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ARTICLE

Dietary Breadth Predicts Toxin Expression Complexity in the Venoms of North American Gartersnakes

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Synopsis Selection on heritable phenotypic variation has played a prominent role in shaping the remarkable adaptations found across the Tree of Life. Complex ecological traits, such as snake venoms, are thought to be the products of selection because they directly link to fitness and survival. Snake venom increases the efficiency of prey capture and processing and is thus likely under intense selection. While many studies of snake venom have investigated the relationship between venom and diet, they have primarily focused on medically relevant front-fanged snakes. However, recent work has suggested that many non-front fanged snakes also rely on venom for subduing prey, despite having reduced toxicity toward humans. Here, we set out to uncover variation in toxin-producing genes, along with the ecological and evolutionary pressures impacting snake venom characteristics in the North American gartersnakes (Squamata: Natricidae: *Thamnophis*), a model group of non-front-fanged snakes. We annotated and analyzed Duvernoy's venom gland transcriptomes from 16 species representing all the major lineages within *Thamnophis*. We then generated measures of complexity of both toxins and dietary breadth. We found strong correlations between the complexity of toxin gene expression and phylogenetic diversity of diet, but no relationship between the complexity of the genetic makeup of the transcriptomes (allelic or sequence variation) and diet complexities. We also found phylogenetic signal associated with venom complexity, suggesting some influence of ancestry on venom characteristics. We suggest that, in non-front-fanged snakes, expression of toxins rather than sequence complexity is under strong selection by dietary diversity. These findings contradict similar studies from front-fanged snakes where increased transcriptomic complexity varies positively with dietary diversity, exposing a potential novel relationship between a complex phenotype—toxin expression—and its selective pressures—diet.

Spanish La selección en la variación fenotípica heredable ha desempeñado un rol prominente en la formación de las adaptaciones encontradas a lo largo del Árbol de la Vida. Se cree que los caracteres ecológicos complejos, como el veneno de las serpientes, son el producto de la selección natural dado que está directamente relacionados con el fitness y la supervivencia. El veneno de las serpientes incrementa la eficiencia en la captura y el procesamiento de las presas por lo que probablemente está sujeto a una selección intensa. A pesar de que varios estudios sobre el veneno de las serpientes han investigado la relación entre el veneno y la dieta, estos se han enfocado en las serpientes medicamente relevantes que poseen colmillos anteriores. Sin embargo, trabajos recientes sugieren que varias serpientes sin colmillos anteriores también dependen del veneno para someter presas, a pesar de que su veneno tiene baja toxicidad hacia los humanos. En el presente trabajo pretendemos descubrir la variación en los genes que producen las toxinas, así como las presiones ecológicas y evolutivas que impactan las características del veneno en las serpientes del género *Thamnophis* (Squamata: Natricidae), que es un grupo modelo de serpientes sin colmillos anteriores. Anotamos y analizamos transcriptomas de las glándulas de Duvernoy de 16 especies representando los linajes principales dentro de *Thamnophis*. Posteriormente, generamos medidas de complejidad tanto de las toxinas como de la dieta. Encontramos correlaciones fuertes entre la complejidad en la expresión de los genes de toxinas y la diversidad filogenética de la dieta,

pero no se observó relación entre la complejidad genética (variación alélica o en la secuencia genética) y la complejidad en la dieta. También encontramos señal filogenética asociada con la complejidad en el veneno, sugiriendo la influencia de la historia filogenética en las características del veneno. Sugerimos que en serpientes sin colmillos anteriores la expresión de toxinas, en vez de la complejidad de la secuencia genética, está sometida a selección por la variación en la dieta. Estos hallazgos contradicen estudios similares hechos en serpientes con colmillos anteriores donde los incremento en la complejidad del transcriptoma varían positivamente con la diversidad en la dieta, exponiendo una posible relación novedosa entre fenotipos complejos (expresión de toxinas) y sus presiones selectivas (dieta).

