Molecular physiology of chemical defenses in a poison frog

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Abstract

Poison frogs sequester small molecule lipophilic alkaloids from their diet of leaf litter arthropods for use as chemical defenses against predation. Although the dietary acquisition of chemical defenses in poison frogs is well-documented, the physiological mechanisms of alkaloid sequestration has not been investigated. Here, we used RNA sequencing and proteomics to determine how alkaloids impact mRNA or protein abundance in the Little Devil Frog (*Oophaga sylvatica*) and compared wild caught chemically defended frogs to laboratory frogs raised on an alkaloid-free diet. To understand how poison frogs move alkaloids from their diet to their skin granular glands, we focused on measuring gene expression in the intestines, skin, and liver. Across these tissues, we found many differentially expressed transcripts involved in small molecule transport and metabolism, as well as sodium channels and other ion pumps. We then used proteomic approaches to quantify plasma proteins, where we found several protein abundance differences between wild and captive frogs, including the amphibian toxin binding protein saxiphilin. Finally, because many blood proteins are synthesized in the liver, we used thermal proteome profiling as an untargeted screen for soluble proteins that bind the alkaloid decahydroquinoline. Using this approach, we identified several candidate proteins that interact with this alkaloid, including saxiphilin. These transcript and protein abundance patterns in chemically defended and alkaloid-free frogs suggest the presence of alkaloids influences frog physiology and that small molecule transport proteins may be involved in toxin bioaccumulation in dendrobatid poison frogs.

Resumen

Las ranas venenosas obtienen moléculas lipofílicas a partir de su dieta de artrópodos que luego usan como una defensa química contra depredadores. Mientras la acumulación de toxinas dietéticas ha sido bien documentada, el mecanismo fisiológico de obtención de alcaloides sigue sin ser investigado. En este estudio usamos secuenciación de RNA y proteómica para determinar cómo la presencia de alcaloides afecta la abundancia de mRNA y proteínas en ranas diablito (*Oophaga sylvatica*) silvestres con defensas químicas en comparación a ranas diablito criadas en cautiverio con una dieta sin alcaloides. Para entender cómo las ranas venenosas mueven los alcaloides de su dieta a las glándulas granulares en su piel, nos enfocamos en medir la expresión de genes en tres tejidos: intestinos, piel e hígado. En estos tejidos, encontramos varios transcriptomas regulados diferencialmente que tienen actividades involucradas con el transporte y metabolismo de pequeñas moléculas, además de canales de sodio y bombas de iones. Luego usamos métodos proteómicos para cuantificar proteínas en plasma, donde encontramos varias diferencias en abundancia de proteínas entre las ranas silvestres y de laboratorio, incluyendo la proteína anfibia de fijación de toxinas, saxifilina. Finalmente, porque muchas proteínas encontradas en la sangre se sintetizan en el hígado, usamos la técnica de perfilación proteómica termal para seleccionar imparcialmente las proteínas solubles que fijan el alcaloide decahydroquinolina. Usando este método, identificamos varias posibles proteínas que interactúan con este alcaloide, incluyendo saxifilina. Estos patrones de cambios en abundancia de transcriptomas y proteínas en ranas con y sin defensas químicas sugieren que la presencia de alcaloides influye en la fisiología de las ranas y que pequeñas moléculas proteicas de transporte podrían estar involucradas en la bioacumulación de toxinas en ranas venenosas dendrobátidos.

73 ## Introduction

74 Many animals, from microbes to vertebrates, have chemical defenses to guard against
75 predation. Although microbes and plants produce secondary metabolites that can serve as toxins
76 [1,2], most animals cannot synthesize small molecule toxins themselves. While some animals like
77 snakes and arachnids produce genetically encoded peptide toxins, other vertebrate animals,
78 including some amphibians, reptiles, and birds, carry small molecule toxins that are acquired from
79 dietary sources [3]. Invertebrates can also sequester small molecule toxins from their diet and this
80 process is well understood in beetles, moths and butterflies [4]. Many decades of research has
81 focused on cataloging the small molecules sequestered by vertebrates and documenting the
82 trophic relationships of these chemically defended animals and their prey [3]. However, much less
83 is known about the physiological adaptations in vertebrates that must occur to resist self-
84 intoxication and uptake small molecule toxins from the diet. In the present study, we explored the
85 physiological changes associated with acquired chemical defenses in a South American poison
86 frog (*Oophaga sylvatica*, family Dendrobatidae) to better understand adaptations that allow toxin
87 bioaccumulation.

88 The ability to sequester small molecule toxins from dietary sources has evolved many
89 times in frogs, including Neotropical dendrobatids and bufonids, Malagasy mantellids, Australian
90 myobatrachids (*Pseudophryne*) and Cuban eleutherodactylids [5]. These frogs uptake lipophilic
91 alkaloids from arthropod prey items, store them in skin granular glands and secrete them as a
92 defensive response [6]. In order to utilize diet-derived toxins, frogs may be resistant to alkaloids
93 they are accumulating through mutations in target proteins. Many alkaloids target voltage-gated
94 sodium channels and nicotinic acetylcholine receptors in the nervous system and some studies
95 have found ion channel mutations that confer toxin resistance in poison frogs [6–9]. Detailed
96 information on binding properties of a wide range of poison frog alkaloids on many different ion
97 channel types is lacking as early studies focused on a few types of alkaloids and ion channels.

98 Despite the progress on understanding the evolution of autoresistance and chemical defenses in
99 poison frogs, no study has examined other molecular aspects of physiological adaptations that
100 allow toxin sequestration.

101 To accomplish the sequestration of alkaloids, poison frogs selectively move toxins from
102 the gastrointestinal tract to the skin glands for storage. In many animals, toxic compounds are
103 unable to pass through the intestinal wall for absorption due to a series of membrane transporters
104 in the gut epithelium. These transporters bind compounds and move them either into circulation
105 or back into the intestinal lumen for excretion [10]. Once absorbed in the intestines, lipophilic
106 compounds can be circulated by a number of different mechanisms, including binding by carrier
107 proteins in the blood circulation or within lipoprotein particles like chylomicrons in the lymph
108 system [11]. In many animals, dietary compounds in the blood circulation are metabolized by the
109 liver and there is some evidence that poison frogs can metabolize dietary alkaloids. Daly and
110 colleagues suggested that some (but not all) dendrobatid poison frog species can metabolize
111 pumiliotoxin **251D** into allopumiliotoxin **267A** [12], although the mechanisms underlying this
112 observation remain unknown. Clearly coordination across several tissue systems is required for
113 alkaloid sequestration in poison frogs, but the physiological basis for alkaloid bioaccumulation
114 remains unexplored.

115 The goal of this present study was to better understand the physiology of acquired
116 chemical defenses in dendrobatid poison frogs. In surveying the alkaloid profiles of wild caught
117 Little Devil frogs (*Oophaga sylvatica*) and juveniles captured in the wild but raised in laboratory
118 on an alkaloid-free diet, we had an opportunity to examine physiological differences associated
119 with chemical defenses. We compared gene expression in the intestines, liver, and skin, as well
120 as plasma protein abundance in wild caught chemically defended frogs and lab-raised alkaloid-
121 free frogs. To further functionally identify proteins that may bind toxins for transport, we performed
122 a thermal proteome profiling assay using liver protein lysate from a lab frog. As toxins are
123 acquired from the diet and transported to the skin granular glands for storage, we expected that

124 the presence of toxins would cause an increase in expression of genes associated with small
125 molecule transport or metabolism.
126

127 ## Materials and Methods

128 ### Field collection of *Oophaga sylvatica*

129 Little Devil (or Diablito) frogs (*Oophaga sylvatica*) were collected during the day near the
130 Otokiki Reserve (N=13) maintained by WIKIRI/Fundación Otonga in northwestern Ecuador in July
131 2013 [13]. Frogs were individually stored in plastic bags with air and vegetation for 2–5 hours. In
132 the evening the same day of capture, adult frogs were anesthetized with a topical application of
133 20% benzocaine to the ventral belly and euthanized by cervical transection. Several juvenile frogs
134 were transported to Centro Jambatu de Investigación y Conservación de Anfibios in Quito,
135 Ecuador (CJ), where they were individually housed and fed a diet of crickets and fruit flies for 6
136 months before being sacrificed as adults as described above. For each individual, the intestines
137 and liver were dissected and stored in RNALater (Life Technologies, Carlsbad, CA, USA). Half of
138 the dorsal skin was stored in RNALater while the other half was stored in methanol for later
139 chemical analysis. Remaining frog tissues were preserved in 100% ethanol and deposited in the
140 amphibian collection of CJ. Additional frogs (N=6) were collected in January 2014 in the Otokiki
141 Reserve and, along with some captive adults raised at CJ (N=6), were treated as described above
142 with one additional procedure: after euthanasia, trunk blood was collected into a heparinized
143 microcentrifuge tube. Blood was centrifuged and plasma was isolated into a fresh tube and flash
144 frozen in liquid nitrogen. Collections and exportation of specimens were done under permits (001-
145 13 IC-FAU-DNB/MA, CITES 32/VS and 37/VS) issued by the Ministerio de Ambiente de Ecuador.

