and scale in
636 poison frogs. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 6175–6180.

637 5. Lawrence JP, Rojas B, Blanchette A, Saporito RA, Mappes J, Fouquet A, Noonan BP. 2023 Linking
638 predator responses to alkaloid variability in poison frogs. *J. Chem. Ecol.* **49**, 195–204.

639 6. AmphibiaWeb. 2024 https://amphibiaweb.org/ University of California, Berkeley, CA, USA.
640 Accessed 14 March 2024.

641 7. Santos JC, Baquero M, Barrio-Amorós C, Coloma LA, Erdtmann LK, Lima AP, Cannatella DC. 2014
642 Aposematism increases acoustic diversification and speciation in poison frogs. *Proc. R. Soc. Lond. B*
643 *Biol. Sci.* **281**, 20141761.

644 8. Darst CR, Menéndez-Guerrero PA, Coloma LA, Cannatella DC. 2005 Evolution of dietary
645 specialization and chemical defense in poison frogs (Dendrobatidae): A comparative analysis. *Am.*
646 *Nat.* **165**, 56–69.

647 9. Santos JC, Coloma LA, Cannatella DC. 2003 Multiple, recurring origins of aposematism and diet
648 specialization in poison frogs. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 12792–12797.

649 10. Toft CA. 1995 Evolution of diet specialization in poison-dart frogs (Dendrobatidae). *Herpetologica*
650 **51**, 202–216.

651 11. Daly JW, Secunda SI, Garraffo HM, Spande TF, Wisnieski A, Cover JF Jr. 1994 An uptake system for
652 dietary alkaloids in poison frogs (Dendrobatidae). *Toxicon* **32**, 657–663.

653 12. Jeckel AM *et al.* 2022 Dose-dependent alkaloid sequestration and N-methylation of
654 decahydroquinoline in poison frogs. *J. Exp. Zool. A Ecol. Integr. Physiol.* **337**, 537–546.

655 13. Jeckel AM, Matsumura K, Nishikawa K, Morimoto Y, Saporito RA, Grant T, Ifa DR. 2020 Use of
656 whole-body cryosectioning and desorption electrospray ionization mass spectrometry imaging to
657 visualize alkaloid distribution in poison frogs. *J. Mass Spectrom.* **55**, 1–6.

658 14. Gonzalez M, Carazzone C. 2023 Eco-Metabolomics Applied to the Chemical Ecology of Poison Frogs
659 (Dendrobatoidea). *J. Chem. Ecol.* (doi:10.1007/s10886-023-01443-0)

660 15. Toft CA. 1980 Feeding ecology of thirteen syntopic species of anurans in a seasonal tropical
661 environment. *Oecologia* **45**, 131–141.

662 16. Toft CA. 1981 Feeding ecology of Panamanian litter anurans: Patterns in diet and foraging mode. *J.*
663 *Herpetol.* **15**, 139–144.

664 17. Caldwell JP. 1996 The evolution of myrmecophagy and its correlates in poison frogs (Family
665 Dendrobatidae). *J. Zool.* **240**, 75–101.

666 18. Daly JW. 1998 Thirty years of discovering arthropod alkaloids in amphibian skins. *J. Nat. Prod.* **61**,
667 162–172.

668 19. Alvarez-Buylla A *et al.* 2023 Binding and sequestration of poison frog alkaloids by a plasma globulin.
669 *Elife* **12**, e85096.

670 20. Tarvin RD, Santos JC, O'Connell LA, Zakon HH, Cannatella DC. 2016 Convergent substitutions in a
671 sodium channel suggest multiple origins of toxin resistance in poison frogs. *Mol. Biol. Evol.* **33**,
672 1068–1081.

673 21. Tarvin RD, Borghese CM, Sachs W, Santos JC, Lu Y, O'Connell LA, Cannatella DC, Harris RA, Zakon
674 HH. 2017 Interacting amino acid replacements allow poison frogs to evolve epibatidine resistance.
675 *Science* **357**, 1261–1266.

676 22. Andreev D, Kreitman M, Phillips TW, Beeman RW, ffrench-Constant RH. 1999 Multiple origins of
677 cyclodiene insecticide resistance in *Tribolium castaneum* (Coleoptera: Tenebrionidae). *J. Mol. Evol.*
678 **48**, 615–624.

679 23. Daborn PJ *et al.* 2002 A single p450 allele associated with insecticide resistance in *Drosophila*.
680 *Science* **297**, 2253–2256.

