Poison frog dietary preference depends on prey type and alkaloid load

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Abstract

The ability to acquire chemical defenses through the diet has evolved across several major taxa, including insects, mollusks, birds and reptiles. Chemically defended organisms may need to balance chemical defense acquisition and nutritional quality of prey items. However, these dietary preferences and potential trade-offs are rarely considered in the framework of diet-derived defenses. Poison frogs (Family Dendrobatidae) acquire defensive alkaloids from their arthropod diet of ants and mites, although their dietary preferences have never been investigated. We conducted prey preference assays with the Dyeing Poison frog (*Dendrobates tinctorius*) to test the hypothesis that alkaloid load and prey traits would influence dietary preferences. We tested size preferences (big vs. small) within each of four prey groups (ants, beetles, flies, and larvae) and found that frogs generally preferred interacting with smaller prey items. Frog taxonomic prey preferences were also tested as we experimentally increased their chemical defense load by feeding frogs decahydroquinoline, an alkaloid compound similar to those naturally found in their diet. Contrary to our expectations, overall preferences did not change during alkaloid consumption, as frogs across groups preferred larvae over other prey. Finally, we assessed the protein and lipid content of prey items and found that small ants have the highest lipid content while large larvae have the highest protein content. Our results suggest that consideration of toxicity and prey nutritional value are important factors in understanding the evolution of acquired chemical defenses and niche partitioning as a whole.

Introduction

Animals must make efficient use of available food to satisfy their energetic and nutritional demands that are necessary for day-to-day function, reproduction, and survival (1). Optimal foraging theory predicts that organisms forage to maximize fitness by reducing the energetic costs associated with consuming low quality prey (1). Chemically defended animals across many taxa acquire toxins through their diet, and therefore their foraging decisions should be based on both chemical defense acquisition and nutritional quality of prey items. Most studies on organisms with acquired chemical defense focus on cataloging prey items from stomach contents, such diet studies in bufonid (*Melanophyriniscus*) toads, *Pitohui* birds and Natricine snakes (2–4). However, quantification of prey availability and nutritional content are rarely considered, leaving a gap in our current understanding of how dietary preferences evolve within the context of acquired chemical defenses. Examining how prey availability and nutritional value may influence selection of prey items contributes to our general understanding of community ecology and the evolution of acquired chemical defense.

Generalist predators must strike a balance between the energetic costs of foraging and prey nutritional value and availability (5,6). For example, *Schizocosa* wolf spiders make choices based on their nutritional and energetic demands by selecting for certain prey, even if they are low in abundance in their environments (7). Some predators must further consider the physiological burden of prey ingestion, especially in species that regularly consume chemically defended prey items. For example, the European Starling (*Sturnus vulgaris*) can learn to avoid toxic prey, but still choose to ingest them in the presence of undefended prey (8). This suggests that defended prey are worth consuming despite their physiological burden, as they may be nutritionally valuable (8). Such a nutritional tradeoff has also been proposed in possums, which regularly consume chemically defended plants that are metabolically costly but nutritionally valuable (9). Choice of prey is arguably even more complex in organisms that acquire chemical

defenses from their prey items. For example, *Acalymma vittatum* beetles have diet-derived chemical defenses and there is a tradeoff between protection from predators and the physiological costs of detoxification (6). These choices are not only important for short term energy needs, but they can also impact development and have long lasting consequences on fitness. In *Battus philenor* butterflies, sequestering defensive chemicals from plants increases survivorship against predation as caterpillars, but at the cost of reduced fat content as adults (5). Together, these examples suggest that environmental availability, chemical defense, and nutritional content of prey items are important considerations for predators with diet-derived defenses (2,3,10). However, the interplay between these factors in foraging decisions made by animals that acquire chemical defenses from their diet is poorly understood.

Diet-derived chemical defenses have evolved multiple times in Central and South American poison frogs (Dendrobatidae), which acquire alkaloid-based chemical defenses from their diets (11,12). Chemical defenses have evolved at least four times within Dendrobatidae, which co-evolved with a dietary specialization on ants and mites in some species (13,14). Stomach content analyses have established that alkaloid-containing ants and mites constitute the majority of diet within all chemically-defended dendrobatids and some non-defended ones, although there is high intra- and inter-specific variation (13,15,16). Consuming an alkaloid-rich diet while also acquiring enough lipids and protein for metabolism and reproduction can be challenging. In particular, lipids are important for gamete production and metabolic maintenance in amphibians (10,17), and yet are a limiting resource among arthropod food webs (18). Chitinous arthropods, such as ants and mites have high levels of insoluble/indigestible carbohydrates (19), and therefore frequent ingestion is expected to incur a nutritional tradeoff (20). Although poison frog stomach content analyses have greatly aided our understanding of diet in the dendrobatid clade, how dietary preferences based on prey phenotype or frog alkaloid load have never been tested. Thus, nutritional analyses paired with prey selectivity assays are

necessary to assess the possible tradeoffs between acquiring chemical defenses and necessary nutrients.

Here, we examined prey preference of Dyeing poison frogs (*Dendrobates tinctorius*). We tested the hypothesis that chemical defense acquisition, prey size and prey type influence dietary preferences in poison frogs. We first tested preference based on size within prey categories, as a study in the Malagasy Mantellidae poison frogs suggests a dietary preference towards smaller prey items (21), and because prey size is known as a crucial factor for dietary selectivity in frogs generally (22,23). We predicted the frogs would show a greater preference towards smaller chitinous prey categories (ants, beetles), but larger versions of non-chinous prey (flies, larvae), as insect larvae tend to have higher protein and lipid content compared to chitinous adult arthropods (24). We next tested preference for prey taxonomic identity and nutritional value and predicted *D. tinctorius* would prefer ants, which make up the majority of poison frog stomach contents in the wild (13,16,25). Finally, we tested whether chemical defense changes prey preference by experimentally manipulating alkaloid load and measuring prey selection. We expected frogs to prefer more nutrient-rich prey as they acquire alkaloids. Together, our work helps to fill the gap in our understanding of how prey size, type and nutritional quality affect the dietary choices of chemically defended predators.

