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Ant and Mite Diversity Drives Toxin Variation in the Little Devil Poison Frog

Jenna R. McGugan[1] · Gary D. Byrd[2] · Alexandre B. Roland[1] · Stephanie N. Caty[1] · Nisha Kabir[3] · Elicio E. Tapia[4] · Sunia A. Trauger[2] · Luis A. Coloma[4,5] · Lauren A. O'Connell[1]

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Abstract Poison frogs sequester chemical defenses from arthropod prey, although the details of how arthropod diversity contributes to variation in poison frog toxins remains unclear. We characterized skin alkaloid profiles in the Little Devil poison frog, *Oophaga sylvatica* (Dendrobatidae), across three populations in northwestern Ecuador. Using gas chromatography/mass spectrometry, we identified histrionicotoxins, 3,5- and 5,8-disubstituted indolizidines, decahydroquinolines, and lehmizidines as the primary alkaloid toxins in these *O. sylvatica* populations. Frog skin alkaloid composition varied along a geographical gradient following population distribution in a principal component analysis. We also characterized diversity in arthropods isolated from frog stomach contents and confirmed that *O. sylvatica* specialize on ants and mites. To test the hypothesis that poison frog toxin variability reflects species and chemical diversity in arthropod prey, we (1) used sequencing of cytochrome oxidase 1 to identify individual prey specimens, and (2) used liquid chromatography/mass spectrometry to chemically profile consumed ants and mites. We identified 45 ants and 9 mites in frog stomachs, including several undescribed species. We also showed that chemical profiles of consumed ants and mites cluster by frog population, suggesting different frog populations have access to chemically distinct prey. Finally, by comparing chemical profiles of frog skin and isolated prey items, we traced the arthropod source of four poison frog alkaloids, including 3,5- and 5,8-disubstituted indolizidines and a lehmizidine alkaloid. Together, the data show that toxin variability in *O. sylvatica* reflects chemical diversity in arthropod prey.

Keywords Poison frog · Alkaloid · Toxin · Ant · Mite · Mass spectrometry · Dendrobatidae

Jenna R. McGugan and Gary D. Byrd contributed equally to this work

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✉ Lauren A. O'Connell
aloconnell@fas.harvard.edu

[1] Center for Systems Biology, Harvard University, Cambridge, MA 02138, USA

[2] Small Molecule Mass Spectrometry Facility, Harvard University, Cambridge, MA 02138, USA

[3] Cambridge Rindge and Latin High School, Cambridge, MA 02138, USA

[4] Centro Jambatu de Investigación y Conservación de Anfibios, Fundación Otonga, Quito, Ecuador

[5] Ikiam, Universidad Regional Amazónica, Muyuna, Tena, Ecuador

Introduction

Many organisms have evolved sophisticated chemical defenses that deter predators. Some defended organisms produce their own chemical defenses, while others sequester toxins from external sources (Casewell et al. 2013; Olivera et al. 1985; Saporito et al. 2009, 2012). Toxin sequestration is best understood in invertebrates, where examples of plant to arthropod toxin transfer has been described in great detail (Heckel 2014; Opitz and Müller 2009). Compared to invertebrates, far less is known about how vertebrate species acquire chemical defenses from external sources. An example of toxin sequestration among vertebrates is poison frogs, which accumulate alkaloid toxins from arthropod prey (Daly et al. 1994a, b; Hantak et al. 2013; Saporito et al. 2009). Although the dietary basis of poison frog toxicity is well established, the relationship between

arthropod prey diversity and frog toxin diversity is not fully understood (Santos et al. 2015; Saporito et al. 2007b, 2012). Here, we characterized the toxins found in the Little Devil frog (*Oophaga sylvatica*) and their arthropod prey, and we traced the dietary source of specific frog alkaloids to ants and mites.

The term "poison frog" refers to anurans that carry alkaloid toxins in their skin. They include several families in Central and South America such as dendrobatids (Dendrobatidae) and bufonids (*Melanophryniscus*), as well as mantellids (*Mantella*) from Madagascar, myobatrachids (*Pseudophryne*) from Australia, and Eleutherodactylids from Cuba (Daly 1995; Daly and Spande 1986; Rodríguez et al. 2011). Many of these frogs carry small molecule alkaloid toxins in granular glands on their skin (Neuwirth et al. 1979). Decades of work have identified over 800 alkaloids organized into over 22 structural classes (Daly et al. 2005; Saporito et al. 2012). Most work on poison frog chemical ecology has focused on the Strawberry poison frog (*Oophaga pumilio*). Over 250 alkaloids have been identified in *O. pumilio* with variation both among and within populations (Saporito et al. 2006, 2007a, 2010). However, the ecological drivers and genetic contributions to variation in chemical profiles still are not understood. More integrative and comparative work is needed in order to comprehend how environmental and genetic variables contribute to the diverse repertoires of defensive chemicals.

Initial reports on poison frog alkaloid toxins suggested frogs synthesized these chemicals. However, the absence of alkaloids in captive-reared frogs and several subsequent feeding experiments led researchers to propose the dietary hypothesis (Daly et al. 1994a, b; Saporito et al. 2009). Ecological studies since have demonstrated that poison frogs ingest mainly ants and mites (Caldwell 1996; Donnelly 1991; Toft 1980), and that this dietary specialization correlates with toxicity (Darst et al. 2005). Remarkably, sequestration of alkaloid toxins from arthropod prey has evolved independently at least four times in the Dendrobatidae family (Santos et al. 2003). As frogs sequester their toxins from arthropod prey, it has been proposed that the toxin diversity observed is reflective of arthropod diversity in the tropics (Saporito et al. 2009, 2012). However, little is known about how variation in ant and mite chemistry influences frog toxin profiles.

Most research efforts to identify the dietary source of frog alkaloids have focused on arthropods collected from leaf litter. Ants have been identified as a source of many frog alkaloids including pumiliotoxins, histrionicotoxins, 5,8 and 3,5-disubstituted indolizidines, decahydroquinolines, and pyrrolizidines (Heckel 2014; Jones et al. 1999; Saporito et al. 2004). Despite their small size, mites seem to confer the greatest diversity of alkaloids. Oribatid mites in particular have been explored as the source of many frog toxins, including pumiliotoxins and indolizidines (Saporito et al. 2007b, 2011; Takada et al. 2005). Although there is a clear connection between alkaloid-containing arthropods and poison frog dietary specialization on these ants and mites, little is known about the specific arthropod species that frogs ingest and the alkaloids that these arthropods carry. This is especially the case for mites, whose taxonomy is understudied compared to ants (Saporito et al. 2015). Although broad leaf litter collections of arthropods and subsequent chemical analyses are useful (Daly et al. 2002; Saporito et al. 2004, 2009), integrative analyses that include arthropod identification from frog stomachs, chemical analyses of arthropod prey items, and correlation analyses of arthropod and poison frog chemical diversity are needed as the next step toward understanding frog skin toxins and their dietary source.

To determine how arthropod diversity contributes to toxin variation in a poison frog, we characterized the alkaloid composition and diet of the Little Devil poison frog (or Diablito poison frog, *Oophaga sylvatica*) and traced the dietary source of toxins to ants or mites. We tested the hypothesis that ingested ant and mite species differ across frog populations, and that the chemical diversity of ingested prey contributes to corresponding differences in alkaloid variation across frog populations. We first use gas chromatography/mass spectrometry (GC/MS) to profile the chemical diversity of three *O. sylvatica* populations. Then, to test the hypothesis that variation in frog toxin profiles reflects the chemical diversity of arthropod prey, we used liquid chromatography/mass spectrometry (LC/MS) to chemically profile ants and mites recovered from stomach contents. We also identified prey items isolated from frog stomachs using the DNA barcode cytochrome oxidase 1 (CO1 or *cox1*) (Meusnier et al. 2008). Finally, we compared the chemical profiles of frogs and their prey items in order to identify either ants or mites as the source of specific alkaloids found in different frog populations.