681 24. Chiu T-L, Wen Z, Rupasinghe SG, Schuler MA. 2008 Comparative molecular modeling of *Anopheles*
682 *gambiae* CYP6Z1, a mosquito P450 capable of metabolizing DDT. *Proc. Natl. Acad. Sci. U. S. A.* **105**,
683 8855–8860.

684 25. Richard H, Carroll L. 2013 The molecular genetics of insecticide resistance. *Genetics* **194**, 807–815.

685 26. Dobler S, Petschenka G, Pankoke H. 2011 Coping with toxic plant compounds - The insect's
686 perspective on iridoid glycosides and cardenolides. *Phytochemistry* **72**, 1593–1604.

687 27. Agrawal AA, Petschenka G, Bingham RA, Weber MG, Rasmann S. 2012 Toxic cardenolides: Chemical
688 ecology and coevolution of specialized plant-herbivore interactions. *New Phytol.* **194**, 28–45.

689 28. Arbuckle K, Rodríguez de la Vega RC, Casewell NR. 2017 Coevolution takes the sting out of it:
690 Evolutionary biology and mechanisms of toxin resistance in animals. *Toxicon* **140**, 118–131.

691 29. Whitehead A, Clark BW, Reid NM, Hahn ME, Nacci D. 2017 When evolution is the solution to
692 pollution: Key principles, and lessons from rapid repeated adaptation of killifish (*Fundulus*
693 *heteroclitus*) populations. *Evol. Appl.* **10**, 762–783.

694 30. Savitzky AH, Mori A, Hutchinson DA, Saporito RA, Burghardt GM, Lillywhite HB, Meinwald J. 2012
695 Sequestered defensive toxins in tetrapod vertebrates: Principles, patterns, and prospects for future
696 studies. *Chemoecology* **22**, 141–158.

697 31. Tarvin RD, Pearson KC, Douglas TE, Ramírez-Castañeda V, Navarrete MJ. 2023 The diverse
698 mechanisms that animals use to resist toxins. *Annu. Rev. Ecol. Evol. Syst.* **54**, 283–306.

699 32. Spurgeon D, Lahive E, Robinson A, Short S, Kille P. 2020 Species sensitivity to toxic substances:
700 evolution, ecology and applications. *Front. Environ. Sci. Eng. China* **8**.
701 (doi:10.3389/fenvs.2020.588380)

702 33. Douglas TE, Beskid SG, Gernand CE, Nirtaut BE, Tamsil KE, Fitch RW, Tarvin RD. 2022 Trade-offs
703 between cost of ingestion and rate of intake drive defensive toxin use. *Biol. Lett.* **18**, 20210579.

704 34. Daly JW, Garraffo HM, Spande TF, Clark VC, Ma J, Ziffer H, Cover JF Jr. 2003 Evidence for an
705 enantioselective pumiliotoxin 7-hydroxylase in dendrobatid poison frogs of the genus *Dendrobates*.
706 *Proc. Natl. Acad. Sci. U. S. A.* **100**, 11092–11097.

707 35. Alvarez-Buylla A, Payne CY, Vidoudez C, Trauger SA, O'Connell LA. 2022 Molecular physiology of
708 pumiliotoxin sequestration in a poison frog. *PLoS One* **17**, e0264540.

709 36. Karageorgi M *et al.* 2019 Genome editing retraces the evolution of toxin resistance in the monarch
710 butterfly. *Nature* **574**. (doi:10.1038/s41586-019-1610-8)

711 37. Petschenka G, Fandrich S, Sander N, Wagschal V, Boppré M, Dobler S. 2013 Stepwise evolution of
712 resistance to toxic cardenolides via genetic substitutions in the Na+/K+-ATPase of milkweed
713 butterflies (Lepidoptera: Danaini). *Evolution* **67**, 2753–2761.

714 38. Yang L *et al.* 2023 Predatory fireflies and their toxic firefly prey have evolved distinct toxin
715 resistance strategies. *Curr. Biol.* **33**, 5160-5168.e7.

716 39. Coleman JL, Cannatella DC. 2023 The molecular basis and evolution of toxin resistance in poison
717 frogs. *Evol. Ecol.* (doi:10.1007/s10682-023-10258-0)

718 40. Abderemane-Ali F *et al.* 2021 Evidence that toxin resistance in poison birds and frogs is not rooted
719 in sodium channel mutations and may rely on "toxin sponge" proteins. *J. Gen. Physiol.* **153**,
720 e202112872.

