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

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	++	+++	++	—
	+	+++	+++	- - ·

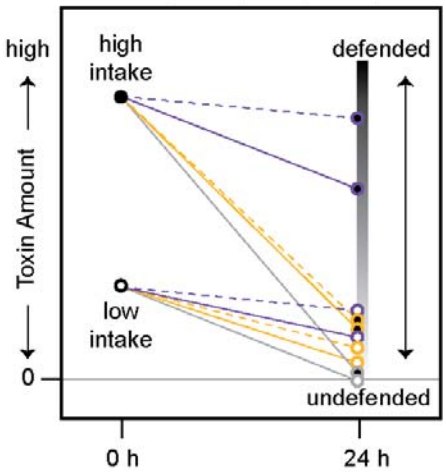

Figure 1. Major processes involved in the transition from the undefended to defended phenotype: 1) toxin intake, here visualized with two discrete points representing low and high rates; 2) toxin elimination rate (Elim. Rate), e.g., via toxin metabolism; 3) toxin sequestration rate (Seq. Rate), i.e.,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

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.

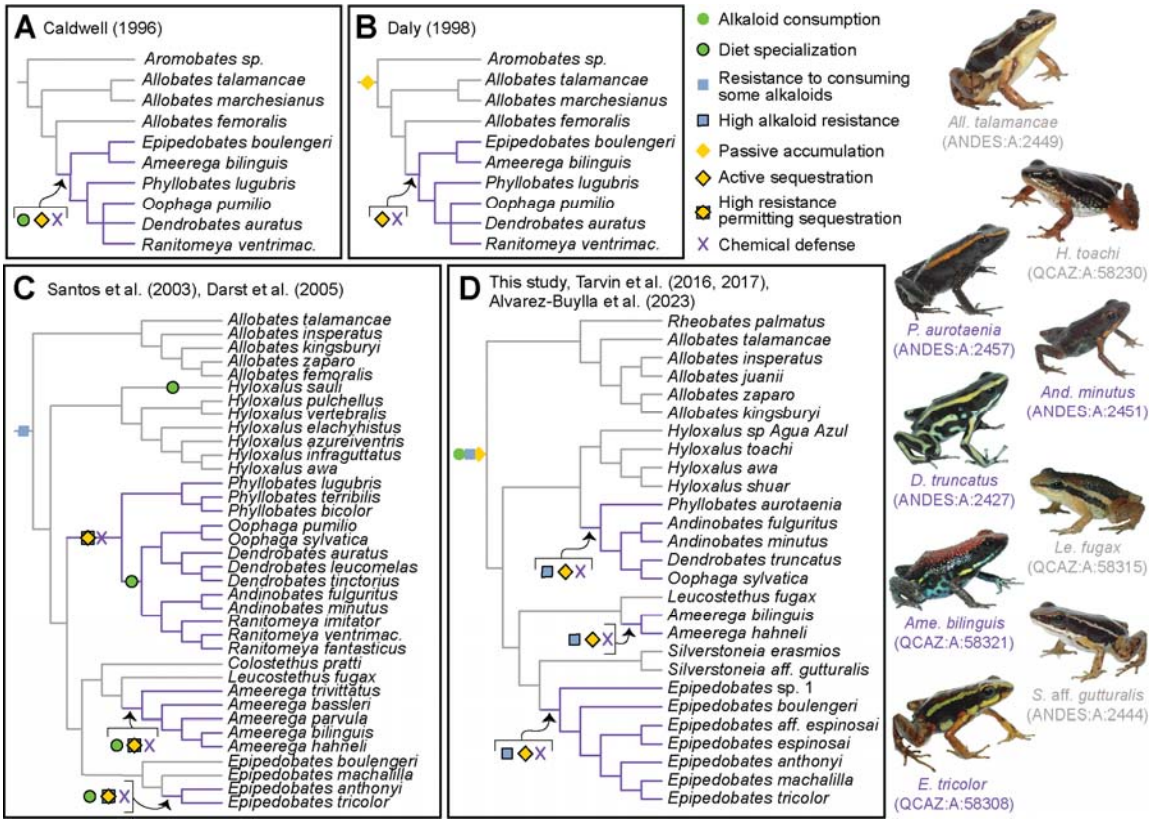

Figure 2. Evolutionary models of toxin sequestration in Dendrobatidae have changed over time. A) When several species of aposematic dendrobatids (purple lines) were found to have narrower dietary niches than undefended dendrobatids and other frogs [10,15,16], researchers hypothesized that diet specialization 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

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].

