Passive accumulation of alkaloids in inconspicuously colored frogs refines the evolutionary paradigm 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. Thus, our data suggest that diet is insufficient to explain the defended phenotype. Our data support the existence of a phenotypic intermediate between toxin consumption and sequestration — passive accumulation — that differs from 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. In light of ideas from pharmacokinetics we incorporate new and old data from poison frogs into an evolutionary model that could help explain the origins of acquired chemical

defenses in animals and provide insight into the molecular processes that govern the fate of ingested toxins.

Resumen

Comprender los orígenes de fenotipos novedosos y complejos es un objetivo central en biología evolutiva. Las ranas venenosas de la familia Dendrobatidae han desarrollado una novedosa habilidad para adquirir alcaloides de su dieta como defensas químicas, al menos tres veces. Sin embargo, el muestreo de taxones en busca de alcaloides ha estado sesgado hacia las especies coloridas, sin prestar atención similar a las poco conspicuas que a menudo se presume, no tienen defensas. Como resultado, nuestra comprensión de cómo evolucionan las defensas químicas en este grupo es incompleta. Aquí, proporcionamos nuevos datos que muestran que, en contraste con estudios anteriores, las especies de cada clado de ranas venenosas no defendidas tienen cantidades bajas pero cuantificables de alcaloides. Confirmamos que los dendrobátidos no defendidos consumen regularmente ácaros y hormigas, que son fuentes conocidas de alcaloides. Por lo tanto, nuestros datos sugieren que la dieta es insuficiente para explicar el fenotipo defendido. Nuestros datos respaldan la existencia de un fenotipo intermedio entre consumo y secuestro de toxinas (acumulación pasiva), que difiere del secuestro en que no implica formas derivadas de mecanismos de transporte y almacenamiento, pero da lugar a bajos niveles de acumulación de toxinas. Discutimos el concepto de acumulación pasiva y su potencial rol en el origen de defensas químicas en ranas venenosas y otros organismos que secuestran toxinas. Considerando ideas de farmacocinética, incorporamos datos nuevos y antiguos de ranas venenosas dentro de un modelo evolutivo que podría ayudar a explicar los orígenes de defensas químicas adquiridas en animales, y proporcionar una visión de los procesos moleculares que regulan el destino de las toxinas ingeridas.

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

1. Introduction

(a) Overview

Complex phenotypes can evolve by leveraging phenotypic plasticity in existing traits with concerted change in developmental modules (West-Eberhard, 2003). 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 (e.g., Martin and Wainwright, 2013). Nevertheless, phenotype diversity can help us unravel origins of novel traits and reveal the physiological trade-offs associated with their evolutionary trajectory (Tarvin et al., 2017).

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 (Berenbaum, 1995; Santos et al., 2016). Although human interest in poisonous plants and animals is old — dating back millennia (Charitos et al., 2022) — we have only recently begun to elucidate the specific mechanisms involved in acquired chemical defenses (Beran and Petschenka, 2022). This persisting gap in knowledge may be partly explained by a historical lack of integration between systems biology and pharmacology (Rostami-Hodjegan, 2012). Here we incorporate ideas from pharmacokinetics with data from poison frogs (Anura: Dendrobatidae) into an evolutionary model that could help explain the origins of acquired chemical defenses in poison frogs and more generally in other animals.

In the following text, we use the terms alkaloid and toxin interchangeably, although the toxicity of each poison frog alkaloid is not always known or very straightforward (Lawrence et al., 2023). Similarly, for simplicity we broadly bin species as defended (high alkaloid content) or undefended (low or zero

alkaloid content), although little information exists regarding the defensive efficacy of specific alkaloids. In this context, we use the term alkaloid to refer to compounds with nitrogen-containing rings, specifically the subset of lipophilic alkaloids representing classes previously described in anuran integument, e.g., "N-methyldecahydroquinolines" or "lehmizidines" (e.g., Daly et al., 2009, 2005).

(b) The history of research leading to the current paradigm: the diet-toxicity hypothesis

In the 1980s, Toft characterized several types of foraging behaviors in neotropical frogs and found that active foraging for ants was common in poisonous frogs (Dendrobatidae and Bufonidae), while sit-and-wait predation on larger prey was common in non-poisonous species (Toft, 1981, 1980). Toft hypothesized that chemical defenses protected poisonous species from the greater predation risk incurred by active foraging. At the time, it was thought that poisonous dendrobatids synthesized their own alkaloids (the biosynthetic hypothesis; reviewed by Saporito et al., 2009), so differences in diet were not considered mechanistically relevant to differences in levels of chemical defense. However, Daly and others (1994a) later demonstrated that chemically defended dendrobatid frogs obtained alkaloids from their diet. This dietary hypothesis led researchers to reevaluate the evolutionary importance of active foraging and hypothesize that specialization on ants promoted the evolution of chemical defense in Dendrobatidae (Caldwell, 1996). Later, a more detailed phylogenetic analysis of Dendrobatidae revealed that chemical defense and diet specialization co-evolved independently several times (Santos et al., 2003). The new information helped generate the diet-toxicity hypothesis, which posits that shifts from a generalist to a specialist diet are correlated with origins of alkaloid uptake (Darst et al., 2005; Santos and Cannatella, 2011). Since then, many studies have focused on the diet of poison frogs in an effort to directly connect diet with chemical defense in specific species (e.g., McGugan et al., 2016; Osorio et al., 2015; Sanches et al., 2023; Sánchez-Loja et al., 2023) and to identify sources of poison frog alkaloids (e.g., Saporito et al., 2004, 2007b). In general, most of the studies of poison-frog ecology since the 1990s emphasize or assume that diet is a primary determinant of defense.

(c) A new paradigm: the passive-accumulation hypothesis

Although in the 1990s Daly and his colleagues proposed that an alkaloid uptake system was present in the ancestor of Dendrobatidae and is overexpressed in aposematic species (Daly, 1998; Daly et al., 1994b; Saporito et al., 2009), no details about this purported system were given, and little focus was placed on the physiological processes of alkaloid sequestration in poison frogs for nearly 20 years. Santos et al. (2016) noted that the study of acquired chemical defenses is "essentially a study in pharmacokinetics." Pharmacokinetics (or toxicokinetics, for toxins; Spurgeon et al., 2020) is the study of how bioactive compounds are processed by animals. Organismal processes are often binned into four categories together known as ADME, which stands for Absorption, or movement into the bloodstream, Distribution, or movement into and out of body compartments, Metabolism, or biotransformation of the compound, and Excretion, or elimination from the body (Ruiz-Garcia et al., 2008). Herein we use similar terms that are more directly relevant to the study of acquired chemical defenses: toxin intake, or the amount of toxin consumed; toxin elimination, or the metabolic detoxification and/or elimination of toxins from the body (equivalent to Metabolism+Excretion); toxin sequestration, or the transport and storage of toxins to a specific location such as the skin (a modified version of Distribution); and toxin accumulation, or the retention of toxins in an animal, whether or not it is by sequestration processes.

Applying ideas from pharmacokinetics to acquired chemical defenses leads us to propose a four-phase evolutionary model, which we call the passive-accumulation hypothesis: 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 leads to its prolonged retention, hereafter passive accumulation; and 4) adaptation of molecular pathways to transport and store the compound in a specific location,

hereafter sequestration, which results in the chemical defense phenotype. Phases 3 and 4 may both select for increased toxin resistance, initiating a positive feedback loop that could intensify chemical defense and resistance over time. Note that while we focus on the physiological processes underlying toxin resistance and sequestration, other selection pressures including predators may influence these patterns (see section 2d).

Savitzky et al. (2012) defined "sequestration" as "the evolved retention within tissues of specific compounds, not normally retained in the ancestors of the taxon in question, which confers a selective advantage through one or more particular functions." We define passive accumulation as a type of toxin accumulation that is temporary and results from the delay between toxin intake and elimination; an example would be the temporary accumulation then clearance of ibuprofen in blood plasma in humans following ingestion (Albert and Gernaat, 1984). We differentiate passive accumulation from sequestration, a term that we argue implies the existence of a derived form of a transport or storage mechanism absent in the ancestor of the taxon, which would permit greater levels of and more long-term toxin accumulation than passive accumulation. In other systems such as insects, mechanisms of sequestration are sometimes described as passive (occurring by diffusion) or active (energy-consuming) (Petschenka and Agrawal, 2016). Given the general lack of data regarding the mechanisms underlying sequestration in frogs, we refrain from applying these modifiers to the sequestration term.