Introduction

Darwin first hypothesized that natural selection could give rise to novel, complex traits, while maintaining others, leading to an interconnected network of traits, functions, and selective pressures (Darwin 1859). Complex traits are thought to be the result of multiple selective pressures acting on the underlying genetics and expressed molecular functions, resulting in a wide range of phenotypic possibilities (Adami et al. 2000; Wolf et al. 2018). Biotic and abiotic conditions provide the selective pressures shaping traits, especially traits that allow for increased resource utilization—such as dietary adaptations (Fernald 2000; Friedman 2008). Venom is a particularly salient dietary adaptation because it allows predators to subdue and process prey that might otherwise be too large or dangerous to handle (Greene 1997). In addition to the ecological role of venom, this complex trait has also been the focal point of many studies due to its medical significance and has garnered the attention of research groups and governing bodies across the globe (Chippaux 2006; Fry et al. 2009; Arbuckle 2017).

Venom has arisen more than one hundred times across the Tree of Life (TOL) and can be found in various organisms including cnidarians, insects, fish, spiders, scorpions, squamate reptiles, and mammals (Casewell et al. 2013; Whittington and Belov 2014; Moreau and Asgari 2015; Phuong et al. 2016; Arbuckle 2017). This complex cocktail of proteins and peptides produces pharmacologic effects with a wide range of characteristics across taxa, but it can generally be summarized as a biological substance produced by one organism that impacts the biological processes of another organism upon envenomation (Fry et al. 2009; Casewell et al. 2013; Arbuckle 2017). While venoms in many taxa may play a defensive role, the most common function is a predatory role that increases proficiency in prey capture and processing (Casewell et al. 2013). Venom can vary ontogenetically (Hogan et al. 2024), between individuals in a population (Rosales-García et al. 2023), across populations (Margres et al. 2017), and across the TOL (Arbuckle 2017). As such, researchers seek to understand both the proximate and ultimate causes of variation in venom.

One of the most well-known groups of venomous animals are snakes. In snakes, it is hypothesized that venom evolved with a primary function of prey immobilization and digestion (Hayes 1992; Daltry et al. 1996; Fry et al. 2006; Fry et al. 2009; Mackessy 2010; Casewell et al. 2013). Research on venom characteristics and the underlying genetic basis of venom suggests a relationship between venom and diet at the genetic level in terms of underlying genetic complexity (transcriptomic complexity) and how that genetic complexity is expressed in protein production (expression complexity) (Holding et al. 2021; Schaeffer et al. 2023). Variation in snake venom characteristics and effects can be seen across multiple scales, both inter- and intraspecifically, supporting the hypothesis that venom is evolving with dietary preferences at local and broad scales (Daltry et al. 1996; Davies and Arbuckle 2019; Holding et al. 2021; Mason et al. 2022; Heptinstall et al. 2023; Schaeffer et al. 2023). For example, some snakes and their prey are players in an evolutionary arms race, where snakes are evolving more potent venom to combat prey that are evolving increasing degrees of venom resistance (Holding et al. 2016; Robinson et al. 2021). However, due to historical assumptions that non-front-fanged colubriform snake venom was medically unimportant, previous research has almost entirely focused on the causes of this variation in front-fanged snakes such as vipers and elapids, ignoring much of the species and venom diversity found in non-front-fanged colubriform snakes (Junqueira-de-Azevedo et al. 2016; Modahl and Mackessy 2019). Thus, the ecological and evolutionary importance of venom complexity in non-front-fanged snakes remains poorly understood.