Methods

Animals

Dendrobates tinctorius (N=20) were purchased from Josh's Frogs (Owosso, MI, USA) and housed in pairs in glass terraria with sphagnum moss, live philodendrons, shelters, egg laying sites, and a water pool. Frogs were fed three times per week with fruit flies dusted with vitamin supplements. Terraria were misted with water ten times per day and maintained in a

temperature-controlled room with a 12h:12h light-dark cycle immediately preceded and followed by a 10 min period of dim lighting to simulate dawn and dusk. The Institutional Animal Care and Use Committee at Stanford University approved all frog experiments (Protocol #33839).

The arthropods used in this study are comparably sized to wild prey items (26), although they are not the same species, which are not commercially available. Four groups of arthropod prey of two sizes each were used in frog feeding assays as follows (large, small): Ants: *Camponotus vicinus* [7.0-10.0 mm, ~5 mg], *Linepithema humile* [2.2-2.6 mm, ~1.5 mg], Flies: *Drosophila hydei* [3.0 mm, ~2.5 mg], *Drosophila melanogaster* [1.6 mm, ~1.3 mg], Beetles: *Stethorus punctillum* [1.5 mm, 400-500 µg], *Dalotia coriaria* [3.0-4.0 mm, 78-80 µg], Larvae (fly): *Callifora vomitoria* [10 mm, ~66 mg], *Musca domestica* [3-5 mm, ~12 mg] (27,28). Ants were collected the morning of frog feeding trials on the Stanford University campus by sweeping ants off their nesting trees with a bristle brush into a clean ziploc bag. All beetles and larvae were purchased from online vendors (Arbico Organics: Tucson, Arizona, USA; Evergreen Growers Supply: Clackamas, Oregon, USA; Nature's Good Guys: Ventura County, California, USA; Speedyworm Bait Suppliers, Alexandria, Minnesota, USA). Live larvae were kept refrigerated at 4ºC and neither fed nor hydrated to prevent them from pupating into adult flies. Flies were maintained at room temperature in 32 oz. plastic deli cups with aerated lids using fruit fly media (Josh's Frogs). Beetles were stored in 32 oz. plastic deli cups with aerated lids with packaging bedding and cotton balls soaked with a 1:1 table sugar:water solution. The beetles were misted with water three times per week and occasionally were fed grain mites (*Acarus siro*) sourced from the lids of mite-infested fly cultures. To our knowledge, none of the eight prey species chosen are chemically defended.

Behavior

All dietary preference assays were conducted in the same room where frogs are housed, in which temperature and humidity replicate conditions of their native habitats. One week before

feeding trials began, all frogs were exposed to arthropod prey items six times over three weeks to reduce neophobia. Trials were conducted in a small transparent plastic bin (33 cm x 20 cm x 12 cm) stacked within an identical secondary bin with a layer of dried magnolia leaves to simulate a forest-floor environment while keeping the texture even throughout the arena. Inner sides of the arena were coated with Fluon™ liquid teflon (Bioquip Products Inc, Rancho Dominguez, CA, USA) to prevent the arthropod prey from escaping.

Size-mediated dietary preference assay

Adult frogs (11 female, 9 male; age unknown) were chosen at random and placed in the arena with 10 individual insects (five big, five small) of one of the given prey groups (ants, beetles, flies, or larvae). Each trial was conducted for 5 min and recorded with a GoPro camera (GoPro Inc, San Mateo, CA, USA) mounted above the arena. Each frog participated in one trial with each prey group and the order in which frogs and prey were tested was randomized. Intertrial intervals were roughly 10 min and the arena was emptied and wiped down with a moist paper towel during this time. Videos of all behavior trials were scored using BORIS (Behavior Observation Research Interaction Software) (29). The number of “eat” events and “attempt to eat” events were scored for each size prey item within a trial.

Alkaloid feeding and taxon dietary preference assay

Dietary preference trials were conducted to determine frog preference for different insect taxa and how this preference may change with chemical defense (Figure 1A). Only female frogs (N=10, age unknown) were used in this behavioral assay as male frogs consistently exited the arena before trial completion. Frogs were orally administered 15 µl of a vehicle control solution (0.85% sodium chloride, 1% ethanol, 98.15% deionized water). One week later, frogs were chosen at random and placed in the arena with 20 individual insects based on preferences in the size trials described above: five large larvae (*Callifora vomitoria*), five small ants

(*Linepithema humile*), five small beetles (*Dalotia coriaria*) and five small flies (*Drosophila melanogaster*). Each trial was conducted for 5 minutes and recorded with a GoPro camera mounted above the arena. The arena was emptied and wiped down with a moist paper towel between trials.

To test how preferences may change as frogs acquire chemical defenses, the same 10 frogs were orally administered 15 µl of 0.01% Decahydroquinoline (DHQ) in vehicle solution three times over the course of one week. The following week, these “low-DHQ” frogs were re-tested in the taxon dietary preference trials described above. One week later, frogs were orally administered 15 µl 0.01% DHQ three times per week over two consecutive weeks, representing a “high-DHQ” treatment. The following week, frogs were retested in a final preference trial. Previous work has established this alkaloid-feeding paradigm as appropriate in creating low and high DHQ groups (30,31).