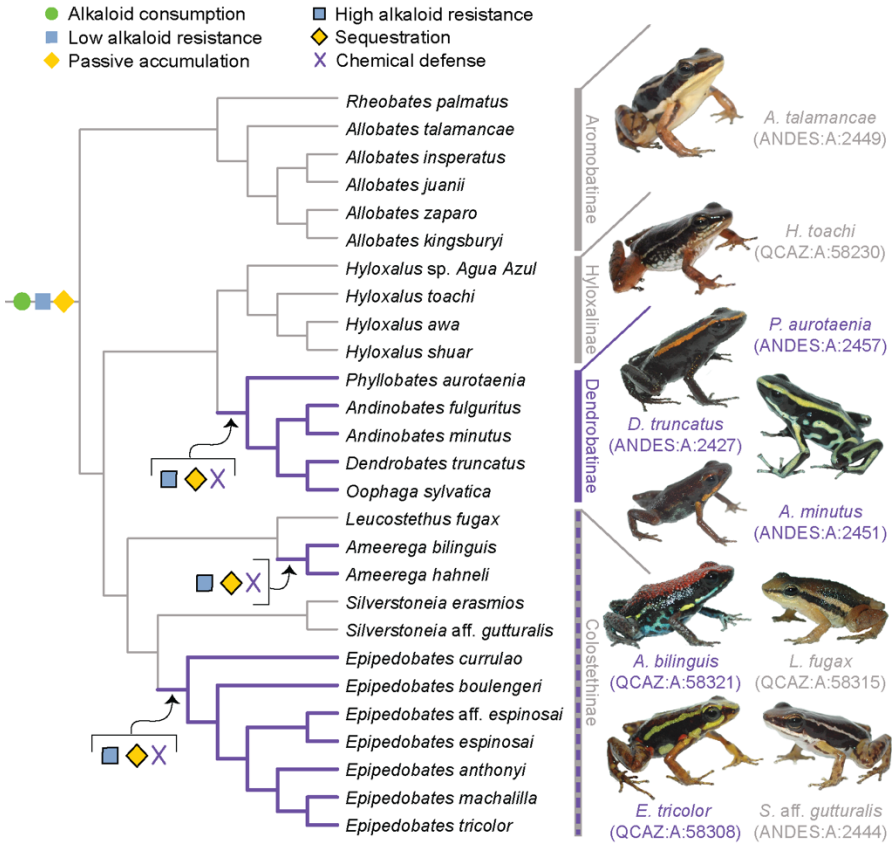

Figure 1. A new evolutionary model of toxin sequestration in Dendrobatidae. We propose that alkaloid consumption, some level of alkaloid resistance, and passive accumulation were present in the most recent common ancestor of Dendrobatidae; enhanced resistance and sequestration mechanisms then arose later, resulting in the chemical defense phenotype. Our model places less emphasis on dietary changes compared to prior studies, and more strongly emphasizes novel molecular mechanisms (e.g., binding proteins and target-site

insensitivity; Alvarez-Buylla et al., 2023; Tarvin et al., 2017, 2016). Purple lines indicate lineages with chemical defense. Gray lines indicate lineages that putatively lack chemical defense. All images of frogs were taken by RDT and are identified by their museum number.

To develop and refine this hypothesis, we gathered diet and toxin data from a broad selection of aposematic and inconspicuously colored poison-frog species. Approximately 100 of the 345 dendrobatid poison-frog species (AmphibiaWeb, 2023) fall into three conspicuously colored and alkaloid-sequestering (aposematic) clades: *Ameerega, Epipedobates,* and Dendrobatinae. The other 245 species compose several other primarily inconspicuously colored clades that for the most part have been assumed to lack alkaloid defenses: that is, all Aromobatinae (e.g., *Allobates, Rheobates, Anomaloglossus*, and *Aromobates*), all Hyloxalinae (*Ectopoglossus*, *Hyloxalus*, *Paruwrobates*), and some Colostethinae (*Colostethus, Silverstoneia, Leucostethus*) (Fig. 1). According to the phylogenetic placement of defended and undefended species within Dendrobatidae, poison frogs have evolved sequestration of lipophilic alkaloids from consumed arthropods at least three times (Santos et al., 2014, 2003), making them a suitable group to study complex phenotypic transitions like the evolution of chemical defense.

In total we surveyed 104 animals representing 32 species of Neotropical frogs including 28 dendrobatid species, 2 bufonids, 1 leptodactylid, and 1 eleutherodactylid (see Methods). Each of the major undefended clades in Dendrobatidae (Fig. 1, Table 1) is represented in our dataset, with a total of 14 undefended dendrobatid species surveyed. Next, we review old and new evidence from poison frogs in the context of the four-phase model (sections 2a and 2b). Then we describe major predictions that need further testing to validate and/or revise the proposed model (section 2c). Finally, we discuss other factors that might influence the evolution of chemical defenses (section 2d), the passive accumulation phenotype in a broader evolutionary context (section 2e), and possible limitations of this study (section 2f). Overall, we propose that further integrating ideas from pharmacokinetics into studies of acquired chemical defenses will lead to new insight in the field, with clear applications to human and ecosystem health. In that vein, we suggest that evolutionary changes in toxin resistance and metabolism are critical physiological shifts that facilitate origins of acquired chemical defenses in animals.

2. Results and Discussion

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

Several of the lipophilic alkaloids found in dendrobatid frogs have been traced to arthropod sources, specifically mites (Saporito et al., 2007b), ants (Saporito et al., 2004), and beetles (Dumbacher et al., 2004), although the extent to which such arthropod prey vary in alkaloid diversity and quantity remains relatively unstudied. Regardless, broad-scale shifts in diet content towards a higher proportion of ants and mites have been hypothesized to play an important role in the origin of chemical defense in poison frogs (Darst et al., 2005; Santos and Cannatella, 2011).

We quantified gut contents for 32 species of Neotropical frogs. Both undefended and defended dendrobatid species consume a large proportion of ants and mites (Fig. 2; Table S1). Although the defended dendrobatid clades tend to consume proportionally more ants and mites, as in other studies, the undefended lineages do consume a high proportion of ants and mites. Other data support this general pattern: ants and mites respectively constituted up to 51% and 60% of the stomach contents of the undefended dendrobatids *Allobates talamancae* (Mebs et al., 2018) and *Hyloxalus sauli* (Darst et al., 2005). Ants and mites compose nearly 50% of the arthropods (36% and 10%, respectively) found in the *Silverstoneia flotator* stomachs we analyzed (Table S1). Sympatric populations of the undefended *Hyloxalus awa* and defended *Epipedobates espinosai* (formerly *E. darwinwallacei* (López-Hervas et al.,

2024) are both diet specialized, with the former consuming mostly ants and beetles and the latter consuming mostly mites and springtails (Sánchez-Loja et al., 2023). In a lab experiment, the defended species *Dendrobates tinctorius* preferred fruit fly larvae over ants when given the choice (Moskowitz et al., 2022b), suggesting that even in defended species, consumption of possible alkaloid-containing prey is not necessarily a preference. Another study revealed that *Oophaga sylvatica* alkaloid quantity is inversely correlated with ant and mite stomach contents; however, this species consumed more mites and ants than sympatric *Hyloxalus elachyhistus* (Moskowitz et al., 2022a). The few bufonids that we assessed also show a high proportion of ants and mites in their diet (Fig. 2). Thus, if we assume that many ants and mites contain alkaloids, it is likely that most if not all dendrobatids and their most recent common ancestors have long been exposed to toxins through their diet.

Few if any experiments have been done to quantify the relationship between natural toxin exposure and toxin resistance in poison frogs. Given the broad diversity of alkaloid classes found in poison frogs (Daly et al., 2005), it is very difficult to predict or quantify all possible types or variations of alkaloid resistance that exist across species, or in their ancestors. In animals, the general mechanisms of toxin resistance are avoidance, metabolism, and target modification (Tarvin et al., 2023). 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 Tarvin et al., 2023 for more details). Given their diet, dendrobatids clearly do not completely avoid toxin exposure, and thus they are likely to survive exposure using some manner of toxin metabolism or target modification. Indeed, target-site resistance to some alkaloids evolved in several defended dendrobatid clades and in some undefended species (Tarvin et al., 2017, 2016). A few defended species have alternative target mechanisms including binding proteins like alpha-binding globulin (Alvarez-Buylla et al., 2023) and saxiphilin (Abderemane-Ali et al., 2021) that might prevent alkaloids from accessing their molecular targets (e.g., ion channels). Other mechanisms may also exist. For example, poison frogs may biotransform alkaloids into less toxic forms until they can be eliminated from the body, e.g., using cytochrome p450s (Caty et al., 2019). The mechanism of resistance employed might differ between undefended and defended species, but more research is necessary to understand these patterns.

Although more data are necessary to understand the evolution of toxin resistance in dendrobatids (Coleman and Cannatella, 2023), existing data suggest that all or nearly all dendrobatids are exposed to alkaloids (Fig. 2) and that alkaloid resistance varies among lineages.