Non-front-fanged snakes account for an overwhelming majority of snake diversity, and many are potentially valuable sources of novel toxins and models for understanding venom evolution (Junqueira-de-Azevedo et al. 2016; Uetz and Stylianou 2018). In addition to an assumed lack of medical relevance (though there are exceptions; see Schramer et al. 2022), the venom of non-front-fanged snakes remains understudied due to difficulties in collecting the venom and venom gland. Many non-front-fanged snakes possess a reduced Duvernoy's venom gland (DVG hereafter), which is thought to be

homologous to the larger and more specialized venom gland of front-fanged snakes (Kardong 2002; Jackson et al. 2017). The DVG lacks the storage and delivery system found in front-fanged snakes and produces lower venom yields, leading to a slow flow of toxins across enlarged rear-teeth rather than the quick, high-pressure injections found in front-fanged species (Kardong 2002). Due to debate over what characteristics of enlarged rear teeth constitute a true rear-fang, we refer to gartersnakes as "non-front fanged" rather than "rear-fanged," as this distinction between these is outside the scope of our current study. The characteristics of the DVG and enlarged-rear teeth make it challenging to study the venom of these snakes; however, advancements in "-omics" technologies have allowed for the generation of large amounts of data from small samples (Modahl and Mackessy 2019). Specifically, transcriptomic technologies offer a time snapshot of relative gene expression and the characterization and quantification of the underlying messenger ribonucleic acid (RNA) which get translated into amino acids during protein production (McGivern et al. 2014; Modahl et al. 2018; Modahl et al. 2021). Snake venom, in particular, provides a model phenotypic trait to examine via venom gland transcriptomics due to the strong correlation between RNA transcription and protein translation in venom glands (Rokyta et al. 2012; Rokyta et al. 2015). These molecular technologies have been used to discover both novel and conserved toxins within non-front-fanged snake species and have demonstrated that there is extensive variation among species (Modahl et al. 2018; Modahl and Mackessy 2019; Hofmann et al. 2021; Cerda et al. 2022; Schramer et al. 2022; Heptinstall et al. 2023). However, few investigations have explored the evolutionary pressures impacting this variation using comparative methods (Modahl and Mackessy 2019).

To investigate the evolutionary pressures impacting non-front-fanged snake venom evolution, we set out to characterize the DVG transcriptome of multiple species from the genus *Thamnophis* and compare their toxin characteristics to a primary selective force of toxin diversity: dietary breadth. Garter and ribbon snakes (*Thamnophis*) are a relatively common, well-studied group of snakes found throughout North America (Rossman et al. 1996). The genus *Thamnophis* contains 38 currently recognized species with diverse trophic ecologies (Rossman et al. 1996; Nuñez et al. 2023). For example, the Common Gartersnake (*T. sirtalis*) is a generalist predator throughout its range, but species such as the Short-headed Gartersnake (*T. brachystoma*) are extreme dietary specialists, feeding almost exclusively on earthworms (Carpenter 1952; Asplund 1963; Heptinstall et al. 2024). However, intraspecific variation in diet is also common in the genus. The Terrestrial Gartersnake (*T. elegans*) complex contains 3 currently recognized subspecies. *Thamnophis e. elegans* and *T. e. vagrans* feed on a generalist diet, while some populations of *T. e. terrestris* feed almost exclusively on slugs (*Ariolimax* spp.) (Britt et al. 2006). This broad dietary variation, paired with molecular evidence of venom production from the genus (Jansen and Foehring 1983; Hayes and Hayes 1985; Kardong and Luchtel 1986; Gomez et al. 1994; Perry et al. 2018) make *Thamnophis* an ideal clade to explore the evolution of venom in a non-front-fanged group.

Here, we generated the first *de novo* DVG transcriptomes with venom gene characterizations for 16 species from across the *Thamnophis* phylogeny. We then tested for relationships between toxin sequence complexity and toxin expression complexity with varying measures of dietary breadth. We hypothesized that dietary ecology drives venom evolution in non-front-fanged snakes and results in a positive correlation between venom complexity and dietary breadth—as seen in front-fanged snakes. The lack of such a correlation would suggest that either (1) other processes are at work, such as evolutionary constraints or unmeasured selective pressures impacting the venom of gartersnakes or (2) that gartersnake venom is evolving largely through neutral processes.

