Poison frog diet and chemical defense are influenced by availability and selectivity for ants

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Abstract

The ability to use small molecule alkaloids as defensive chemicals has evolved in many organisms, often via trophic interactions due to dietary specialization. Animals with diet-derived defenses must balance food choices to maintain their defense reservoirs along with other physiological needs. However, environmental availability of prey and dietary preferences of vertebrate animals with acquired defenses remains largely unexplored. Here, we test the hypothesis that poison frogs that sequester alkaloids from their arthropod prey display prey preference. We collected alkaloid-defended Diablito frogs (*Oophaga sylvatica*) and leaf litter samples in five localities in northwestern Ecuador. Additionally, we collected the undefended Chimbo rocket frogs (*Hyloxalus infraguttatus*) from one locality in which it is living in sympatry with *O. sylvatica*. We found that both diets and skin alkaloids of *O. sylvatica* frogs were distinct among localities, and that undefended and sympatric *H. infraguttatus* ate fewer ants and mites. Unexpectedly, across *O. sylvatica* populations, we found a negative correlation between their summed skin alkaloid content and number of ants and mites they consumed. Finally, we compared leaf litter ants to frog stomach contents, and found that frogs eat a small subset of the available ant genera found in surrounding leaf litter, as only 13% of all the ants recovered from leaf litter samples belong to the 16 ant genera consumed by the frogs. Our results suggest that the consumption of diet-acquired defenses depends on preference-informed food choices in addition to environmental availability. This impacts our understanding of chemical defenses as a whole, as behavioral reinforcement may be an understudied, yet important selection factor in the evolution of acquired defenses.

Resumen

La capacidad de usar moléculas pequeñas alcaloides como productos químicos defensivos ha evolucionado en muchos organismos, a menudo a través de interacciones tróficas debido a la especialización dietética. Los animales con defensas derivadas de la dieta deben equilibrar la selección de los alimentos para mantener sus reservorios de defensa junto con otras necesidades fisiológicas. Sin embargo, la disponibilidad ambiental de presas y las preferencias dietéticas de los animales vertebrados con defensas adquiridas son aspectos en gran parte desconocidos. Aquí, probamos la hipótesis de que las ranas venenosas que obtienen alcaloides de sus presas de artrópodos muestran preferencia por las presas. Recolectamos especímenes de ranas diablito (*Oophaga sylvatica*), con alcaloides defensivos, y muestras de hojarasca en cinco localidades del noroeste de Ecuador. Adicionalmente, recolectamos la rana cohete de Chimbo (*Hyloxalus infraguttatus*), sin alcaloides defensivos, de una localidad en la que vive en simpatría con *O. sylvatica.* Encontramos que tanto las dietas como los alcaloides de la piel de las ranas *O. sylvatica* eran distintas entre localidades, y que la simpátrica y no venenosa *H. infraguttatus* comía menos hormigas y ácaros. Inesperadamente, entre poblaciones de *O. sylvatica,* encontramos una correlación negativa entre la sumatoria del contenido de alcaloides cutáneos y el número de hormigas y ácaros que consumieron. Finalmente, comparamos las hormigas de hojarasca con el contenido del estómago de las ranas y descubrimos que las ranas comen un pequeño subconjunto de los géneros de hormigas disponibles que se encuentran en la hojarasca circundante, ya que solo el 13% de todas las hormigas obtenidas de las muestras de hojarasca pertenecen a los 16 géneros de hormigas consumidos por las ranas. Nuestros resultados sugieren que el consumo de defensas adquiridas a través de la dieta depende de la selección preferencial de alimentos, además de la disponibilidad ambiental. Esto influye en nuestra comprensión de las defensas químicas en su conjunto, ya que el refuerzo conductual puede ser un factor de selección poco estudiado, pero importante, en la evolución de las defensas adquiridas.

Introduction

Many organisms use chemical defenses to protect themselves from predators or pathogens, a trait that has evolved multiple times across most major taxa (1). Alkaloids are small molecules made by plants or microbes that are often used for chemical defense, but can also be acquired trophically by other taxa (2-4). For example, some insects that specialize on a specific plant species can accumulate certain plant secondary metabolites for chemical communication or defense (2). However, many of these ecological relationships remain unexplored among arthropods and their vertebrate predators that sequester their chemical defenses. Whether general ecological principles underlying arthropod specialization on plants transfer to vertebrates that sequester their chemical defenses from arthropods remain understudied.

Chemical profiles of alkaloid-defended species tend to vary more across populations within a species, while also showing substantial within population variation. This pattern can be seen in both alkaloid-producing species and organisms that accumulate alkaloids from their diet. For example, the coniferous Norwegian spruce (*Picea abies*) produces alkaloids, but the composition and concentration differ by location (5). In *Brachymyrmex* ants, the amount and types of pumiliotoxins, a class of poison frog alkaloids, varies across colonies and seasons (6). Similarly, Ecuadorian leaf litter ant alkaloids also show between-species variation in alkaloid content and within-species colony variation with some alkaloids (7). Many studies in amphibians that acquire their chemical defenses from their diet have demonstrated geographic variability in chemical defense profiles. For example, Malagasy *Mantella laevigata* poison frogs (Mantellidae) and Panamanian *Oophaga pumilio* (Dendrobatidae) frogs have seasonal variation in skin alkaloids that may be linked to variation in environmental availability of alkaloid-containing prey items (8, 6). Taken together, these studies suggest that the varied availability and alkaloid load of prey items should be considered within the framework of diet-acquired defense evolution.

Consumer dietary preferences may play an important and understudied role in shaping alkaloid repertoire in organisms protected by diet-acquired chemical defenses. For example, when presented with seven dietary choices, chemically defended *Stylocheilus longicauda* sea hares showed a strong preference for *Microcoleus lyngbyaceus* cyanobacteria, their dietary source for anti-predator chemical defense (9). Still, organisms must also maintain other physiological needs, such as metabolism and reproduction, and therefore must make dietary decisions based on energy acquisition in addition to food availability and maintenance of chemical defenses. This is demonstrated in a study on *Grammia geneura* caterpillar dietary preferences, in which the caterpillars did not choose to eat plants which maximized their growth efficiency, but instead chose a mixed diet including toxic plants from which they could acquire defenses (10). Similarly, when lab-reared *Dendrobates tinctorius* poison frogs were presented with ants, mites, beetles and fly larvae, they consistently chose larvae, which have a significantly higher protein content than the other prey (11). These studies demonstrate the importance of considering food preference among organisms with diet-acquired defenses, which should be further investigated to more deeply understand how defense acquisition from diet evolved.

Poison frogs are native to Central and South America, and many carry small molecule alkaloids in their skin for defense against predation. Within the Dendrobatidae family, chemical defenses have evolved independently four times, which coincides with a dietary specialization on ants and mites, as these frogs do not synthesize alkaloids *de novo*, but acquire them from alkaloid-containing arthropods (12-13). Since poison frog alkaloid defenses are environmentally-dependent, their chemical profiles likely serve as a proxy for past trophic interactions. Dendrobatids generally show interpopulation variation in alkaloid profiles (14-15), reflecting the immense biological diversity of available prey within soil ecosystems. Indeed, our previous work demonstrates that variation in dendrobatid chemical defenses reflects chemical diversity of their local ant and mite communities (7, 15), implying that dendrobatid chemical

profiles are tightly linked to their diet of leaf litter arthropods. Yet, it is unclear if preference for alkaloid-containing prey plays a role in the ability of poison frogs to dietarily acquire their chemical defenses, as most studies focus on diet without assessing environmental availability. Thus, the framework for the evolution of diet-acquired defenses and dietary choice is incomplete since prey availability is unknown.