146 The Institutional Animal Care and Use Committee of Harvard University approved all procedures
147 (Protocol 15-02-233).

148 **Gas chromatography / mass spectrometry (GC/MS)**

149 Frog dorsal skin samples (N=5 wild caught and N=3 lab raised frogs) were used to
150 characterize alkaloid profiles as previously described [14]. As an internal standard, 25 µg of D3-
151 nicotine in methanol (Sigma-Aldrich, St. Louis, MO) was added to each sample prior to alkaloid
152 extraction. After extraction in methanol, the samples were evaporated to dryness under nitrogen
153 gas and reconstituted in 0.5 mL of methanol by vortexing. The samples were transferred to a
154 microcentrifuge tube and spun at 12000 rpm for 10 min. A 200 µL aliquot of the supernatant was
155 transferred to a 0.3 mL glass insert in an amber sample vial and stored at -20°C until analysis.

156 GC/MS parameters were conducted as previously described [14], which is based on a
157 slight modification of the method reported by Saporito and colleagues [15]. Briefly, analyses were
158 performed on a Waters Quattro Micro system (Beverly, MA) with an Agilent 6890N GC (Palo Alto,
159 CA). Each total ion chromatogram was reviewed and alkaloid responses were characterized from
160 their mass spectra and retention times. The extensive database developed by Daly and
161 colleagues [16] was used to identify alkaloids by matching major mass spectrum peaks and
162 relative retention times using D3-nicotine as a reference. AMDIS (NIST) was used in conjunction
163 with the database to identify candidate alkaloids peak. Those peaks were then manually inspected
164 and the confirmed alkaloids peaks were then integrated. Unidentifiable compounds were removed
165 from data analysis. The relative abundance values were normalized by nicotine and were
166 visualized using the heatmap.2 R function in the gplots package. A representative extracted ion
167 chromatograms for each group was normalized to the same scale to create a visual overview. All
168 mass spectrometry data is available at DataDryad (submission pending).

169

170 **Reference transcriptome**

171 To build a reference transcriptome for gene expression analyses, we sequenced tissues
172 from a single *O. sylvatica*. We isolated intestines, liver and dorsal skin from a single lab-bred *O.*
173 *sylvatica* frog (Indoor Ecosystems Whitehouse, Ohio, USA) for the purpose of *de novo*
174 transcriptome assembly. Flash frozen tissue samples were placed in Trizol (Life Technologies,
175 Grand Island, NY) where RNA was extracted according to manufacturer instructions. Poly-
176 adenylated RNA was isolated from each sample using the NEXTflex PolyA Bead kit (Bioo
177 Scientific, Austin, TX, USA) according to manufacturer instructions. Lack of contaminating
178 ribosomal RNA was confirmed using the Agilent 2100 Bioanalyzer. Strand specific libraries for
179 each sample (intestines, liver, and skin) were prepared using the dUTP NEXTflex RNAseq kit
180 (Bioo Scientific), which includes a magnetic bead-based size selection. Libraries were pooled in
181 equimolar amounts after library quantification using both quantitative PCR with the KAPA Library
182 Quantification Kit (KAPA Biosystems, Wilmington, MA, USA) and the fluorometric Qubit dsDNA
183 high sensitivity assay kit (Life Technologies), both according to manufacturer instructions.

184 Libraries were sequenced on an Illumina HiSeq 2500 over a full flow cell to obtain
185 433,951,467 paired-end 250 bp reads. We first corrected errors in the Illumina reads using
186 Rcorrector ([17]; parameters: run_rcorrector.pl -k 31) and then applied quality and adaptor
187 trimming using Trim Galore! (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/;
188 parameters: trim_galore --paired --phred33 --length 36 -q 5 --stringency 5 --illumina -e 0.1). We
189 used Trinity to *de novo* assemble the *O. sylvatica* transcriptome ([18], parameters: --seqType fq
190 --SS_lib_type RF --normalize_reads). The Trinity assembly produced 2,111,230 contigs (N50:
191 738 bp). To reduce redundancy in the assembly, we ran cd-hit-est ([19]; parameters: -c 0.98)
192 resulting in 1,596,373 contigs (N50: 789 bp). For the purpose of our current study, we were
193 specifically interested in putative protein coding transcripts to gain insight into the physiological
194 mechanisms of toxin sequestration. We used blastx to compare contigs in the assembly to

195 proteins in the Uniprot Swiss-Prot database (e-value threshold of 1e-5) and retained 241,116
196 contigs (N50: 2169 bp) with homology to annotated proteins. As we were particularly interested
197 in frog transcripts, we removed contigs that had homology to non-vertebrate proteins in the
198 Uniprot Swiss-Prot database, including microbes, nematodes, and arthropods. These
199 contaminants that likely represent parasites, pathogens, and prey items represented roughly 20%
200 of the contigs in the draft assembly. Our final *O. sylvatica* draft assembly contained 194,309
201 contigs (N50=2280 bp). We then assessed the completeness of this filtered assembly by
202 examining vertebrate ortholog representation using BUSCO (Benchmarking Universal Single-
203 Copy Orthologs) [20]. BUSCO estimated the completeness of our assembly at 84% (700)
204 complete single-copy BUSCOs, with 61% (1862) duplicated BUSCOs, 5.9% (181) fragmented
205 BUSCOS and 9.2% (280) missing BUSCOs out of 3023 total BUSCO groups searched.

206 We annotated the transcriptome using Trinotate [21] and used this final assembly for
207 downstream gene expression analyses. We used TransDecoder
208 (https://github.com/TransDecoder/TransDecoder/wiki) to generate a peptide file populated by the
209 longest open reading frame for each contig, which was then annotated using blastp to the UniProt
210 database and hmmscan to the PFam-A database. The results of these database queries were
211 loaded into an SQlite database.

212 **RNA sequencing and analysis**

213 The alkaloid differences between wild and lab raised frogs presented a unique opportunity
214 to examine gene expression differences across chemically defended and alkaloid-free frogs (see
215 results). We focused on the liver and skin, as alkaloid compounds have been detected in these
216 tissues in other poison frog species previously [5]. We also analyzed gene expression in the
217 intestines as a likely location of alkaloid absorption [22]. We isolated RNA from the intestines,
218 liver, and dorsal skin of the lab raised (alkaloid-free) and wild caught frogs, including the same

219 individuals used for the GC/MS analysis. Illumina libraries were constructed as described above
220 and sequenced on an Illumina HiSeq 2000 over 6 lanes to obtain 910,220,544 paired-end 100bp
221 reads. All Illumina data is available on the Sequence Read Archive (submission pending).