Materials and methods

Sample collection

Fifty-one *Thamnophis* individuals from 16 species were collected from various locations throughout the United States and Mexico from 2017 to 2023. We collected a range of one to eight individuals per species (mean of 3.19 individuals per species). Sampling details including sampling locality, collection permit details, IACUC protocol numbers, SRA accession numbers, museum deposition numbers, and other metadata can be found in Supplementary Material 1. To ensure sampling across the phylogeny, we targeted species of multiple clades and distributions, obtaining at least one representative species of almost every major clade (Fig. 1).

DVG transcriptomics

The DVG tissue collection and sequencing process followed protocols previously proven successful and repeatable for producing high-quality snake venom transcriptomes (Rokyta et al. 2011; Hofmann et al. 2018; Holding et al. 2018; Hofmann et al. 2021). In short, individuals were injected with pilocarpine at a dosage of 10 ug/g (Morais-Zani et al. 2018) to stimulate venom secretion and sedated with isoflurane. Venom was extracted by placing the enlarged back tooth of each top jaw onto a capillary tube (Hill and Mackessy 1997;

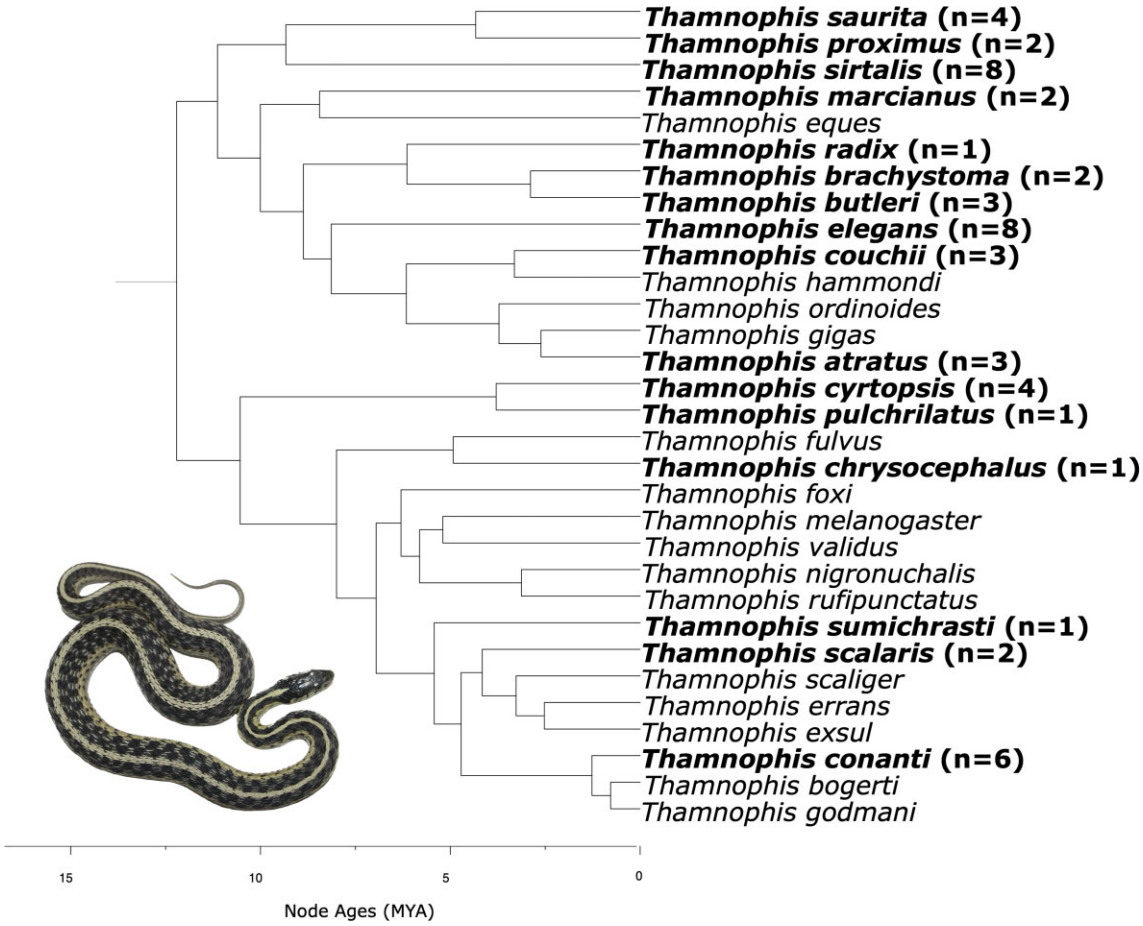

Fig. 1 Species sampling for this project displayed on the phylogeny (Hallas et al. 2022) of the genus *Thamnophis*. Sampled species are in bold, with the number of individuals collected for each species listed in parentheses. Photo of *T. sirtalis* by T.C.H.

Morais-Zani et al. 2018). Four days later, when transcription was at its peak (Rotenberg et al. 1971), individuals were anesthetized using a 1% MS222 (tricaine methanesulfonate) solution and euthanized using a 50% solution of MS222 at quantities dependent on the individual's weight (Conroy et al. 2009). Immediately following euthanasia, DVGs were extracted and placed into RNALater and stored temporarily at 4°C before being transferred to −80°C for long-term storage.