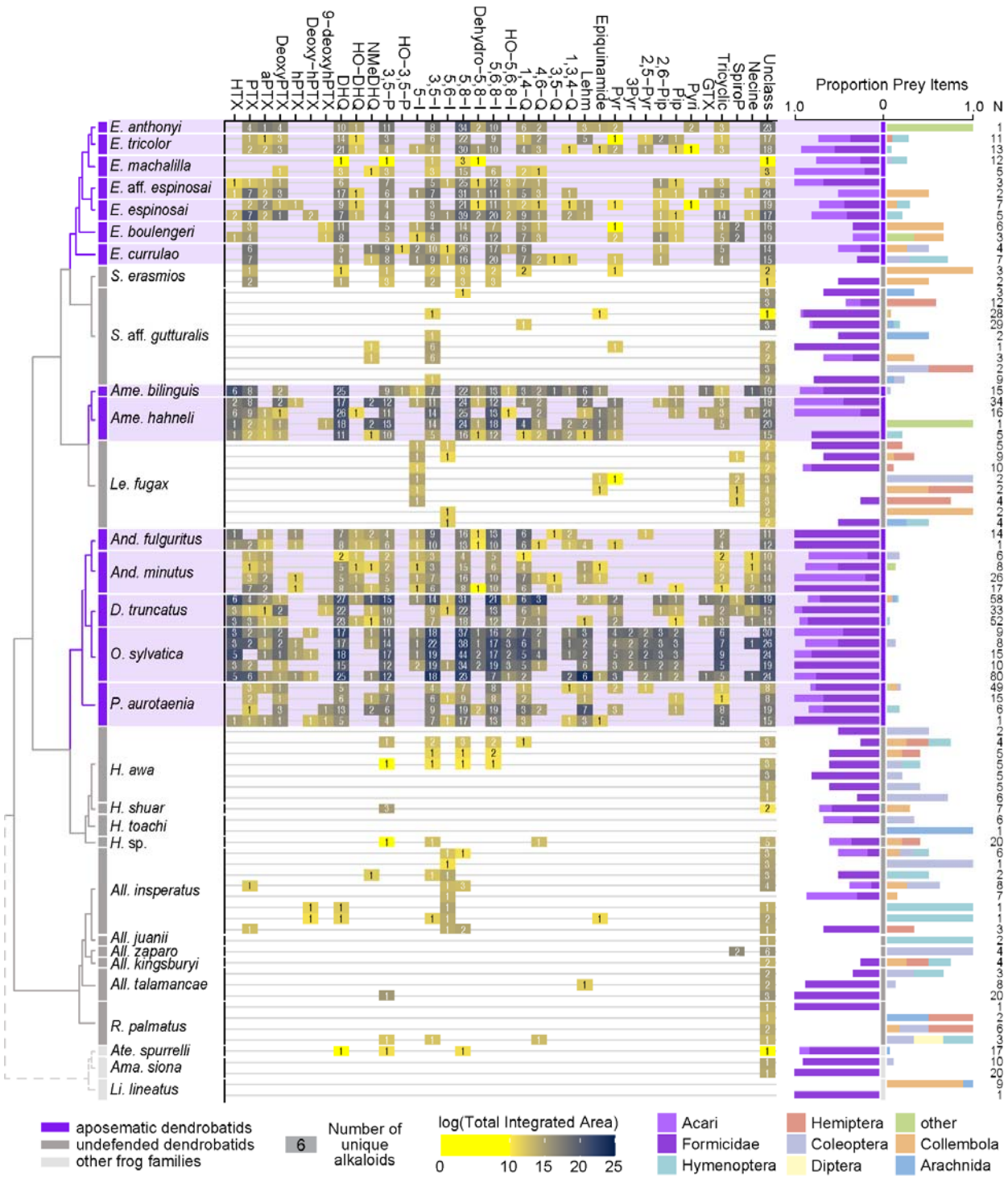

Figure 2. From left to right: an ultrametric tree showing phylogenetic relationships inferred previously (Wan et al., 2023) among sampled species with the three defended poison frog clades highlighted in purple, the undefended clades in dark gray, and non-dendrobatids in light gray (Bufonidae: *Amazophrynella 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 GC-MS 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 S1 for raw diet data and Table S4 for full alkaloid 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 (Daly et al., 2009, 2005) 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.

(b) Phases 3 and 4: Evidence for passive accumulation and sequestration in poison frogs

To understand the major evolutionary transition from consuming to sequestering toxins, it is essential to characterize the metabolism and sequestration of alkaloids in defended and undefended dendrobatid lineages (Gonzalez and Carazzone, 2023). However, many of the undefended lineages have not been carefully evaluated for the presence or absence of chemical defense. By reviewing existing data, we found that only 31 of the 245 inconspicuous poison frog species described to date (AmphibiaWeb, 2023) have been assessed for toxins, sometimes using methods that would not necessarily detect lipophilic alkaloids (Table S2). Further, prior studies have sometimes misinterpreted or not fully incorporated these data (Table S2, and see below). Our review and reassessment of these studies suggest that at least 11 undefended species might have lipophilic alkaloids: *Allobates femoralis*, *Allobates kingsburyi*, *Allobates zaparo*, *Colostethus ucumari*, *H. elachyhistus*, *Hyloxalus nexipus, Hyloxalus vertebralis, Hyloxalus yasuni*, *Leucostethus fugax, Paruwrobates erythromos,* and *Silverstoneia punctiventris* (Daly et al., 1987; Darst et al., 2005; Gonzalez et al., 2021; Grant, 2007; Moskowitz et al., 2022a; Santos and Cannatella, 2011).

We tested for possible alkaloid presence in additional aposematic and inconspicuously colored poison-frog lineages. Using Gas-Chromatography Mass-Spectrometry (GC-MS), we surveyed 89 animals representing 30 species of Neotropical frogs including 27 dendrobatid species, 1 leptodactylid, and 2 bufonids (Fig. 2). We also performed a highly sensitive, untargeted analysis —ultra-high-performance liquid-chromatography heated-electrospray-ionization tandem mass spectrometry (UHPLC-HESI-MS/MS) — of a dendrobatid from an undefended clade (*S. flotator;* 12 individuals) and a species of eleutherodactylid (*Eleutherodactylus cystignathoides*; 3 individuals), in which alkaloid diversities and types, but not quantities, were assessed. Each of the major undefended clades in Dendrobatidae (Fig. 1, Table 1) is represented in our dataset with a total of 13 undefended dendrobatid species surveyed with GC-MS and 1 undefended dendrobatid species surveyed with UHPLC-HESI-MS/MS. As far as we are aware, we provide alkaloid data for the first time for six undefended dendrobatid species (*Rheobates palmatus, Allobates juanii, Hyloxalus shuar, Hyloxalus* sp. Agua Azul*, Silverstoneia* aff. *gutturalis*, and *Silverstoneia erasmios*) and one defended species (*Epipedobates currulao*). We also provide the first alkaloid data for the non-dendrobatids *Amazophrynella siona*, *El. cystignathoides*, and *Lithodytes lineatus* (but see de Lima Barros et al., 2016). Because chemical standards for most poison frog alkaloids do not exist, it is not possible to provide absolute quantification of alkaloids. Reported values for GC-MS

284 data are in units of integrated area, which do not directly correspond to alkaloid quantity because of
285 differences in ion yield. Nevertheless, qualitative comparisons of integrated areas can provide insight
286 into how species differ in degrees of magnitude.

287 Overall, we detected alkaloids in skins from 13 of the 14 undefended dendrobatid species included in
288 our study, although often with less diversity and relatively lower quantities than in defended lineages
289 (Fig. 2, Table 1, Table S3, Table S4). The pervasiveness of low alkaloid levels in undefended dendrobatid
290 lineages (Aromobatinae, Hyloxalinae, some species of Colostethinae) contrasts with the mixed or
291 opposing evidence from previous analyses (Table S2). In addition, our GC-MS assessment revealed
292 substantially higher diversities of alkaloids in defended dendrobatid species than previously reported
293 (Cipriani and Rivera, 2009; Daly et al., 1987; Lawrence et al., 2023; Moskowitz et al., 2022a, 2020), and
294 expands knowledge on major classes of alkaloids within genera.

295 The large number of structures that we identified is in part due to the way we reviewed GC-MS data: in
296 addition to searching for alkaloids with known fragmentation patterns, we also searched for anything
297 that could qualify as an alkaloid mass spectrometrically but that may not match a previously known
298 structure in a reference database. Similarly, the analysis of UHPLC-HESI-MS/MS data was untargeted,
299 and thus enables a broader survey of chemistry compared to that from prior GC-MS studies. Structural
300 annotations in our UHPLC-HESI-MS/MS analysis were made using CANOPUS, a deep neural network that
301 is able to classify unknown metabolites based on MS/MS fragmentation patterns, with 99.7% accuracy
302 in cross-validation (Dührkop et al., 2021).

303 Although contamination across samples is possible, it is unlikely to invalidate the identification of
304 alkaloids in undefended species based on the following. 1) At several sites, we only sampled undefended
305 species, and these individuals were found to contain alkaloids (e.g., Las Brisas: *R. palmatus*; El Valle: *S.*
306 aff. *gutturalis*; Santa Maria: *H.* sp. Agua Azul); i.e., these cannot possibly have come from contamination
307 by defended species. 2) At one site where we collected both undefended and defended species, the
308 undefended species shows no alkaloids (Lita: *Hyloxalus toachi*); i.e., the preparation of both types does
309 not imply cross-contamination of samples. 3) At two sites where the undefended species were prepared
310 on a different day from the defended species (Valle Hermoso: *H. awa* and *Epipedobates boulengeri*;
311 Canelos: *L. fugax* and *Ameerega hahneli*) and could not have been cross-contaminated, the undefended
312 species still show evidence of alkaloids. 4) All chromatograms in the GC-MS sequence and integration
313 data were inspected manually. Peaks with low areas or following samples with high areas and subject to
314 carryover were excluded from further analysis. 5) Data collected by a different team and analyzed with
315 different methods also identify alkaloids in an undefended dendrobatid (*S. flotator*) from Panama.