222 We first applied quality and adaptor trimming using Trim Galore!
223 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/; parameters: trim_galore --
224 paired --phred33 --length 36 -q 5 --stringency 5 --illumina -e 0.1). We analyzed tissues separately
225 because some individuals were removed from specific comparisons because these samples had
226 fewer than 6 million reads. We aligned the reads to the *O. sylvatica* reference transcriptome (see
227 above) using kallisto [23]. Differences in gene expression levels between samples were
228 calculated using edgeR [24] and DESeq2 [25] within the *in silico* Trinity pipeline ($p < 0.05$, 4-fold
229 change). We planned on retaining significant differences in transcript abundance if this difference
230 was present in both the edgeR and DESeq2 analyses. However, all transcripts with significant
231 differences in DESeq2 were present in the edgeR significant gene set, but not vice versa, where
232 there were 2-3 times the number of significant genes in the edgeR analysis. Thus, only DESeq2
233 results are presented in the manuscript to be more conservative. All gene expression results from
234 the DESeq2 analysis were loaded into the SQlite database created from the Trinotate pipeline.
235 The R package GOseq [26] was used to examine gene ontology enrichment in transcript
236 expression for each tissue within the *in silico* Trinity pipeline. A subset of differentially expressed
237 genes were visualized using boxplots, which were made with the R package *ggplot2* (R version
238 3.4.3).

239 **Plasma proteomics**

240 As alkaloids can potentially be transported through the blood circulation to the skin glands
241 for storage, we quantified plasma protein abundance in chemically defended wild caught frogs
242 and alkaloid-free lab-reared frogs. To have enough protein concentration for tandem mass tag

243 labeling and quantitative proteomics, plasma was pooled for two frogs to create three biological
244 replicates per group. Tandem Mass Tag (TMT; Thermo Scientific, Waltham, MA, USA) labeling
245 was done according to manufacturer's instructions. Samples were off-line fractionated prior to
246 liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis performed on a LTQ
247 Orbitrap Elite (Thermo Scientific) equipped with a Waters NanoAcquity high performance liquid
248 chromatography (HPLC) pump (Milford, MA, USA). Peptides were separated onto a 100 μm inner
249 diameter microcapillary trapping column packed first with approximately 5 cm of C18 Reprosil
250 resin (5 μm, 100 Å, Dr. Maisch GmbH, Germany) followed by analytical column ~20 cm of Reprosil
251 resin (1.8 μm, 200 Å, Dr. Maisch GmbH, Germany). Separation was achieved through applying a
252 gradient from 5–27% ACN in 0.1% formic acid over 90 min at 200 nl min−1. Electrospray
253 ionization was enabled through applying a voltage of 1.8 kV using a home-made electrode
254 junction at the end of the microcapillary column and sprayed from fused silica pico tips (New
255 Objective, Woburn, MA, USA). The LTQ Orbitrap Elite was operated in data-dependent mode for
256 the mass spectrometry methods. The mass spectrometry survey scan was performed in the
257 Orbitrap in the range of 395 –1,800 m/z at a resolution of 6 × 104, followed by the selection of the
258 twenty most intense ions (TOP20) for CID-MS2 fragmentation in the ion trap using a precursor
259 isolation width window of 2 m/z, AGC setting of 10,000, and a maximum ion accumulation of 200
260 ms. Singly charged ion species were not subjected to CID fragmentation. Normalized collision
261 energy was set to 35 V and an activation time of 10 ms. Ions in a 10 ppm m/z window around ions
262 selected for MS2 were excluded from further selection for fragmentation for 60 s. The same
263 TOP20 ions were subjected to HCD MS2 event in Orbitrap part of the instrument. The fragment
264 ion isolation width was set to 0.7 m/z, AGC was set to 50,000, the maximum ion time was 200
265 ms, normalized collision energy was set to 27V and an activation time of 1 ms for each HCD MS2
266 scan. All mass spectrometry data is available at DataDryad (submission pending).

267 Raw data were analyzed using Proteome Discoverer 2.1.0.81 software (Thermo
268 Scientific). Assignment of MS/MS spectra was performed using the Sequest HT algorithm by
269 searching the data against the *O. sylvatica* protein sequence database and other known
270 contaminants such as human keratins and common lab contaminants. To create an mRNA-based
271 protein reference database for peptide matching, we used the PHROG tool (**P**roteomic Reference
272 with **H**eterogeneous **R**NA **O**mitting the **G**enome; [27];
273 http://kirschner.med.harvard.edu/tools/mz_ref_db.html). We used as input the Trinity fasta file
274 that had cd-hit-est applied (see above) containing hits to the Uniprot Swiss-Prot database (both
275 vertebrates, invertebrates, and other potential contaminants like microbes). Briefly, this tool
276 identifies ORF frames, frameshift corrections and annotation using blastx to a deuterostome
277 database. The longest peptide is retained and database redundancy is removed with cd-hit. This
278 output is then used as the protein reference for peptide matching. Sequest HT searches were
279 performed using a 20 ppm precursor ion tolerance and requiring each peptides N-/C termini to
280 adhere with Trypsin protease specificity, while allowing up to two missed cleavages. 6-plex TMT
281 tags on peptide N termini and lysine residues (+229.162932 Da) was set as static modifications
282 while methionine oxidation (+15.99492 Da) was set as variable modification. A MS2 spectra
283 assignment false discovery rate (FDR) of 1% on protein level was achieved by applying the target-
284 decoy database search. Filtering was performed using Percolator [28]. For quantification, a 0.02
285 m/z window centered on the theoretical m/z value of each the six reporter ions and the intensity
286 of the signal closest to the theoretical m/z value was recorded. Reporter ion intensities were
287 exported in result file of Proteome Discoverer 2.1 search engine as an excel table. The total signal
288 intensity across all peptides quantified was summed for each TMT channel, and all intensity
289 values were adjusted to account for potentially uneven TMT labeling and/or sample handling
290 variance for each labeled channel. Sequest found 453 protein matches within the *O. sylvatica*
291 protein database across all six plasma samples. We omitted from our analysis proteins that had

292 only one peptide match. The data was then imported into Perseus proteomics analysis software
293 [29] and a t-test was used to examine differences between groups.

294 **Thermal proteome profiling**

295 A single lab-reared (alkaloid-free) *O. sylvatica* frog was purchased from the Indoor
296 Ecosystems (Whitehouse, Ohio, USA) and sacrificed as described above to conduct the thermal
297 proteome profiling experiment [30]. This assay relies on thermodynamic principles of protein-
298 ligand binding, where a protein that has bound its small molecule target has increased thermal
299 stability, a shift detectable by tandem mass spectrometry. The liver was dissected, flash frozen in
300 liquid nitrogen, and stored at -80°C for 2 weeks. On the day of processing, the liver was crushed
301 in liquid nitrogen with a mortar and pestle. Powdered tissue was then scraped into a Dounce
302 tissue homogenizer and 1.2 mL of ice-cold 1X PBS supplemented with EDTA-free cOmplete
303 protease inhibitor cocktail (Roche, Indianapolis, IN, USA) was added. After the tissue powder was
304 homogenized with 10 plunges of the homogenizer, the cell suspension was transferred to a chilled
305 microcentrifuge tube. For cell lysis, the sample was placed in liquid nitrogen for 1 min followed by
306 a brief thaw at room temperature, and then this cycle was repeated twice. Cell lysate was
307 separated from cellular debris by spinning 20,000xg for 20 min at 4°C. The sample was then
308 divided in half (~400 uL) and either 4 uL of 10mM DHQ (decahydroquinoline, Sigma) in DMSO
309 (100 mM final concentration) or 4 uL of DMSO (vehicle treatment) was added. Lysate was
310 incubated for 30 min at room temperature and then 35 uL of each samples was aliquoted into
311 each of 10 PCR tubes. PCR tubes were heated at different temperatures (67°C, 64.3°C, 58.5°C,
312 56.2°C, 53°C, 50°C, 47.4°C, 45.1°C, 39.9°C, 37°C) for 3 min followed immediately by 2 min room
313 temperature incubation. Immediately after this incubation, samples were flash frozen in liquid
314 nitrogen and stored at -80°C overnight. The following morning, samples were thawed on ice and
315 transferred to 0.2 mL polycarbonate thick-wall tubes (Beckman Coulter, Danvers, MA, USA) and
316 spun at 100,000xg for 20m at 4°C in Optima Max ultracentrifuge (Beckman Coulter). Tubes were

317 carefully removed from the ultracentrifuge with forceps and 30 uL of supernatant was removed to
318 a PCR tube with every care taken to avoid the protein pellet. Soluble protein lysate was flash
319 frozen in liquid nitrogen and stored at -80°C until downstream processing. Samples were then
320 TMT labeled and analyzed using LC MS/MS as described above and following [30]. Data was
321 analyzed using the TPP package in R, which fits protein abundances to a curve and generates p-
322 values for proteins with a significant thermal shift when incubated with the ligand of interest
323 compared to the vehicle control. Thermal shift graphs were output using the TPP package and
324 customized using ggplot in R.