721 41. Caty SN *et al.* 2019 Molecular physiology of chemical defenses in a poison frog. *J. Exp. Biol.* **222**,
722 jeb204149.

723 42. Daly JW, Myers CW, Whittaker N. 1987 Further classification of skin alkaloids from neotropical
724 poison frogs (Dendrobatidae), with a general survey of toxic/noxious substances in the Amphibia.
725 *Toxicon* **25**, 1023–1095.

726 43. Gonzalez M, Palacios-Rodriguez P, Hernandez-Restrepo J, González-Santoro M, Amézquita A,
727 Brunetti AE, Carazzone C. 2021 First characterization of toxic alkaloids and volatile organic
728 compounds (VOCs) in the cryptic dendrobatid *Silverstoneia punctiventris*. *Front. Zool.* **18**, 39.

729 44. Cipriani I, Rivera M. 2009 Detección de alcaloides en la piel de cuatro especies de anfibios
730 ecuatorianos (Anura: Dendrobatidae). *Rev. Ecuat. Med. Cienc. Biol.* **30**, 42–49.

731 45. Moskowitz NA *et al.* 2020 Land use impacts poison frog chemical defenses through changes in leaf
732 litter ant communities. *Neotropical Biodiversity* **6**, 75–87.

733 46. Moskowitz NA, Alvarez-Buylla A, Morrison CR, Chamba A, Rentería J, Tapia EE, Coloma LA, Donoso
734 DA, O'Connell LA. 2022 Poison frog diet and chemical defense are influenced by availability and
735 selectivity for ants. *bioRxiv*. , 2022.06.14.495949. (doi:10.1101/2022.06.14.495949)

736 47. Sanchez E *et al.* 2019 Transcriptomic signatures of experimental alkaloid consumption in a poison
737 frog. *Genes* **10**, 733.

738 48. Saporito RA, Grant T. 2018 Comment on Amézquita et al. (2017) "Conspicuousness, color
739 resemblance, and toxicity in geographically diverging mimicry: The pan-Amazonian frog *Allobates*
740 *femoralis* ." *Evolution* , 1009–1014.

741 49. Amézquita A, Ramos Ó, González MC, Rodríguez C, Medina I, Simões PI, Lima AP. 2017
742 Conspicuousness, color resemblance, and toxicity in geographically diverging mimicry: The pan-
743 Amazonian frog *Allobates femoralis*. *Evolution* **71**, 1039–1050.

744 50. Daly JW, Ware N, Saporito RA, Spande TF, Garraffo HM. 2009 N-methyldecahydroquinolines: An
745 unexpected class of alkaloids from Amazonian poison frogs (Dendrobatidae). *J. Nat. Prod.* **72**, 1110–
746 1114.

747 51. Daly JW, Garraffo HM, Spande TF. 1999 Alkaloids from amphibian skins. In *Alkaloids: Chemical and*

748 *Biological Perspectives* (ed SW Pelletier), pp. 1–161. New York: Pergamon.

749 52. Guillory WX *et al.* 2020 Phylogenetic relationships and systematics of the Amazonian poison frog
750 genus *Ameerega* using ultraconserved genomic elements. *Mol. Phylogenet. Evol.* **142**, 106638.

751 53. Darst CR, Cummings ME. 2006 Predator learning favours mimicry of a less-toxic model in poison
752 frogs. *Nature* **440**, 208–211.

753 54. Jones TH, Gorman JST, Snelling RR, Delabie JHC, Blum MS, Garraffo HM, Jain P, Daly JW, Spande TF.
754 1999 Further alkaloids common to ants and frogs: Decahydroquinolines and a quinolizidine. *J.*
755 *Chem. Ecol.* **25**, 1179–1193.

756 55. Rodríguez C, Rollins-Smith L, Ibáñez R, Durant-Archibold AA, Gutiérrez M. 2017 Toxins and
757 pharmacologically active compounds from species of the family Bufonidae (Amphibia, Anura). *J.*
758 *Ethnopharmacol.* **198**, 235–254.

759 56. Prates I, Antoniazzi MM, Sciani JM, Pimenta DC, Toledo F, Haddad FB, Jared C. 2011 Skin glands,
760 poison and mimicry in dendrobatid and leptodactylid amphibians. *Journal of Morphology* **273**, 279–
761 290.