Each frog was assayed once at each toxicity level for a total of three trials. Some Videos could not be scored due to camera malfunction. Both ingestion and an attempt to pursue or ingest prey of each type were scored using BORIS software. The scorer was not aware of the treatment group at the time of scoring.

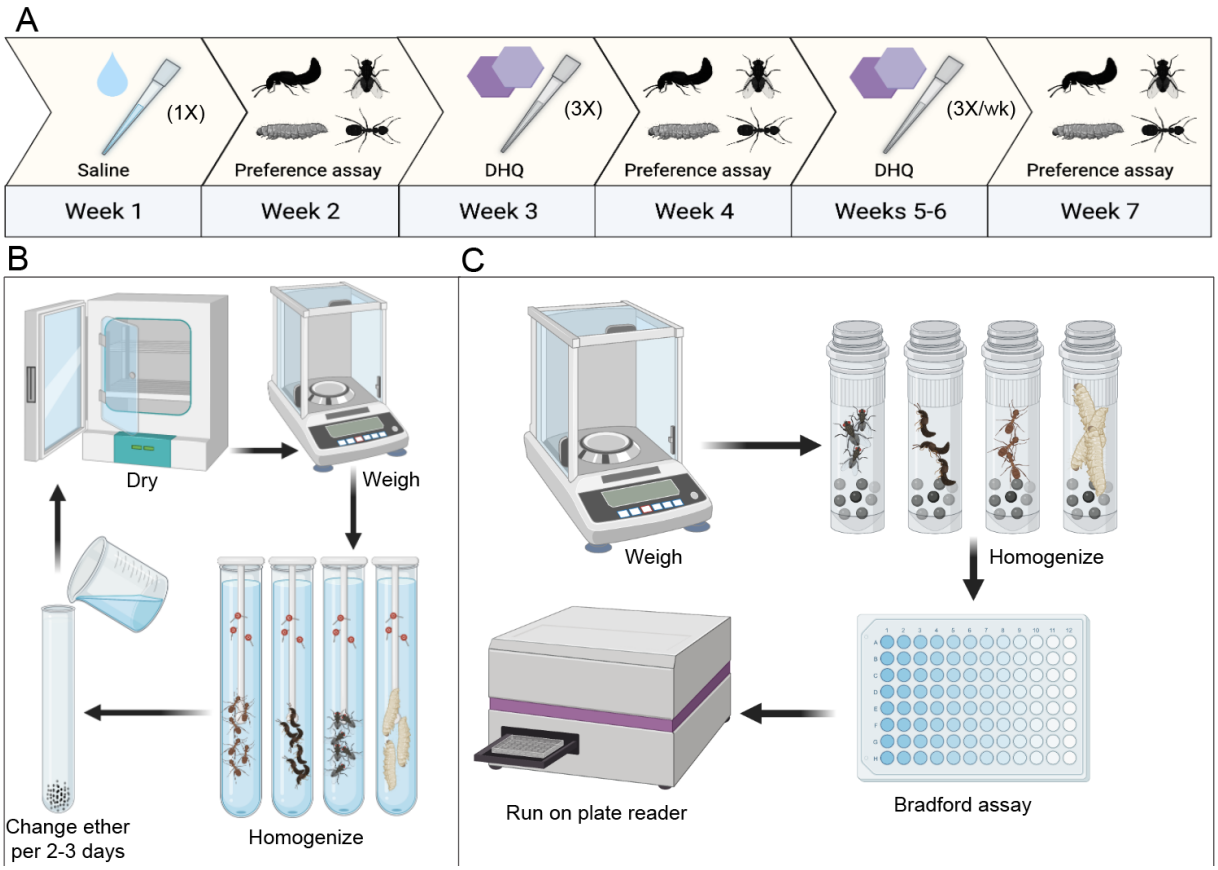

Figure 1: Experimental workflows of diet preference and nutritional assays. (A) The workflow of the alkaloid-mediated diet preference assay is depicted on a 7-week timeline. **(B)** Workflow of ether-soluble lipid quantification of prey items are shown. After the 6-8 day ether step, the arrow points to the drying and weighing steps, as they must be repeated to obtain lipid-free weight. **(C)** Workflow of the Bradford Protein assay of prey items are shown. Although our experiment used 5 replicates per prey group in lipid and protein assays, only single replicates are depicted here.

Nutritional content assays

Crude lipid content was quantified across the eight prey groups used in the dietary preference behavior experiments using an ether-soluble lipid extraction method (Figure 1B) (24). All arthropods were fresh-frozen at -80ºC and stored for 1-2 weeks. After thawing, specimens were separated into five 1-2 mL biological replicates per prey group. The number of specimens used per prey type varied due to density and size. Each sample was placed in an incubator at 60ºC until they were dried to a constant mass, defined as less than a 0.10 percent

change after an additional 20 minutes of drying (24). One replicate per arthropod sample group was weighed once after 2 hours, and again after three hours, when a constant mass was reached for all prey groups except for larvae, which reached a constant mass after ~12 hours of drying. Once a constant mass was reached for all prey groups, the final mass was recorded and samples were placed in a glass culture tube filled with 5 ml of petroleum ether (Thermofisher Scientific, Waltham, MA, USA) and ground with a Dounce Homogenizer. Specimens were then incubated in ether for 8 days at room temperature. Every two days during this period, each ether was carefully decanted from each tube to prevent loss of non-lipid mass and then replaced with fresh ether. Specimens were then left in their tubes uncovered for ≥24 h in a fume hood to allow for evaporative removal of the remaining ether. Samples were then placed into an incubator at 60ºC for ≥3 h for additional drying. Once specimens were completely dried, they were weighed and lipid-free mass was subtracted from total mass, indicating the biological portion made up of ether-soluble lipids.