To understand if dietary preference covaries with diet-derived chemical defenses, we sampled two sympatric species, including the cryptic, chemically undefended *Hyloxalus infraguttatus (*Family: Dendrobatidae) and the aposematic, chemically defended *Oophaga sylvatica.* We hypothesized that their diets would differ despite access to the same prey, and predicted that *O. sylvatica* would consume prey more selectively. In addition, we collected several allopatric populations of *O. sylvatica* and sampled both locally available ants and frog stomach contents. We hypothesized that *O. sylvatica* frogs have different diets across localities, and predicted these differences would be reflected in population variation between skin alkaloids, ant consumption, and ant availability. Together, our between and within species comparisons of poison frog stomach contents and prey availability help disentangle the relationship between dendrobatid diet and alkaloid acquisition, which has broader implications for our general understanding of trophic interactions and the evolution of chemical defenses across taxa.

Methods

Frog sampling

Diablito frogs (*Oophaga sylvatica*) were collected in May 2019, during the day near the towns of Cristóbal Colón (N=11), Ceiba (N=10), La Maná (N=20), Puerto Quito (N=11), and Santo Domingo de los Tsáchilas (N=10) in Northwestern Ecuador (Figure 1A). As *O. sylvatica* suffers from illegal poaching, coordinates for collection can be obtained from the corresponding

author. Chimbo rocket frogs (*Hyloxalus infraguttatus*) were collected during the day from the La Maná locality (N=9). Frogs were anesthetized with 20% benzocaine gel applied to the ventral skin and euthanized by decapitation. For each individual, the dorsal skin was dissected and stored in methanol in glass vials. The stomach contents were stored in 100% ethanol in 1.5 ml plastic tubes. Remaining frog tissues were either preserved in 100% ethanol or RNAlater (Thermo Scientific, Waltham, MA, USA), or deposited in the amphibian collection of Centro Jambatu de Investigación y Conservación de Anfibios in Quito, Ecuador. Collections and exportation of specimens were done under permits (Collection permit: No. 0013-18 IC-FAU-DNB/MA; Export permit: No. 214-2019-EXP-CM-FAU-DNB/MA; CITES export permit No. 19EC000036/VS) issued by the Ministerio de Ambiente de Ecuador to LAC. The Administrative Panel on Laboratory Animal Care of Stanford University approved all frog-related procedures (Protocol #34153).

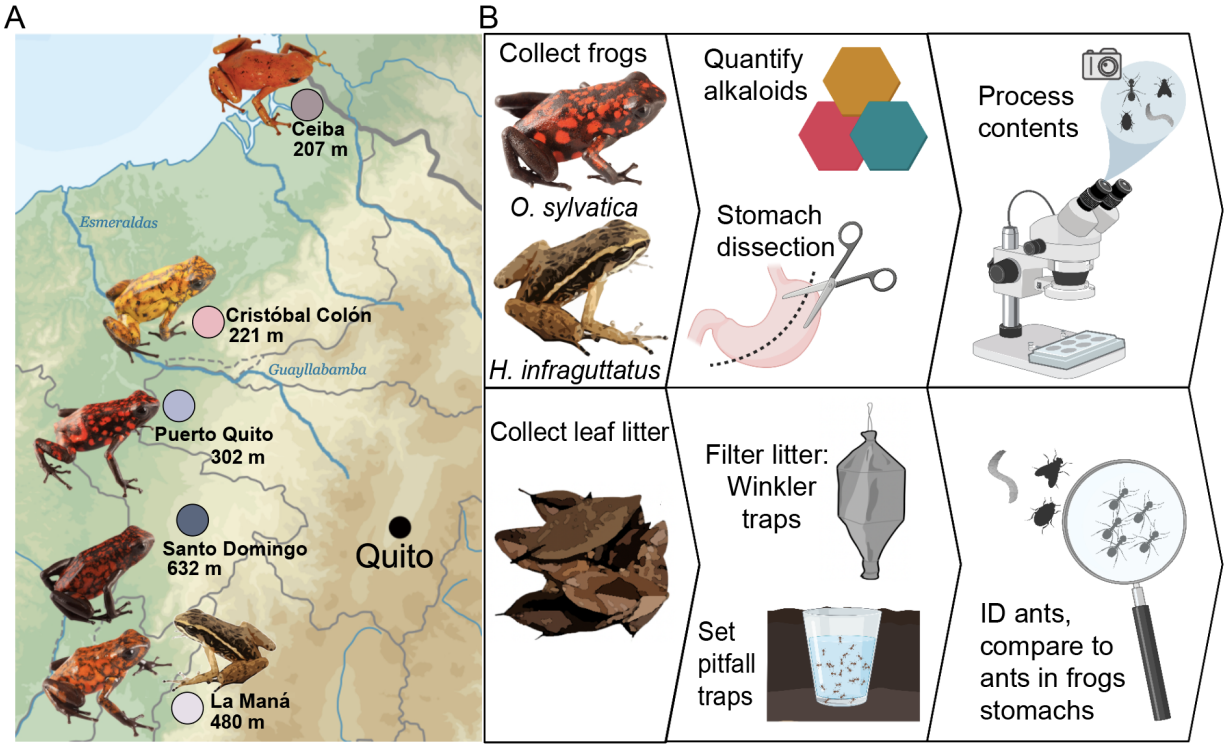

Figure 1. Map of frog populations and experimental workflow. (A) Collection sites are shown on a topographic map of western Ecuador. Note that both *Hyloxalus infraguttatus* and *Oophaga sylvatica* were collected from La Maná. **(B)** A flowchart depicts the main steps of data collection for frog stomach content and leaf litter samples, in order to compare ant genera abundances between these groups.

Alkaloid extraction and quantification

Skins were taken out of methanol with forceps and weighed. From the methanol in which the skin was stored, 1 ml was syringe filtered through a 0.45 um PTFE syringe filter (Thermo Scientific, 44504-NP) into the new glass vial (Wheaton, PTFE caps, 60940A-2) supplemented with 25 ug (-)-nicotine (Sigma Aldrich, N3876-100ML). All tubes were then capped, vortexed and stored for 24 hours at -80ºC to precipitate lipids and proteins. After precipitating for 24 hours, the supernatant was filtered through a 0.45 um PTFE syringe filter again into a new glass vial. A 100 uL aliquot was added to a gas chromatography / mass spectrometry (GC/MS) autosampler vial, and the remaining solution was stored in the original capped vial at -80ºC.

GC/MS analysis was performed on a Shimadzu GCMS-QP2020 instrument with a Shimadzu 30m x 0.25 mmID SH-Rxi-5Sil MS column closely following the protocol outlined in Saporito et al, 2010. In brief, separation of alkaloids was achieved with helium as the carrier gas (flow rate: 1 mL/min) using a temperature program from 100 to 280°C at a rate of 10°C/minute. This was followed by a 2 minute hold and additional ramp to 320°C at a rate of 10°C/minute for column protection reasons, and no alkaloids appeared during this part of the method. Compounds were analyzed with electron impact-mass spectrometry (EI-MS). The GC-MS data files were exported as CDF files and the Globar Natural Products Social Molecular Networking (GNPS) software was used to perform the deconvolution and library searching against the AMDIS (NIST) database to identify all compounds (16-17). For deconvolution (identification of peaks and abundance estimates) the default parameters were used. Through the deconvolution process, molecular features were reported as rows/observations, while m/z intensities were

reported as columns/variables. Automatic library search was obtained from reference libraries of natural products (NIST, Wiley, University of CORSICA, GNPS), and our resulting dataset was filtered to keep only our nicotine standard and alkaloids previously found in poison frogs from the Daly 2005 database, or compounds with the same base ring structure and R group positions as the classes defined in Daly 2005 (18). Once the feature table from the GNPS deconvolution was filtered to include only poison frog alkaloids and nicotine, the abundance values (ion counts) were normalized by dividing by the nicotine standard and skin weight, and we included the 10 top database hits in our library search. The resulting filtered and normalized feature table was used for all further analyses and visualizations. All normalization steps were carried out with R version 4.0.4.