325

326 # Results

327 ## Alkaloid profiles of wild and captive frogs

328 Wild caught frogs contain many alkaloid chemicals (Figure 1, Supplementary Excel File),
329 the most abundant of which include four decahydroquinolines (**219A**, **219C**, **223Q**, **251A**), several
330 3,5,-disubstituted indolizidines (**223AB** and **249A**), a 5,8-disubstituted indolizidine (**235B**), two
331 lehmizidines (**277A** and **275A**), and an unclassified alkaloid with unknown structure (**283C**).
332 Control frogs captured as juveniles and housed in laboratory for 6 months on a diet of fruit flies
333 and crickets contained only trace or undetectable quantities of alkaloids compared to wild caught
334 frogs. The major compounds detected in lab-reared frogs were nicotine-D3 (spiked internal
335 reference), benzocaine (anesthetic), and cholesterol remaining from the extraction process.

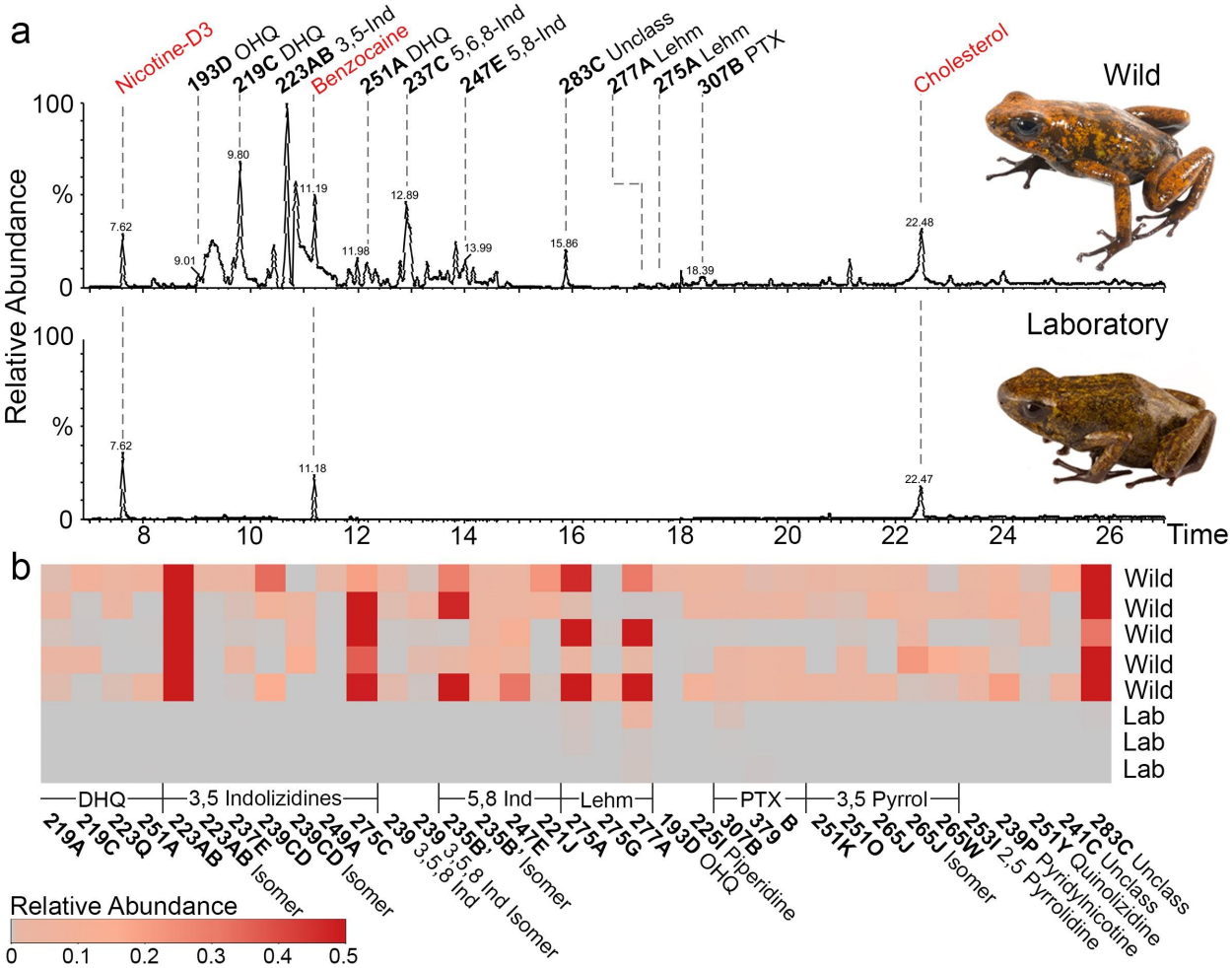


Figure 1. Skin alkaloid abundance differs between wild caught and laboratory-reared frogs from the Otokiki population of *Oophaga sylvatica*. Alkaloid profiles assayed by gas chromatography / mass spectrometry (GC/MS) show chemical defenses in wild caught (top) and laboratory (bottom) frogs. **(a)** Ion chromatograms show small molecule peaks (labels at the top); compounds common to both groups are highlighted in red. **(b)** Heatmap shows relative abundance of alkaloid compounds in each frog, highlighting the zero or trace alkaloid quantities in lab-reared frogs compared to wild caught frogs. Abbreviations: DHQ, decahydroquinoline; 5,8 Ind, 5,8, disubstituted indolizidines; Lehm, lehmizidine; PTX, pumiliotoxin; 3,5 Pyrrol, 3,5 pyrrolizidine.

Gene expression changes associated with chemical defenses

We next examined how chemical defenses acquired through differences in diet impact physiology by comparing gene expression in several tissues of chemically defended wild caught frogs and captive frogs reared on an alkaloid-free diet. As alkaloids are acquired from the diet and stored in the skin granular glands, we used RNA sequencing to measure gene expression in the intestines, skin, and liver.

Intestines. Comparing the intestinal gene expression profiles of wild caught and lab-raised frogs, 47 transcripts were differentially expressed between the two groups (Figure 2a). Chemically defended wild frogs had increased expression of 9 transcripts and decreased expression of 38 transcripts compared to the lab-reared frogs (See Supplementary Excel File). Gene ontology (GO) analyses showed enrichment of several interesting processes: molecular function included ligand gated sodium channel activity (p = 0.002), sodium:chloride symporter activity (p = 0.011), and sodium channel activity (p = 0.029). Enriched cellular compartments included sodium channel complex (p = 0.003), extracellular region part (p = 0.004), and apical plasma membrane (p = 0.004). There were many biological processes enriched in the differentially expressed genes, with the top two being sodium ion transmembrane transport (p = 0.0007) and small molecule metabolic process (p = 0.002). Given the prominence of sodium channels and small molecule metabolism in the GO processes, we further explored the expression of transcripts whose proteins are involved in small molecule transport (sodium channels and solute carrier proteins) and metabolism (cytochrome p450s) (Figure 2b), which mostly show a decrease in expression in wild caught animals compared to lab-reared alkaloid-free animals.