762 57. de Lima Barros A, López-Lozano JL, Lima AP. 2016 The frog *Lithodytes lineatus* (Anura:
763 Leptodactylidae) uses chemical recognition to live in colonies of leaf-cutting ants of the genus *Atta*
764 (Hymenoptera: Formicidae). *Behav. Ecol. Sociobiol.* **70**, 2195–2201.

765 58. Rodríguez A, Poth D, Schulz S, Gehara M, Vences M. 2013 Genetic diversity, phylogeny and
766 evolution of alkaloid sequestering in Cuban miniaturized frogs of the *Eleutherodactylus limbatus*
767 group. *Mol. Phylogenet. Evol.* **68**, 541–554.

768 59. Wan YC *et al.* 2023 Selection on visual opsin genes in diurnal Neotropical frogs and loss of the SWS2
769 opsin in poison frogs. *Mol. Biol. Evol.* **40**. (doi:10.1093/molbev/msad206)

770 60. Daly JW, Spande TF, Garraffo HM. 2005 Alkaloids from amphibian skin: A tabulation of over eight-
771 hundred compounds. *J. Nat. Prod.* **68**, 1556–1575.

772 61. Saporito RA, Donnelly MA, Norton R a., Garraffo HM, Spande TF, Daly JW. 2007 Oribatid mites as a
773 major dietary source for alkaloids in poison frogs. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 8885–8890.

774 62. Saporito RA, Garraffo HM, Donnelly MA, Edwards AL, Longino JT, Daly JW. 2004 Formicine ants: An
775 arthropod source for the pumiliotoxin alkaloids of dendrobatid poison frogs. *Proc. Natl. Acad. Sci.*
776 *U. S. A.* **101**, 8045–8050.

777 63. Dumbacher JP, Wako A, Derrickson SR, Samuelson A, Spande TF, Daly JW. 2004 Melyrid beetles
778 (*Choresine*): a putative source for the batrachotoxin alkaloids found in poison-dart frogs and toxic
779 passerine birds. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 15857–15860.

780 64. Mebs D, Yotsu-Yamashita M, Pogoda W, Vargas Alvarez J, Ernst R, Köhler G, Toennes SW. 2018 Lack
781 of alkaloids and tetrodotoxin in the neotropical frogs *Allobates* spp. (Aromobatidae) and
782 *Silverstoneia flotator* (Dendrobatidae). *Toxicon* **152**. (doi:10.1016/j.toxicon.2018.07.027)

783 65. López-Hervas K, Santos JC, Ron SR, Betancourth-Cundar M, Cannatella DC, Tarvin RD. 2024 Deep
784 divergences among inconspicuously colored clades of Epipedobates poison frogs. *Mol. Phylogenet.*
785 *Evol.* , 108065.

786 66. Sánchez-Loja S, Donoso DA, Páez-Vacas MI. 2023 Conspicuous and cryptic poison frogs are picky
787 and prefer different meals in syntopy. *Evol. Ecol.* (doi:10.1007/s10682-023-10282-0)

788 67. Moskowitz NA, D'Agui R, Alvarez-Buylla A, Fiocca K, O'Connell LA. 2022 Poison frog dietary

789 preference depends on prey type and alkaloid load. *PLOS ONE* **17**, e0276331.

790 68. Waters KR, Dugas MB, Grant T, Saporito RA. 2023 The ability to sequester the alkaloid epibatidine is
791 widespread among dendrobatid poison frogs. *Evol. Ecol.* (doi:10.1007/s10682-023-10260-6)

792 69. Saporito RA, Spande TF, Garraffo HM, Donnelly MA. 2009 Arthropod alkaloids in poison frogs: A
793 review of the 'Dietary Hypothesis.' *Heterocycles* **79**, 277–297.

794 70. ffrench-Constant RH, Daborn PJ, Goff GL. 2004 The genetics and genomics of insecticide resistance.
795 *Trends Genet.* **20**, 163–170.

796 71. Clayton DH, Wolfe ND. 1993 The adaptive significance of self-medication. *Trends Ecol. Evol.* **8**, 60–
797 63.

798 72. Singer MS, Mace KC, Bernays EA. 2009 Self-medication as adaptive plasticity: increased ingestion of
799 plant toxins by parasitized caterpillars. *PLoS One* **4**, e4796.

800 73. Poyet M, Eslin P, Chabrerie O, Prud'homme SM, Desouhant E, Gibert P. 2017 The invasive pest
801 *Drosophila suzukii* uses trans-generational medication to resist parasitoid attack. *Sci. Rep.* **7**, 43696.