316 **Table 1.** Range and median of alkaloid quantity (estimated by the sum of integrated areas) and alkaloid diversity
317 (number of different compounds) by species from the GC-MS assessment. The presumed chemical defense
318 phenotype for poison frogs is given according to Santos and Cannatella (2011). Purple rows highlight defended
319 species. *From a UHPLC-HESI-MS/MS dataset for which alkaloids were not quantified. Note that the UHPLC-HESI-
320 MS/MS and GC-MS assays differed in both instrument and analytical pipeline, so "Alkaloid Number" values from
321 the two assay types should not be compared to each other directly.

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	0 – 1	0.0
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 currulao*	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	0 – 0	0.0
Leptodactylidae	Leptodactylinae	*Lithodytes lineatus*	NA	2	0.00 – 0.00	0.00	0 – 0	0.0

Aromobatinae. 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 18 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 *Al. juanii* while two were found in one individual of *Al. kingsburyi*. At least two alkaloids were identified in each of the three sampled individuals of *Al. talamancae* (including the lehmizidine **277A** and five new alkaloids). Eight alkaloids were identified in the single surveyed *Al. 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* (Santos and Cannatella, 2011), but none in 12 *Al. insperatus* (Darst et al., 2005). Four studies (Table S2) failed to identify any alkaloids in *Al. talamancae. Allobates zaparo* was shown to possibly have trace alkaloids, although the interpretation of these data was absence of alkaloids (Darst et al., 2005). There are no known defended species from this subfamily, although we note conflicting evidence for the presence of alkaloids in *Al. femoralis* (Amézquita et al., 2017; Daly et al., 1987; Sanchez et al., 2019; Saporito and Grant, 2018) (Table S2).

Colostethinae. Within Colostethinae, we surveyed individuals from two undefended clades, *Leucostethus* and *Silverstoneia*, and from two defended clades, *Epipedobates* and *Ameerega.* From *L. 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 *L. fugax* tested positive for

skin compounds (Santos and Cannatella, 2011), though prior interpretations of these data were different (Table S2). We also surveyed two species of *Silverstoneia* with GC-MS. We found alkaloids in all nine *S.* aff. *gutturalis*, with a total of 14 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**). *Silverstoneia erasmios* and *S.* aff. *gutturalis* had not been surveyed for alkaloids previously, but thirteen alkaloids were found in eight individuals of a congener (*S. punctiventris*) (Gonzalez et al., 2021). In addition, our more conservative UHPLC-HESI-MS/MS analysis of *S. flotator*, from which we only report compounds previously known as lipophilic frog alkaloids (Daly et al., 2005), we identified the presence of alkaloids in 5 of 12 sampled individuals (a quinolizidine and an epibatidine; Table S5, S6). When we expand our analysis to include any compound assigned to the "alkaloid pathway" by NPClassifier (>99% alkaloid pathway probability; Table S5), we identified a total of 67 compounds, some of which were present in each individual (Tables S5). Although the assignments made by this pipeline are broad and include diverse nitrogen-containing metabolites such as biogenic amines (Table S6), it is possible that some represent additional lipophilic alkaloids whose structures and formulae are undescribed. Note that UHPLC-HESI-MS/MS data should not be directly compared to GC-MS data (see Table 1 legend).

In terms of the defended clades of Colostethinae that we sampled, most of the individual skins of *Epipedobates* and *Ameerega* contained dozens to more than one hundred unique alkaloids (see Table S4 for full details). For *Ameerega*, we surveyed five individuals representing two species, all of which had integrated areas that were more than 75,000x greater compared to individuals of its sister clade, *Leucostethus* (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* (Daly et al., 2009); here we also found high levels of indolizidines (Fig. 2). Patterns for *Epipedobates* as compared to sister genus *Silverstoneia* were similar, although less extreme. We surveyed 13 individuals representing seven 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* (Darst et al., 2005; Santos and Cannatella, 2011). However, the quantity and diversity of alkaloids in *Epipedobates machalilla* was substantially lower than in other *Epipedobates* species, occurring at levels similar to *Silverstoneia* spp. (Table 1, Fig. 2). Except for *E. machalilla*, each *Epipedobates* species had about 10x greater 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. Epibatidines have also been detected in *E. espinosai, Ameerega silverstonei*, *S. flotator* (Daly et al., 1999; this study), and *Ameerega petersi* or a closely related, undescribed species (reported as *Dendrobates pictus* from Loreto, Peru in (Daly et al., 1987), but see taxonomic revision by (Guillory et al., 2020)).

Hyloxalinae. Hyloxalinae is generally considered an undefended clade (Table S2). We surveyed four species of *Hyloxalus*, three of which had detectable levels of alkaloids. We identified 17 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 0 to 12 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* is consistent with the observations that avian predators consume *H. awa* (Darst and Cummings, 2006). No alkaloids were detected in two individuals of *H. toachi*, the only undefended dendrobatid species from which we failed to detect alkaloids. Previously, a GC-MS assessment previously revealed that *P. erythromos* contained 5,8-disubstituted indolizidine

394 **251B**, allopumiliotoxin **267A**, and unclassified alkaloid **281D** (Daly et al., 1987). *Hyloxalus azureiventris* is
395 also thought be able to accumulate alkaloids (Daly, 1998; Saporito et al., 2009) and thin-layer
396 chromatography suggested the presence of alkaloids in two assessed *H. yasuni* (previously identified as
397 *Hyloxalus maculosus*), one of three *H. nexipus*, and two of five *H. vertebralis* (Santos and Cannatella,
398 2011), though prior interpretation of these data differed (Table S2). Our data support the widespread
399 presence of low levels of alkaloids in this group.

400 **Dendrobatinae**. According to the most recent phylogenetic reconstructions (Santos et al., 2014), the
401 sister clade to Hyloxalinae is Dendrobatinae (Fig. 1). Dendrobatinae contains exclusively (or near
402 exclusively) toxic species. From this subfamily, we surveyed 18 individuals representing five species
403 using GC-MS. We identified a total of 187 unique alkaloids from four *Phyllobates aurotaenia*, 316
404 alkaloids from five *O. sylvatica*, and 213 alkaloids from three *Dendrobates truncatus*. These three species
405 are all relatively large poison frogs (snout-vent lengths 20–35 mm; Table S3), which may in part explain
406 their high alkaloid diversities and quantities (Jeckel et al., 2015; Saporito et al., 2010). In *Andinobates*
407 *minutus* and *Andinobates fulguritus*, which are members of the same subfamily but are much smaller in
408 size (11–15 mm; Tables 1 and S3), we detected 129 and 109 alkaloids, respectively. Three of the *An.*
409 *minutus* individuals were juveniles. The total alkaloid quantities (integrated areas) in *D. truncatus* and *O.*
410 *sylvatica* were comparable to those of *Ameerega* but were higher than quantities detected in
411 *Epipedobates*. We also report for the first time, to the best of our knowledge, the occurrence of N-
412 methyldecahydroquinolines outside of the genera *Adelphobates*, *Ameerega*, *Dendrobates*, *Oophaga*,
413 and *Ranitomeya* (in *E.* aff. *espinosai*, *E. currulao*, *S.* aff. *gutturalis*, *An. minutus*, *An. fulguritus*, *P.*
414 *aurotaenia*, and *Al. insperatus*) (Daly et al., 2009; Hovey et al., 2018; Jeckel et al., 2019; Lawrence et al.,
415 2019; Stuckert et al., 2014). The ability to N-methylate decahydroquinoline (demonstrated
416 experimentally in *Adelphobates galactonotus*, *Dendorbates auratus*, and *Ranitomeya ventrimaculata*
417 (Jeckel, 2021; Jeckel et al., 2022)) may thus be conserved in dendrobatids, or, non-exclusively, arthropod
418 sources of the alkaloid class (likely myrmicine ants (Jones et al., 1999)) are widespread.