Frog stomach content processing

Whole stomachs were stored in 100% ethanol at -20ºC until processing. Stomach contents were isolated into the central well of the six-well dish, which contained 2 ml of 1x phosphate-buffered saline (PBS). To avoid missing prey items that were stuck inside of the stomach membrane, empty stomachs were rinsed in a plate well of 1x PBS until no particles were found after rinsing. Using sterile forceps, stomach contents were sorted and photographed. Prey items in the photographs were identified to the lowest possible taxonomic rank and then ultimately grouped into broad diet categories: ants, mites, larvae, beetles, and “other”. The “other” category constitutes any non-larval arthropod not described by the other four groups. Note that most larvae belong to Dipteran or Coleopteran taxa, but are considered in our distinct “larvae” category given their differences in appearance and physiology from adults. The vast majority of identifiable prey remained whole, with the exception of ants, whose heads frequently detached from their bodies upon ingestion. To prevent overcounting, only whole ant specimens, partial ant specimens with heads, or individual ant heads were counted. Ant

specimens were identified to genus using visual inspection against a previously described reference collection of Ecuadorian ants (19-21).

Leaf litter collection and ant identification

Sixty pitfall trap samples and one hundred leaf litter samples extracted using the Winkler method were collected in secondary forest sites in May–June 2019 within one square meter of where a frog had been previously collected that day. Twenty Winkler samples were collected from each locality and twenty pitfall trap samples were collected from all localities except for Puerto Quito and Cristóbal Colón due to time and resource limitations. Pitfall traps were set up by placing a small, transparent plastic drinking cup filled half-way with 70% ethanol in a cup-depth sized hole, hidden under a leaf. Traps were left for 24 hours. Arthropods were extracted from leaf litter using the Winkler method, where sacs were loaded and hung for 24 hours, during which the arthropods were collected into 70% ethanol. Only ants were identified, as described above. Collection of ant specimens were done under permits issued by the Ministerio de Ambiente de Ecuador to Museo de Historia Natural Gustavo Orcés at Escuela Politécnica Nacional (MAE-DNB-CM-2017-0068 to DAD).

Data analysis and visualization

Statistical analyses were run in R version 4.1.1 using the packages ‘glmmTMB’ (23) and ‘vegan’ version 2.5-7 (24). To test for differences in the general structure of frog diet categories, we performed a permutational multivariate analysis of variance (PERMANOVA) on Bray-Curtis dissimilarities, which simultaneously considers the number of ants, mites, beetles, larvae and “other” prey items consumed across all individuals and compares these differences between groups. To assess differences in the number of consumed ants between populations, we used generalized linear mixed models (GLMM) based on a Poisson distribution. We compared the

number of ants found in stomach contents across all *O. sylvatica* frogs for a population-based diet comparison and between sympatric *H. infraguttatus* (undefended) and *O. sylvatica* frogs for a species-based diet comparison. To account for individual variation in the subjects, frog ID was used as a random effect. Total prey consumed was used as an offset to account for individual differences in the total number of consumed prey recovered for each frog. As population was not a reliable predictor of the consumption of the non-ant prey groups, we were unable to appropriately fit a GLMM for these comparisons. Therefore, the consumption of non-ant prey categories across populations was assessed using non-parametric Kruskal-Wallis tests. General diet differences based on the number of prey belonging to broad categories (ants, mites, beetles, larvae, other) were tested. *Oophaga sylvatica* consumed 16 different ant genera across localities, and the consumption of these genera were compared across populations to one another, and to local leaf litter ant availability, using PERMANOVA as described above.

As necessitated by our multivariate approach, differences between frog skin alkaloid profiles were also assessed using PERMANOVA as described above, by considering the relative abundance of 79 known frog alkaloids across localities. Each of the 79 alkaloids fell into one of ten structural classes: allopumiliotoxins, decahydroquinolines, histrionicotoxins, indolizidines, lehmizidines, piperidines, pumiliotoxins, pyrrolidines, pyrrolizidines, or quinolizidines. We additionally grouped and summed alkaloids based on these ten structural groups to make broad comparisons regarding locality, alkaloids and prey consumption using PERMANOVA and Spearman Rank correlations. Finally, we performed Spearman Rank correlations to compare both total summed alkaloid content and summed alkaloids within each of the 10 alkaloid classes to prey category consumption.

P-values were adjusted using the Benjamini-Hochberg procedure to correct for multiple hypothesis testing across all analyses. Data were visualized using images from BioRender.com, and boxplots and barplots were created using the package ‘ggplot2’ in R version 4.1.1 (25).

Results

Alkaloids differ between diablito frog populations

We first examined the broad patterns of skin alkaloid composition across five *O. sylvatica* populations. The summed abundance of all 79 detected alkaloids varied between populations (PERMANOVA; F: 10.178, p = 0.0001) (Figure 2A). A post hoc pairwise comparison indicates significant differences between all possible population pairs for individual alkaloids (Table 1). Individual alkaloids belong to different classes based on their structure and the most abundant ten structural classes in this *O. sylvatica* dataset include allopumiliotoxins, decahydroquinolines, histrionicotoxins, indolizidines, lehmizidines, piperidines, pumiliotoxins, pyrrolidines, and pyrrolizidines. After we compared alkaloid profile composition across populations using these ten alkaloid classes, we found significant population differences (PERMANOVA; F: 9.265, p = 0.0001) (Figure 2), and our post hoc pairwise comparisons indicate significant differences between all possible population pairs (Table 2, Figure 2C). Histrionicotoxins and indolizidines are the most abundant alkaloid classes in all populations. Finally, we confirmed *Hyloxalus infraguttatus* does not have detectable alkaloids in their skin (Figure 2A), representing an undefended dendrobatid species for which we could examine prey preference.

