Pumiliotoxin metabolism and molecular physiology in a poison frog

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ABSTRACT

Poison frogs bioaccumulate alkaloids for chemical defense from their arthropod diet. These small molecules are sequestered from their gastrointestinal tract and transported to the skin for storage. Although many alkaloids are accumulated without modification, some poison frog species can metabolize pumiliotoxin (PTX **251D**) into the more potent allopumiliotoxin (aPTX **267A**). Despite extensive research characterizing the chemical arsenal of poison frogs, the physiological mechanisms involved in the sequestration and metabolism of individual alkaloids is unknown. We performed a feeding experiment with the Dyeing poison frog (*Dendrobates tinctorius*) to ask if this species can metabolize PTX **251D** into aPTX **267A** and what gene expression changes are associated with PTX **251D** exposure in the intestines, liver, and skin. We found that *D. tinctorius* can metabolize PTX **251D** into aPTX **267A**, and that PTX **251D** exposure changed the expression of genes involved in immune system function and small molecule metabolism and transport. These results show that individual alkaloids can modify gene expression across poison frog tissues and suggest that different alkaloid classes in wild diets may induce specific physiological changes for accumulation and metabolism.

1. INTRODUCTION

Poison frogs (Family Dendrobatidae) are chemically defended against predators [1–3] using alkaloids that are sequestered from dietary arthropods [4,5]. Many poison frog alkaloids have been found in the ants and mites they consume, suggesting most alkaloids are sequestered unchanged [6–9]. However, poison frogs can also metabolize specific alkaloids into more potent forms [10], although the underlying physiological mechanisms of this are unknown. Controlled alkaloid-feeding experiments have been crucial in understanding alkaloid metabolism in poison frogs [10–12]. Although many alkaloids are sequestered unmodified, some dendrobatids metabolize pumiliotoxin (PTX) **251D** into the more potent allopumiliotoxin (aPTX) **267A** [10]. After PTX **251D** feeding, both PTX **251D** and its metabolite, aPTX **267A,** were detected on the skin of *Dendrobates auratus*, but only PTX **251D** was detected in *Phyllobates bicolor* and *Epipedobates tricolor* [10]. These results suggest that an unidentified enzyme performs the 7'-hydroxylation of PTX **251D** into aPTX **267A** in certain species. Whether other poison frog species can metabolize PTX **251D** into aPTX **267A** remains unknown, along with the metabolic mechanisms involved.

We conducted an alkaloid feeding study with the Dyeing poison frog (*Dendrobates tinctorius*) to test whether this species can metabolize PTX **251D** into aPTX **267A** and to explore gene expression changes associated with PTX **251D** exposure. To test whether PTX **251D** elicited specific gene expression changes, we fed control frogs decahydroquinoline (DHQ) and treatment frogs a mixture of PTX **251D** and DHQ. We predicted metabolic enzymes involved in the hydroxylation of PTX **251D** into aPTX **267A** may be upregulated in response to their metabolic target. Furthermore, if specific alkaloid sequestration pathways exist for PTX **251D**, the proteins involved in that process may also be enriched upon exposure.

2. MATERIALS AND METHODS

Alkaloid feeding

Lab-reared (non-toxic) *Dendrobates tinctorius* were housed in terraria with live plants, a water pool, and a shelter. Ten adult females were size-matched, randomly assigned to control or experimental groups (N=5 per group), and then housed individually. To measure the specific effects of PTX **251D** compared to a background toxicity, the control group was fed 0.01% DHQ (Sigma-Aldrich, St. Louis, USA) in a solution of 1% EtOH and the experimental group was fed a solution of 0.01% DHQ and 0.01% PTX (PepTech, Burlington, MA, USA) in a solution of 1% EtOH. Each frog was fed 15 µL each day for five days by pipetting the solution directly into the mouth between 10am-12pm. On the afternoon of the fifth day, frogs were euthanized by cervical transection and the dorsal skin, liver, intestines, and eggs were dissected into Trizol (Thermo Fisher Scientific, Waltham, USA). All procedures were approved by the Institutional Animal Care and Use Committee at Stanford University (protocol number #32870).