Crude proteins were extracted from each of the eight prey groups. All arthropods were fresh-frozen live at -80ºC and stored for 1-2 weeks. Five replicates of 10-150 mg of each arthropod type were put into 2 ml tubes, each containing 2.4 mm stainless steel beads (Omni Life Science Inc, Raynham, Massachusetts, USA, SKU: 19-640) and 0.8 ml of cold 1x phosphate-buffered saline (PBS, pH 7.4). Samples were homogenized at 4ºC using a Beadmill 24 Homogenizer (Thermofisher Scientific) with the following settings: Speed in m/s (S): 4.5, Number of cycles (C): 10, Cycle time (T): 30s, Interval paused between cycles (D): 10s. Samples were then centrifuged at 2,000 rpm at 4ºC for five minutes. The supernatant containing the protein lysate was moved into a new 1.5 mL microfuge tube and stored for 1 week at -80ºC. Proteins were then quantified using a Bradford Protein assay according to manufacturer's instructions (Pierce Coomassie Plus Bradford Protein Assay Reagent, Thermofisher Scientific). Samples and albumin standards were run in triplicate on a 96-well optical plate and quantified on a BioTek Synergy H1 96-well plate reader (Winooski, Vermont, USA). Absorbance was

measured at 595 nm and an endpoint absorbance value was calculated for each standard and sample. Crude protein concentrations of arthropod samples were calculated based on the absorbance values of samples compared to the standard curve.

Data analysis and visualization

Statistical analyses were run in R version 4.1.1 using the glmmTMB package (32). To assess differences in large and small prey consumption, we fit zero-truncated generalized linear mixed models (GLMM) for each of the four prey assays (large vs. small ants, beetles, flies, larvae) based on a truncated Poisson distribution of the number of “eats” or “attempts” across all 19 frogs. To control for individual variation in the subjects, frog ID was used as a random effect, and since each frog differed in its total number of prey interactions across the four size preference assay categories, “total interactions with prey” was used as an offset in order to account for these differences in consumption. Sex was also used as a covariate because of previous knowledge of differences in diet between male and female dendrobatids (33).

To assess differences of prey interactions across control, low-DHQ, and high-DHQ groups, we used our taxonomic prey preference assay data to fit a zero-truncated generalized linear mixed models (GLMM) to compare the number of prey interactions for each prey type: ants, beetles, flies, larvae based on a truncated Poisson distribution of the number of “eats” or “attempts” across the 10 female frogs that were assayed. To account for individual variation in the subjects, frog ID was used as a random effect, and since each frog differed in its total number of prey interactions across the four prey choices, we used “total interactions with prey” as an offset in order to account for these differences in consumption. To check for differences in the general structure of frog diet categories across the three groups, we performed a permutational multivariate analysis of variance (PERMANOVA) on Bray-Curtis dissimilarities. We used a zero-truncated GLMM (glmmTMB package, R version 4.1.1) to compare total frog interactions (eats or attempts of all prey items) across the three groups: control, low and high

DHQ. The model was based on a truncated Poisson distribution as described above. To control for repeated measures in the subjects, frog ID was again used as a random effect. P-values were adjusted using the Benjamini-Hochberg procedure to correct for multiple hypothesis testing across all dietary preference and nutritional analyses. Data were visualized with boxplots and barplots created using the package 'ggplot2' in R version 4.1.1 and infographics were created with images from BioRender.com.

Results

Frogs prefer smaller prey items but large larvae

Overall, frogs interacted (ate or attempted) more with the smaller versions of prey items (Figure 2). Frogs interact more with small compared to large flies (GLMM, Z: 16.02, BH-adjusted $p < 0.0001$) and small compared to large beetles (GLMM, Z: 2.558, BH-adjusted $p = 0.021$). There was no preference for small versus large ants or larvae within this behavioral assay (Generalized linear mixed model (GLMM), Ants: Z: 0.844, BH-adjusted $p = 0.477$; Larvae: Z: 0.712, BH-adjusted $p = 0.477$). The relative frequency of prey interactions did not differ between prey groups (Kruskal-Wallis chi-squared = 2.6556, df = 3, p-value = 0.448).