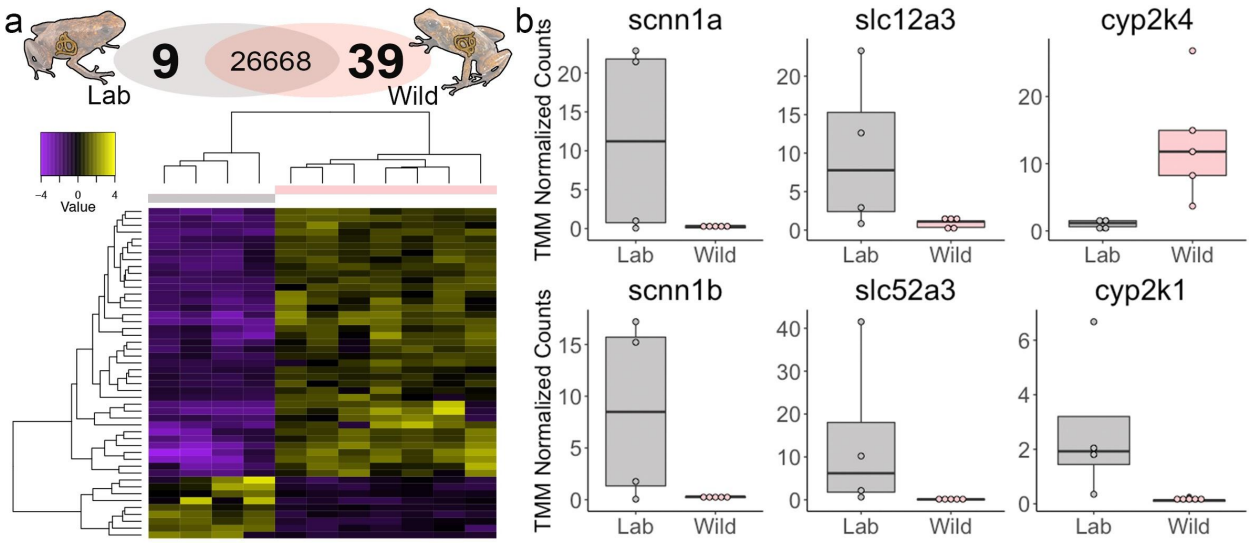


Figure 2. Intestinal gene expression differences between lab-reared and wild poison frogs. (a) Number of transcripts differentially expressed in captive reared (grey) and wild caught (pink) Little Devil

frogs is shown on the top. The heatmap shows clustering of individual frogs (columns) and differentially expressed transcripts (rows, yellow is relative increased expression and purple is relative decreased expression). **(b)** Differentially expressed genes involved in sodium or small molecule transport or metabolism. Gene name abbreviations: scnn1a, sodium channel epithelial 1 alpha subunit; scnn1b, sodium channel epithelial 1 beta subunit; slc12a3, solute carrier family 12 member 3; slc52a3, solute carrier family 52 member 3; cyp2k4, cytochrome P450 2K4; cyp2k1, cytochrome P450 2K1.

Skin. A total of 48 transcripts had significant differential expression, with chemically defended wild caught frogs having increased expression of 39 transcripts and decreased expression of 9 transcripts compared to the lab-raised frogs (Figure 3a). Only two GO enrichment terms survived false discovery rate correction, both with molecular function enrichment in RNA polymerase II function (p = 0.046). Of particular note, we found differential expression of the beta 2 subunit of the sodium potassium ATPase pump. Mutations in the alpha subunit of this ion pump are well known to be associated with chemical defenses in a number of animals [31].

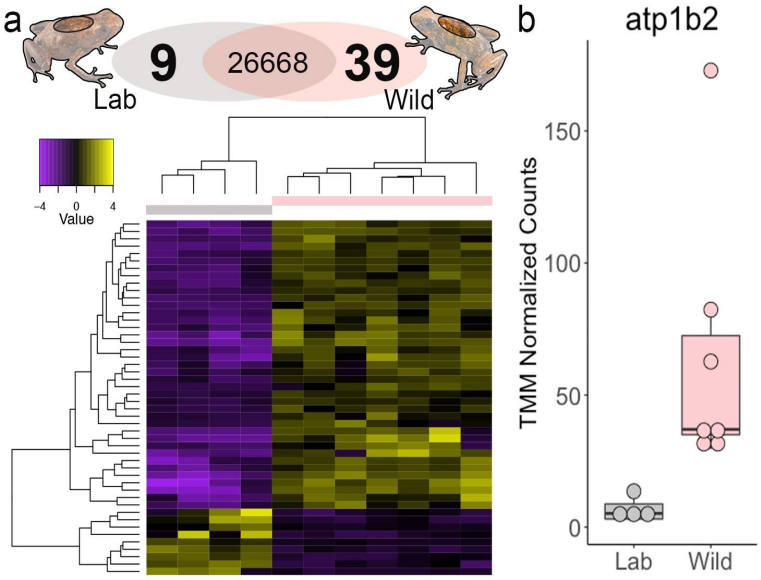


Figure 3. Skin gene expression differences between lab-reared and wild poison frogs. (a) Number of transcripts differentially expressed in captive reared (grey) and wild caught (pink) Little Devil frogs is shown on the top. The heatmap shows clustering of individual frogs (columns) and differentially expressed transcripts (rows, yellow is relative increased expression and purple is relative decreased expression). **(b)** Differentially expressed genes include the sodium-potassium-transporting ATPase subunit beta 2 (atp1b2), which is involved in sodium transport.

Liver. In the liver, 75 transcripts had significant differential expression, with chemically defended wild caught frogs having increased expression of 54 transcripts and decreased expression of 21 transcripts compared to the lab-reared frogs (Figure 4a). Although there were more transcripts differentially expressed in the liver with better group clustering compared to the intestines (see heatmap in Figure 2a), no GO enrichment terms survived false discovery rate

correction. Despite this, we noted several functional themes among the differentially expressed transcripts. There were several cytochrome p450s involved in small molecule metabolism, several small molecule transport proteins, including apolipoprotein A-IV and solute carrier proteins, and several molecular chaperones (heat shock proteins) (FIgure 4b). Most of these transcripts involved in small molecule metabolism and transport had higher expression in the wild caught chemically defended frogs compared to the lab-reared frogs.