419 **Other frog families.** Outside of Dendrobatidae, we detected a new unclassified alkaloid, New159, in
420 each of two *A. siona* (Bufonidae) and four alkaloids in one individual of *Atelopus* aff. *spurrelli* (Bufonidae;
421 3,5-disubstituted pyrrolizidine **237R**-1, decahydroquinoline **243A**-3, 5,8-disubstituted indolizidine **251B**-
422 2, and an unclassified alkaloid, New267-2). As far as we know, the detection of a decahydroquinoline
423 and a 3,5-disubstituted pyrrolizidine in a bufonid frog other than *Melanophryniscus* (Rodríguez et al.,
424 2017) is novel and may provide useful context for understanding the evolution of chemical defense in
425 the family. We detected no alkaloids in two *Li. lineatus* (Leptodactylidae) individuals, which is surprising
426 because *Li. lineatus* has been hypothesized to be a Müllerian mimic of poison frogs, though the
427 composition of its chemical defenses may be primarily proteinaceous (Prates et al., 2011). These
428 findings are also interesting in light of the fact that *Li. lineatus* live and breed in ant colonies using
429 chemical signals that provide camouflage (de Lima Barros et al., 2016). In addition, while we recovered
430 no alkaloids in three sampled individuals of the frog *El. cystignathoides* (Eleutherodactylidae) with
431 UHPLC-HESI-MS/MS when we applied our stringent search criteria, we identified 55 metabolites
432 assigned to the alkaloid pathway at >99% probability. Forty of these appear to be identical to those
433 identified in *S. flotator* according to our analyses (Tables S5, S6). Some of these could be widespread
434 byproducts of frog metabolism (or symbiont metabolism). A few species of *Eleutherodactylus* frogs from
435 Cuba are thought to sequester alkaloids (Rodríguez et al., 2013) and alkaloid sequestration evolved in
436 the bufonid genus *Melanophryniscus* (Daly et al., 1984; Hantak et al., 2013). The presence of low levels
437 of alkaloids in other (non-sequestering) species of Bufonidae and the possibility of some exogenous but
438 as of yet undescribed alkaloids in *El. cystignathoides* reflect that passive accumulation may have evolved
439 in an older ancestor shared by the three families, predating convergent origins of sequestration in all
440 three groups.

(c) Predictions arising from the passive-accumulation hypothesis

Data from this and other studies point to the ubiquity of mites and ants in nearly all dendrobatid diets, and possibly more generally in other leaf-litter dwelling frogs (Fig. 2). 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). The total amount of alkaloids accumulated is a result of multiple processes including toxin intake, elimination, and sequestration (Fig. 3) — not just intake alone.

For example, dendrobatid species vary in their ability to eliminate alkaloids. Some appear to lack specific transport and storage mechanisms for consumed alkaloids ("sequestration"), yet they have detectable levels of alkaloids in their skin; we refer to this phenotype as passive accumulation and suggest that it is an evolutionary intermediate between toxin consumption (with no sequestration) and sequestration (Fig. 3). We predict that the ancestral state of poison frogs and potentially other clades with alkaloid-sequestering species (e.g., Bufonidae: *Melanophryniscus*, Eleutherodactylidae: *Eleutherodactylus*, and Mantellidae: *Mantella*) is alkaloid consumption and low levels of alkaloid resistance, accompanied by passive alkaloid accumulation (e.g., see Figs. 1 and 3). Alternatively, passive accumulation may have arisen in an even earlier ancestor. That we detected alkaloids in two genera of bufonid toads could reflect a single origin of passive accumulation in the ancestor of the clade that includes Dendrobatidae and its sister group (the clade comprised of the Terranana [including Eleutherodactylidae], Bufonidae, Leptodactylidae, and Odontophrynidae) (AmphibiaWeb, 2023; Blackburn and Wake, 2011; Feng et al., 2017; Streicher et al., 2018; Yuan et al., 2019). Further sampling for alkaloids within Eleutherodactylidae and Leptodactylidae could reveal whether passive accumulation has persisted in these clades. Discriminating a single origin — no matter the timing — from multiple ones would require more extensive alkaloid surveys, as we only assessed four non-dendrobatid species.

	Process			
Phenotype	Elim.	Seq.	Acc.	Line
No Accumulation	high	0	0	- - -
Passive Accumulation	med	0	low	—
Sequestration	low	med/ high	med/ high	—

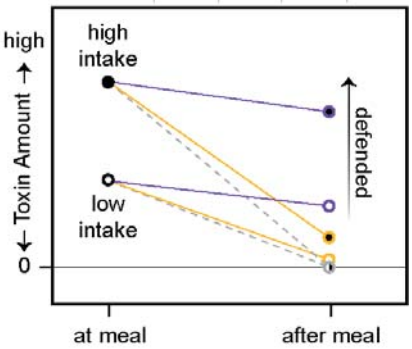

Figure 3. Hypothesized physiological processes that interact to determine the defense phenotype: toxin intake, toxin elimination (Elim.), and toxin sequestration (Seq.). See section 1c for definitions. Although toxin intake sets a maximum for the total possible amount of toxin accumulation (Acc.), it cannot fully explain the defensive phenotype. We hypothesize that an undefended "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 (dashed gray lines); this phenotype is a likely ancestral state for many animals. In contrast, we hypothesize that an undefended passive-accumulation phenotype is characterized by lower elimination than the no accumulation phenotype, leading to a low amount of toxin accumulation (yellow lines). We hypothesize that a defended sequestration phenotype evolves from an intermediate passive-accumulation phenotype through the addition of novel sequestration mechanisms, and possibly even lower elimination rates, that result in high toxin accumulation and the defended phenotype (purple lines).

Here we propose and discuss three additional predictions arising from the passive-accumulation hypothesis that would help further evaluate the validity of a four-phase model.

Prediction 1) We predict that some toxin resistance evolves prior to or in concert with passive

accumulation, and that it increases or changes once sequestration mechanisms evolve.

Alkaloid resistance is associated with alkaloid sequestration in dendrobatid poison frogs (Tarvin et al., 2017, 2016). We anticipate that some alkaloid resistance evolved in the ancestor of Dendrobatidae or in an even older ancestor, but is yet to be described (Darst et al., 2005; Santos et al., 2016) (Fig. 1). Such resistance may be difficult to characterize using the comparative method if it involves mutations of small effect (ffrench-Constant et al., 2004), pleiotropic processes, or undescribed physiological adaptations (e.g., Alvarez-Buylla et al., 2023). Regardless, it appears that arthropods likely to contain alkaloids are widespread among the regular diets of defended and undefended dendrobatid poison frogs (Fig. 2; Darst et al., 2005; Santos and Cannatella, 2011; Toft, 1995). Short-term alkaloid feeding experiments (e.g., Daly et al., 1994b; Sanchez et al., 2019) demonstrate that both defended and undefended frogs can survive the immediate effects of alkaloid intake, although the degree of resistance and the alkaloids that different species can resist vary. An experiment conducted by Abderemane-Ali and colleagues (2021) showed that both aposematic and (presumably) undefended frogs can withstand three highly toxic alkaloids in quantities greater than what the frogs are likely to experience in nature. Two aposematic dendrobatids (*D. tinctorius* and *Phyllobates terribilis*), an aposematic *Mantella* (*Mantella aurantiaca*), and the putatively undefended rhacophorid *Polypedates leucomystax* (a congener is reported to contain TTX (Tanu et al., 2001)) — recovered from injections of the highly toxic alkaloids batrachotoxin and tetrodotoxin delivered at 20x the mouse LD_{50} value. The three aposematic species also survived a third potent alkaloid (saxitoxin), but *P. leucomystax* did not. Other work revealed no signs of intoxication in two undefended hylids (*Hyla cinerea* and *Boana bandeirantes*) after two weeks of oral administration of histrionicotoxin **235A** and decahydroquinoline (Jeckel, 2021). In an epibatidine-feeding experiment with five aposematic dendrobatid species (*E. anthonyi, Ranitomeya variabilis, Ranitomeya imitator*, *Phyllobates vittatus*, and *D. tinctorius*), Waters et al. (2023) found that *E. anthonyi* was adversely affected by the initial dose of epibatidine, reflecting either a body-size effect or species-level variation in epibatidine resistance.

Different types of resistance may be important during different evolutionary phases leading to chemical defense. For example, many mechanisms of toxin metabolism are common to all animals and were likely used by the ancestors of most if not all animals that eventually evolved toxin sequestration, including poison frogs (Tarvin et al., 2023). Although one might expect that toxin metabolism may also prevent toxin sequestration, the ability to metabolize toxins can in some cases augment toxin defenses (Douglas et al., 2022), increase the toxicity of a compound (e.g., pumiliotoxin to allopumiliotoxin in the poison frogs *Ad. galactonotus*, *Adelphobates castaneoticus*, *D. auratus*, *D. tinctorius*, and *R. ventrimaculata* (Alvarez-Buylla et al., 2022; Daly et al., 2003; Jeckel, 2021)), or result in some amount of passive accumulation through increased toxin exposure (Douglas et al., 2022; Karageorgi et al., 2019). If toxin intake increases or is sustained over long evolutionary periods, selection may favor other mechanisms of resistance, such as target-site resistance, which can eliminate the cost of toxin exposure by making the targeted protein insensitive to the toxin (Tarvin et al., 2017). Indeed, other toxin-sequestering animals often have specialized mechanisms of toxin resistance when compared to toxin-free relatives (Tarvin et al., 2023). For example, three amino acid replacements in the ATPα protein evolved in association with cardenolide sequestration in Danainae butterflies (Karageorgi et al., 2019; Petschenka et al., 2013) and predatory fireflies that sequester lucibufagins have ATPα gene duplications that enhance lucibufagin resistance (Yang et al., 2023).

These data suggest that some dendrobatids and other frog species have a minimal level of resistance to alkaloids, yet more data from undefended frogs will be necessary to reconstruct the evolutionary history of the trait.

Prediction 2) We predict that in species with passive accumulation the rate of toxin elimination is

slower than in those with no accumulation and faster than in those with sequestration.