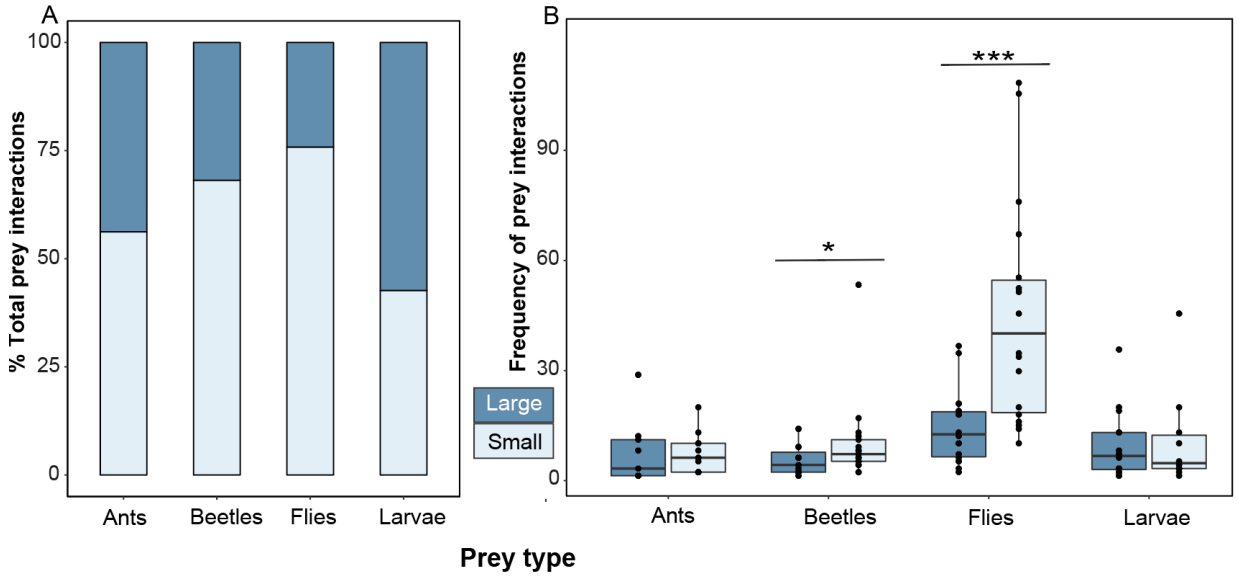

Figure 2. Dietary choices based on prey size. *Dendrobates tinctorius* frogs mainly interacted with (ate or attempted) small prey items in prey size preference assays. Each frog was presented with one prey group at a time and given the choice of small and large versions of ants, beetles, flies and larvae. **(A)** Percent breakdown of all interactions across prey groups are plotted in a stacked bar chart and **(B)** the frequencies of individual frog interactions among large and small prey are visualized as box plots, where each dot represents one frog**.**

Alkaloid ingestion changes dietary preference

When given the choice between multiple prey taxa, frogs without DHQ interact more with larvae than any other prey item (GLMM, Z: 4.95, p<0.00001), but there are significant preferences among other three prey categories. However, frogs change their dietary preferences as they acquire DHQ. Although the three groups did not differ in their interaction frequency with prey types when compared simultaneously (PERMANOVA; F: 0.8932, p= 0.470), high DHQ frogs interact more with larvae compared to control and low DHQ frogs (GLMM, Z: -2.598, BH-adjusted p= 0.036), although interactions with larvae were extremely variable across groups. There were no differences found among ant (GLMM, Z: 0.326, BH-adjusted p= 0.744),

fly (GLMM, Z: 0.879, p= 0.379, BH-adjusted p= 0.744), or beetle interactions (z: -0.409, BH-adjusted p: 0.744).

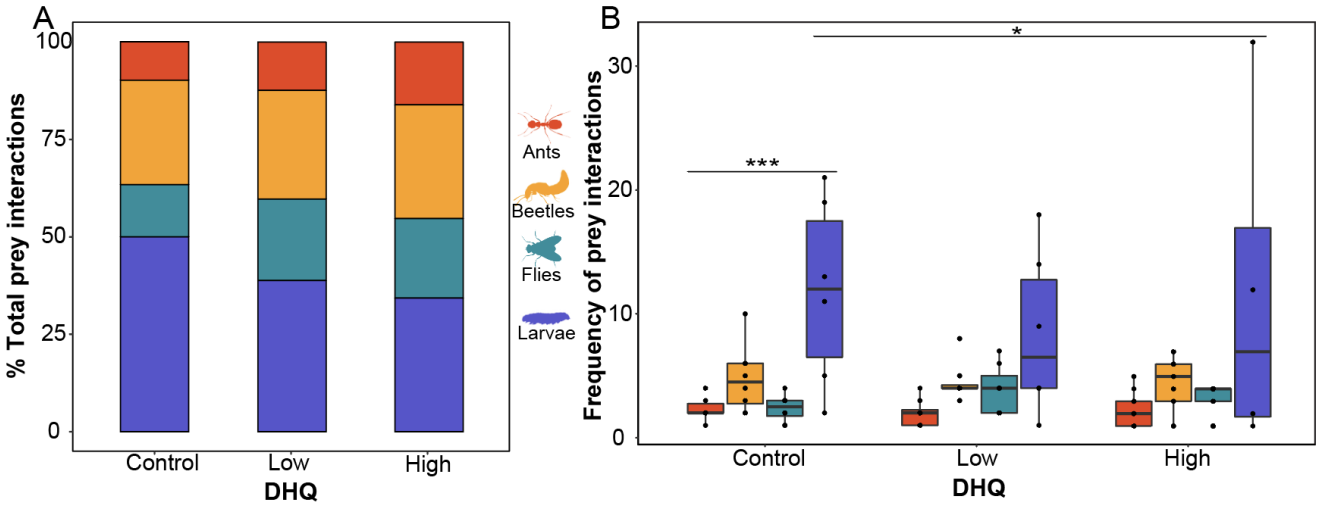

Figure 3. Frog prey preference changes with increasing chemical defense **(A)** Percent breakdown of all prey interactions (eats or attempts) across DHQ-fed and vehicle control groups are plotted in a stacked bar chart and **(B)** the raw frequencies of prey interactions among frog toxicity groups are visualized as box plots to show the variation of prey interactions across individuals, where each dot represents a frog.

Prey items differ in lipid and protein content

All eight prey species were evaluated for two important dietary components: lipids and proteins (Figure 4). Overall, lipid content differed across all prey types (Kruskal-Wallis χ^2 = 29.564, df = 7, p-value = 0.0001). Small ants, beetles and larvae had a higher proportion of lipids than their larger counterparts, while large flies had a higher proportion of lipids than small flies (Figure 4, Table 1). Protein content also differed across species (Kruskal-Wallis χ^2 = 29.206, df = 7, p-value = 0.0001), where large larvae have more protein than most prey items, followed by small beetles, which have more protein than small or large ants and flies (Figure 4, Table 2). Protein content did not differ within taxa for large compared to small prey. In summary, small ants have the most lipids, while large larvae have the most protein of all other prey categories.