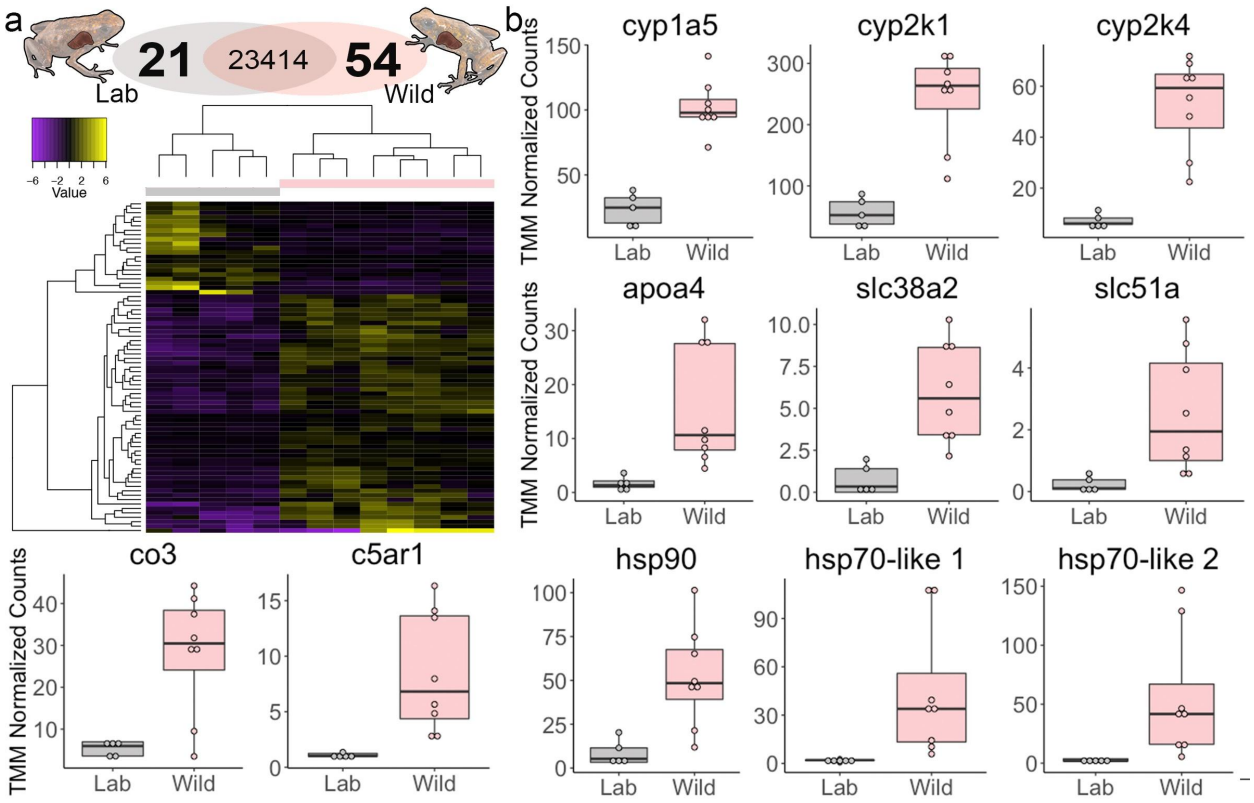
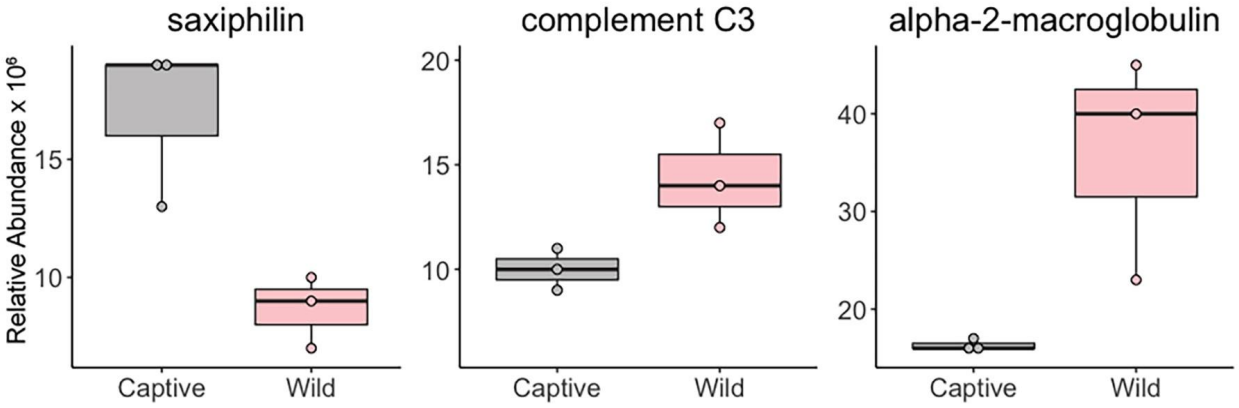


Figure 4. Liver gene expression differences between lab-reared and wild poison frogs. (a) Number of transcripts differentially expressed in lab-reared (grey) and wild caught (pink) Little Devil frogs is shown on the top. The heatmap on the bottom shows clustering of individual frogs (columns) and differentially expressed transcripts (rows, yellow is relative increased expression and purple is relative decreased expression). **(b)** Differentially expressed genes involved in small molecule transport or metabolism. Gene name abbreviations: cyp1a5, cytochrome P450 1A5; cyp2k1, cytochrome P450 2K1; cyp2k4, cytochrome P450 2K4; apoa4, apolipoprotein A-IV; slc38a2, solute carrier family 38 member 2; slc51a, solute carrier family 51 member a; co3, complement C3; c5ar1, C5a anaphylatoxin chemotactic receptor 1; hsp90, Heat shock protein HSP 90-alpha; hsp70-like 1, heat shock 70 kDa protein 1-like; hsp70-like 2, heat shock 70 kDa protein 2-like.

412 **Proteomic analyses identify candidate toxin-binding proteins**

413 We reasoned that plasma proteins may shuttle these lipophilic small molecules to their
414 final destination. Therefore, we quantified plasma proteins in chemically defended wild caught
415 frogs and lab-reared frogs reared on an alkaloid-free diet. The most abundant plasma proteins
416 identified were fetuins (fetuin-B and alpha-2-macroglobulins), apolipoproteins (A-I and A-IV),
417 albumin, hemoglobin, transferrins (serotransferrin and saxiphilin), and immune-related proteins
418 (complement factors and immunoglobulins). Ten proteins were identified as having significant
419 differences in abundance between groups, although these did not survive false discovery
420 adjustments, likely due to the small samples size (See Supplementary Excel File for data and
421 statistics). Among these proteins was saxiphilin (p=0.02), an amphibian transferrin-like protein
422 that binds the neurotoxin saxitoxin [32], which was more abundant in lab frogs (Figure 5).
423 Complement C3 was significantly higher in wild frogs compared to lab frogs raised on an alkaloid-
424 free diet (p=0.04), supporting gene expression patterns in the liver (Figure 4b). Finally, plasma
425 alpha-2-macroglobulin protein was also higher in chemically defended wild frogs compared to lab
426 frogs (p=0.05).



427

428 **Figure 5. Plasma protein abundance differences between lab-reared and wild poison frogs.** Lab-
429 reared (grey) and wild caught (pink) Little Devil frogs have abundance differences of saxiphilin, complement
430 C3, and alpha-2-macroglobulin proteins.
431

432 The liver makes many of the proteins found in blood. To identify candidate toxin-binding
433 proteins that may bind alkaloids for transport in the circulation, we used an untargeted thermal

proteome profiling approach to identify proteins that interact with unlabeled small molecules (Figure 6a) [30]. The small molecule used in this assay was decahydroquinoline, a commercially available alkaloid that we have detected in the wild caught frogs (present study) and in other *O. sylvatica* frog populations previously [14]. Using this assay, we identified several soluble proteins whose thermal profile shifted more than 4C that parallels other proteins of interest identified from above (Figure 6b). This includes saxiphilin, which was higher in the plasma of lab frogs compared to wild frogs (Figures 5), and heat shock protein 90, which had higher transcript abundance in the livers of chemically defended wild frogs compared to lab frogs raised on an alkaloid-free diet (Figure 4b).