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

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

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

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

186 **Table 1.** Range and median of alkaloid quantity (estimated by the sum of integrated areas) and alkaloid diversity
187 (number of different compounds) by species from the GCMS assessment. The presumed chemical defense
188 phenotype for poison frogs is given according to Santos and Cannatella [4]. Purple rows highlight defe species.
189 *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

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

220 13 alkaloids in *S. punctiventris* [43]. In addition, we conducted a highly sensitive, untargeted analysis
221 (UHPLC-HESI-MS/MS) of *S. flotator*, which revealed that alkaloids were present in all 12 sampled
222 individuals (>99% alkaloid pathway probability; Table S6). At this probability level, we found 67 alkaloids
223 including one quinolizidine, two pyridines, and an analog of epibatidine (Tables S5 and S6).

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

243 Within Hyloxaline, a generally undefended clade, we surveyed four species of *Hyloxalus*, three of which
244 had detectable levels of alkaloids. We identified seventeen different alkaloids in *H. awa* (**197D**, **197H**,
245 **199B**, **217B**, **221P**, **223AB**, **231A**, **231C**, **247E**, and eight previously undescribed alkaloids), with the seven
246 sampled individuals having zero to twelve alkaloids each. We detected five alkaloids in a single individual
247 of *H. shuar* (**197D**, **199B**, **237G**, and two isomers of **239K**) and eight alkaloids in a single individual of *H.*
248 sp. Agua Azul (**195C**, **197D**, **199B**, **251K**, and four new alkaloids). Our detection of low levels of alkaloids
249 in *H. awa* are consistent with the observations that avian predators consume *H. awa* [53]. No alkaloids
250 were detected in two individuals of *H. toachi*, the only undefended species from which we failed to
251 detect alkaloids.

252 According to the most recent phylogenetic reconstructions [7], the sister clade to Hyloxalinae is
253 Dendrobatinae. Dendrobatinae contains exclusively (or near exclusively) toxic species. From this
254 subfamily, we surveyed 18 individuals representing 5 species. We identified a total of 187 unique
255 alkaloids from four *Phyllobates aurotaenia*, 316 alkaloids from five *Oophaga sylvatica*, and 213 alkaloids
256 from three *Dendrobates truncatus*. These three species are all relatively large poison frogs (snout-vent
257 lengths 20–35 mm; Table S2), which may in part explain their high alkaloid diversities and quantities. In
258 *Andinobates minutus* and *Andinobates fulguritus*, which are members of the same subfamily but are
259 much smaller in size (11–15 mm; Table S2), we detected 129 and 109 alkaloids, respectively. Three of
260 the *Andinobates minutus* individuals were juveniles. The total alkaloid quantities (integrated areas) in *D.*
261 *truncatus* and *O. sylvatica* were comparable to those of *Ameerega* but were higher than quantities
262 detected in *Epipedobates*. We also report for the first time, to the best of our knowledge, the
263 occurrence of N-Methyldecahydroquinolines outside the genus *Ameerega* (in *E.* aff. *espinosai*, *E.* sp. 1, *S.*
264 aff. *gutturalis*, *Andinobates minutus*, *Andinobates fulguritus*, *D. truncatus*, *O. sylvatica*, *P. aurotaenia*,
265 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.