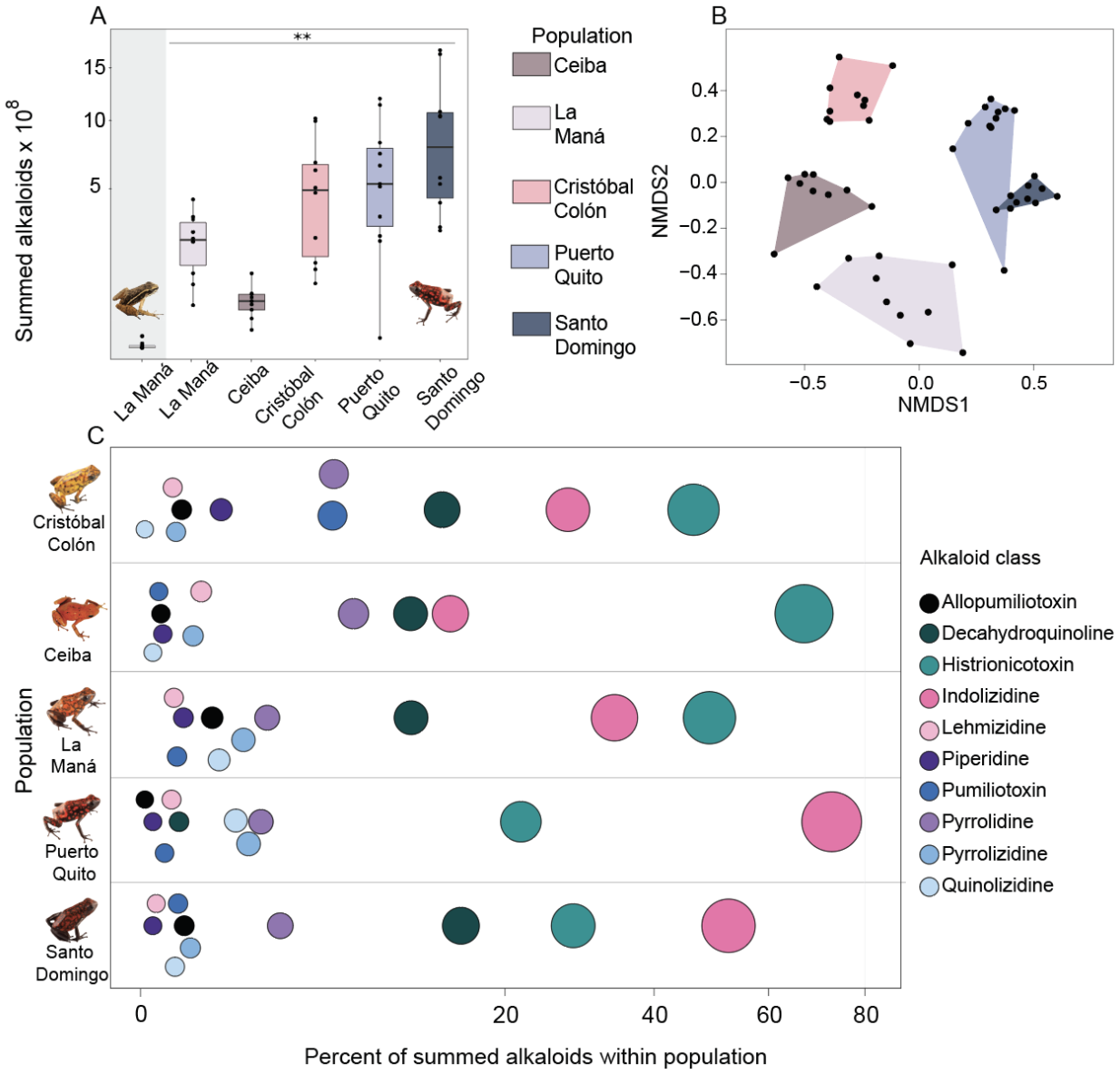

Figure 2. Alkaloid profiles differ between *Oophaga sylvatica* populations. (A) The summed relative abundances of 79 alkaloids are visualized as box plots to show the variation across individuals, where each dot represents a frog. The y axis is square-root transformed for visual clarity. Populations are represented with different colors. *Hyloxalus infraguttatus*, collected near the town of La Maná, is shown on the far left. **(B)** Non-metric multidimensional scaling (NMDS) biplots based on Bray-Curtis dissimilarities show alkaloid profile differences between populations based on the relative abundances of 79 unique alkaloids (Stress = 0.156). Each dot represents a single frog's alkaloid profile. **(C)** Percent of all summed alkaloids grouped by alkaloid class and population are plotted as bubble plots, where each bubble represents an alkaloid class, and bubble size corresponds to the percentage of total summed alkaloids composed by the respective alkaloid class. The x axis is square-root transformed for visual clarity.

Table 1. Post-hoc pairwise comparison of 79 individual alkaloid abundances between populations

Pairwise comparison	F	R2	BH-adjusted p-value
Ceiba vs. Santo Domingo	17.447	0.506	0.001
Ceiba vs. Puerto Quito	14.277	0.442	0.001
Cristóbal Colón vs. Santo Domingo	12.564	0.411	0.001
Cristóbal Colón vs. Puerto Quito	10.071	0.346	0.001
Ceiba vs. Cristóbal Colón	9.249	0.352	0.001
La Maná vs. Cristóbal Colón	8.283	0.315	0.001
Santo Domingo vs. Puerto Quito	8.013	0.297	0.001
La Maná vs. Santo Domingo	7.972	0.307	0.001
La Maná vs. Puerto Quito	7.933	0.295	0.001
La Maná vs. Ceiba	7.806	0.315	0.001

Table 2. Post-hoc pairwise comparison of the abundance of 10 alkaloid structural classes between populations

Pairwise comparison	F	R2	BH-adjusted p-value
La Maná vs. Ceiba	10.545	0.383	0.001
La Maná vs. Cristóbal Colón	2.514	0.123	0.053
La Maná vs. Santo Domingo	9.929	0.356	0.001
La Maná vs. Puerto Quito	7.461	0.282	0.001
Ceiba vs. Cristóbal Colón	13.544	0.443	0.001
Ceiba vs. Santo Domingo	24.736	0.593	0.001
Ceiba vs. Puerto Quito	16.969	0.485	0.001
Cristóbal Colón vs. Santo Domingo	5.05	0.219	0.004
Cristóbal Colón vs. Puerto Quito	4.943	0.206	0.001
Santo Domingo vs. Puerto Quito	2.033	0.097	0.07

Diet differences between species and populations

After confirming that *O. sylvatica* are chemically defended with alkaloids and *H. infraguttatus* is not, we examined species differences in diet. Overall consumption of prey categories (ants, mites, beetles, larvae, other; Figure 3A) differed between *O. sylvatica* (N=10) and *H. infraguttatus* (N=9) from the La Maná region (PERMANOVA; F: 31.234, p = 0.0001; Figure 3B). In this location, *O. sylvatica* ate more ants and more mites than *H. infraguttatus* (ants, GLMM: Z: 2.674, p = 0.0075; mites, Wilcoxon, W: 15, p=0.014). These frog species did not differ in their consumption of the remaining prey categories (larvae: Wilcoxon, W: 42, p=0.813; beetles: Wilcoxon, W: 38, p=0.588; other prey items: Wilcoxon, W: 64, p = 0.121).