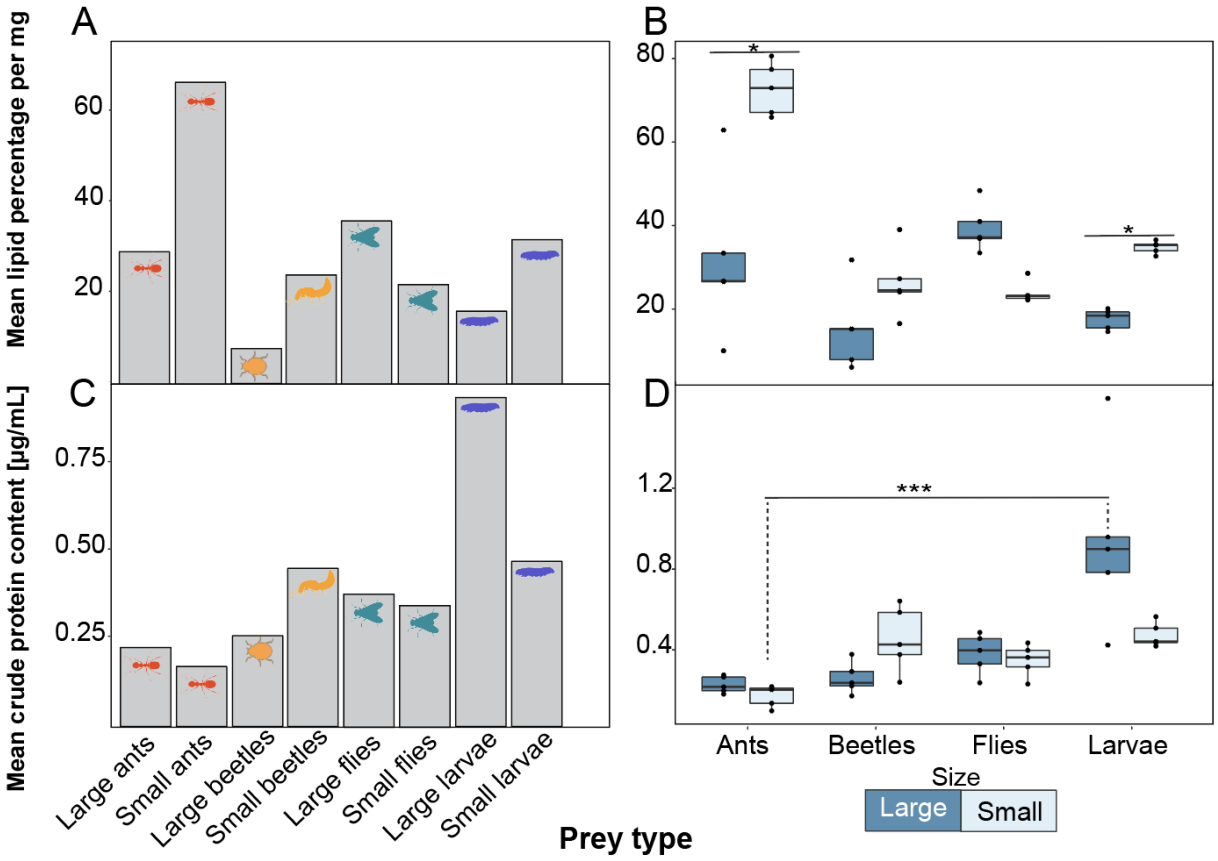

Figure 4. Nutritional content of prey from dietary preference assays. (A) Average crude lipid contents across replicates of the eight arthropod species are depicted in a bar chart, and **(B)** Variation of these values across replicates is shown in a box plot, where each dot represents a replicate. **(C)** Average crude protein content across replicates of the eight arthropod species are depicted in a bar chart, and **(D)** variation of these values across replicates is shown in a box plot, where each dot represents a replicate.

Table 1. Differences in crude lipid content (% dry weight, mg) between arthropods. Statistics of arthropod taxa differences in crude lipid content using Kruskal-Wallis test with a Dunn Multiple Comparison Posthoc test. P-values were adjusted using the Benjamini-Hochberg procedure to correct for multiple hypothesis testing

Comparison	Z	Raw P	BH-adjusted P
large larvae - small ants	-4.0307	0.0001	0.0008
large beetles - small ants	-4.1659	0.0000	0.0009
small ants - small flies	3.1109	0.0019	0.0131
large flies - large larvae	3.0298	0.0025	0.0137

large beetles - large flies	-3.1650	0.0016	0.0145
small ants - small beetles	2.6240	0.0087	0.0348
large beetles - small larvae	-2.6240	0.0087	0.0406
large ants - small ants	-2.4617	0.0138	0.0430
large larvae - small larvae	-2.4887	0.0128	0.0449
large flies - small flies	2.1100	0.0349	0.0976

Table 2. Differences in crude protein content (conc [µg/ml]) between arthropod prey. Statistics of arthropod taxa differences in crude protein content using Kruskal-Wallis test with a Dunn Multiple Comparison Posthoc test. P-values were adjusted using the Benjamini-Hochberg procedure to correct for multiple hypothesis testing.