The RNA extraction process followed a standard Trizol extraction procedure (Rokyta et al. 2013; Hofmann et al. 2018; Strickland et al. 2018). We extracted RNA from each individual's left and right DVG and then combined RNA from each gland in equal concentration to create comprehensive DVG RNA pools for each individual. Using a Qubit RNA Assay (Thermo Fisher Scientific, Waltham, MA, USA), RNA was quantified and subsequently quality checked on a BioAnalyzer RNA Pico chip or a 4150 TapeStation System (Agilent Technologies, Santa Clara, CA, USA).

We processed the quantified RNA samples into separate, uniquely indexed cDNA libraries per individual snake. For cDNA library preparation, we used the NEBNext Ultra II RNA Library Prep Kit (E7775) (New England Biolabs, Ipswich, MA, USA) with the Ploy(A) mRNA Magnetic Isolation Module (E7490) (New England Biolabs, Ipswich, MA, USA). After mRNA isolation and cDNA synthesis, we targeted RNA inserts with a size of 300bp by using a fragmentation incubation time of 15 minutes and 15 PCR cycles to yield the desired cDNA concentration. Purification of cDNA samples was done using NEBNext Sample Purification Beads. A Qubit DNA BR Assay (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine DNA concentration. Quality control of samples was done using a Bioanalyzer with a HS DNA kit according to the manufacturer's instructions or a 4150 TapeStation System (Agilent Technologies, Santa Clara, CA, USA). Libraries were sequenced with Illumina SBS technology at Florida State University or Clemson University.