Methods and Materials

Field Collection Little Devil (*O. sylvatica*) poison frogs were collected during the day near the villages of Cristóbal Colón ($N = 10$; 0.005 km^2 area), Simón Bolívar ($N = 10$; 0.0008 km^2 area), and along the Felfa River ($N = 12$; 0.003 km^2 area) near the village Montalvo in the northwestern Esmeraldas province of Ecuador in July 2014. Collections and exportation of specimens were done under permits (001–13 IC-FAU-DNB/MA, CITES 17 V/S) issued by the Ministerio de Ambiente de Ecuador. Frogs were stored individually in plastic bags with air and vegetation for 3–8 h. In the evening the same day of capture, frogs were anesthetized with a topical application of 20 % benzocaine to the ventral belly, and euthanized by cervical transection. The dorsal skin (from the back of the head but not including the legs; 35.94 ± 13.03 mg) was isolated and stored in plastic vials containing 1 ml of 100 % methanol. Stomachs were dissected and their contents sorted by

arthropod type (as genera could not be visually determined) into separate tubes of ants, mites, and other arthropods. Arthropods were stored in 1 ml of 100 % methanol in plastic vials at 4 °C for a few weeks. Remaining frog tissues were preserved in RNAlater (Life Technologies, Carlsbad, CA, USA) or 100 % ethanol, and were deposited in the amphibian collection of Centro Jambatu de Investigación y Conservación de Anfibios in Quito, Ecuador (CJ 3089–3139). The Institutional Animal Care and Use Committee of Harvard University approved all procedures (Protocol 15–02-233). In order to protect the vulnerable *O. sylvatica* populations that are highly targeted by illegal poaching, specific GPS coordinates of frog collection sites can be obtained from the corresponding author.

Quantification of Arthropods in Stomach Contents Each arthropod was photographed individually with a Lumenera Infinity 2 camera mounted on an Olympus dissection microscope (SZ40), and was assigned a unique seven digit identification number with the first four digits being the voucher specimen number of the frog from which the arthropod was taken and the last three digits being the number assigned to the arthropod in increasing order from which it was removed from the stomach contents. Ant and mite samples from the stomach contents of 5 frogs from each population were selected for alkaloid analysis using LC/MS based on the largest quantity of mites recovered. Mites are extremely small, and we selected frogs with the largest number of mites to increase the likelihood that any alkaloids would be at detectable levels. These samples for LC/MS were pooled by arthropod type (ants, mites, or other) into 1 ml of 100 % methanol in glass vials until alkaloid extraction. The arthropod samples from the remaining frogs were placed individually in vials of 100 % ethanol for later molecular identification by PCR.

Diet was quantified both by quantity of each arthropod type (ants, mites, beetles, or "other") and by volume of each arthropod type to account for the wide range of prey size. To determine volume, the photographs of each arthropod were analyzed using Image J (National Institute of Health, Bethesda, MD, USA) to determine their dimensions. Length measurements were taken from the tip of the mandible and extended to the rearmost point of the arthropod. The width measurement was taken at the midpoint of the arthropod and excluded the extra girth added by appendages. If the prey item was fragmented, the measurements were taken from the nearest identifiable body part. The length and width measurements were used to calculate the volume of each prey item. The equation of a prolate sphere was used for the volume calculation of the ant, beetle, and other arthropods: $V = (4\pi/3) * (Length/2) * (Width/2) \wedge 2$. The different body shapes among the mites taken from the stomach contents required the use of three different formulas to accurately describe their volume and shape. In addition to the equation for a prolate sphere, the equations of a hemicylinder (VHC) and a sphere (VS) also were used based on individual mite shape (see Online Resource Fig. 1 for examples): $VHC = (4\pi/6) * (Length/2) * (Width/2) \wedge 2$ and $VS = (4\pi/3) * (Diameter/2) \wedge 3$.

Isolation of Alkaloids A set of samples including frog skins, stomach-isolated ants, and stomach-isolated mites from five frogs across populations of Felfa, Simón Bolívar, and Cristóbal Colón were used to characterize alkaloid profiles (45 samples in total). The contents of each sample vial (including arthropods and 100 % methanol) were emptied into a sterilized Dounce homogenizer. The empty vial was rinsed with 1 ml of methanol and added to the homogenizer to ensure the full transfer of all materials. As an internal standard, 25 μg of D3-nicotine in methanol (Sigma-Aldrich, St. Louis, MO, USA) were added to each vial. The sample (either frog skin or arthropods) was ground with the piston ten times in the homogenizer before being transferred to a glass vial. The homogenizer and piston were rinsed with 1 ml of methanol that then was also added to the glass vial to collect any alkaloid residue. The equipment was cleaned with a triple rinse of methanol before being used to process another sample. A 200 μl aliquot of sample was removed for later LC/MS analysis. The remainder of the frog skin samples was evaporated to dryness under nitrogen gas and reconstituted in 0.5 ml of methanol. Samples were transferred to a microcentrifuge tube and spun at 12,000 rpm for 10 min. A 200 μl aliquot of the supernatant was transferred to a 0.3 ml glass insert in an amber sample vial for later GC/MS analysis. All samples were stored at −20 °C until GC/MS (frog skin only) or LC/MS (frog skin, ants, and mites) analyses. Some background impurities from the sampling, storage, and processing steps were noted. These included siloxanes, fatty acids, and methylated fatty acids, which are easily distinguishable from alkaloids by their mass spectra. We cannot rule out that some alkaloids may be masked by GC/MS responses of plasticizers or fatty acids. However, our goal was to identify differences in alkaloids profiles among frog populations and the putative arthropod source of these alkaloids. Future work aimed at extensively identifying all alkaloids in these frogs will include the use of glass vials in the field as well as an alkaloid extraction protocol that specifically removes fatty acids as described recently (Saporito et al. 2010).

Gas Chromatography/Mass Spectrometry (GC/MS) GC/MS analysis was based on a slight modification of the method reported by (Saporito et al. 2010). Analyses were performed on a Waters Quattro Micro system (Beverly, MA, USA) with an Agilent 6890 N GC (Palo Alto, CA, USA). A J&W DB5ms 30 m × 0.25 mm column with a 0.25 μm film thickness (Agilent Technologies, Palo Alto, CA, USA) was used for separations. Helium carrier gas was held constant at 1 ml/

min and split injection (split flow ratio of 12) was used with an injector temperature of 280 °C. A 2 μl injection of the concentrated and reconstituted methanol extracted samples was used for GC/MS analysis. The column temperature gradient began with a 1 min hold at 100 °C, then increased at 8 °C/min to 280 °C, and held for 2.5 min. The interface temperature was 280 °C. The mass spectrometer was operated in the 70 eV electron ionization mode and scanned continuously in the range m/z 20–616 at a rate of 0.4 s/scan. Each total ion chromatogram was reviewed, and alkaloid responses were characterized from their mass spectra and retention times. Nominal molecular weights were identified from the $M^{+\cdot}$ and/or the $(M\text{-}CH_3)^+$ ions that were visible for the major alkaloids. The extensive database developed by Daly and colleagues (Daly et al. 2005) was used to tentatively identify alkaloids by matching major mass spectrum peaks and relative retention times using D3-nicotine as a reference. This database is the cornerstone of all poison frog GC/MS chemistry, as frog toxins are not available commercially to serve as standards. Assignment of alkaloid identity is tentative without pure standards available.

Liquid Chromatography/Mass Spectrometry (LC/MS) LC/MS analyses were performed primarily on a Bruker maXis Impact Q-TOF system (Billerica, MA, USA) with an Agilent 1290 LC (Palo Alto, CA, USA). Product ion scans of the ant/mite/skin correlation study were performed on an Agilent 6550 Q-TOF (Palo Alto, CA, USA) configured with the same LC system, and operated with similar MS parameters. A reversed-phase LC gradient method was developed using a Phenomenex Gemini C18 3 μm 2.1 × 100 mm column (Torrance, CA, USA). Mobile phase A was composed of water with 0.1 % formic acid, and mobile phase B was composed of acetonitrile with 0.1 % formic acid. The flow rate was 0.2 ml/min. The gradient began with 0 % B for one min, then increased linearly to 100 % B at 15 min, and held until 18 min. LC/MS is more sensitive than GC/MS so that a 1 μl injection volume of either the un-concentrated arthropod samples or a 20-fold dilution (with methanol) of the frog skin samples could be used for LC/MS analysis. The Bruker mass spectrometer was tuned for standard mass range analysis, and data were continually acquired in the range *m/z* 50–3000; each run was recalibrated using a post-run injection of a sodium formate solution. Electrospray positive mode ionization was used with a source drying gas of 10 L/min at 200 °C, nebulizer at 30 psi, and capillary set at 4000 V with an endplate offset of 500 V. For the Agilent Q-TOF, the Ion Funnel electrospray positive mode source used drying gas of 14 L/min at 200 °C with nebulizer at 35 psi, a sheath gas flow of 11 L/min at 350 °C, capillary set at 3500 V, nozzle voltage of 1000 V, and Fragmentor set at 175 V. Collision energies were set at 15 and 30 eV, and data were continually acquired in the range *m/z* 50–1700 using a reference lock mass. Alkaloid responses were identified by retention times and high-resolution mass spectra. Although elemental composition determinations with less than 5 ppm accuracy were possible, and some MS/MS data were acquired, there is no LC/MS database for frog alkaloid compounds. The occurrence of numerous alkaloid isomers made tentative identifications by LC/MS challenging. Comparisons with GC/MS data from frog skin were required for tentative identification of alkaloids given that standards for frog alkaloids are not commercially available.