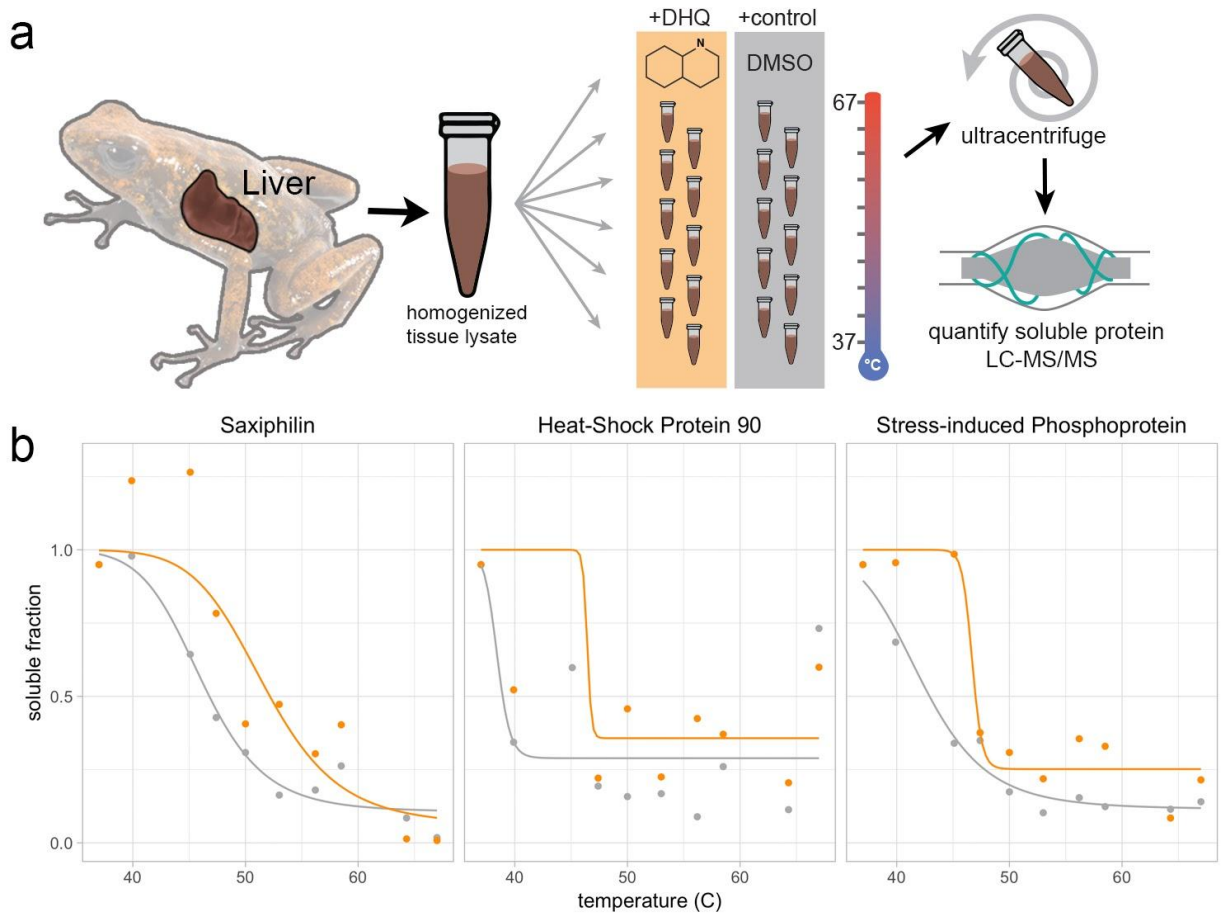


Figure 6. Thermal proteome profiling provide candidates for toxin-binding proteins in poison frogs. (a) Liver lysate was treated with either decahydroquinoline (DHQ, orange) or DMSO (vehicle control, grey) and incubated at a range of temperatures prior to ultracentrifugation followed by quantitative proteomics using liquid chromatography tandem mass spectrometry (LC MS/MS). **(b)** Examples of proteins with a greater than 4C shift in thermal stability when treated with DHQ (orange) compared to vehicle (grey).

449 # Discussion

450 By comparing the physiology of wild caught chemically defended frogs to lab-raised frogs
451 reared on an alkaloid-free diet, we were able to quantify differences in gene expression and
452 protein abundance that may be involved in pathways linked to chemical defenses. In these
453 comparisons, we found several themes in the differentially expressed transcripts in the intestines,
454 liver and skin, as well as the plasma proteome, including involvement in small molecule
455 metabolism. Moreover, several proteins were identified as possible candidate alkaloid-binding
456 proteins across gene expression and ligand-binding experiments. Here we discuss these results
457 in the context of possible physiological mechanisms of toxin sequestration and highlight the
458 limitations of the present study with suggestions for future research priorities.

459 **Dietary alkaloid compounds**

460 Many of the alkaloids identified in the Otokiki population were indolizidines and
461 lehmizidines. These small molecules have been identified in other *O. sylvatica* populations and
462 many other dendrobatid poison frogs [14,33]. All alkaloids described here have been noted in *O.*
463 *pumilio* over 30 years of intense chemical ecology research [34]. Research in the 1970s identified
464 histrionicotoxins as the major alkaloid group in *O. sylvatica* ([33], referred to as *D. histrionicus*).
465 However, our present data from the Otokiki population, as well as previous work on three other
466 *O. sylvatica* populations, did not include histrionicotoxins as the major alkaloid [14], but rather
467 lehmizidines and several classes of indolizidines. There could be several reasons for the absence
468 of histrionicotoxins in *O. sylvatica* in this study. Myers and Daly (1976) sampled *O. sylvatica* in
469 the southernmost part of their Ecuadorian range and did not examine northern populations (e.g.
470 Otokiki); perhaps southern populations contain histrionicotoxins whereas northern Ecuadorian
471 populations do not. Alternatively, since diet is a major component of poison frog alkaloid profiles
472 [35], arthropod diversity within the *O. sylvatica* range may have shifted in the last 40 years to

473 chemically distinct prey items that make indolizidines the dominant alkaloid group. Population
474 variation in toxin profiles is well established among various poison frogs species [36]-[37] and so
475 broader sampling of many populations would reveal a more complete picture of dominant
476 alkaloids in this species.

477 We detected zero to trace amounts of alkaloids in frogs moved from the wild as juveniles
478 and into laboratory for six months on a diet of crickets and fruit flies. This came as a surprise, as
479 there are some reports of poison frogs retaining toxins for long periods of time in terrariums,
480 including years for the Golden poison frog (*Phyllobates terribilis*) [38]. It is possible that there are
481 species differences in alkaloid retention duration in captivity, although a robust toxin loss
482 experiment had never been reported. It is also conceivable that different toxin classes
483 (batrachotoxin versus indolizidines) are retained for varying amounts of time. For example, there
484 were trace amounts of pumiliotoxin **307B** and some lehmizidines in the lab-reared frogs and
485 perhaps these are retained for longer periods of time than indolizidines. On the other hand, it may
486 be the age at which the lab frogs in our study were collected from the wild that influenced this
487 result. Juvenile and subadult poison frogs tend to have a lower diversity and abundance of toxins
488 in their skin [39] as well as smaller granular glands for storage [40]. It is possible that the lab frogs
489 used in this study did not contain many alkaloids at the time they were collected and were then
490 subsequently lost in the laboratory. The rate of alkaloid uptake and loss in adult poison frogs is
491 understudied and such pharmacokinetics studies would lend insight into the dynamics of toxin
492 sequestration and loss. Regardless, tissue samples from chemically defended and alkaloid-free
493 frogs collected from a single population enabled us to examine the physiology of alkaloid
494 sequestration across various tissues.

495 **Sodium transporters**

496 Sodium transport proteins were a common class of differentially expressed genes. In the
497 intestines, we found significant downregulation of the epithelial sodium ion channel subunit

498 transcripts (scnn1a and scnn1b; also called amiloride-sensitive sodium channel). This non-
499 voltage sensitive sodium ion channel is inhibited by the diuretic alkaloid amiloride and is
500 responsible for sodium influx across the apical membrane of intestinal epithelial cells. It is
501 regulated by a number of intrinsic and extrinsic factors like diet and hormones [41], which makes
502 it difficult to determine how the various differences between the frogs groups studied here most
503 influenced this result. Voltage gated-sodium channels are a well known target of frog alkaloids
504 [6], but to our knowledge the epithelial sodium channel has not been tested with poison frog
505 alkaloids. Another transporter of note is the sodium-potassium-ATPase pump, whose beta 2
506 subunit expression was elevated in chemically defended wild caught frogs compared to lab frogs.
507 The sodium-potassium-ATPase pump alpha subunit is a frequent target in the evolution of toxin
508 resistance in a number of herbivorous invertebrates [42][43] and vertebrates [31]. Investigations
509 of the function and evolution of the beta subunit in the context of toxin resistance is lacking. An
510 analysis of sodium-potassium-ATPase pump sequence evolution across chemically defended
511 and non-defended amphibians would likely yield interesting results. Binding studies with these
512 channels and poison frog alkaloids as well as protein sequence evolution analyses are an
513 important future goal in understanding potential interactions.

514 **Small molecule transport and metabolism**

515 Vertebrates use two physiological mechanisms for transport of exogenous compounds
516 depending on the degree of hydrophobicity: the portal venous system and the lymph system [11].
517 Cholesterol-derived bile acid molecules, which are made in the liver, are secreted to the
518 gallbladder and then deposited into the intestines through the bile duct. With the help of bile acids
519 and phospholipids, lipophilic molecules form micelles that are shuttled through the intestinal cells
520 and excreted into the the portal venous system. The transporter responsible for bile acid-
521 associated export from intestinal cells into the portal blood is solute carrier protein 51a (slc51a,
522 also known as organic solute transporter subunit alpha) [44], which has increased expression in

523 the liver of chemically defended wild frogs compared to lab frogs. Bile acid-associated pathways
524 are of particular interest, as bile acid derivatives have been observed in the skin of mantellid
525 poison frogs [45], suggesting a bile acid-based transport system for some poison frog alkaloids.
526 Alkaloids can be also be carried by a number of transport proteins in the blood circulation. One
527 protein of particular interest that was increased in lab frogs compared to chemically defended wild
528 frogs is saxiphilin. Saxiphilin is an amphibian specific transferrin that has high specificity for the
529 alkaloid neurotoxin saxitoxin [32]. We also found evidence through thermal proteome profiling that
530 saxiphilin may also bind other alkaloid toxins, like decahydroquinoline. It is currently unknown if
531 saxiphlin binds additional alkaloid toxins and further studies on the poison frog saxiphilin protein
532 as a more general alkaloid carrier are warranted.