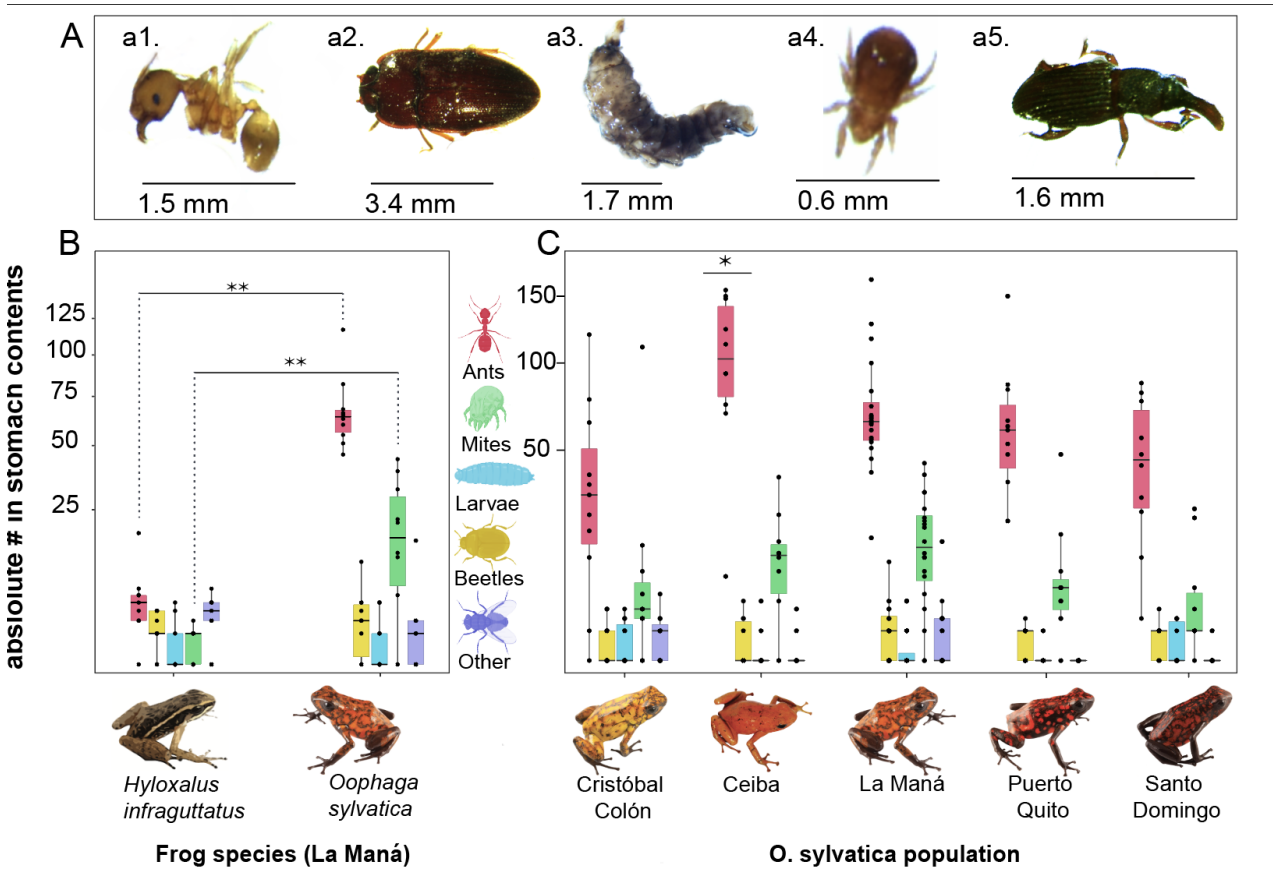

Figure 3. Diet differs between and within frog species. (A) Photographs of specimens found in *Oophaga sylvatica* stomach contents; a1 - *Wasmannia auropunctata* ant, a2 - Buprestidae beetle, a3 - beetle larvae, a4 - Sarcoptiformes mite, a5 - Weevil beetle. **(B)** Absolute number of prey found in stomach contents between undefended *Hyloxalus infraguttatus* and defended *O.*

sylvatica frogs are visualized as box plots to show the variation across individuals, where each dot represents a frog. The y axis is square-root transformed for visualization clarity. Ants are red, mites are green, larvae are blue, beetles are yellow and "other" is purple in both B and C. **(C)** Absolute number of prey found in stomach contents across five *O. sylvatica* frog populations are visualized as box plots to show the variation across individuals, where each dot represents a frog. The y axes are square-root transformed for visual clarity.

Given *O. sylvatica* populations vary in diet-derived alkaloid profiles, we next asked if there were population differences in diet. We found the consumption of prey categories differed across the five populations (PERMANOVA; F: 3.104, p = 0.001; Figure 3C). A post hoc pairwise comparison showed Cristóbal Colón is significantly different than both La Maná (F: 6.001, p = 0.01) and Ceiba (F: 4.387, p = 0.036), while Ceiba is different than Santo Domingo (F: 3.727, p = 0.045). Frogs from the Ceiba population consumed more ants than the other populations (GLMM: Z: 2.188, p = 0.029). Generally, the number of "other" prey items consumed differed among populations (Kruskal-Wallis, X^2; 11.376, p = 0.023; Figure 3C), but a post hoc pairwise comparison showed no differences between any two specific populations. Further, when the consumption of "other" categories were compared by suborder abundance, no differences were found between any two populations (PERMANOVA; F: 1.199, p = 0.248). Mite, larvae and beetle consumption did not differ among populations (mites: Kruskal-Wallis, X^2 : 8.726, p = 0.068; larvae: Kruskal-Wallis, X^2: 4.8378, p=0.304; beetles: Kruskal-Wallis, X^2:1.823, p=0.768; Figure 3C).

Alkaloids correlate with dietary components and altitude

As *O. sylvatica* populations differed in both chemical defenses and diet, we tested for relationships between alkaloids and prey categories. The total number of consumed ants and summed alkaloid values were negatively correlated across all *Oophaga sylvatica* individuals (rho = -0.386, S = 28864, p = 0.006). Similarly, the total number of consumed mites and summed alkaloids were also negatively correlated (rho = -0.310, S = 27271, p = 0.03). Since

altitude is known to strongly impact the abundance and distribution of ants (26), we next asked if there is a relationship between skin alkaloid load and locality altitude. Indeed, we found a positive relationship between the altitudes of frog localities and their summed alkaloids (rho = 0.481, S = 10813, p-value = 0.0004).

We next examined the relationship between ant and mite consumption and the most abundant ten alkaloid classes. The strongest relationship between a diet variable and alkaloid class is between ants and pumiliotoxin (rho = -0.544, S = 32146, $p < 0.001$), suggesting frogs with higher levels of pumiliotoxins consumed fewer ants. A similar negative relationship was found with ants and lehmizidines (rho = -0.428, S = 29733, p = 0.002). Negative correlations were also detected between the following prey group/alkaloid family pairs: ants and histrionicotoxin, (rho = -0.320, S = 27479, p-value = 0.02), ants and indolizidine (rho = -0.305 S = 27188, p-value = 0.03), ants and pyrrolidine (rho = -0.324, S = 27563, p-value = 0.02), mites and decahydroquinoline (rho = -0.304, S = 27157, p-value = 0.032), mites and lehmizidine (rho = -0.331, S = 27721, p-value = 0.02), mites and pyrrolidine (rho = -0.314, S = 27370, p = 0.026).

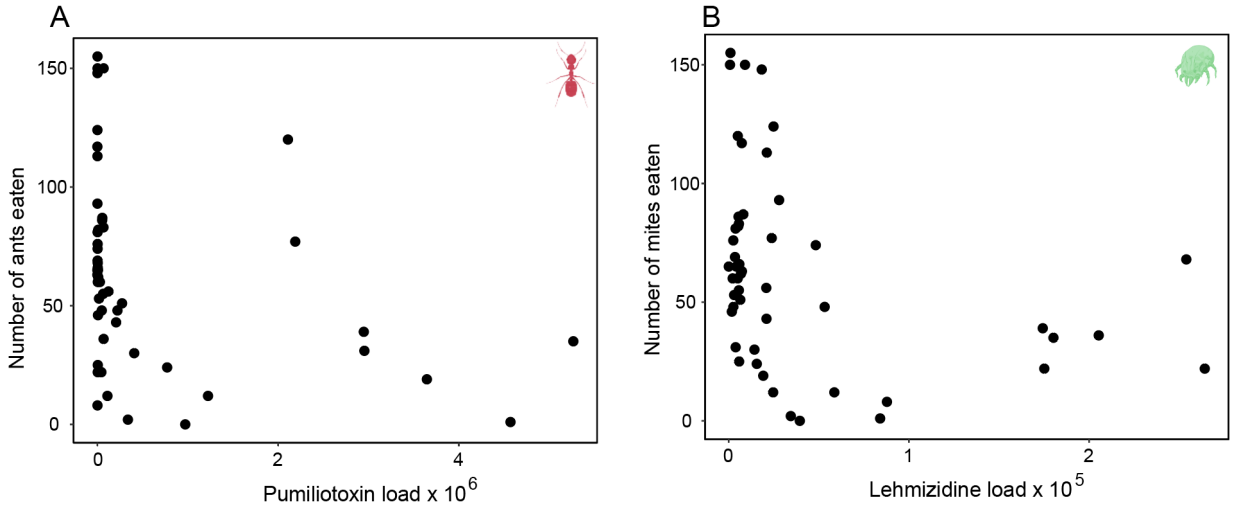

Figure 4. Ant and mite consumption negatively correlate with alkaloid abundance (A) Scatterplot of Pumiliotoxin alkaloid class abundance in relation to ant consumption, where each dot represents an *Oophaga sylvatica* frog. **(B)** Scatterplot of Lehmizidine alkaloid class

abundance in relation to mite consumption, where each dot represents an *Oophaga sylvatica* frog.