Comparison	Z	Raw P	BH-adjusted P
large larvae - small ants	4.1932	0.0000	0.0008
large ants - large larvae	-3.5709	0.0004	0.0050
small ants - small larvae	-3.4086	0.0007	0.0061
large beetles - large larvae	-3.1246	0.0018	0.0125
small ants - small beetles	-2.9217	0.0035	0.0195
large ants - small larvae	-2.7864	0.0053	0.0249
large flies - small ants	2.5159	0.0119	0.0475
large ants - small beetles	-2.2995	0.0215	0.0668
large beetles - small larvae	-2.3401	0.0193	0.0675
large larvae - small flies	2.1507	0.0315	0.0882

Discussion

We tested the hypothesis that frog chemical defenses and prey traits like size and nutritional value would influence diet choices in poison frogs. We expected laboratory *D. tinctorius* to prefer small prey items, reflecting their wild diet, which was generally true. However, we were surprised to find frogs did not prefer ants over other prey items, as these frogs are thought to have a dietary specialization on ants and mites in the wild. Rather, *D. tinctorius* generally prefers prey items with high lipid and protein content and frog alkaloid load can influence prey preferences. We discuss these laboratory results in the context of field studies in amphibians and the importance of replicating these studies under natural conditions in the future.

Prey selectivity of arthropods

Predators' ability to consume and obtain energy from prey is primarily limited by the prey size, making size an important factor in dietary selection (1). In our study, *D. tinctorius* frog dietary preference was assessed using prey size mediated assays, where frogs preferred small flies and beetles over large ones, with no preference for size in ants or larvae. Our findings are similar to a study conducted with *Mantella aurantiaca* poison frogs (Mantellidae), which have a slight preference for smaller prey items (21). We did not find a size preference for ants or larvae, which has been documented in *Chiasmocleis mehelyi* (Microhylidae) frogs that show a preference for small ants and avoidance of large ants relative to environmental abundance (23). However, we did not test ants from the natural range of *D. tinctorius* and testing such species may reveal other preferences based on size or other prey traits like alkaloid content. Additionally, these captive frogs have never consumed a wild-like diet, as they have been exclusively fed *Drosophila melanogaster* (small flies) until the start of this experiment, which

may have influenced their dietary preferences. Despite these limitations, our study and previous literature show that prey size is a crucial factor for dietary choices of predators.

Taxonomic dietary preference was assayed using the most frequently chosen species (either large or small) from each prey group (small ants, small beetles, small flies, large larvae), and frogs preferred large larvae over other prey items. Although poison frogs consume a diverse array of non-ant prey items (13,14,34), the stomach contents of *D. tinctorius* and many other chemically defended poison frogs in the wild are composed of over 50% ants (13,26,34). Unexpectedly, we found that *D. tinctorius* frogs preferred larvae over other prey items tested, including ants, beetles, and flies. It is possible that frogs' foraging behavior would have been different when presented with ants from their natural habitat. For example, a field study with *Chiasmocleis mehelyi* (Microhylidae) frogs, which are hypothesized to sequester chemical defenses from their diet, showed preferential selection for ants over other arthropod categories (23,35). Frogs in general can be selective predators, as the stomach contents of bufonid toads (*Bufo cognatus* & *Bufo woodsii*) differ from local arthropod abundance (36). However, it may be prey availability that contributes to ant abundance in poison frog diets, as the frogs occupy microhabitats of the forest with high ant abundance. For example, a study that compared stomach contents of chemically defended Malagasy poison frogs (mantellidae) to environmental availability of arthropods found no significant differences between them, suggesting that their diet may reflect environmental availability rather than prey selectivity (37). Given the known importance of prey availability, we encourage future studies on organisms with diet-derived defenses to consider comparing environmental availability of prey items' effect on dietary patterns and preferences.

Chemical defenses and prey preference

While the acquisition of chemical defenses from diet can be physiologically burdensome, defended prey may also be nutritionally valuable. We tested the hypothesis that acquiring

chemical defenses would change prey preferences and predicted that alkaloid-associated prey, such as ants, would become less desirable due to low nutritional value reported in the literature (19,24). Contrary to our predictions, as frogs accumulated alkaloids they increased their preference for larvae, with high DHQ frogs showing a higher preference for larvae over low DHQ and control groups. As preference changes with defense consumption, it is possible that alkaloids can change foraging behavior as described in other animals. For example, in Red Knot birds (*Calidris canutus*), bodily levels of ingested toxins slow their feeding rates (38,39). Another example comes from *Mytilus* mussels, where previous ingestion of toxins slows their toxin uptake rate and feeding rate (40,41). Further, although not chemically defended themselves, possums consistently eat toxic, metabolically costly plants for their nutritional value (42). Based on these studies, a positive feedback relationship could be expected between frog alkaloid levels and ingestion of alkaloid-associated prey up to a physiological threshold of chemical burden. In the future, using ant species with alkaloids that poison frogs sequester may give more ecologically-relevant insight into how behavior changes with alkaloid uptake.

Behavior can change over time with repeated exposure to prey items (43–45). Our experimental design utilizes within-subject testing as frogs went from non-toxic to various loads of DHQ levels and thus repeated exposure and testing may instead reflect learning through experience rather than changes associated with alkaloid levels. We cannot currently verify that learning does not influence the prey preference patterns observed in our study. Although all eight prey species in the present study are not known to possess small-molecule alkaloids (46,47), ants found in stomach frog contents carry alkaloids and are presumed to be the main dietary source for frog defenses (12,48). We suggest that future studies should include alkaloid extractions on all prey items, as the possible presence of previously undetected defenses could influence preference assay results. Future studies on distinct groups of chemically defended and undefended frogs being presented with native prey items that contain alkaloids the frogs

sequester are needed to disentangle these caveats. The results from our captive study suggest that this would be a worthwhile line of research to pursue in the future.