Arthropod Genomic DNA Extraction Ant genomic DNA was isolated using a prepGEM Insect kit (ZyGEM, Hamilton, New Zealand) for smaller fragments or a NucleoSpin Tissue kit (Macherey-Nagel, Bethlehem, PA, USA) for full ants. Exoskeletons were crushed individually in a microcentrifuge tube with a sterile plastic pestle before following the manufacturers' instructions. Mite genomic DNA was isolated using a phenol-chloroform extraction based on protocols from (Doyle 1987) and (Navajas et al. 1998) with some modifications. To crush the exoskeleton, each mite was pulverized in a microcentrifuge tube filled with 200 μl of extraction buffer (2 % cetyltrimethyl ammonium bromide [CTAB], 1.4 M NaCl, 0.2 % 2-β mercaptoethanol, 20 mM EDTA, 100 mM TRIS-HCl pH 8) with a sterile plastic pestle before being incubated for 30 min at 60 °C. Cold isopropanol was added to precipitate the DNA. After centrifugation, the DNA pellet was washed with 75 % ethanol before resuspension in water. Purified genomic DNA was stored at −20 °C until PCR.

Molecular Identification of Stomach Contents To identify mites and ants collected from frog stomachs, we used PCR to amplify the cytochrome oxidase 1 (CO1 or *cox1*) region of genomic DNA, which is often used for taxonomic barcoding (Meusnier et al. 2008; Smith et al. 2005; Young et al. 2012). For all reactions, we used 2 μl of each primer (10 μM) and 25 μl of 2X Phusion High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs, Ipswich, MA, USA) in a total reaction volume of 50 μl. Samples were amplified with either the primers LCO-1490 (5′-GGTCAACAAATCATAAAGATATTGG) and HCO-2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA) from (Folmer et al. 1994) or the primers CI-J-1632 (5′-TGATCAAATTTATAAT) and CI-N-2191 (GGTAAAATTAAAATATAAACTTC) from (Kambhampati and Smith 1995). A touchdown PCR program was used as follows: one round of 95 °C for 5 min; 5 rounds of 95 °C for 40 s, 45 °C for 40 s with −1 °C per cycle, 72 °C for 1 min; 40 rounds of 95 °C for 40 s, 40 °C for 40 s, 72 °C for 1 min; one round of 72 °C for 5 min. Successful reactions with a single band of the expected size were purified with the E.Z.N.A. Cycle Pure Kit (Omega Bio-Tek, Norcross, GA, USA) and Sanger sequenced by GeneWiz Inc. (Cambridge, MA, USA).

We amplified CO1 in 45 out of 137 ant samples and 9 out of 20 mite samples. Nucleotide BLAST of the NCBI

Genbank nr database was used to identify the resulting CO1 sequences (Online Resource Tables 1 and 2). For ants and mites, we assigned a family or genus level taxonomic identity based on the results of the BLAST search, where we considered greater than 96 % sequence similarity sufficient to assign species or genera (Hebert et al. 2003, 2004). For less than 95 % similarity, we assigned specimens to a family based on BLAST similarity. For some ant specimens, we identified the species based on the greater wealth of DNA barcoding information available for ants as compared to mites. We used the software MEGA 6.0 (Tamura et al. 2013) to separately analyze the ant and mite CO1 sequences. ClustalW was used to align the CO1 sequences from this study with other closely related species retrieved from GenBank. A nearest-neighbor joining tree then was constructed with a bootstrap of 5000 replications. All arthropod CO1 sequences have been submitted to GenBank (Accession numbers: ants KU128453–97; mites KT947980–8).

Data Analysis and Statistics GC/MS analysis of alkaloids across populations were based on the extensive dataset by John Daly and colleagues (Daly et al. 2005) for identification of the major alkaloid responses. First, a general overview of the data was created to show variations and similarities in major alkaloid features among populations. Electron ionization mass spectrometry produced spectra of frog skin alkaloids with base peak fragment ions that are specific diagnostic ions based on the GC/MS database (Daly et al. 2005). Seven diagnostic ions were selected from their occurrence in the most abundant alkaloids across several frog populations. These extracted ion chromatograms were overlaid and normalized to the same scale to create a visual overview of major alkaloid distribution in different populations. This normalization process allowed us to visualize lesser alkaloids along with major alkaloids, which in some cases were expanded off scale, creating the "squared off" top of some major peaks in the total ion chromatogram visualization. Individual samples from each population also were processed in the same manner to visually compare individual variation within each frog population (See Online Resource Figs. 2–4). GC/MS and LC/MS alkaloid data were analyzed using a principal component analysis in the XCMS software maintained by the Scripps Center for Metabolomics (Tautenhahn et al. 2012). In XCMS, a nonlinear alignment was performed on the ions, and integrated areas were compared among different populations to obtain a relative abundance. A principal component analysis was performed on the frog alkaloids (GC/MS and LC/MS) and arthropod chemical profiles (LC/MS only) using the relative abundance of every feature as input. All raw mass spectrometry data are available at DataDryad (doi:10.5061/dryad.34rs7).

We used LC/MS for analysis of alkaloids common to frogs and arthropods. For each frog skin, ant, and mite sample, the most abundant alkaloids (five to seven in most comparisons) were selected for generation of an extracted ion chromatogram (EIC). Major ant or mite responses in the EIC were analyzed by molecular ion $(M + H)^+$ product scans of both the ant or mite samples and the corresponding frog skin sample to compare both retention times and mass spectra. Although the other peaks in the insect samples could be alkaloids, we analyzed only similar peaks present in both the frog skin sample and corresponding arthropod samples. To confirm putative shared compounds with similar mass-to-charge ratios (m/z), 15 eV collision energy product ion scan total ion chromatograms (TIC) were compared to confirm the presence of a co-eluting compound in both the frog skin and the arthropod sample of interest (ant or mite). Finally, we compared of 15 eV product ion mass spectra from each putative compound shared by the frog and corresponding arthropod sample to confirm the spectra closely match, suggesting the compound of interest is identical in both frogs and arthropods.

To compare stomach contents across *O. sylvatica* populations, we quantified the number and volume of ants, mites, beetles, and other arthropods. Each diet variable was normally distributed, and an ANOVA was used to determine population differences in different diet categories. A Tukey's honest significance difference (HSD) test was used *post-hoc* to determine population differences. A Benjimini Hochberg false discovery rate correction was applied to adjust for multiple hypothesis testing.

Results

Little Devil Poison Frog Populations Vary in Alkaloid Profiles We found that each frog population had a unique profile of alkaloids (Fig. 1a). A principal component analysis (PCA) showed clustering among groups in principal component 1, which accounts for 25 % of the alkaloid variation. In the PCA, the Cristóbal Colón population is centered between Felfa and Simón Bolívar (Fig. 1b), indicating Cristóbal Colón is more similar to both populations rather than just one, possibly due to its geographic location (Fig. 1b). Relatively little within-population alkaloid variation was observed with the exception of frogs collected from Cristóbal Colón (Online Resource Figs. 2–4). The most common compounds identified were 3,5-disubstituted indolizidines, 5,8-disubstituted indolizidines, decahydroquinolines, and lehmizidines (Table 1). Major alkaloids (the most abundant 5–6 alkaloids in each population) generally were unique to a specific population, with overlap only between Cristóbal Colón and Simón Bolívar with the 5,8-disubstituted indolizidine **231C.** Overall the results show each population has a distinct composition with little overlap among populations. This observation was replicated with liquid chromatography/mass spectrometry (LC/MS) (Online Resource Fig. 5).