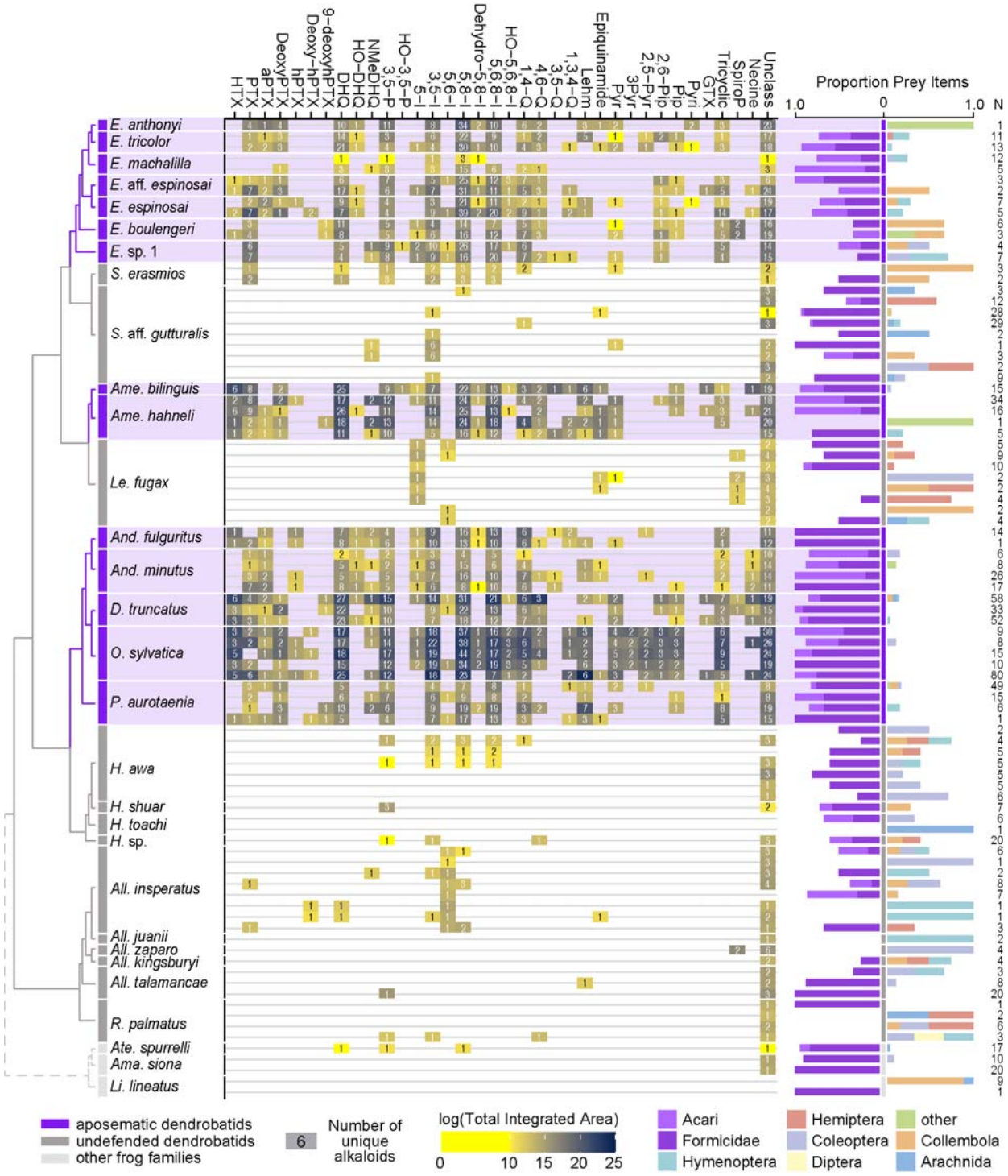

Figure 3. From left to right: an ultrametric tree showing phylogenetic relationships inferred previously [55] among sampled species with the three defended poison frog clades highlighted in purple, the undefended clades in dark gray, and non-dendrobatids in light gray (Bufonidae: *Amazophrynella siona*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