Frogs eat a subset of ant genera from leaf litter communities

As the diet of *O. sylvatica* is mainly composed of ants (Figure 3C), we next asked if the frogs eat specific ant genera preferentially or if their ant diet reflects the genera of the surrounding leaf litter communities. We recovered a total of 46 genera of ants from leaf litter communities, and the genera recovered differed across locations (PERMANOVA; F: 5.27, p = 0.0001; Figure 5A, Table 5). A post hoc pairwise comparison showed significant differences among all locality pairs (Table 4). In contrast, only 16 genera of ants were consumed by frogs across those same localities, and the genera recovered from frog stomach contents differs across populations (PERMANOVA; F: 3.209, p = 0.0002; Figure 5B). Specifically, a post hoc pairwise comparison revealed the following the population pairs differ in their ant consumption: Cristóbal Colón and Ceiba (F: 4.654, p = 0.007), Cristóbal Colón and Puerto Quito (F: 5.200, p = 0.007), Ceiba and Santo Domingo (F: 5.337, p = 0.007), Cristóbal Colón and La Maná (F: 3.602, p = 0.008), Puerto Quito and Santo Domingo (F: 4.288, p = 0.044).

We next compared the abundance of these 16 ant genera in both the leaf litter and frog stomach contents and found significant variation across populations (PERMANOVA; F: 51.058, p = 0.0001; Figure 5C, Figure 6). Stomach content and leaf litter abundances of the 16 ant genera within each of the five populations differ significantly in all locations [Ceiba (F: 11.26, p = 0.0001), Cristóbal Colón (F: 12.705, p = 0.0001), La Maná (F: 14.242, p = 0.0001), Santo Domingo (F: 14.963, p = 0.0001), Puerto Quito (F: 21.936, p = 0.0001) (Figure 5C], suggesting that frogs show preference for specific ant genera. To further explore this idea, we compared the relative abundance of three common ant genera in leaf litter and stomach contents: *Pheidole*, *Solenopsis*, and *Wasmannia* (Figure 5D). Overall, the consumption of the following ant genera differ compared to leaf litter prevalence of ants: *Pheidole* (W = 4296.5, p = 0.0004), *Wasmannia*

(W = 4066, p = 0.0014850) and *Solenopsis* (W = 5457, p = 2.9013e-14). *Solenopsis* was the only genus that differed in prevalence between frog stomachs and local leaf litter samples within all five populations (Table 3). *Wasmannia* and *Pheidole* ant prevalence differed between frog stomachs and leaf litter samples in three of the five populations (Table 3).

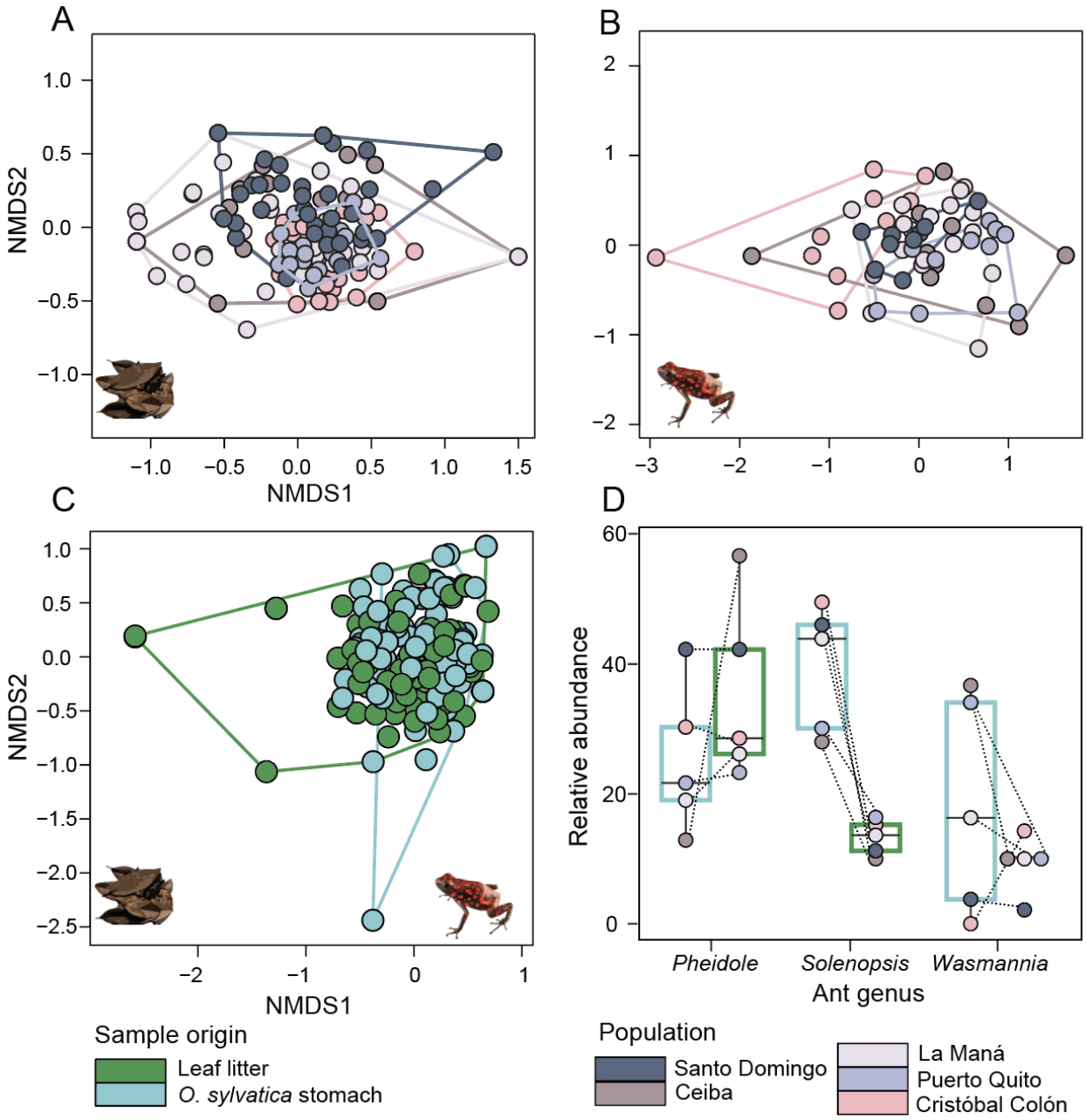

Figure 5: Relative abundance of ant genera differs across localities. (A) Non-metric multidimensional scaling (NMDS) biplots based on Bray-Curtis dissimilarities show differences in relative abundances of all 46 ant genera found within leaf litter samples (Stress = 0.207).

Each dot represents a leaf litter sample and space between them represents overall differences in composition of the 16 ant genera among all samples. **(B)** Non-metric multidimensional scaling (NMDS) biplots based on Bray-Curtis dissimilarities show differences in relative abundances of the 16 ant genera found within frog stomach samples (Stress = 0.179). Each dot represents a single frog stomach, and space between them represents overall difference in consumption of the 16 ant genera. **(C)** Non-metric multidimensional scaling (NMDS) biplots based on Bray-Curtis dissimilarities show differences in relative abundances of the 16 ant genera found within both leaf litter and frog stomach samples (Stress = 0.220). Each dot represents either a leaf litter sample (green) or a frog stomach sample (blue), and space between them represents overall differences in the composition of the 16 ant genera among all samples. **(D)** Across all leaf litter and stomach samples from *Oophaga sylvatica* localities, the relative abundances of the three most commonly eaten ant genera within our dataset are visualized as box plots. Each dot's color corresponds to locality, and signifies the abundance of an ant genus, relative to the other 15 shared genera found in frogs. Relative abundance values were calculated both within their respective groups (leaf litter or frogs) and populations. Dotted lines connect populations between groups in order to draw direct comparisons in relative abundance.

Table 3. Mann-Whitney U comparison of ant genera prevalence between frog stomachs and leaf litter samples within populations. P values were adjusted using the Benjamini-Hochberg method.