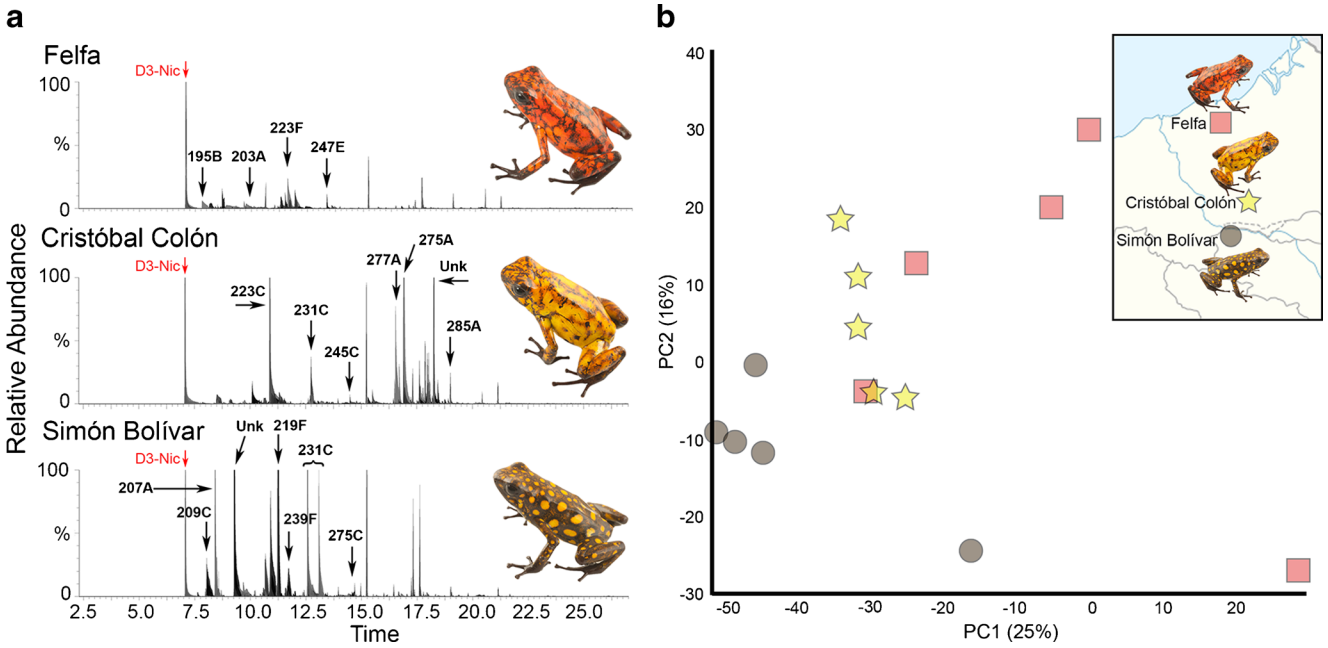


Fig. 1 Little Devil poison frog (*Oophaga sylvatica*) population variation in chemical defenses. **a** Alkaloid toxin profiles assayed by gas chromatography/mass spectrometry (GC/MS) show population variation in chemical defenses where each peak is a chemical and peak amplitude represents abundance ($N = 5$ per group). Major alkaloids are identified with arrows. Red arrows indicate spiked D3-nicotine as an internal reference. Individual plots can be seen in Online Resource materials. **b** Principal component analysis (log scaled and centered) of GC/MS profiles reveals clustering of populations along a latitudinal gradient (see geographic distribution in legend)

Dietary Differences among Little Devil Poison Frog Populations To gain a better understanding of how diet contributes to alkaloid profiles, we determined the quantity and identity of arthropods collected from frog stomachs by using both morphometric and molecular methods. We first grouped them into broad categories of ants, mites, beetles, and "other" (Table 2), and calculated the number and volume of each type. With a subset of ants and mites from each population ($N = 5$ frogs per population), we used an untargeted metabolomics approach with LC/MS to characterize the chemical signature

Table 1 Major alkaloids identified in *Oophaga sylvatica*

Alkaloid	Felfa	Cristóbal Colón	Simón Bolívar
Histrionicotoxin 285 A		+	
3,5-Disubstituted indolizidine 275C			+
5,8-Disubstituted indolizidines			
203 A	+		
207 A			+
219F			+
231C		+	+
247E	+		
245C		+	
Decahydroquinolines			
195B	+		
223F	+		
5,6,8-Trisubstituted indolizidine 209C			+
Lehmizidines			
277 A		+	
275 A		+	
Izidine 239F			+
Unknown structures 223C		+	

Table 2 Broad diet characterization of Felfa, Cristóbal Colón, and Simón Bolívar populations of *Oophaga sylvatica*

Population	*N*	No. prey per frog (mean (min & max))	% ants (num)	% ants (vol)	% mites (num)	% mites (vol)	% beetles (num)	% beetles (vol)
Felfa	10	12.6 (6–33)	78.72	85.81	15.86	6.825	3.693	7.317
Cristóbal Colón	10	9.6 (4–31)	54.37	70.15	35.10	19.69	8.545	6.392
Simón Bolívar	12	12.5 (1–40)	66.45	52.55	16.81	22.96	14.26	18.08

N number of frogs per population, *num* number of arthropods, *vol* volume of arthropods

of consumed arthropods across frog populations. For the remaining ants and mites, we sequenced the CO1 gene to characterize species diversity.

Species and Chemical Diversity in Consumed Ants When characterizing broad patterns in ant consumption (Table 2), we found that the percentage of ants by volume varied among populations (ANOVA, $F_{2,32} = 4.480$, $P = 0.020$), but the percentage of ants by number did not. Felfa had a higher percentage by volume compared to Simón Bolívar (Tukey's HSD, $P = 0.015$) but not compared to Cristóbal Colón (Tukey's HSD, $P = 0.382$). Cristóbal Colón and Simón Bolívar did not differ in the percentage by volume.

To determine if consumed ants (pooled by individual frog) vary in their chemical profiles across populations, we performed a PCA of ant LC/MS data (Fig. 2a). We found that ant prey chemical profiles also cluster by the frog population from which they were isolated, suggesting the three frog populations have access to different ant prey with distinct chemical profiles.

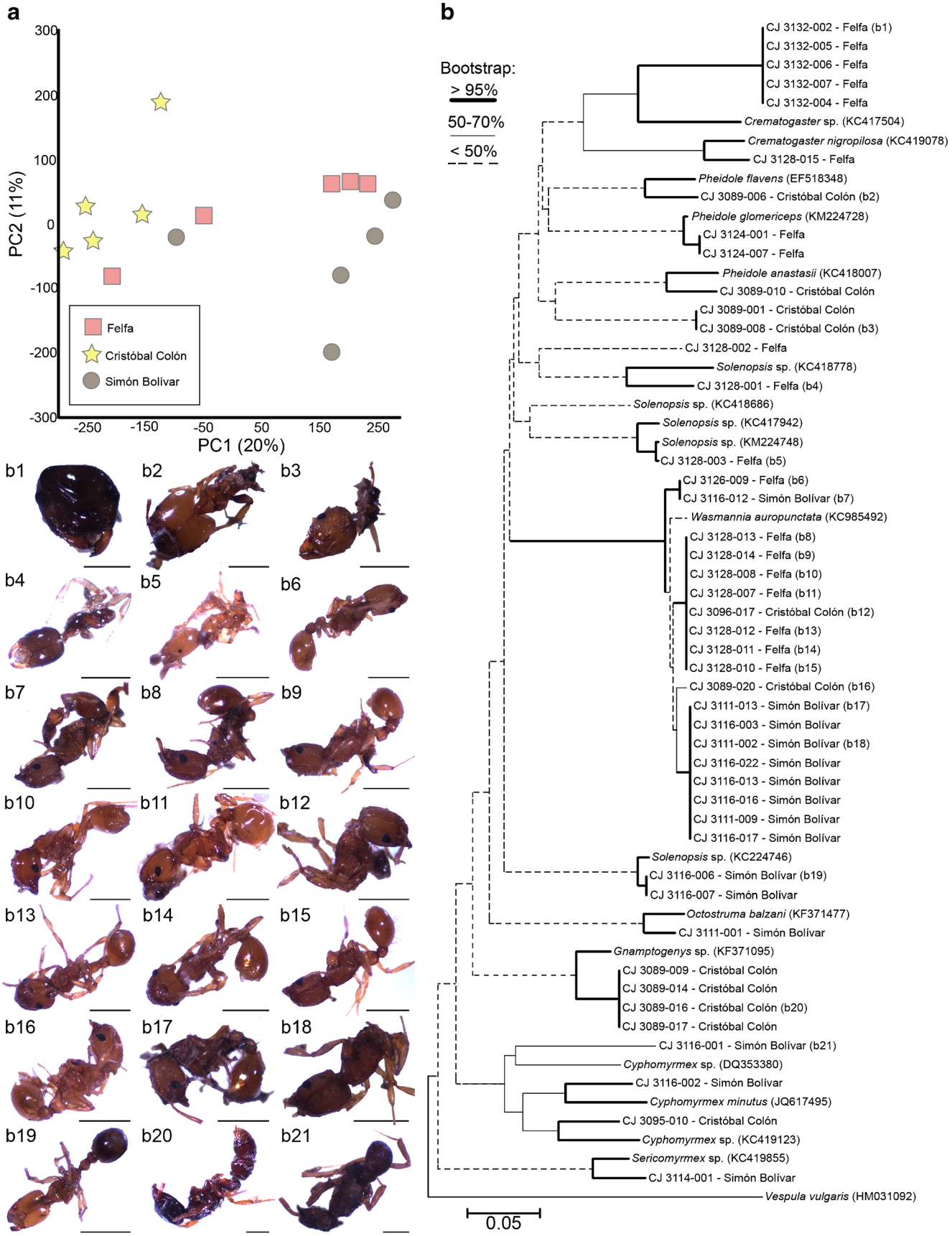
To better understand how ant prey may differ in these geographically distinct frog populations, we obtained CO1 sequence for 45 out of 137 ant specimens isolated from frog stomachs. Consumed ants belong to seven genera of the Myrmicinae sub-family (tribe Attini [fungus-growing ants], genera: *Cyphomyrmex*, *Octostruma*, *Pheidole*, *Sericomyrmex*, *Wasmannia*; tribe Crematogastrini, genus: *Crematogaster* [acrobat ants]; tribe Solenopsidini, genus: *Solenopsis* [stinging ants]), and from one genus of the Ectatomminae sub-family (tribe Ectatommini, genus: *Gnamptogenys* [predatory ants]) (Fig. 2b, Online Resource Table 1). All ant CO1 sequences have more than 80 % sequence similarity with their top BLASTn hit matches in GenBank, and 22 have more than 96 % similarity, sometimes allowing resolution at the species level. However, some specimens have close BLASTn matches (80–90 %) for different ant genera (usually undescribed species), which do not allow confident assignment of some specimens. Many specimens only had a match of up to 85 % homology in CO1 sequence to records in the GenBank database, suggesting these may be undescribed ant species.