RNA extraction and library preparation

RNA extraction followed the protocol outlined in Caty *et al.* 2019 [13] and according to the manufacturer's instructions. After the first spin, the organic layer was saved for alkaloid extraction (see below). Poly-adenylated RNA was isolated using the NEXTflex PolyA Bead kit (Bioo Scientific, Austin, USA) following manufacturer's instructions. RNA quality and lack of ribosomal RNA was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). Each RNA sequencing library was prepared using the NEXTflex Rapid RNAseq kit (Bioo Scientific). Libraries were quantified with quantitative PCR (NEBnext Library quantification kit, New England Biolabs, Ipswich, USA) and an Agilent Bioanalyzer High Sensitivity DNA chip, both according to manufacturer's instructions. All libraries were pooled at equimolar amounts

and were sequenced on four lanes of an Illumina HiSeq 4000 machine to obtain 150 bp paired-end reads.

Transcriptome assembly and differential expression analysis

All scripts are detailed in supplementary materials. We created a reference transcriptome using Trinity [14], and cleaned the raw assembly by removing contigs with BLAST hits belonging to microorganisms and invertebrates in the Swiss-Prot database [15]. Overlapping contigs were clustered using cd-hit-est [16,17] and contigs that were less than 250bp long were removed from the assembly. We mapped the paired quality-trimmed sequences to the reference transcriptome using kallisto [18]. Samples were compared across treatment groups (DHQ vs DHQ+PTX) for the skin, liver, and intestines, as these tissues contained higher levels of PTX. Differences in gene expression levels were calculated using DESeq2 [19] [P<0.05 false discovery rate (Benjamini–Hochberg FDR), 4-fold change]. Contigs with significant expression differences were BLAST-ed to the non-redundant (nr) database using an E-value cutoff of 1e-5. Many contigs did not have a BLAST hit, or aligned to hypothetical or non-vertebrate proteins. Therefore, BLAST annotations were visually inspected and contigs of interest were chosen based on candidates from existing literature. Boxplots were made with R package ggplot2 (R version 3.6.3) using TMM (trimmed mean of M-values) normalized expression.

Alkaloid extraction and detection

To isolate alkaloids, 0.3 mL of 100% EtOH was added to 1mL of organic layer from the Trizol RNA extraction, inverted 10 times, and stored at room temperature for 2-3 minutes to precipitate genomic DNA, which was pelleted by centrifugation at 2000g for 5 minutes at 4°C. Then, 300 µL of supernatant was transferred to a new microfuge tube. Proteins were precipitated by adding 900 µL of acetone, mixing by inversion for 10-15 seconds, incubating at

room temperature for 10 min, and centrifuging at max speed for 10 min at 4°C. Then, 1 mL of supernatant was moved into a glass vial and stored at -20°C until dried down completely under a gentle nitrogen gas flow.

Samples were resuspended in 200 µl of methanol:chloroform 1:1 and 1 µM Nicotine-d3 (used as an internal standard). A 10-point standard curve was prepared in the same solution with DHQ and PTX. A QE+ mass spectrometer coupled to an Ultimate3000 LC (ThermoFisher) was used for analysis. Five µl of each sample were injected on a single Gemini C18 column (100x2mm, Phenomenex). The mobile phases were A: water and B: acetonitrile, both with 0.1% formic acid. The gradient was 0% B for 1min, then increased to 100% B in 14 min, followed by 5 min at 100% B and 3.5 min at 0% B. Data were quantified using accurate mass, and specific transitions for DHQ and PTX used the standard curve for absolute quantification. aPTX was identified by accurate mass and MS/MS fragmentation similarity to PTX.

Data analysis of liquid chromatography/tandem mass spectrometry (LC-MS/MS) data

R version 3.6.3 was used for all statistical analyses. There were instances in the LC-MS/MS data where the molecules of interest (DHQ, PTX **251D**, or aPTX **267A**) were not found, and these were converted to zeros prior to statistical analyses. A generalized linear mixed model was used (glmmTMB package in R) [20] to test for differences in alkaloid abundance across tissues and treatment type, with the frog as a random effect and a negative binomial error distribution. PTX **251D** and DHQ were analyzed separately. The aPTX **267A** abundance was approximated using the area-under-the-curve divided by the internal nicotine standard, as there is no standard for aPTX **267A,** and therefore exact pmol values could not be calculated. A Wilcoxon rank-sum test (wilcox.test) was used to compare the aPTX values in the skin between treatment groups and the Kruskal-Wallis test (kruskal.test) was used to compare the aPTX values across tissues.