Only a few studies have reviewed toxin metabolism and elimination (clearance from the body) in dendrobatids. One study demonstrated that the undefended *Al. femoralis* and undefended hylid *Hy. cinerea* accumulated less than 1% of orally administered alkaloids into the skin, yet the alkaloids were absent (or present in only trace amounts) in the feces (Jeckel, 2021). In the same experiment, the defended dendrobatids *Ad. galactonotus* and *D. tinctorius* efficiently sequestered the alkaloids, with only trace quantities detected in the feces. These results hint at an unknown but possibly conserved mechanism for metabolism of alkaloids in anurans. Even among defended dendrobatids, there appears to be species-level variation and plasticity in the metabolism and elimination of alkaloids. *Epipedobates anthonyi, Ra. variabilis,* and *Ra. imitator* accumulate more than twice as much ingested epibatidine compared to *P. vittatus* and *D. tinctorius* (Waters et al., 2023). *Oophaga sylvatica* and *D. tinctorius* upregulate detoxification genes such as cytochrome p450s upon alkaloid consumption (Alvarez-Buylla et al., 2022; Caty et al., 2019). *Adelphobates galactonotus* sequesters the alkaloids histrionicotoxin **235A** and decahydroquinoline less efficiently at higher doses (Jeckel et al., 2022). Some species metabolically alter the structure of alkaloids: *Ad. galactonotus*, *Ad. castaneoticus*, *D. auratus*, *D. tinctorius*, and *Ra. ventrimaculata* can hydroxylate pumiliotoxin **251D** (Alvarez-Buylla et al., 2022; Daly et al., 2003; Jeckel, 2021), making it more toxic (to mice); *Ad. galactonotus*, *D. auratus*, and *Ra. ventrimaculata* can also N-methylate decahydroquinoline (Jeckel, 2021; Jeckel et al., 2022). These studies indicate that alkaloid elimination rate and metabolism vary among defended species, but not enough information exists to infer much about elimination rates in undefended lineages with or without passive accumulation. Given the experimental demonstration of less efficient alkaloid uptake in undefended frogs — in combination with our data that show that despite likely ingesting alkaloid-bearing prey regularly in the wild, undefended frogs show much lower levels of alkaloids in the skin (Fig. 2) — we hypothesize that toxin elimination rates in undefended lineages are faster or more efficient than rates in defended lineages and are slower than lineages with no accumulation (e.g., Fig. 3). More nuanced versions of this model could also be envisioned. For example, elimination rates in defended species could still modulate the amount of toxins ultimately accumulated, with lower elimination rates resulting in a higher proportion of toxin accumulation overall. Additional data are necessary regarding toxicokinetics of consumed alkaloids in several tissues.

Prediction 3) We predict that sequestration mechanisms are absent in undefended lineages.

Daly and colleagues (Daly, 1998; Daly et al., 1994b) hypothesized that there was an alkaloid uptake system present in the ancestor of Dendrobatidae that is “overexpressed” in the defended lineages. This hypothesis remains to be tested. Our model posits that sequestration mechanisms (Figs. 1 and 3) are unique to chemically defended species. Alternatively, if the mechanisms of toxin transport and/or storage exist in undefended species, they seem to be expressed at such a low level that they only result in a trace level of toxin accumulation. In order to distinguish between these two possibilities, we first will need to better understand the molecular mechanisms underlying toxin sequestration.

To date, little is known regarding the mechanisms of toxin sequestration in poison frogs or in other toxin-sequestering animals. An alkaloid-binding globulin was recently characterized in the poison frog *O. sylvatica* (Alvarez-Buylla et al., 2023). While plasma assays demonstrated that the defended species *O. sylvatica*, *Epipedobates tricolor*, and *D. tinctorius* can bind and sequester a pumiliotoxin-like photoprobe, plasma from the undefended *Al. femoralis* showed no binding activity. In addition, the evolutionarily distant mantellid species *M. aurantiaca*, which sequesters alkaloids, did not show binding activity. These data hint at variation in molecular mechanisms for alkaloid uptake across lineages, which may be tuned to availability of specific alkaloids in each species’ diet.

The potential absence of sequestration mechanisms in the undefended *Al. femoralis* are consistent not

580 only with our alkaloid data from wild-caught frogs, but also with experimental data. Using GC-MS,
581 researchers did not detect any alkaloids in the skins of two undefended dendrobatids (*Al. talamancae*
582 and *Colostethus panamansis*) after the frogs consumed fruit flies dusted with 5,8-disubstituted
583 indolizidine **209B**, decahydroquinoline **195A**, and histrionicotoxin **285C** for five weeks (Daly et al.,
584 1994b). Other unpublished data suggest that the brightly colored but undefended *H. azureiventris* are
585 unable to accumulate alkaloids from fruit flies (the sample size and alkaloid identities are unknown),
586 though *H. azureiventris* apparently accumulated four distinct alkaloids from a methanol-saline solution
587 (Saporito et al., 2009). After oral administration of decahydroquinoline and histrionicotoxin **235A**, the
588 undefended hylid *Hyla cinerea* cleared almost all consumed alkaloids (accumulating between 0.01 to
589 0.1%), the undefended *Al. femoralis* accumulated only trace amounts of decahydroquinoline (~1%), and
590 the defended *Ad. galactonotus* and *D. tinctorius* sequestered on average ~10% (decahydroquinoline) or
591 ~50% (histrionicotoxin **235A**) (Jeckel, 2021). Sparteine, a quinolizidine structurally similar to the common
592 "izidine" alkaloids in poison frogs, was detected in the skin of a single *Al. femoralis* individual after the
593 frog was fed sparteine-dusted fruit flies for over a month, but the experimental methods prohibited
594 quantification of the alkaloid (Sanchez et al., 2019).

595 Additional data on potential uptake mechanisms in dendrobatids exist for benzocaine, a synthetic
596 lipophilic compound that is used for anesthesia and euthanasia in amphibians. Benzocaine is readily
597 taken up orally to the skin in the defended poison frog *D. auratus*, the undefended ranid (*Lithobates*
598 *clamitans*), and the alkaloid-sequestering bufonid *Melanophryniscus moreirae* (Saporito and Grant,
599 2018). Although the same amount of benzocaine was injected into each frog, twice as much benzocaine
600 was recovered from *D. auratus* than *Li. clamitans* and three times as much was recovered from *Me.*
601 *moreirae* (see their Fig. 2), suggesting that lipophilic compound uptake occurs without specialized
602 mechanisms of sequestration in *Li. clamitans* (e.g., possibly passive accumulation) but that *D. auratus*
603 and *Me. moreirae* likely have distinct sequestration mechanisms that result in much higher levels of
604 benzocaine accumulation.

605 In contrast to sequestration, passive accumulation would be expected to result in the diffusion of
606 alkaloids across many tissues, rather than concentration of alkaloids within a specific tissue. Desorption
607 electrospray ionization mass spectrometry imaging data indicate that alkaloids diffuse across various
608 tissues in the defended dendrobatid *D. tinctorius* immediately following intake, possibly an evolutionary
609 trace of the low elimination rates that may have initially evolved in an ancestor with the passive
610 accumulation phenotype (Jeckel et al., 2020). It would be beneficial to conduct a time-series study to
611 show how tissue-specific accumulation patterns change after feeding in different species. Clearly, more
612 data will be necessary to evaluate phylogenetic patterns and mechanisms of sequestration, and to test
613 the hypotheses presented here regarding passive accumulation as an intermediate evolutionary phase.

614 **(d) Other factors that may shape the evolution of acquired chemical defenses**

615 Many animals occasionally or frequently consume toxins, and a multitude have evolved toxin resistance.
616 Some invertebrate pests resist pesticides (Andreev et al., 1999; Chiu et al., 2008; Daborn et al., 2002;
617 Richard and Carroll, 2013), many insect herbivores resist plant toxins (Agrawal et al., 2012; Dobler et al.,
618 2011), some predators resist toxic prey (Arbuckle et al., 2017), and many animals resist environmental
619 pollutants (Whitehead et al., 2017). Our model predicts that some or many of these may be on their way
620 towards evolving acquired chemical defenses. Yet, not all toxin-exposed or toxin-resistant species
621 inevitably evolve chemical defenses, presumably because the ecological context or physiology that
622 favors accumulation is absent or because resisting and accumulating toxins is too costly.

623 Acquired chemical defenses usually evolve within the context of a tri-trophic interaction: animals in the
624 middle of the food web accumulate toxins from their prey, and possible predators or parasites are

625 deterred by the accumulated toxin (Agrawal, 2000). This phenomenon is referred to as enemy-free
626 space, i.e., escape from parasitism or predation (Jeffries and Lawton, 1984). If there is no predator or
627 parasite present to exert selection on a toxin-consuming animal, there may be no benefit for the animal
628 to accumulate the toxins. Furthermore, some chemicals may not be able to be accumulated because of
629 how they interact with the physiology of an animal (e.g., Mebs et al., 2016). Thus, the evolution of
630 chemical defenses may be constrained by the specific chemicals present in an ecosystem, the existing
631 trophic interactions among species, and the physiology of predators and parasites in relation to the
632 chemicals in question.