533 Not all molecules are transported in the blood however, as highly lipophilic exogenous
534 compounds are transported by lipoproteins that move lipophilic cargo in chylomicron particles
535 through the lymphatic system [11]. Apolipoproteins are required for chylomicron assembly and
536 play some role in determining the types of compounds that are transported in their cargo. In our
537 study, chemically defended frogs had increased expression of an apolipoprotein A-IV (apoa4)
538 transcript in the liver compared to captive alkaloid-free frogs. We should also note that these
539 changes in gene expression could also be due to differences in diet between our two groups,
540 where wild frogs eat mostly ants and mites and lab frogs were fed *Drosophila* and crickets, which
541 differ not only in alkaloid content but also in lipid and protein content [46]. A more controlled diet
542 experiment and additional biochemical testing would be required to completely understand the
543 involvement of these candidate transport mechanisms in diet and chemical defenses of poison
544 frogs.

545 Solute carrier proteins are of special interest as they aid in the transport of ions and small
546 molecules across cell membranes, which is likely important for alkaloid transport in both the blood
547 and the lymphatic system. In the intestines, two solute carrier family transcripts had decreased
548 expression in wild caught frogs compared to lab-reared frogs. One of the transporters is slc12a3,

549 a sodium and chloride cotransporter that is sensitive to a number of thiazide-like alkaloid diuretics
550 that are used to treat hypertension [47]; the role of this transporter in the intestines is not well
551 understood. The other differentially expressed transporter in the intestines is slc52a3, which
552 transports riboflavin (vitamin B2, also an alkaloid) and is well-studied as loss of function due to
553 mutations cause Brown-Vialetto-Van Laere syndrome [48]. In both cases, whether these proteins
554 can transport other compounds is not known, although their expression can be regulated by diet,
555 such as riboflavin intake [49]. As these transporters interact with plant alkaloids naturally, it
556 remains an open question as to whether poison frog alkaloids also influence their activity and
557 gene expression. In the liver, the other small molecule transport protein differentially expressed
558 between groups was slc38a2 (also called SNAT2), which had increased transcript abundance in
559 wild frogs compared to alkaloid-free frogs. This protein functions as a sodium-dependent amino
560 acid transporter and its expression is heavily regulated by nutritional factors, where amino acid
561 withdrawal induces slc38a2 expression in vitro [50]. As ants have lower amino acid content within
562 a hard exoskeleton compared to softer fruit flies and crickets [51], slc38a2 expression in these
563 ant-specialist frogs is likely correlated with diet variation between frog groups rather than the
564 presence of alkaloids.

565 Several cytochrome P450s transcripts were differentially expressed in the intestines and
566 liver. This large protein family is well known for small molecule metabolism and has important
567 clinical implications in drug metabolism and toxin breakdown [52]. We found three cytochrome
568 P450s were differentially expressed between chemically defended wild caught frogs and lab-
569 reared alkaloid-free frogs. In both the intestines and liver, two transcripts in the cytochrome P450
570 2 family (tentatively cyp2k1 and cyp2k4) were differentially expressed between groups. These
571 enzymes were originally identified in rainbow trout [53] and their specific chemical function has
572 not been investigated in detail. In the liver, a cytochrome P540 1A enzyme (tentatively cyp1a5)
573 was also differentially expressed between groups. This enzyme is well described in birds and is
574 located in liver microsomes where it oxidizes a number of structurally unrelated compounds,

575 including xenobiotics and steroids [54,55]. It is important to note that many biological factors can
576 influence the expression of cytochrome P450s including diet, sex, and temperature [56]. We
577 cannot conclude based on this study design if the presence of alkaloids induced these changes
578 in gene expression and further experiments would be necessary to determine the dynamic
579 regulation of these important enzymes. However, some poison frog species are thought to
580 metabolize alkaloids, where John Daly and colleagues hypothesized a yet unidentified
581 "pumiliotoxin 7-hydroxylase" was responsible for the metabolism of PTX **251D** into the more
582 potent aPTX **267A** [12]. Moving forward, a better understanding of alkaloid metabolism is needed
583 in order to link poison frog alkaloid profiles to metabolism by cytochrome P450s.

584 **Other proteins classes of interest**

585 Two other classes of proteins were different in many of the comparisons between wild
586 caught chemically defended frogs and lab-reared frogs described here: proteins involved in
587 immune system function and heat shock proteins. Two proteins in the complement immune
588 system showed differences between groups. Complement C3 had increased transcript
589 abundance in the liver and higher protein abundance in the blood in wild chemically defended
590 frogs compared to lab-reared frogs. The membrane bound C5a receptor also showed higher
591 transcript abundance in livers of wild frogs compared to lab frogs. Although complement factors
592 can bind alkaloids [57], abundance can also be influenced by pathogens and other environmental
593 factors [58]. Thus, further experiments with more controlled groups are necessary to determine if
594 these immunity factors are important for chemical defenses. The other class of molecules with
595 differences in transcript abundance between groups was liver heat shock proteins. Although
596 expression of these proteins can be modulated by stress and environmental variables [59], we
597 also found evidence that heat shock protein 90 may bind the poison frog alkaloid
598 decahydroquinoline. Other alkaloids have also been found to both decrease and increase the
599 protein folding of the heat shock protein chaperones [60] and, therefore, it is possible that poison

600 frog alkaloids also modify heat shock protein activity. Alternatively, the alkaloids themselves could
601 destabilize liver lysate proteins which then leads to an increased thermal shift of heat shock
602 proteins in the thermal proteome profiling assay, where heat shock proteins are binding to proteins
603 as they begin to unfold in the presences of decahydroquinoline. More thorough *in vitro* studies
604 are needed to test these hypotheses.

605 **Conclusions and future directions**

606 Our study has provided many candidate proteins and biological processes that may be
607 involved in alkaloid sequestration in poison frogs. This includes candidate mechanisms for
608 alkaloid transport like plasma carrier proteins and chylomicrons as well as genes involved in small
609 molecule metabolism. However, a limitation of our study is that we compared gene expression or
610 differences of chemically defended and alkaloid-free frogs that were in different environments
611 (wild caught in the rainforest compared to laboratory housing). Future studies should be
612 conducted in identical environmental conditions with controlled toxin feeding regimes. Finally,
613 although our thermal proteome profiling experiment highlighted candidate proteins, this method
614 exclusively tests soluble proteins and further studies should be done to test membrane bound
615 proteins as well. Overall, we have provided the first organismal perspective into how poison frogs
616 bioaccumulate small molecule chemical defenses from their diet. This work provides a foundation
617 for future mechanistic and comparative work that will be able to uncover how poison frogs have
618 altered their physiology to acquire chemical defenses.
619

620 **Data Accessibility**

621 The Illumina fastq files, GC/MS data from the alkaloid analysis, LC-MS/MS files from
622 plasma proteomics, the *O. sylvatica* transcriptome and proteome fasta files, the Trinotate SQLite
623 database that includes the gene expression analysis, and an Excel File containing all data and
624 analyses (Supplementary Excel File) is available at DataDryad (submission pending).

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Author Contributions

LAO and LAC designed the research; LAO, SNC, ABR, EET and LAC collected frog samples in the Ecuador; EET and LAC maintained the lab frogs; LAO performed the laboratory research and RNAseq analysis; GDB and SAT performed the small molecule mass spectrometry; GDB and CV performed the alkaloid GC/MS analysis; BB conducted the plasma proteomics experiments; AAB performed the thermal proteome profiling analysis; SNC and LAO wrote the paper with contributions from all authors.

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