3. RESULTS

The Dyeing poison frog metabolizes PTX to aPTX

We conducted a feeding experiment to determine if the Dyeing poison frog can metabolize PTX **251D** into aPTX **267A** (Figure 1A). Alkaloids were most abundant in the skin and liver, followed by the intestines. Alkaloid abundance in the eggs was very low. DHQ abundance did not differ by treatment group (GLMM treatment, $p = 0.377$), confirming that both groups were fed equal amounts. DHQ abundance differed across tissue types (GLMM tissue, $X^2(3) = 203.642$, $p < 2e\text{-}16$), with the highest levels occurring in the liver and skin (Figure 1B). PTX **251D** abundance differed by tissue and treatment (GLMM tissue:treatment, $X^2(3) = 57.265$, $p < 2e\text{-}12$), with the highest levels in the liver and skin in the DHQ+PTX feeding group (Figure 1C). We detected aPTX **267A** in the skin of all individuals in the DHQ+PTX feeding group at higher levels than the DHQ-fed group (Wilcoxon test, $W = 0$, $p = 0.012$, Figure 1D). The amount of aPTX **267A** differed across tissues (Kruskal-Wallis, $X^2(3) = 13.727$, $p = 0.003$), with the abundance in the skin greater than eggs (post-hoc Dunn test, $p = 0.001$) and intestines (post-hoc Dunn test, $p = 0.035$, Figure 1E). These data show *D. tinctorius* can metabolize PTX **251D** into aPTX **267A** and that some alkaloid metabolism may occur in the liver and intestines.