633 Origins of chemical defenses are also shaped by the cost of resisting and accumulating toxins, which can
634 change over evolutionary time as animals adapt to novel relationships with toxins. In poison frogs and
635 other toxin-accumulating animals, it is common to observe a few amino acid substitutions in ion
636 channels that provide target-site resistance to alkaloids but adversely affect the function of the protein;
637 these substitutions are often accompanied by additional, compensatory substitutions that restore
638 protein function without affecting resistance (Karageorgi et al., 2019; Mohammadi et al., 2021; Reid et
639 al., 2016; Tarvin et al., 2017; Zhang et al., 2016). It is rare but possible to observe species that lack
640 (known) compensatory substitutions (Tarvin et al., 2017), suggesting that species are under strong
641 selection to overcome some costs of target-site resistance. In one species of garter snake (*Thamnophis*
642 *sirtalis*), the cost of target-site resistance in a voltage-gated sodium channel is not completely offset as
643 animals with target-site resistance have reduced crawl speeds (Hague et al., 2018). In some insects,
644 resistance to insecticides comes with a cost in fecundity or survival (Kliot and Ghanim, 2012). For
645 example, the aphid *Aphis nerii* experiences trade-offs between population growth and defense
646 effectiveness (Züst et al., 2018). As far as we are aware, the possible lifetime fitness costs (e.g., in
647 reproductive success) of alkaloid consumption in dendrobatids have not been measured.

648 Once chemical defenses evolve, they are often further shaped by co-evolution between the defended
649 prey and their predators (Brodie and Brodie, 1990; Bucciarelli et al., 2022), which can result in the
650 appearance of visual or morphological signals, mimicry, and even the loss of defenses in the prey if the
651 predator evolves sufficient resistance (Brodie and Brodie, 1991; Brown and Trigo, 1994; Crothers et al.,
652 2016). These additional ecological factors in turn shape the physiology of an animal in ways that may
653 further promote evolutionary innovation (Loeffler-Henry et al., 2023; Przeczek et al., 2008; Santos et al.,
654 2014). In sum, various factors interact in a dynamic equilibrium over short and long timeframes to shape
655 chemical defenses.

656 **(e) The passive-accumulation phenotype in a broader evolutionary context**

657 Passive accumulation of toxins is not a novel concept, as it has been discussed previously in terms of
658 self-medication (Clayton and Wolfe, 1993; Singer et al., 2009) and bioaccumulation (e.g., of
659 environmental pollutants; Butler, 1978; Spurgeon et al., 2020; Streit, 1992), and we propose that it is
660 also conceptually analogous to some medical treatments in humans such as chemotherapy. Any
661 organism that consumes something toxic might simultaneously suffer from toxin exposure yet benefit
662 from the compound's effect on disease, infection, parasites, or predators. For example, in the presence
663 of parasitoids, *Drosophila suzukii* flies preferentially lay their eggs on the insecticide atropine, which
664 protects them from being parasitized but prolongs development (Poyet et al., 2017). Mechanisms that
665 likely underlie passive accumulation may also be analogous to key organismal functions (Duffey, 1980).
666 For example, humans accumulate vitamin E in the liver (Violet et al., 2020) and use a transfer protein
667 abundant in liver cells to shuttle the vitamin into the plasma where it becomes bioavailable (Arita et al.,
668 1995). The transition from passive accumulation to sequestration in poison frogs may similarly rely on
669 the use of proteins that bind to and transport alkaloids (Alvarez-Buylla et al., 2023).

670 If toxin accumulation is both low-cost and beneficial, slow toxin elimination rates could evolve quite
671 readily, resulting in passive accumulation. Two recent studies support the idea that some toxin
672 resistance permits toxin intake and results in passive accumulation. In one, nicotine-resistant *Drosophila*
673 *melanogaster* fruit flies that were fed nicotine accumulated measurable amounts of the toxin in their
674 bodies, more than nicotine-sensitive flies (Douglas et al., 2022). In another study, ouabain-resistant *D.*
675 *melanogaster* flies that were fed ouabain accumulated measurable amounts of ouabain in their bodies,
676 more than ouabain-sensitive flies (Karageorgi et al., 2019). In a another example, the sawfly *Athalia*
677 *rosae* shows constant turnover of its glucosinolate toxins, suggesting that these insects cannot
678 effectively store glucosinolates, yet their metabolic clearing is inefficient enough that they still maintain
679 a high level of toxins in the hemolymph (Müller and Wittstock, 2005). It is conceivable, then, that in
680 some cases, passive accumulation could result in chemical defense through a mechanism that enables
681 high net toxin intake, followed by evasion of elimination mechanisms, passive entry into the
682 bloodstream, and diffusion into other tissues.

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

690 **(f) Limitations**

691 Our study presents a novel alkaloid dataset for dendrobatid frogs and some relatives, yet it is limited in
692 the following ways. For some species we only sampled one or two individuals, which may paint an
693 incomplete picture of toxin diversity, toxin quantity, and diet in the group. Poison frogs vary
694 substantially over time, space, and seasons in their alkaloid profiles and diets (Agudelo-Cantero et al.,
695 2015; Saporito et al., 2007a), yet we did not conduct serial sampling over a broad geographic range for
696 each species. Standards are unavailable for most frog alkaloids and thus we could not measure absolute
697 quantity. Relative quantitation of GC-MS data was performed based on integration of the extracted ion
698 chromatogram of the base peak for each alkaloid for maximum sensitivity and selectivity. The nature of
699 these data means that qualitative comparisons may be meaningful but quantitative comparisons across
700 alkaloid structures could be misleading, especially given our small sample sizes for some species. Finally,
701 batrachotoxin and tetrodotoxin are too heavy to study using GC-MS; we cannot exclude the possibility
702 that they occur in the sampled species.

703

704 3. Conclusion

705 The large-scale evolutionary transition from consuming to sequestering toxins has occurred in a plethora
706 of invertebrates (Duffey, 1980) and vertebrates (Savitzky et al., 2012). Here we provide new evidence
707 showing that undefended poison frogs and frogs in a closely related family (Bufonidae) contain
708 measurable amounts of alkaloids, and we confirm that they consume some amount of toxic arthropod
709 prey. We propose that passive accumulation of consumed alkaloids is an ancestral state in
710 Dendrobatidae, and possibly in related taxa, and that selection acts on toxin elimination and resistance
711 to result in toxin accumulation and chemical defense. Future studies of the toxicokinetics of alkaloids in
712 different tissues of both defended and undefended poison frogs will shed light on these putative
713 intermediate evolutionary steps. In turn, insights from poison frog physiology will provide a novel
714 perspective for the development of human therapeutics, which modulate some of the same

715 pharmacokinetic processes.

716

717 4. Methods

718 (a) Field collection

719 *Silverstoneia flotator* and *El. cystignathoides* were collected and euthanized with benzocaine in 2022 in
720 Gamboa, Panama (9.1373, -79.723183) and in 2024 in Austin, Texas, USA (30.285, -97.736 and
721 30.292487, -97.737874), respectively. Dorsal and ventral skins were removed and placed separately in
722 ~1-mL MeOH in 1-dram glass vials for UHPLC-HESI-MS/MS analyses (see below). All other species were
723 collected in 2014 and euthanized with an overdose of lidocaine. Whole skins were removed and placed
724 in ~1-mL MeOH in glass vials with PTFE-lined caps. Stomachs of all species were removed and placed in
725 95% ethanol. Instruments and dissection surfaces were cleaned with 95% ethanol between dissections.

726 (b) Alkaloid identification and quantification

727 For samples from Ecuador and Colombia, a 100-µL aliquot of the MeOH was sampled from each vial and
728 transferred to a 200-µL limited volume insert and analyzed directly by GC-MS. The system used was a
729 Thermo AS-3000 autosampler interfaced to a Trace GC Ultra interfaced to an iTQ 1100 ion trap mass
730 spectrometer autotuned with FC-43 (PFTBA) operating in positive ion mode. AS conditions were as
731 follows: 2 pre-wash cycles of 5 µL MeOH, then 3 plunger strokes and withdrawal of 1.00 µL sample with
732 1-µL air gap, injection with no pre- or post-injection dwell followed by 3 post wash cycles of 5 µL MeOH.
733 GC conditions were as follows: splitless injection, splitless time 1.00 min with surge (200 kPa for 0.70
734 min, to sharpen early peaks), split flow 50 mL/min; injector temperature 250C, oven temperature
735 program 100C for one minute, then ramped at 10C/min to 280C and held 10 min; transfer line
736 temperature 300C. MS conditions were as follows: for electron ionization (EI), collection mode profile, 1
737 microscan, 25 µsec max ion time, range 35–650 µ, source temperature 250C, solvent delay 3.00 min,
738 source voltage 70 eV; for chemical ionization (CI), reagent gas NH3 (1.8 mL/min). Samples for CI were
739 run in ddMS2 mode (3 precursor ions) with 1 microscan, 50 ms max ion time, 0.450 µ precursor width
740 and dynamic exclusion duration 0.2 min.