Based on CO1 sequence similarity among ant specimens, we estimate 20 different species of ants were recovered. Two genera of Myrmicinae Attini ants were recovered from frog stomach contents in all three *O. sylvatica* populations, including *Solenopsis* and *Wasmannia*. A unique species to the Felfa group is a Myrmicinae Crematogastrini *Crematogaster* species, which was not observed in the two other frog populations. These *Crematogaster* ants were found in the same Felfa frog (CJ 3132), and the closest match in the GenBank database exhibits only 86 % similarity, suggesting these specimens represent an undescribed ant species. A different *Crematogaster* species was isolated in a separate Felfa frog (CJ 3138), and has 94 % similarity in CO1 sequence with *C. nigropilosa*. Four ant specimens collected from both Felfa (CJ 3124) and Cristóbal Colón (CJ 3089) cluster with Myrmicinae Attini *Pheidole* genera, although there is not an exact match with any described ant CO1 sequences in the GenBank database. Finally, four specimens recovered from a Cristóbal Colón frog (CJ 3089) were the only non-Myrmicinae ants recovered. These specimens have identical CO1 sequence with each other, and are 95 % similar to the Ectatomminae *Gnamptogenys* genera.

Species and Chemical Diversity in Consumed Mites The amount of mites consumed among *O. sylvatica* populations varied (Table 2). The percentage of mites in the stomach contents by number differed among frog populations (ANOVA, $F_{2,32} = 4.232$, $P = 0.024$) where the Cristóbal Colón population had a higher percentage of mites by number compared to both Felfa (Tukey's HSD, $P = 0.042$) and Simón Bolívar (Tukey's HSD $P = 0.044$). The percentage of mites by volume also varied among populations (ANOVA, $F_{2,25} = 7.063$, $P = 0.004$), where Simón Bolívar frogs had a higher percentage of mites by volume in the stomach contents compared to Felfa (Tukey's HSD, $P = 0.003$).

To determine if the chemical profiles of consumed mites were distinct among *O. sylvatica* populations, we performed a PCA of the mite LC/MS data (Fig. 3a). We found clustering of mite samples according to frog population in principal component 2 (12 % of variance) but not principal component 1 (13 % of variance).

We hypothesized that diversity in consumed mite species was responsible for the clustering observed in the PCA. To test this hypothesis, we obtained CO1 sequence for 9 out of 20 mites individually isolated from frog stomachs. Based on the top BLASTn hit for each mite, all specimens are likely



◀ **Fig. 2** Identification and metabolomics of ants found in the stomachs of Little Devil poison frogs (*Oophaga sylvatica*). **a** Principal component analysis clustering of ant liquid chromatography/ mass spectrometry data colored by frog population. **b** A phylogenetic tree with nearest neighbor clustering shows relationships in cytochrome oxidase 1 sequence among ants isolated from *O. sylvatica* stomachs compared to other known ants. Ants are numbered by the four-digit frog specimen identifier followed by a three-digit number assigned to the arthropods in the order they were isolated from a single stomach. The frog population each ant was isolated from is listed beside each identifier. Genbank IDs of known ants are shown after the species name. The common wasp (*Vespula vulgaris*) was used as an outgroup. (b1–21) Photos of some specimens are shown; Scale bar is 5 mm

Oribatid (Order Oribatida) mites (Fig. 3b, Online Resource Table 2). However, due to the low percent similarity of mite CO1 sequence to the records of mites in the GenBank nr database (78–84 %), we were unable to assign mites to particular genera, and many of these specimens likely represent undescribed species. An exception is one mite that has a 99 % similarity to the mite *Archegozetes longisetosus*, and is likely the same species. Additionally, three mites isolated from two Simón Bolívar frogs had identical morphology and CO1 sequence, suggesting they are the same undescribed species. Thus, we identified 5 undescribed and 1 described species of mites from the diet of three *O. sylvatica* populations. We did not obtain enough sequence information from separate mite specimens to determine if different frog populations have access to distinct mite species as prey items. In the future that type of information could shed light on the mite LC/MS chemistry clustering.

Other Dietary Arthropods In addition to ants and mites, which together represent over 80 % of the *O. sylvatica* diet, we also evaluated the number and volume of beetles and "other" arthropods isolated from frog stomach contents (Table 2). However, the number of arthropods in these categories was so low that we did not evaluate statistical differences among frog populations. Nearly all frogs from Cristóbal Colón had eaten at least one beetle (8 out of 10 frogs) whereas only two Simón Bolívar and three Felfa frogs had. Few arthropods were isolated that did not group into the categories of ants, mites, or beetles (two in Felfa, five in Simón Bolívar, and four in Cristóbal Colón). These specimens were usually flies, wasps, or stinkbugs, although we did not use molecular methods to identify these samples. We did not examine the chemical profiles of non-mite or non-ant arthropods using LC/MS.

Poison Frog Alkaloids in Ants and Mites While the dietary specialization of poison frogs on ants and mites has been well documented, less is known about which alkaloid compounds are derived from specific prey categories. We compared three samples (frog skin, ants, and mites) for five individuals from each *O. sylvatica* population using LC/MS, which provides increased sensitivity compared to GC/MS. By comparing all three samples within each individual, we were able to trace the dietary source of four alkaloids.

Alkaloids Detected in Ants We compared the LC/MS responses in frog skin and their corresponding prey samples in