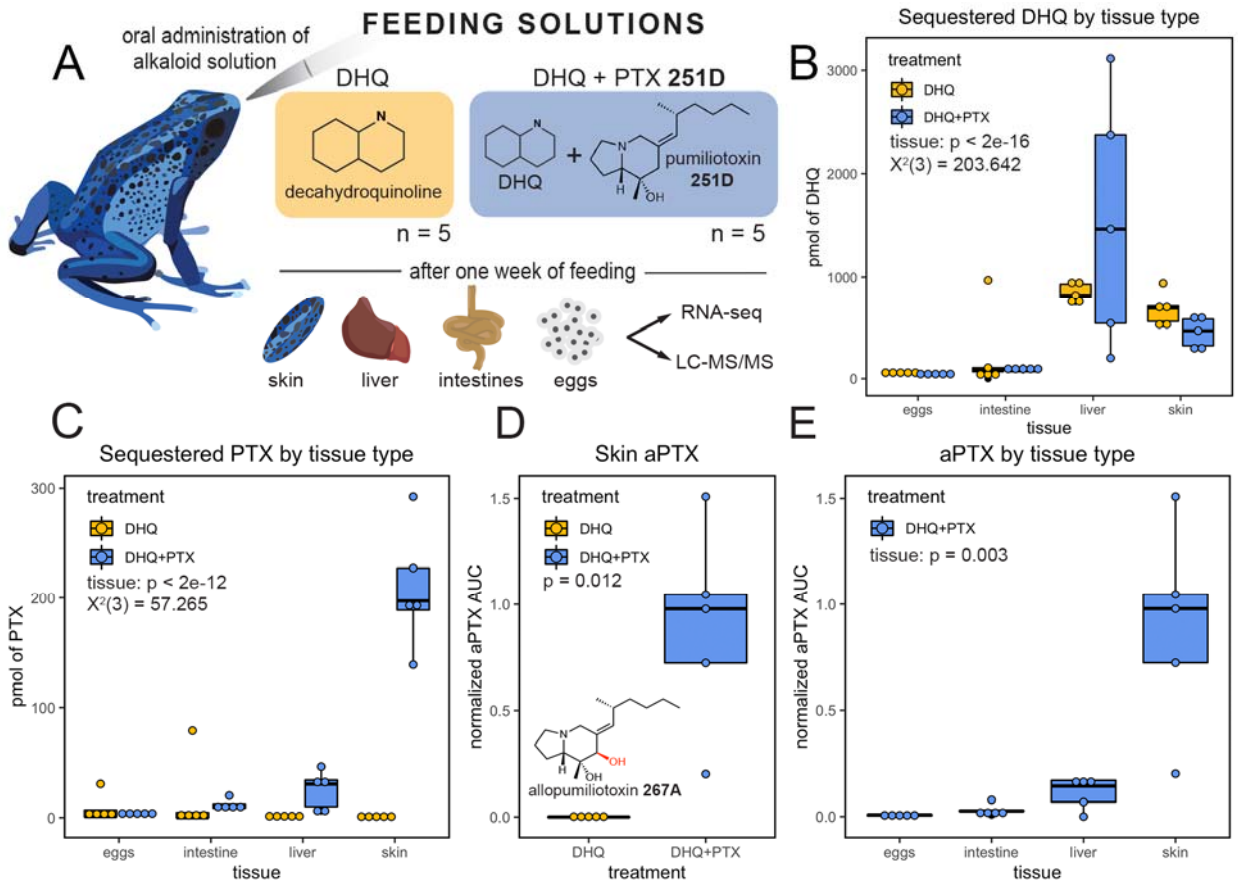

Figure 1: Alkaloid sequestration in different tissue types. (A) Frogs were orally administered either DHQ or DHQ+PTX once a day for five days. **(B)** DHQ abundance differed by tissue but not treatment group, and was highest in the liver and skin (GLMM tissue, X^2 (3) = 203.642, p < 2e-16). **(C)** PTX levels differed by tissue and treatment, and were higher in the liver and skin of the DHQ+PTX fed group (GLMM tissue:treatment, X^2 (3) = 57.265, p < 2e-12). **(D)** The hydroxylated metabolite aPTX was found in the DHQ+PTX fed frogs (Wilcoxon test, W = 0, p-value = 0.012, n = 5). **(E)** aPTX abundance differed across tissues within the DHQ-PTX group (Kruskal-Wallis, X^2 (3) = 13.727, p = 0.003), and was found primarily in the skin, with some in the liver.

PTX alters gene expression across tissues

We next quantified gene expression changes associated with PTX sequestration and metabolism. Although hundreds of genes were differentially expressed in each tissue, most did not have annotations, or aligned with unknown, hypothetical, or non-vertebrate proteins. Cytochrome P450 (CYP3A29), an enzyme family well-known for their involvement in small molecule hydroxylation, was upregulated in the intestines (log2FC = 5.72, p = 0.0045; Figure

2A). In the liver, vitellogenin 2 (VTG2) was downregulated in the PTX feeding group (log2FC = -7.73, p = 0.0421, Figure 2B). MHC Class I was upregulated in both the liver (log2FC = 3.49, p = 0.0005) and intestines (log2FC = 5.41, p = 0.0001) in the presence of PTX **251D** (Figure 2A,B). In the skin, Syntaxin 1A (STX1A) was upregulated (log2FC = 2.58, p = 0.0385) and a Solute Carrier Family 2 protein (SLC2) was downregulated (log2FC = 6.73, p = 0.0496) in response to PTX **251D** (Figure 2C).