The subsequent analyses used GNU parallel 20,220,722 (Tange 2015) for multiprocess computing. Demultiplexed sequences were trimmed using `Trim Galore 0.6.7` (https://github.com/FelixKrueger/TrimGalore) and paired-end reads were merged using `PEAR 0.9.11` (Zhang et al. 2014). Merged reads were then assembled using `Extender` (Rokyta et al. 2012) and `Trinity 2.14.0` (Haas et al. 2013). Assembly outputs were combined and `cd-hit-est` (Li and Godzik 2006; Fu et al. 2012) was used at 100% to cluster redundant transcripts. This complete assembly was then annotated using `ToxCodAn` (Nachtigall et al. 2021) and custom annotation scripts (Rokyta et al. 2012; Hofmann et al. 2018), then manually annotated using `Geneious Prime 2022.2.2` (https://geneious.com). Finally, to check for any potentially missing, highly-expressed transcripts, we followed the open-reading frame identification and classification pipeline detailed in Schramer et al. (2022). Non-toxin transcripts were annotated using `BLAST` with the UniProt animal database (The UniProt Consortium 2023) and `ToxCodAn's CodAn` (Nachtigall et al. 2021). All duplicates and ambiguities were removed. `Cd-hit-est` (Li and Godzik 2006) was used at 99% on all samples to cluster transcripts and reduce redundancy. A final species-level consensus transcriptome was created by combining all individual transcriptomes and clustering at 97%. To determine gene expression, we used `bowtie2` (Langmead and Salzberg 2012) to map merged reads from each individual to the consensus transcriptome and used `RSEM` (Li and Dewey 2011) to generate the expected counts of reads mapping to the transcripts. There is much debate over the actual toxicity of many toxin families discovered in our transcriptomes, but for simplicity, we refer to these putative toxins collectively as "toxins" or "venom components" (Modahl et al. 2016; Modahl and Mackessy 2019).

Due to the small size of several of our study specimens and, therefore, the DVG, we wanted to confirm that all transcripts originated from the DVG. Thus, we compared the pairwise relationship between non-toxin gene transcript expression among all species post-assembly and annotation using linear regressions of the centered log ratio transformed transcripts per million (TPM). We expected that if non-toxin expression significantly differed from a 1:1 relationship, it would provide evidence that tissue types other than DVG were sequenced, as non-toxin genes are expressed differently in different tissue types.

To investigate homology between toxin genes found within our *Thamnophis* transcriptomes and transcriptomes of other venomous snake species, phylogenetic trees of the cystine-rich secretory protein (CRISP) and snake venom metalloproteinase (SVMP) toxin families were constructed. All unique CRISP and SVMP sequences were extracted from the *Thamnophis* transcriptomes, and additional CRISP (Sunagar et al. 2012) (Supplementary Material 2) and SVMP (Reyes Velasco et al. 2014) (Supplementary Material 3) sequences were gathered. This resulted in a dataset of 52 CRISP sequences and 40 SVMP sequences for analyses. Using Geneious Prime 2025.0.3 (https://www.geneious.com), sequences were aligned with MAFFT (Katoh and Standley 2013), and trees were generated using PhyML (Guindon et al. 2010).

Intraspecific variation

We primarily focused on 2 taxa (*T. elegans* and *T. sirtalis)* with multiple wide-ranging subspecies representing distinct populations to test for intraspecific toxin variation within the genus. For *T. elegans*, 3 subspecies are currently recognized: *T. e. elegans*, *T. e. vagrans*, and *T. e. terrestris*. We gathered samples from all 3 subspecies to examine intraspecific variation across the entire species complex. *Thamnophis sirtalis* has 12 currently recognized subspecies (but see Jones et al. 2023); however, due to sampling restrictions, we investigated only 3: *T. s. sirtalis*, *T. s. fitchi*, and *T. s. infernalis*. Additionally, our sampling of *T. saurita* and *T. cyrtopsis* allowed for investigation for intraspecific variation between the *T. s. septentrionalis* and *T. s. saurita* subspecies of *T. saurita*, and the *T. c. cyrtopsis*, the *T. c. occelatus*, and the *T. c. collaris* populations of *T. cyrtopsis*, thus, they were included in these analyses. Using the `DESeq2 1.44.0` R package (Love et al. 2014), we tested for intraspecific differences in expression across the subspecies classification of both *T. sirtalis* and *T. elegans*. To test for significance, we used a Wald significance test with a local fit dispersion and a false-discovery rate of <0.05 as the significance threshold (Heptinstall et al. 2023; Rosales-García et al. 2023). For visualization, we utilized the log TPM of the differentially expressed toxin, and plotted a heatmap using the R package `pheatmap 1.0.12` (Kolde 2019).