802 74. Violet P-C *et al.* 2020 Vitamin E sequestration by liver fat in humans. *JCI Insight* **5**.
803 (doi:10.1172/jci.insight.133309)

804 75. Arita M, Sato Y, Miyata A, Tanabe T, Takahashi E, Kayden HJ, Arai H, Inoue K. 1995 Human alpha-
805 tocopherol transfer protein: cDNA cloning, expression and chromosomal localization. *Biochem. J*
806 **306 (Pt 2)**, 437–443.

807 76. Petschenka G, Agrawal AA. 2015 Milkweed butterfly resistance to plant toxins is linked to
808 sequestration, not coping with a toxic diet. *Proc. Biol. Sci.* **282**, 20151865.

809 77. Müller C, Wittstock U. 2005 Uptake and turn-over of glucosinolates sequestered in the sawfly
810 *Athalia rosae*. *Insect Biochem. Mol. Biol.* **35**, 1189–1198.

811 78. Saporito RA, Donnelly MA, Jain P, Martin Garraffo H, Spande TF, Daly JW. 2007 Spatial and
812 temporal patterns of alkaloid variation in the poison frog *Oophaga pumilio* in Costa Rica and
813 Panama over 30 years. *Toxicon* **50**, 757–778.

814 79. Duffey SS. 1980 Sequestration of plant natural products by insects. *Annu. Rev. Entomol.* **25**, 447–
815 477.

816 80. Daly JW, Brown GB, Mensah-Dwumah M, Myers CW. 1978 Classification of skin alkaloids from
817 neotropical poison-dart frogs (Dendrobatidae). *Toxicon* **16**, 163–188.

818 81. Sedio BE *et al.* 2021 Chemical similarity of co-occurring trees decreases with precipitation and
819 temperature in North American forests. *Frontiers in Ecology and Evolution* **9**.
820 (doi:10.3389/fevo.2021.679638)

821 82. Schmid R *et al.* 2023 Integrative analysis of multimodal mass spectrometry data in MZmine 3. *Nat.*
822 *Biotechnol.* **41**, 447–449.

823 83. Wang M *et al.* 2016 Sharing and community curation of mass spectrometry data with Global
824 Natural Products Social Molecular Networking. *Nat. Biotechnol.* **34**, 828–837.

825 84. Nothias L-F *et al.* 2020 Feature-based molecular networking in the GNPS analysis environment. *Nat.*
826 *Methods* **17**, 905–908.

827 85. Dührkop K, Fleischauer M, Ludwig M, Aksenov AA, Melnik AV, Meusel M, Dorrestein PC, Rousu J,
828 Böcker S. 2019 SIRIUS 4: a rapid tool for turning tandem mass spectra into metabolite structure

information. *Nat. Methods* **16**, 299–302.

86. Dührkop K, Shen H, Meusel M, Rousu J, Böcker S. 2015 Searching molecular structure databases with tandem mass spectra using CSI:FingerID. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 12580–12585.

87. Dührkop K *et al.* 2021 Systematic classification of unknown metabolites using high-resolution fragmentation mass spectra. *Nat. Biotechnol.* **39**, 462–471.

88. Djoumbou Feunang Y *et al.* 2016 ClassyFire: automated chemical classification with a comprehensive, computable taxonomy. *J. Cheminform.* **8**, 61.

89. Kim HW *et al.* 2021 NPClassifier: A Deep Neural Network-Based Structural Classification Tool for Natural Products. *J. Nat. Prod.* **84**, 2795–2807.

90. R Core Team. 2023 *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing. See https://www.R-project.org/.

91. Wickham H. 2016 ggplot2: Elegant Graphics for Data Analysis.

92. Wilke CO. 2020 *cowplot: Streamlined plot theme and plot annotations for "ggplot2."* See https://cran.r-project.org/package=cowplot.

93. Wickham H, François R, Henry L, Müller K, Vaughan D. 2023 *dplyr: A grammar of data manipulation*. See https://cran.r-project.org/package=dplyr.

94. Paradis E, Schliep K. 2019 ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics*. **35**, 526–528. (doi:10.1093/bioinformatics/bty633)

95. Revell LJ. 2012 phytools: An R package for phylogenetic comparative biology (and other things). *Methods in Ecology and Evolution*. **3**, 217–223. (doi:10.1111/j.2041-210X.2011.00169.x)