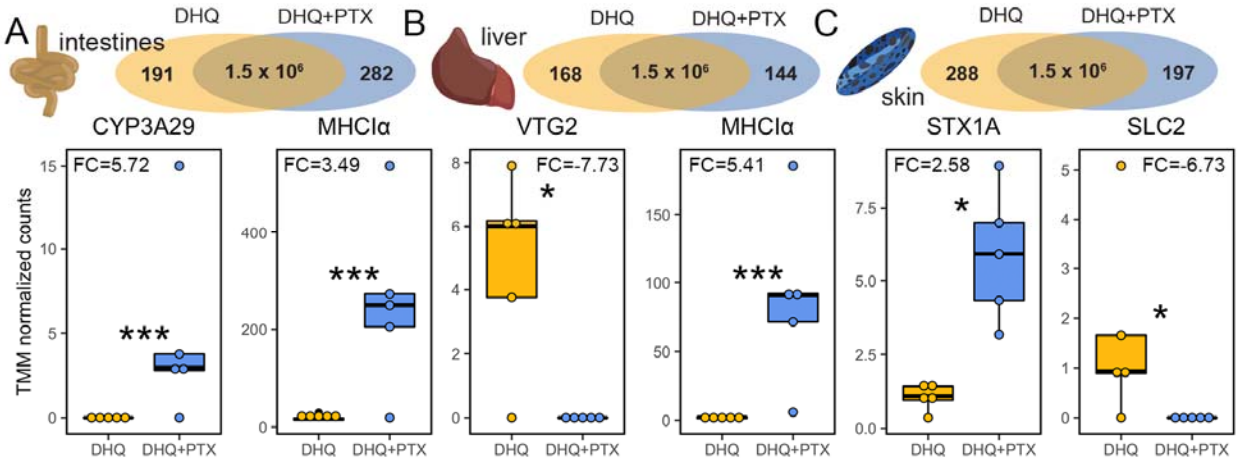

Figure 2: Differentially expressed genes in different tissues*. (A) Differentially expressed genes in the intestines include CYP3A29, Cytochrome P450 Family 3 Protein 29 and MHCI, MHC Class I alpha. **(B)** Differentially expressed genes in the liver included VTG2, vitellogenin 2 and MHCI, MHC Class I alpha again. **(C)** Differentially expressed genes in the skin included STX1A, syntaxin 1A and SLC2, solute carrier family 2. (FC indicates log2 fold change values, * indicates adjusted p-value < 0.05, *** indicates adjusted p-value < 0.005) **Note: y-axis of individual plots have different scales*

4. DISCUSSION

Performing a controlled feeding study with DHQ and PTX **251D** allowed us to determine that *D. tinctorius* can metabolize PTX **251D** into aPTX **267A**. Although previous studies have documented wild *D. tinctorius* with aPTX on their skin [2,6], this is the first experimental evidence that this species metabolizes PTX **251D** into aPTX **267A**. The accumulation of both DHQ and PTX **251D** in the liver and intestines, along with the skin, indicates that these tissues play an important role in the sequestration of alkaloids. Indeed, the liver and intestines are

189 important sites of alkaloid metabolism in mammals due to high levels of Cytochrome P450s [21–
190 23]. Together, these results show that *D. tinctorius* is able to metabolize PTX **251D** into aPTX
191 **267A** and that the tissue distribution of alkaloids includes the skin, liver, and intestines. In the
192 future, a better understanding of alkaloid pharmacokinetics could be achieved through finer
193 time-course feeding experiments.

194 PTX **251D** feeding changed gene expression in the intestines, liver, and skin, suggesting
195 a single alkaloid can change poison frog physiology. Specifically, the upregulation of CYP3A29
196 in response to PTX in the intestines implicates this enzyme in the metabolism of PTX **251D** into
197 aPTX **267A**, or of PTX into a metabolic byproduct to be later discarded. Although we originally
198 expected to identify metabolism enzymes in the liver, it is possible the liver instead acts as a
199 detoxification site. In the dendrobatid *Oophaga sylvatica*, feeding DHQ compared to a non-
200 alkaloid vehicle control led to a downregulation of CYP3A29 in the intestines, suggesting that
201 expression is regulated differently by specific alkaloids [24]. The upregulation of MHC class I
202 proteins in the intestines and liver in response to PTX **251D** supports previous findings that frog
203 immune systems respond to alkaloids [13,25]. We also found VTG2 (vitellogenin-2) was
204 downregulated in response to PTX **251D**. Although vitellogenins are typically thought to be egg-
205 yolk proteins, they also play regulatory roles and protect cells from reactive oxygen species that
206 may arise from alkaloid metabolism [26–28]. Finally, SLC2 (solute carrier family 2) which
207 encodes for the GLUT family of glucose transporters, was downregulated in the skin with PTX
208 feeding. Alkaloids have been found to be potent inhibitors of GLUTs in mammalian cell lines,
209 and the downregulation of GLUTs in this case may be due to the presence of concentrated PTX
210 **251D** in the frog skin [29]. Together, these data support an argument for physiological “fine-
211 tuning” of gene expression in response to certain alkaloids.

212 We provide evidence that *D. tinctorius* can metabolize PTX **251D** into aPTX **267A** and
213 that PTX **251D** exposure changes gene expression across tissues, demonstrating that specific
214 alkaloids can change poison frog physiology [24,25]. Following up on candidate genes with

biochemical studies is needed in order to fully characterize the genetics of alkaloid sequestration and metabolism. In the wild, where chemically defended dendrobatids carry many different alkaloids, subtle alkaloid differences may induce distinct gene expression changes. More broadly, modulating gene expression in response to specific alkaloids may set the stage for local adaptation to environmental resources.

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6. DATA ACCESSIBILITY

All LC-MS/MS data from the alkaloid analysis is available from the Dryad Digital Repository (pending). All Illumina fastq files are available on the Sequence Read Archive (pending). All data and code is available in the supplementary material.

7. AUTHOR CONTRIBUTIONS

AAB and LAO designed the experiment. AAB and CYP carried out the experimental procedures. CV and SAT quantified alkaloids. AAB analyzed the data. AAB and LAO wrote the manuscript with contributions from all authors.

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