Population	**Ant genus**	**p-value**
La Maná	*Solenopsis*	8.745E-05
Santo Domingo	*Solenopsis*	0.0002
Ceiba	*Wasmannia*	0.0004
Cristóbal Colón	*Wasmannia*	0.0006
Cristóbal Colón	*Pheidole*	0.0036
Puerto Quito	*Solenopsis*	0.0051
Cristóbal Colón	*Solenopsis*	0.0064
Santo Domingo	*Pheidole*	0.0072
Puerto Quito	*Wasmannia*	0.0184
Ceiba	*Solenopsis*	0.0191
La Maná	*Pheidole*	0.0825
La Maná	*Wasmannia*	0.1440
Ceiba	*Pheidole*	0.1440
Santo Domingo	*Wasmannia*	0.2780
Puerto Quito	*Pheidole*	0.7600

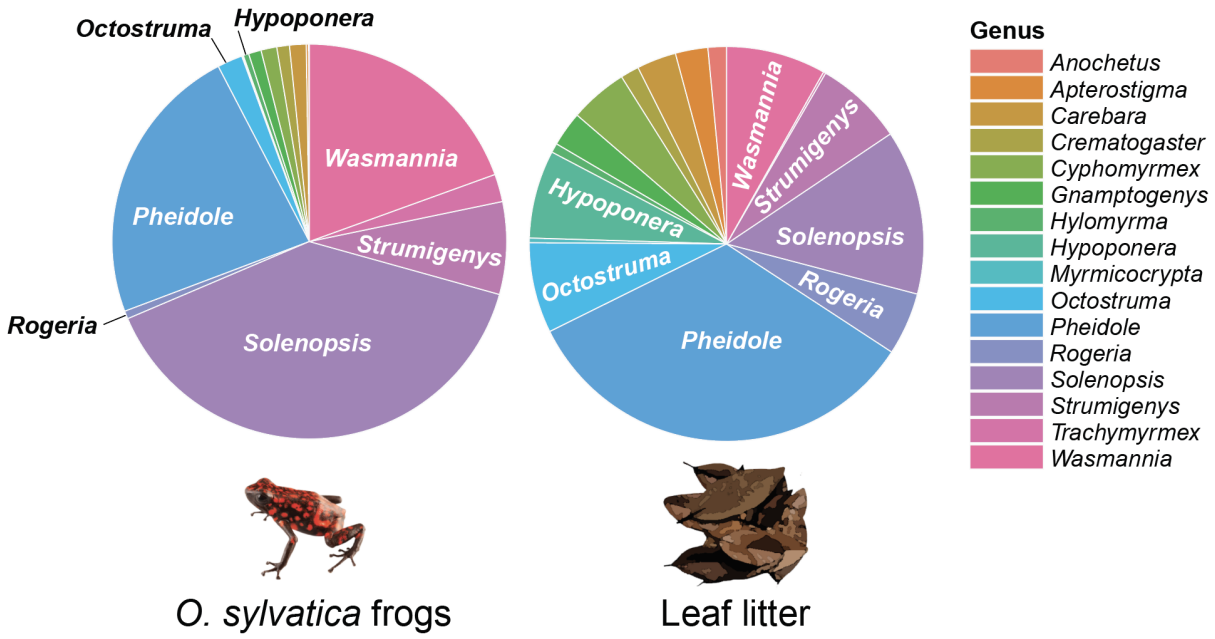

Figure 6. Relative abundance of dietary ant genera differs between frog stomachs and leaf litter samples. Pie charts show the relative abundances of all 16 genera consumed by *Oophaga sylvatica* frogs, across all leaf litter and all *O. sylvatica* samples. Each circle represents all ant specimens belonging to the 16 genera, found within a group, converted to relative abundance for visualization.

Table 4. Post-hoc pairwise comparison of the consumption of all 16 ant genera consumed by frogs between populations. P values were adjusted using the Benjamini-Hochberg method.

Pairwise comparison	**F**	**R2**	**p-value**
Cristóbal Colón vs. Ceiba	4.654	0.205	0.007
Cristóbal Colón vs. La Maná	3.602	0.126	0.008
Cristóbal Colón vs. Puerto Quito	5.200	0.215	0.007
Cristóbal Colón vs. Santo Domingo	1.861	0.099	0.114
Ceiba vs. La Maná	2.123	0.078	0.081
Ceiba vs. Puerto Quito	1.225	0.061	0.275
Ceiba vs. Santo Domingo	5.337	0.239	0.007
La Maná vs. Puerto Quito	2.631	0.092	0.081
La Maná vs. Santo Domingo	1.998	0.077	0.111
Puerto Quito vs. Santo Domingo	4.288	0.192	0.044

Table 5. Post-hoc pairwise comparison of the leaf-litter-only prevalence of all 46 ant genera between populations

Pairwise comparison	**F**	**R2**	**BH-adjusted p-value**
Cristóbal Colón vs. Ceiba	6.814	0.167	0.001
Cristóbal Colón vs. La Maná	6.730	0.109	0.001
Cristóbal Colón vs. Puerto Quito	2.873	0.074	0.008
Cristóbal Colón vs. Santo Domingo	5.892	0.098	0.001
Ceiba vs. La Maná	2.736	0.051	0.008
Ceiba vs. Puerto Quito	7.581	0.192	0.001
Ceiba vs. Santo Domingo	3.833	0.071	0.001
La Maná vs. Puerto Quito	6.148	0.104	0.001
La Maná vs. Santo Domingo	5.526	0.072	0.001
Puerto Quito vs. Santo Domingo	5.094	0.089	0.001

Discussion

We tested the hypothesis that poison frog ant consumption is influenced by dietary preferences in addition to environmental availability. We found that sympatric defended *O. sylvatica* and non-defended *H. infraguttatus* frogs differ in diet, and *O. sylvatica* diet and alkaloid profiles vary among localities. Surprisingly, we also found that frog skin alkaloid load is negatively correlated with the number of ants and mites consumed, but not in beetles, larvae or other prey groups. We discuss these results in the context of diet-acquired chemical defenses across vertebrates, and the importance of replicating these studies under both natural and laboratory conditions in the future.

Alkaloid defenses vary within and between localities

We found that *O. sylvatica* alkaloid profiles varied widely between populations, which matches findings on dendrobatid poison frogs and organisms with diet-derived alkaloid defenses (14-15). For example, studies in *Ranitomeya imitator* and *Oophaga pumilio* show significant alkaloid profile variation across localities, and less variation within them (14-15). This general pattern has also been observed in alkaloid-acquiring toad species (genus *Melanophyrniscus*) where frogs living in closeby sites show many similarities compared to conspecifics living in distant populations (27). The demonstrated variation of alkaloid profiles across populations signifies the importance of examining the environmental availability of alkaloids in the future. Such studies would strengthen our understanding of the interplay between dietary preferences and environmental availability of prey in organisms with diet-acquired defenses.

Dietary selectivity of animals with diet-acquired chemical defenses

Organisms with diet-derived defenses must maintain their defense reservoirs through balancing food choice and availability. In our study, sympatric groups of defended *O. sylvatica* ate more ants and mites than undefended *H. infraguttatus*, demonstrating dietary differences between closely related species with similar prey access. Additionally, although *O. sylvatica* differed overall in diet and alkaloid profiles across populations, the majority of their stomach contents were constituted by ants. This finding is consistent with our previous studies of *O. sylvatica* diet (7, 15). Our results are also consistent with a broader dietary study on undefended and defended Dendrobatidae species, in which defended species, such as *Epipedobates anthonyi, Dendrobates auratus, O. pumilio,* showed a greater degree of dietary specialization on ants than undefended species, such as *Allobates femoralis, Allobates zaparo, Hyloxalus infraguttatus* (13). However, there was an exception to this trend, as they found that a cryptically

colored, undefended dendrobatid species, *Hyloxalus sauli* had eaten mainly ants (13). These broad trends are also found in other amphibians with diet-derived defenses, such as *Melanophryniscus* toads and Rhinella toads (27, 28). Our comparison of defended and undefended frog diet from the same location suggests that diet can be specialized in chemically defended frogs even with similar prey availability. Food availability should be systematically assessed in future studies focusing on organisms whose defenses are environmentally-dependent.