741 EI spectra were manually compared with published data (Daly et al., 2005, 1999, 1978) to identify class
742 and likely ID. A set of known standards was run to give accurate retention times across the range of
743 alkaloids and normalized to literature data using linear regression. Sample retention times were then
744 normalized, and molecular weights were obtained from CI MS1 spectra. These were then directly
745 compared to archival Daly GC-MS data where possible. CI MS2 spectra were also used where possible to
746 confirm functional groups such as alcohols by loss of water, etc. Kovats retention indexes (semi-standard
747 nonpolar) are also provided based on retention times and published indexes for background silicone
748 impurities. Accuracy of index assignments was confirmed based on fatty acid methyl esters from skin
749 lipids present in extracts. Epibatidine coelutes with the lipid methyl palmitoleate and the latter caused a
750 number of false positives in the GC-MS data. We thus reviewed LC-HRMS data at the known elution time
751 relative to a known standard. Epibatidine was only found in one sample in trace quantities and is
752 marked as such.

753 Samples from Panama and Texas were extracted on separate occasions, then filtered and run in tandem
754 with UHPLC-HESI-MS/MS, following an untargeted metabolomics protocol, with conditions optimized
755 specifically for retention and subsequent identification of alkaloids (Sedio et al., 2021). Briefly, for
756 extraction, methanol was evaporated and skins were homogenized with stainless steel beads in a
757 TissueLyser II (QIAGEN Sciences, Germantown, MD, USA) and resuspended in 1800 µL of extraction

758 solvent (9:1 MeOH:H_2O). Samples were then extracted for 3 hr at 4˚C in a ThermoMixer (Eppendorf US,
759 Enfield, CT, USA), followed by evaporation of the methanol component with a SpeedVac concentrator
760 (Thermo Fisher Scientific, Waltham, MA, USA). Next, samples were freeze-dried with a lyophilizer
761 overnight and resuspended in 500 µL extraction solvent. Resuspended extracts were then filtered and
762 diluted 1:7 in 100% MeOH. The metabolomic extracts were run on a Thermo Fisher Scientific (Waltham,
763 MA, United States) Vanquish Horizon Duo UHPLC system with an Accucore C18 column with 150 mm
764 length, 2.1 mm internal diameter, and 2.6-µm particle size, and a Thermo Fisher Scientific Q Exactive
765 hybrid quadrupole-orbitrap mass spectrometer. The instrumental methods (e.g., the separation of
766 metabolites by UHPLC, the volumes of buffers and their use in solvent gradients, and the use of heated
767 electrospray ionization [HESI] run in positive ion mode with full-scan MS1 and data-dependent
768 acquisition of MS2 [dd-MS2]) were identical to those described by (Sedio et al., 2021). A positive
769 reference of 1 µg/µL ≥98% (±)-epibatidine dihydrochloride hydrate (Sigma-Aldrich, St. Louis, MO, USA)
770 was included in the run, but injected last in the instrument so as to avoid possible carryover in the
771 column.

772 Following UHPLC-HESI-MS/MS, chromatographic data were processed using MZmine 3 (v3.9.0) (Schmid
773 et al., 2023), applying a stringent MS1 noise threshold parameter >100000 used by other workers (e.g.,
774 (Sedio et al., 2021)). So as to avoid additions of false positive metabolite observations, we did not use a
775 gap filling algorithm, a step often used in analysis of chemically homogeneous datasets to backfill
776 overlooked metabolite occurrences. MZmine 3 assigns chromatographic features to putative
777 compounds based on mass-to-charge (*m/z*) ratio and retention time. MZmine 3 feature tables and MS2
778 data were then uploaded to the Global Natural Products Social Molecular Networking (GNPS) platform
779 (Wang et al., 2016) for Feature-Based Molecular Networking (Nothias et al., 2020). We used SIRIUS
780 v5.8.6 (Dührkop et al., 2019) and CSI:FingerID (Dührkop et al., 2015) to infer molecular formulae and
781 predict structures including the elements H, C, N, O, P, and S. CANOPUS was used to classify metabolites
782 (Dührkop et al., 2021), following the ClassyFire (Djoumbou Feunang et al., 2016) and NPClassifier
783 molecular taxonomies (Kim et al., 2021). Only compounds assigned to the alkaloid pathway with an
784 NPClassifier pathway probability score >99% were retained in the feature table, which was generated in
785 R v4.2.2 (R Core Team, 2023) At >99% confidence, epibatidine was detected in three *S. flotator* skin
786 samples. Its presence was confirmed by manual inspection; the retention time, peak shape, isotope
787 pattern and MS2 are consistent with the epibatidine standard. We note that MS2 fragments were only
788 present in one of the three samples because of abundance.

789 With respect to the compounds exclusive to the positive reference sample (i.e., not present in the frog
790 skins), at >99% confidence, the algorithms implemented in SIRIUS also predicted annotations consistent
791 with an epibatidine alkaloid for a feature only detected in the positive reference sample, at the levels of
792 most specific class ("epibatidine analogues": ClassyFire) and class and superclass ("pyridine alkaloids''
793 and "nicotinic acid alkaloids": NPClassifier). The *m/z* ratio and structural prediction for this feature are
794 consistent with the epibatidine homolog "homoepibatidine" (Table S6). However, this annotation seems
795 at odds with the true identity of the feature (the retention time is at 0.5 minutes, the approximate void
796 volume with the highly polar compounds, and the isotope pattern is not correct for Cl, matching better
797 with silicon). Instead, the feature may represent a silicone derivative that, based on results from
798 multiple runs of the instrument (unpublished), we suspect could be an impurity consistently co-
799 occurring with and mistaken for homoepibatidine. In another run, we recovered a feature exclusive to
800 the positive reference sample with annotations identical at all levels to those for our "homoepibatidine"
801 feature, but with epibatidine's expected *m/z* ratio (~209) and structure (SMILES). In the run we publish
802 here, what is likely this same feature (with an *m/z* ratio of ~209 and annotated as (+/-)-epibatidine by
803 GNPS) was also recovered at the 99% confidence level. Assuming this feature is our positive reference —
804 (+/-)-epibatidine — the molecule was annotated as expected at class and superclass levels ("pyridine

alkaloids'' and “nicotinic acid alkaloids”, respectively) but annotated incorrectly at the level of most specific class (as a “pyrimidinethione”). Our results suggest that SIRIUS sometimes correctly annotates at all pathway levels our (+/-)-epibatidine positive reference.

(c) Diet identification

Stomach contents were inspected under a stereomicroscope 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 (R Core Team, 2023) using the packages *ggplot2* (Wickham, 2016), *cowplot* v1.1.1 (Wilke, 2020), and *dplyr* v1.1.2 (Wickham et al., 2023). The UHPLC-HESI-MSMS pipeline used on the samples from Panama and Texas allows for higher sensitivity to detect a broader array of compounds compared to our GC-MS methods but has lower retention-time resolution and produces less reliable structural predictions. Furthermore, due to the lack of liquid-chromatography-derived references for poison-frog alkaloids, precise alkaloid annotations from the UHPLC-HESI-MSMS dataset could not be obtained. Therefore, the UHPLC-HESI-MSMS and GC-MS datasets are not directly comparable, and UHPLC-HESI-MSMS data are not included in Fig. 2. Phylogenies were subsetted from (Wan et al., 2023) using *ape* v5.7.1 (Paradis and Schliep, 2019) and *phytools* v1.9.16 (Revell, 2012). Co-eluting compounds in the GC-MS and having the same base peak could not be discerned with the parameters we used in the Xcalibur processing method, 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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Ethics. Collection in Colombia and Ecuador 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). Collection in and export from Panama were performed under 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), and collection in Texas was performed under scientific research permit SPR-0922-131 issued by the Texas Parks and Wildlife Department. The animal use protocols were approved by the University of Texas at Austin (IACUC AUP-2012-00032, AUP-2021-00042, and AUP-2024-00003) 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), the Museo de Historia Natural C.J. Marinkelle (ANDES) at the Universidad de los Andes in Bogotá, Colombia, the Museo de Vertebrados de la Universidad de Panamá (MVUP), and within the Herpetology division of the of the University of Texas at Austin Biodiversity Collections.

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. GC-MS and UHPLC-HESI-MS/MS data are deposited on Global Natural Product Social Molecular Networking as MassIVE datasets under accession numbers MSV000095866 and MSV000094961, respectively. Other raw data are available here as supplementary tables.

Supplementary Information

Table S1. Stomach content data for every individual.

Table S2. A summary of data available on alkaloid detection in undefended lineages of poison frogs.

Table S3. Collection localities, specimen numbers, size, sex, and summary of alkaloid quantities and diversity for each individual.

Table S4. Alkaloid-level data for every individual analyzed by GC-MS.

Table S5. S5a) A feature table including information on *Silverstoneia flotator* and *Eleutherodactylus*

892 *cystignathoides* skin alkaloids; S5b) identifying information for samples corresponding to run numbers
893 listed in Table S5a columns.

894 **Table S6.** List of the subset of classes and most specific classes of compounds in *Silverstoneia*
895 *flotator* annotated as alkaloids ("Alkaloid Pathway" of NPClassifier) at >99% probability, their
896 presence/absence in *Eleutherodactylus cystignathoides*, whether the compound is from one of the
897 classes of lipophilic alkaloids listed in the Daly database, and whether the molecular formula for the
898 metabolite is found in the Daly database.

899

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