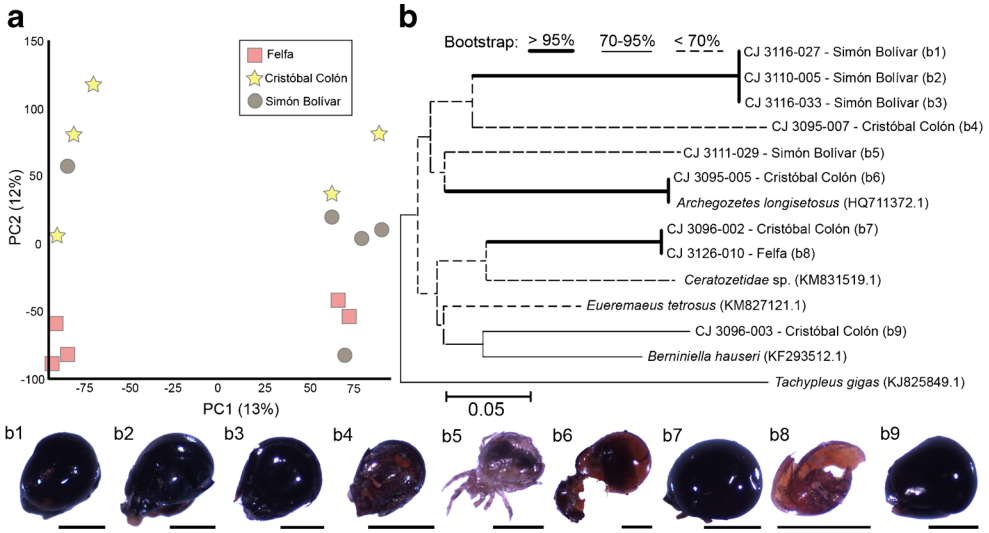


Fig. 3 Identification of mites found in the stomachs of Little Devil poison frogs (*Oophaga sylvatica*). **a** Principal component analysis clustering of mite liquid chromatography/mass spectrometry data colored by frog population. **b** A phylogenetic tree with nearest neighbor clustering shows relationships in cytochrome oxidase 1 sequence among mites isolated from *O. sylvatica* stomachs compared to other mites. Isolated mites are numbered by the four-digit frog identifier followed by a three-digit number assigned to the arthropods in the order they were isolated from a single frog stomach. The frog population each mite was isolated from is listed beside each identifier. Genbank IDs of known mites are shown after the species name. A horseshoe crab (*Tachypleus gigas*) was used as an outgroup. (b1–9) Photos of each specimen are shown; Scale bar is 5 mm

search of compounds that had similar mass to charge ratios in both the frog skin and the ants, but not the mites. We then further examined these compounds with tandem mass spectrometry to confirm the alkaloids found in frog skin and ants had similar fragmentation mass spectra patterns, which suggests the compound of interest is identical in both samples.

Using this approach, we identified ants as the dietary source of two frog alkaloids (Fig. 4; Online Resource Fig. 6). In a Cristóbal Colón frog skin (CJ 3093) and the corresponding sample of consumed ants (Fig. 4a), we observed a compound with a mass to charge ratio of 278 that also had identical fragmentation patterns in both samples, suggesting these compounds are identical. Comparing the LC/MS data to the GC/MS data from the same frog skin sample, we concluded that this ant-derived alkaloid is lehmizidine **277-A**. We also identified two similar LC/MS responses in a Felfa frog (CJ 3129) and consumed ants isolated from that same frog's stomach (Fig. 4b). This compound has a mass to charge ratio of 204, and the fragmentation patterns show the mass spectra closely match, suggesting identical compounds in both frog and ant samples. From comparison of GC/MS responses with *m/z* 204 in this same frog skin sample, this alkaloid is likely 5,8-disubstituted indolizidine **203-A**.

Alkaloids Detected in Mites We took a similar approach to detect common LC/MS responses in frog skin and their corresponding mite samples, where compounds should have similar mass to charge ratios in both the frog skin and the mites, but not the ants. Further examination of these compounds with tandem mass spectrometry confirmed the alkaloids found in frog skin and mites are identical.

Using this comparative LC/MS approach, we identified mites as the dietary source of two poison frogs alkaloids (Fig. 5, Online Resource Fig. 6). In a Cristóbal Colón frog (CJ 3091), a similar LC/MS response with a mass to charge ratio of 224 was observed in both the frog skin and the pooled mite sample (Fig. 5a). Comparison of the tandem mass spectrometry fragmentation pattern shows the spectra closely match, suggesting the compounds detected in these samples are identical. From comparison of the GC/MS responses with a mass to charge ratio of 224 in this same frog skin sample, this compound is likely 3,5-disubstituted indolizidine **223AB**. In a separate frog-mite comparison, a similar LC/MS response with a mass to charge ratio of 292 was observed in a Simón Bolívar frog (CJ 3112) and the pooled mites from the frog's stomach (Fig. 5b). Fragmentation mass spectra of the compounds suggest this compound is identical. From the GC/MS analysis of the frog skin sample, no corresponding alkaloid with a similar mass to charge ratio was detected, likely due to the lack of sensitivity of GC/MS compared to LC/MS.

Discussion

We showed that *O. sylvatica* populations vary in defensive chemical profiles in a manner that reflects prey arthropod chemical diversity. Overall these results highlight the diversity of chemical defenses found in *O. sylvatica* and Ecuadorian ants and mites, adding to the growing literature of the trophic relationships of poison frog toxins and their arthropod sources.

Little Devil Poison Frog Defensive Chemicals Population differences in alkaloid toxins have been demonstrated previously in nearly all poison frog groups, including dendrobatids (Saporito et al. 2006, 2007a; Stuckert et al. 2014), mantellas (Daly et al. 2008), and bufonids (Daly et al. 2007). Other work has focused on the Strawberry poison frog (*O. pumilio*) in Costa Rica and Panama (Saporito et al. 2006, 2007a, 2010, 2012), and to a lesser extent on the Imitator frog (*Ranitomeya imitator*) in Peru (Stuckert et al. 2014). These studies also have shown similarity in alkaloid profiles associated with geographic proximity of populations within the same species (Saporito et al. 2007a). Variation in alkaloid profiles among populations has been described for some *O. sylvatica* populations (under the name *Dendrobates histrionicus*), as well as a number of other *Oophaga* species, including *O. histrionica* and *O. lehmanni* (Myers and Daly 1976). Dietary environment is a significant component of toxin profiles within *Oophaga*. For example, sympatric populations of *O. granulifera* and *O. pumilio* have more similar alkaloid profiles than the geographically distant populations of *O. granulifera* (Myers et al. 1995). However, this is not always the case as co-mimetic (sympatric) species of *Ranitomeya* differ in toxin profiles (Stuckert et al. 2014), which may suggest variation in micro-habitats, contrasting prey preferences, or genetic differences that impact the efficiency and specificity of alkaloid sequestration. More comparative work across a broader range of species and habitats is required to fully understand how these ecological and genetics factors impact frog chemical defenses.

The most abundant classes of alkaloids in *O. sylvatica* were 3,5- and 5,8-disubstituted indolizidines, although the precise alkaloid in these classes differed among the three populations (Table 1). This is in contrast to toxin profiles for this species collected from Ecuador by Myers and Daly (Daly et al. 1978) who reported only histrionicotoxins as the major alkaloids in several populations. We found histrionicotoxins only in Cristóbal Colón frogs. This discrepancy could be due to sampling location, as *O. sylvatica* in the (Myers and Daly 1976) study were collected in the southernmost part of their range. Another possible explanation for the differences could be changes in dietary arthropod composition over the past 40 years, which may have shifted the most abundant alkaloids in this species from histrionicotoxins to disubstituted