Prey selectivity and nutritional content

Dietary lipids and proteins are important for amphibian reproduction and survival. For example, the survival rate of *Epidalea calamita* toad tadpoles is positively correlated with protein consumption (49), and leopard frog tadpoles given a protein-rich diet are much more likely to overcome *Batrachochytrium dendrobatidis* infection (50), a disease which has ravaged frog populations globally (51). Lipids are also of known importance to frogs, specifically for metabolic demand and gamete production (17). A study that monitored whole-body lipid content in domestically-reared *H. marmoratus taeniatus* frogs showed a significant decrease in whole-body lipid content following their breeding period, suggesting that fat stores are quickly depleted through reproduction (52). Another study conducted in *Rana tigrina* found that frogs with small abdominal fat bodies showed significantly lower fecundity and decreased egg size than those with large abdominal fat bodies, and these differences were caused by diet quality (53). Given the importance of lipids and proteins to gamete production, metabolic demand, immunity and survival, nutrient content of prey items should be quantified when investigating factors that shape prey preference in amphibians.

In the current study, we assessed crude protein and lipid content of eight prey species, and unexpectedly found small ants were high in lipid content. Much of the literature describes ants as being composed primarily of insoluble fibers due to their chitinous exoskeletons (18,19,54) On this basis we expected eating ants to impose a trade-off in poison frog foraging decisions between acquiring alkaloids versus nutrition. Yet, there are very few studies examining ant nutritional value, although our ant lipid results are similar to one study assessing nutrient quality of small ant species, which report lipid contents of 40-60% (55). However, our small ant lipid results are substantially higher than the only study which examined lipid

percentage of the same small ant species as used in our study, *Linepithema humile,* as they report lower lipid contents of 18-20% (56). However, the ants in that study were given strict feeding regimens for the purposes of studying behavioral changes associated with manipulated protein:carbohydrate ratios in the diet. Therefore, those ants are expected to have a different nutritional makeup than the ones in our experiment, which are sourced from the wild. We note that the ants used in this study likely lack alkaloids previously described in poison frogs and a fruitful future direction would be to measure lipid, protein, and alkaloid content from species within the *D. tinctorius* natural range. Lipid and protein content of large larvae (*Calliphora vomitora*) from our current study matched previously reported values (57). Overall, the relative lipid values among orders from our study (Diptera, Coleoptera, Hymenoptera) are quite similar to the only large scale study which quantified and compared lipid content across major arthropod orders (24). However, a recent review of the nutritional quality of insects for animal feed shows that both lipid and protein contents are remarkably variable across studies of all insect taxa, even within single species (58). More studies with larger sample sizes across a wider range of arthropods are necessary to understand their nutritional value as prey items.

Although arthropods are an important dietary component of many taxa (57,58), there are a few studies which quantify lipid or protein content of individual arthropod species. The vast majority of studies which examine arthropod crude protein and lipid content are focused on the benefits of arthropod consumption for humans and domestic animals (60,61), with little focus on wild animals' diets. Within our study, small ants and large larvae seem to be the most nutrient-rich of all prey items. Large larvae have the highest protein content per mg but are lipid-poor, while small ants have the highest lipid content but are protein-poor (Figure 3). Both sizes of beetles and flies have similar protein levels to one another, but flies had a higher lipid content than beetles. Therefore, a nutritional trade-off arises between the decision of *D. tinctorius* individuals to consume either lipid and protein-rich prey items in the presence of both. Assuming the small ants in wild poison frog diet possess similar lipid and protein levels to the

small ants in the present study (*Linepithema humilis*), chemical defense acquisition may not signify a total nutritional tradeoff, given the lipid richness of small ants. However, we encourage longer-term studies to be conducted using a higher sample size, prey items which more accurately reflect poison frog wild diet, and a wider variety of poison frog species and alkaloids, since diets between defended and undefended species indeed vary (13).

Prey choice becomes more complex when considering highly proteinous prey, like larvae, in the presence of lipid-rich small ants, which frogs are likely to associate with chemical defenses. We expected a clearer-cut tradeoff between consuming ants, which have high levels of indigestible carbohydrates (19,57) while missing out on the nutritional value of undefended prey. However, given the lipid richness of small ants and the ant-dependent nature dendrobatid defenses, we propose that poison frogs consume alkaloid-laden prey opportunistically, but are predisposed to interacting more with higher protein prey, considering that protein is a limiting resource in arthropod food webs (18) and required for amphibian health (49,50). Similarly, a particularly high demand for lipids should be expected in neotropical frogs that tend to breed for the majority of the year (62,63) and should have a high lipid demand. Taken together, these wild diet studies paired with our results suggest that typical ant-rich wild poison frog stomach contents likely reflect a combination of their ant-rich habitats and dietary preferences, as proposed previously (13,26,64).

Conclusions

We found that poison frogs prefer prey with high lipid and protein content and that alkaloid uptake influences dietary choices. The known importance of lipids to amphibian reproduction and survival taken together with our prey nutrient and preference assay results show that poison frogs may have nutritionally benefitted from a dietary specialization on ants before they evolved an ability to acquire chemical defenses from them. We suggest that innate prey preferences, the nutritional value of prey and prey availability are all important for our

understanding of how dietary alkaloid sequestration evolved multiple times within the Dendrobatidae clade. The influence of nutritional value on dietary choices is essential for understanding the evolution of acquired chemical defenses and niche partitioning across heterotrophs. Future studies should evaluate the ecological significance of our findings by investigating preference and nutritional content of prey items available to wild frogs across a larger geographical and taxonomic scale that includes defended and undefended species.

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Resources: NAM and LAO
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Writing – review and editing: RD
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