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

322 [62], and beetles [63], but the amount and diversity of alkaloids in each arthropod prey type is generally
323 unknown. Shifts in diet content towards a higher proportion of ants and mites have been hypothesized
324 to play an important role in the origin of chemical defense in poison frogs [4,8]. We quantified gut
325 contents for the same individuals that we analyzed by GCMS and found that both undefended and
326 defended species consume a large proportion of ants and mites (Fig. 3; Table S4). Although the
327 defended clades tend to consume proportionally more ants and mites, as found in other studies, the
328 undefended lineages do consume a high proportion of ants and mites. Other data support this general
329 pattern: ants and mites constituted up to 51% and 60% of the stomach contents of the undefended
330 species *Allobates talamancae* [64] and *H. sauli* [8], respectively. Ants and mites compose nearly 50% of
331 the arthropods (36 and 10%, respectively) found in the *S. flotator* stomachs we analyzed (Table S4).
332 Sympatric populations of the undefended *H. awa* and defended *E. espinosai* (formerly *E. darwinwallacei*
333 [65]) are both diet specialized, with the former consuming mostly ants and beetles and the latter
334 consuming mostly mites and springtails [66]. In a lab experiment, the defended species *D. tinctorius* was
335 shown to prefer fruit fly larvae over ants when given the choice [67], suggesting that even in defended
336 species, consumption of possible alkaloid-containing prey is not necessarily a preference. One study
337 found that *O. sylvatica* alkaloid quantity is inversely correlated with ant and mite stomach contents;
338 however, this species consumed more mites and ants than sympatric *H. infraguttatus* [46]. Although in
339 many cases defended species consume more mites and ants than undefended species, the undefended
340 lineages clearly consume toxic prey items, and some of the consumed alkaloids reach the skin (Fig. 3). In
341 sum, the available data do not strongly support that changes in diet alone are sufficient to explain
342 differences in alkaloid skin quantities.

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

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

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

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

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

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

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

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

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

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

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

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

457 **(d) Limitations**

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

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

469

470 ## 3. Conclusion

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

480

481 ## 4. Methods

482 ### (a) Field collection

483 In the case of *Silverstoneia flotator* and *Eleutherodactylus cystignathoides*, animals were collected and
484 euthanized with benzocaine in 2022 in Gamboa, Panama (9.136, -79.723) and in 2024 in Austin, Texas,
485 USA (30.285, -97.736). Dorsal and ventral skins were removed and placed separately in ~1-mL MeOH in
486 1-dram glass vials for UHPLC-HESI-MS/MS analyses (see below). For all other species, animals were
487 collected in 2014 and euthanized with an overdose of lidocaine. Whole skins were removed and placed
488 in ~1-mL MeOH in glass vials with PTFE-lined caps. Stomachs of all species were removed and placed in
489 95% ethanol.

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

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

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

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

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

531 Samples from Panama and Texas were extracted on separate occasions, then filtered and run in tandem
532 with ultra-high-performance liquid-chromatography heated-electrospray-ionization tandem mass
533 spectrometry (UHPLC-HESI-MS/MS), following an untargeted metabolomics profile, with conditions
534 optimized specifically for retention and subsequent identification of alkaloids [81]. Briefly, for extraction,
535 methanol was evaporated and skins were homogenized with stainless steel beads in a TissueLyser
536 QIAGEN™ and resuspended in 1800 µL of extraction solvent (9:1 MeOH: pH 5 water). Samples were
537 then extracted for 3 hr at 4˚C in a ThermoMixer (Eppendorf US, Enfield, CT, USA), followed by
538 evaporation of the methanol component with a speedvac concentrator (Thermo Fisher Scientific,
539 Waltham, MA, USA). Next, samples were freeze-dried with a lyophilizer overnight and resuspended in
540 500 µL extraction solvent. Resuspended samples were then filtered, diluted 1:7 in 100% MeOH, and
541 analyzed using UHPLC-HESI-MS/MS on a Thermo Vanquish LC and QExactive quadrupole-orbitrap MS.
542 Instrumental methods were identical to those described by [81]. A positive reference of 1 µg/µL ≥98%
543 (±)-epibatidine dihydrochloride hydrate (Sigma-Aldrich, St. Louis, MO, USA) was included in the run.

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

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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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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