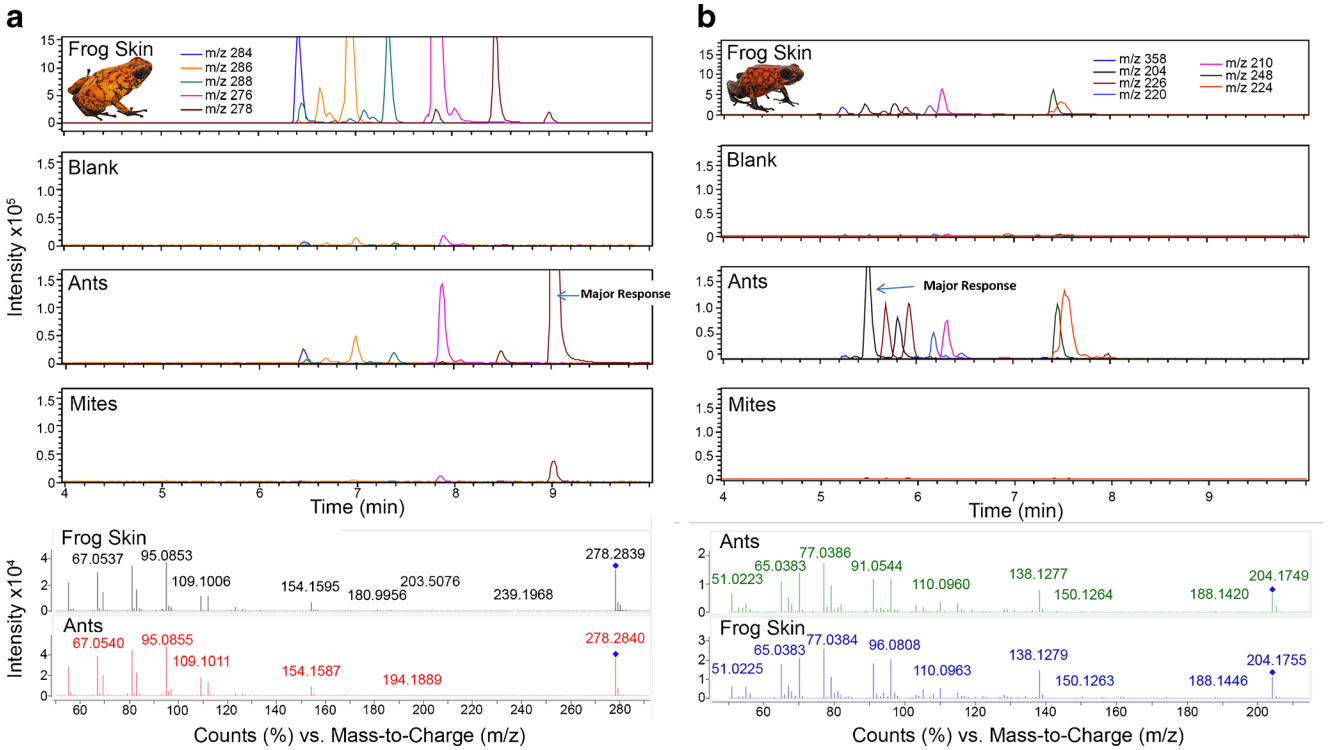


Fig. 4 Identification of ants as a dietary source of lehmizidine **277-A** and 5,8-disubstituted indolizidine **203-A** in the Little Devil frog (*Oophaga sylvatica*). Liquid chromatography/mass spectrometry (LC/MS) was used to analyze alkaloids from *O. sylvatica* skin samples and the ants and mites found in those same frogs' stomachs. Top: Abundant alkaloids in the frogs were selected for extracted ion chromatogram analysis. Each compound is represented as a different color and mass-to-charge (*m/z*) ratios are listed at the top of each panel. In both comparisons from two different frogs, a major alkaloid response (labeled as "Major Response") was detected in the ant sample that was similar to a response found in the frog skin sample. Bottom: Tandem mass spectrometry fragmentation patterns via 15 eV product ion mass spectra shows a close match between the compounds found in the frogs and the ants. **a** A similar response of *m/z* 278 was found in a Cristóbal Colón frog skin sample and the corresponding ant sample. Comparison with the GC/MS data from this frog suggests the identity of this shared alkaloid is lehmizidine **277-A**. **b** A similar response of *m/z* 204 was found in a Felfa frog skin sample and the corresponding ant sample. Comparison with the GCMS data from this frog suggests the identity of this shared alkaloid is 5,8-disubstituted indolizidine **203-A**

indolizidines. The unique major alkaloids in populations from Cristóbal Colón compared to those from Felfa and Simón Bolívar include histrionicotoxin **285-A** and lehmizidines **277-A** and **275-A**. Only Felfa frogs had decahydroquinolines (DHQ **195B** and **223F**) as a major alkaloid group. Many of the alkaloids we identified in *O. sylvatica* also have been detected in *O. pumilio* (Saporito et al. 2007a), with the exception of **5,8-I 245C** and Izidine **239F**, and some frogs in the genus *Mantella* (Garraffo et al. 1993).

Individual variation in alkaloid profiles was limited in the Simón Bolívar and Felfa populations, but was much greater in the Cristóbal Colón population where almost every frog had a distinct profile. Without genetically profiling the stomach contents, which were instead used for LC/MS alkaloid quantification, we were unable to determine dietary species diversity. However, quantification of stomach contents represents a snapshot in time and may not be representative of the full dietary repertoire that contributed to skin alkaloid profiles accumulated over weeks or months. Additionally, five frogs are likely too small a sample size to accurately capture within-population variation. Interestingly, frogs that had high alkaloid peaks around 16–19 min by GC/MS were all male, whereas the other two frogs with low alkaloid levels around this time point were female. There maybe sex differences in toxin sequestration within the population, as has been documented in some *O. pumilio* populations (Saporito et al. 2010). However, this is unlikely as both males and females were included in the Simón Bolívar and Felfa populations, which did not show much variation within the population. There are two more plausible explanations for this individual variation. First, frog age may be a contributing factor as was recently documented in the Brazilian red-belly toad (*Melanophryniscus moreirae*) (Jeckel et al. 2015). We were unable to determine the age of the *O. sylvatica* frogs, although this is likely an important variable to consider. Second, frogs may have been collected in different microhabitats, and this difference in diet availability may influence alkaloid profiles, although we did not note any obvious changes in habitat within the Cristóbal Colón sampling range. Further sampling and broader analyses of potential contributing factors within the Cristóbal Colón population are required.

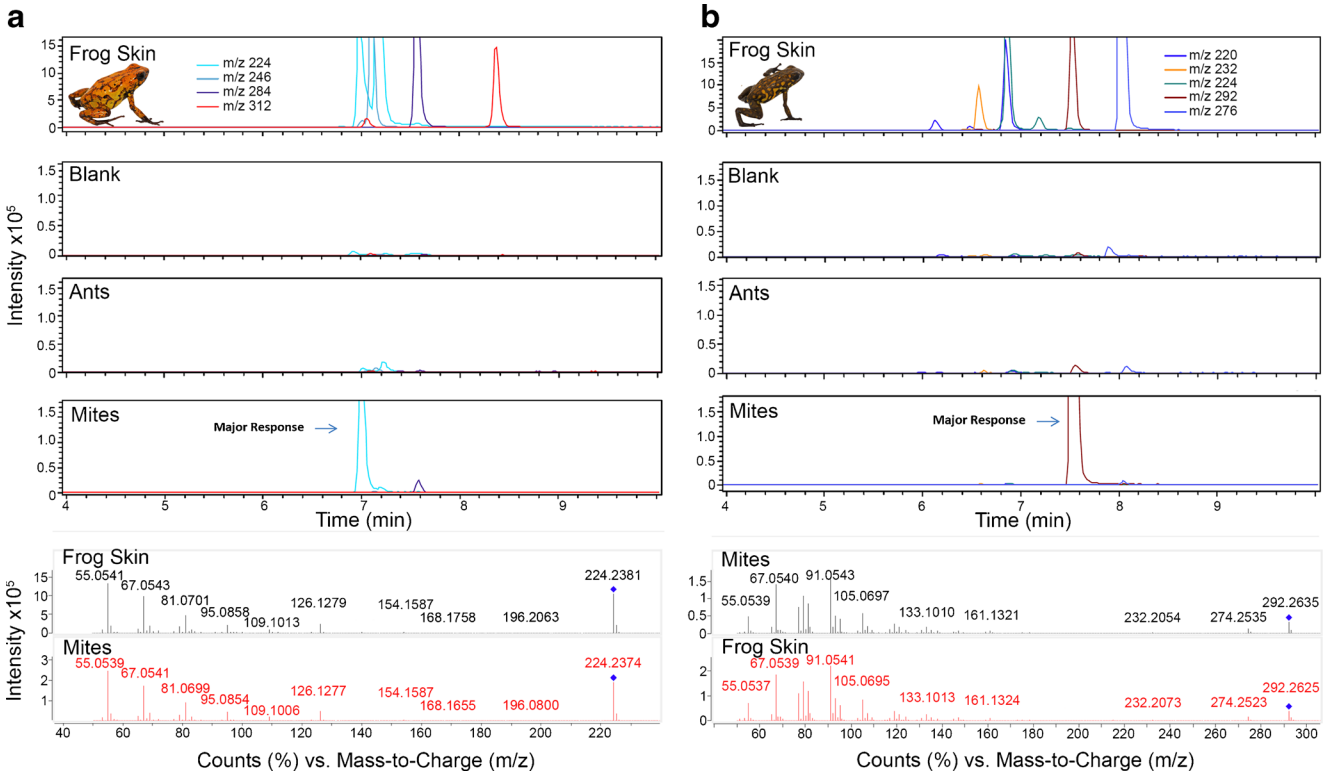


Fig. 5 Identification of mites as dietary source of 3,5-disubstituted indolizidine **223AB** and an unknown alkaloid in the Little Devil poison frog (*Oophaga sylvatica*). Liquid chromatography/mass spectrometry (LC/MS) was used to analyze alkaloids from *O. sylvatica* frog skin samples and the ants and mites found in those same frogs' stomachs. Top: Abundant alkaloids in the frogs were selected for extracted ion chromatogram analysis. Each compound is represented as a different color and mass-to-charge (*m/z*) ratios are listed in the top of each frog panel. In both comparisons from two different frogs, a major alkaloid response (labeled as "Major Response") was detected in the mite sample that was similar to a response found in the frog skin sample. Bottom: Tandem mass spectrometry fragmentation patterns via 15 eV product ion mass spectra shows close match between the compounds found in the frogs and the mites. **a** A similar response of *m/z* 224 was found in a Cristóbal Colón frog skin sample and the corresponding mite sample. Comparison with the GC/MS data from this frog suggests the identity of this shared alkaloid is 3,5-disubstituted indolizidine **223AB**. **b** A similar response of *m/z* 292 was found in a Simón Bolívar frog skin sample and the corresponding mite sample. Identification of this alkaloid is not possible, as it was not observed in GC/MS data for this frog