When the evolution of diet-derived chemical defenses co-occurs with dietary specialization, selection for behaviors favoring prey preference are likely shaped by prior experience and chemical defense load. For example, a study on *Oreina elongata* leaf beetles sourced from non-alkaloidal plants preferred to feed on plants containing a sequestrable alkaloid once they were given the option, despite having never encountered them, while beetles reared on alkaloid-producing plants showed no dietary preferences (29). Despite the preference in the group with novel exposure to the plant, there appears to be a fitness tradeoff, as *O. elongata* beetle larvae grew most efficiently when reared on non-alkaloidal plants (29). Within our present study, our results also suggest a negative feedback mechanism between accumulated alkaloid load and consumption of likely-defended prey items (ants, mites), given we found negative correlations between total summed skin alkaloids and the number of both ants and mites consumed. Indeed, in lab-reared poison frogs (*Dendrobates tinctorius*), frogs which had ingested more of the alkaloid decahydroquinoline ate and attempted to eat more of the highest-protein prey items (fly larvae) than frogs without prior alkaloid ingestion (11). Group disparity related to previous food exposure within these studies demonstrates the entanglement of food preference and environmental availability, which should be simultaneously considered in relation to the evolution of diet-acquired defenses.

In addition to broad categorical differences in prey selectivity, it is also possible that chemically defended species have preferences within a prey category. We focused on genus

selectivity within ants because they are relatively easy to visually identify compared to mites and are known to carry alkaloid compounds also found in poison frogs (6). We compared the relative abundances of all 16 ant genera found in both *O. sylvatica* and leaf litter samples and found that these only account for 13% of all recovered leaf litter ants. Although our results strongly suggest *O. sylvatica* prefer certain ants, we note that certain ant genera may have been absent from frog diet due to other reasons such as ant size or texture that preclude ingestion by the frogs. Another possibility that may exaggerate the breadth of ant availability for frogs is that some genera may be inactive during frog foraging hours, but fall into sample traps at other times. Furthermore, previous research shows that pitfall and winkler traps sample slightly different sets of ants, as winklers sample more subterranean ants compared to pitfalls (18). Therefore, we suggest that future studies continue arthropod sampling using mixed methods, as neither one alone likely encompasses prey availability. Overall, *O. sylvatica* showed the greatest preference for *Solenopsis* ants, as they made up a much larger portion in frog stomach contents than they did in leaf litter. Our results are similar to recent studies of *Oophaga sylvatica* (7), and *Chiasmocleis mehelyi* (Microhylidae) frogs, which are hypothesized to acquire defensive alkaloids from their diet and preferentially select for alkaloid-containing ants, especially *Solenopsis*, and mites, over other arthropod categories (30-31). Together, these studies show that similar mechanisms of dietary selectivity may have evolved in distantly related species. We propose that future studies include similar sampling techniques for other defense-acquiring organisms over a long, continuous time period in order to monitor variation in food availability, and how these changes are reflected in organismal diet and defenses.

Alkaloids and trophic interactions

Through connecting our leaf litter, dietary and alkaloid datasets from wild *O. sylvatica,* we suggest that alkaloids are a reliable proxy for understanding trophic interactions generally, as certain alkaloid classes have known origins in specific taxa. Although ants are considered the

main source of reported poison frog alkaloids, only a few have been directly linked to ants. However, given that the alkaloid profiles of several commonly consumed ant genera have been described, including Solenopsis (32), we are able to make inferences about longer-term dietary patterns based on their skin alkaloids, which they may retain for months at a time (33). For example, skin alkaloids of *O. sylvatica* are primarily composed of histrionicotoxins, indolizidines, decahydroquinolines, and pyrrolidines. These alkaloids have all been detected in multiple species of *Solenopsis* ants, which is the genus of ants eaten most selectively by our frogs compared to their leaf litter abundance (Figure 5D). Some alkaloids are only associated with certain *Solenopsis* species, yet species of different genera may share similar compounds (32). Given these differences, we suggest that future research on dietary alkaloid origins includes taxonomic identification of ants to the species level. High levels of alkaloids belonging to the pumiliotoxin class were also found in *O. sylvatica* (Figure 5D), which have multiple dietary sources including Scheloribatid mites, *Brachymyrmex* and *Patrechina* ants (6). Given the varied, but overall high number of mites eaten by *O. sylvatica* across localities (Figure 3C), our data suggest that mites are also an important defensive alkaloid source for *Oophaga sylvatica*, as previously proposed in *Oophaga pumilio* (6). Mite taxonomy and chemistry is drastically understudied compared to ants and future studies should also make efforts to include mites in their analyses.

The interplay between defense acquisition and physiological demands for *O. sylvatica* may be clarified through examination of the relationships between skin alkaloids and consumed prey. We found negative correlations between total *O. sylvatica* summed skin alkaloids and the number of ants or mites consumed. Further, we found negative correlations between ant consumption and the summed values of pumiliotoxins, lehmizidines, histrionicotoxins, indolizidines and pyrrolidines. Each of these alkaloid families except histrionicotoxins have been detected in *Solenopsis* ants (34-39), which were highly prevalent in *O. sylvatica* stomach contents compared to their relative abundance in leaf litter. However, it is important to note that

alkaloid profiles of most ant species have not been quantified, and that some alkaloids, such as pumiliotoxins have putative origins in multiple ant species as well as mites (40). Similarly, indolizidines and pyrrolidines have been detected in multiple Ecuadorian *Pheidole* species, as well as in mites (7, 40). Negative correlations in all significant prey-alkaloid relationships suggests the ingestion of some alkaloids is controlled by a physiological negative feedback loop, where once a threshold of alkaloid sequestration is reached, the frog no longer seeks alkaloid-laden prey items. However, this is speculatory, and while our correlations have ecological bases, further research incorporating alkaloids and prey preference assays is needed to test this hypothesis. Although our results support the idea that poison frogs rely on ants and mites for their defenses, it is still possible that they rely on other prey for defenses. For example, some Staphylinidae beetles, which were commonly found in the stomachs of *O. sylvatica* in this study, have evolved chemical mimicry to deter ants from attacking (41), and these beetles also made up over 25% of the stomach contents on a prior study of *Dendrobates truncatus* poison frogs (42). As alkaloid profiles of arthropods are understudied, we strongly encourage future studies to focus on extensive chemical quantification of these prey items in order to understand patterns of trophic interactions.

Conclusions

Overall, we found that *O. sylvatica* diet and alkaloids differ between localities, but across these localities, frogs appear selective towards *Solenopsis* ants, given that they made up a higher proportion of frog stomach contents than leaf litter. We also found *O. sylvatica* ate more ants and mites than sympatric, undefended *H. infraguttatus.* Finally, our data show an overall negative relationship between total consumed ants/mites and summed skin alkaloids. To gain a deeper understanding of poison frog alkaloid origin, we suggest similar studies be conducted with the addition of alkaloid quantification of leaf litter ants and mites. Our findings aid in elucidating the complex relationship between poison frog diet and chemical defense, and

highlight our need to assess environmentally available alkaloids at a finer resolution. These studies would have broader implications for understanding the relationship between prey communities and their reliant predators, as well as for our general understanding of the evolution of diet-acquired chemical defenses in other taxa.

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Data availability

Diet and alkaloid data is included in supplementary materials. Gas chromatography / mass spectrometry files and arthropod photos are available on DataDryad (submission pending acceptance).

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