Arthropod Diversity in Diet of Poison Frogs Poison frog dietary specialization on ants and mites is well established (Caldwell 1996; Darst et al. 2005). As poison frogs sequester toxins from their diet, it has been proposed that variation in frog toxins reflects diversity in dietary arthropods (Saporito et al. 2009). The stomach contents of three *O. sylvatica* populations contained over 80 % ants and mites by number. Ants in particular composed at least 50 % of the diet in these populations. Our results are similar to those reported for *O. histrionica* (Osorio et al. 2015), a closely related species in Colombia, whose diet is mainly composed of Formicidae (ants), Acari (mites), and Coleoptera (beetles). These three arthropod families account for over 97 % of the diet (by number) of the *O. sylvatica* populations we investigated. Felfa frogs tended to have more ants in the stomach contents, and ants have been proposed to contain mostly unbranched alkaloids (Saporito et al. 2012). We also found that the major alkaloids in the Felfa frogs were DHQs **195-A** and **223F**, which have an ant origin (Jones et al. 1999). Both Cristóbal Colón and Simón Bolívar populations had higher proportion of mites in their stomach contents (either by number or volume, respectively). Mites have been proposed as the main source of branched-chain alkaloids (Saporito et al. 2007b, 2011).

Ants Ant genera and chemical diversity reflects the heterogeneity of *O. sylvatica* toxin profiles across populations. Ant metabolomics data clustered by frog population in a PCA, suggesting geographic differences in toxin availability within ant prey. We identified ant genera in *O. sylvatica* stomachs that often are consumed by other poison frogs such as *Pheidole*, *Crematogaster*, *Cyphomyrmex*, *Octostruma*, *Gnamptogenys*, *Solenopsis*, *and Wasmannia* (Gómez-Hoyos et al. 2014; Mebs et al. 2014; Osorio et al. 2015). Most consumed ants were in the Subfamily Myrmicinae Tribe Attini, the fungus-cultivating ants. Specimens in the genus *Solenopsis* were present in all frog populations, but likely represent different species or subspecies given their diversity in CO1 sequence and morphology. *Solenopsis* ants have been identified as a source for decahydroquinolines (DHQs) (Daly

et al. 2000; Jones et al. 2012), and we detected DHQ **195B** in Felfa frogs that also consumed *Solenopsis* ants. Although *Solenopsis* species and DHQs were observed in Felfa, we did not detect DHQs in Cristóbal Colón or Simón Bolívar frogs, which also consumed *Solenopsis* ants. This discrepancy between the consumption of *Solenopsis* ants by all three *O. sylvatica* populations and the distinct chemistry among these populations is a caution against assuming every *Solenopsis* ant species is chemically similar or that every ingested arthropod found in a poison frog stomach contributes to the alkaloid repertoire of the frog. Finally, *Pheidole* ants also are known to be predators of Oribatid mites (Wilson 2005), which carry alkaloid toxins identified in frogs. This raises the possibility of toxin transfer from mites through ants to frogs, as well as from mites to frogs directly (Saporito et al. 2011).

Mites We found the genera and chemical diversity of mites partially reflects the variation in toxin profiles across *O.sylvatica* populations. We sequenced the CO1 region from nine mite specimens representing six undescribed species. The closest match in the GenBank nr database for all samples are Oribatid mites, which are a source of many poison frog alkaloids (Saporito et al. 2007b). Interestingly, Saporito et al. (2011, 2015) has shown that Oribatid mites carry many alkaloids found on poison frogs as well as many other non-frog alkaloids. A recent study showed tropical mites carry more alkaloids than temperate mites (Saporito et al. 2015), although it is not clear if poison frog alkaloid variation reflects diversity in Oribatid mite species across a small geographic gradient. Our PCA of mite metabolomics data shows only partial clustering by frog populations, but more chemical profiling of mites is needed to determine how they contribute to geographic variation. Moreover, Oribatid mites are extremely diverse (Franklin et al. 2004; Saporito et al. 2007b), which may explain why PCA clustering of mite chemical data was not clear. Mites are a rich biochemical resource for poison frog chemical defense and should be studied in this context more systematically.

Tracing the Source of Poison Frog Toxins Since the dietary hypothesis of poison frog toxicity was proposed in the 1990s (Daly et al. 1994a, b), there has been a focus on identifying the arthropods harboring these alkaloids. Many of the classes have been identified in either ants or mites (or both) as well as in poison frogs (Santos et al. 2015; Saporito et al. 2009, 2012). For example, histrionicotoxins and pumiliotoxins have been identified in ants (Jones et al. 2012; Saporito et al. 2004). However, it has been historically difficult to identify poison frog alkaloids in arthropods given the small size and low alkaloid content per insect specimen, as well as the difficulty in obtaining species that poison frogs actually ingest from the leaf litter.

To identify the arthropod source of *O. sylvatica* toxins, we took a simple approach of pooling separately the ants and mites found in the frogs' stomachs. This allowed us to examine the chemistry of arthropods the frogs ingested rather than surveying the surrounding leaf litter that may contain many arthropods the frogs do not eat. Moreover, the high sensitivity of LC/MS allowed us to detect small quantities of alkaloids that may be undetectable by GC/MS. However, as the alkaloid data library by Daly and colleagues is based on GC/MS data (Daly et al. 2005), examining frog skin alkaloids must be done with both GC/MS (for alkaloid identification) and LC/MS (for comparison with arthropod data). This dual approach allowed us to identify the arthropod source of three alkaloids (**277-A**, **203-A**, and **223AB**), but for one alkaloid we were not able to tentatively assign an identity given it was not detected using GC/MS. Our experimental design for LC/MS limits our ability to identify the exact species of ant or mite in which each alkaloid was found. Although we photographed the arthropods prior to alkaloid extraction, we cannot reliably identify the species within the pooled sample without genetic testing. Moreover, LC/MS profiling of stomach contents represents a snapshot in time. Many frog alkaloids were not detected in the arthropod samples, which is not surprising since the collected samples do not represent the full repertoire of dietary diversity in these frogs. We cannot rule out contamination among insects in the stomach that may have been partially digested or from a previous eating bout. However, given that detected alkaloids were specific to either ants or mites, the likelihood of cross-insect contamination was low. Finally, many of the frog toxins found on the skin could have been acquired from the diet weeks to months prior to our sampling. A combination of non-invasive and repeated sampling of prey arthropods and frog skin toxins would be a step forward to resolving some of the pitfalls of current methods for identifying the trophic source of frog toxins.

We have provided evidence of the dietary source of three alkaloid toxins. We found lehmizidine **277-A** in frogs of the Cristóbal Colón population and traced the dietary source of this toxin to ants. Ants have already been proposed as the source of lehmizidines for poison frogs, since monosubstituted lehmizidines occur in a myrmicine ant (Jones et al. 2007), but we provide here the first evidence for this trophic relationship. We also were able to identify ants as the source of the 5,8-disubstituted indolizidine **203-A** in frogs from Felfa. This alkaloid (**203-A**) also has been observed in mites, and other 5,8-disubstituted indolizidines have been identified in both ants and mites (Daly et al. 2002; Saporito et al. 2007b). Finally, we were able to trace the source of the 3,5-disubstituted indolizidine **223AB** in Cristóbal Colón frogs to mites. Alkaloid **223-A** has previously been reported in mites, and 3,5-disubstituted indolizidines have been attributed to both ants and mites (Jones et al. 1999; Saporito et al. 2007b). Finally, although we identified an alkaloid (*m/z* 292)

in both frog skin from Simón Bolívar and mites found in that same frogs' stomach, we were unable to identify the alkaloid given the lack of LC/MS library data for these chemicals.

In summary, we have described here the alkaloid profiles of three populations of *O. sylvatica*, the arthropods that compose their distinctly different diets, and have identified the dietary source of four poison frog alkaloids. Moreover, our results highlight how arthropod species and chemical diversity drives variation in poison frog chemical defenses. Future work will focus on identifying the specific arthropod species that harbor these alkaloids and profiling additional *O. sylvatica* populations.

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