Toxin and dietary complexities

To capture aspects of the genetic and expression complexities of toxins, we followed analytical pipelines from both Holding et al. (2021) and Schaeffer et al. (2023), all of which were conducted using R version 2.2.2 (2022–10-31) (R Core Team 2022) and RStudio version 2023.3.0 + 386 (Posit team 2023). For genetic complexity (referred to as transcriptomic complexity henceforth), we followed Holding et al. 2021, and counted appearances of unique k-mer sequences ($k = 21$, $k = 60$, and $k = 97$ base pair sequences), in a randomly sampled subset of 4 million DVG RNA-seq reads mapped to toxin gene transcripts. We utilized the

complexity scores from the 60-mer analysis following recommendations for accurate detection of point mutations (Holding et al. 2021), and referred to these scores as "transcriptomic complexity" unless specified otherwise. To capture the diversity of toxin expression, we employed methods from Schaeffer et al. (2023). Relative abundances of each toxin family present in our species' transcriptomes were calculated using `RSEM` (Li and Dewey 2011) expression results, and Shannon's H index was then used to calculate expression complexity measures. Shannon's H was transformed as exp(H) (Jost 2006) for downstream analyses.

Dietary records for each species were taken from Heptinstall et al. (2024). Dietary complexity metrics were calculated using Shannon's H index (Shannon 1948; Holding et al. 2021; Schaeffer et al. 2023), abundance-weighted mean phylogenetic distance (MPD) (Holding et al. 2021), and through the use of a diversity profile (Chao et al. 2014; Chao and Jost 2015). Shannon's H index and MPD were calculated using the abundance of prey items at the prey genus, family, order, and class taxonomic classification level; however, the results were broadly consistent across all classifications; thus, only results from the genus level classification are presented herein. Additionally, we employed a diversity profile to examine the relationship between toxins and dietary ecology across a spectrum of importance of prey item abundance (Roswell et al. 2023). Using the `rarity` function from the `MeanRarity 0.0.1.0005` R package (Roswell and Dushoff 2023), we varied the "l" exponent from the Hill diversity equation to emphasize a range of rarities of prey items. Following Chao and Jost (2015), we employed values of $l = -2$, $l = -1.5$, $l = -1$, $l = -0.5$, $l = 0$, $l = 0.5$, and $l = 1$. A value of $l = 1$ represents true species richness and is most sensitive to the rarest of species (Chao and Jost 2015). A value of $l = -1$ represents Hill–Simpson diversity and concerns the relative abundance patterns, which is most sensitive to common species (Simpson 1949). A value of $l = 0$ is called Hill–Shannon diversity and represents a balance between the emphasis of rarer vs. more common species (Kempton 1979). This method allowed us to determine if rarely consumed prey items or commonly consumed prey items were a stronger source of selection on toxin characteristics.

To test for phylogenetic signaling of transcriptomic sequence and expression complexity data, we used the `phylosig` function from `phytools` (Revell 2012) and calculated Blomberg's K (Blomberg et al. 2003) and Pagel's λ (Pagel 1993). Due to a moderate-to-low phylogenetic signal, we tested for relationships between toxin and dietary complexities using phylogenetic linear regression via `phylolm 2.6.2` (Ho and Ané 2014) R package. A phylogenetic tree of the genus *Thamnophis* was extracted from Hallas et al. (2022 and matched to our data using the `treedata` function from the `Geiger 2.0.11` R package (Pennell et al. 2014). Phylogenetic linear models were then run to calculate relationships between all combinations of toxin and dietary complexity measures. Lastly, we tested for relationships between expression and transcriptomic sequence complexities across our sampling. *Thamnophis brachystoma* and *T. butleri* were excluded from regression analyses due to an extremely low abundance of toxins being expressed in the transcriptome (<1% total expression) and displaying outlying toxin complexity values.

To investigate the relationship between *Thamnophis* toxins and dietary breadth within individual paralogous toxin gene families, we investigated transcriptomic complexities of 3 commonly expressed toxin families: CRISPs, SVMPs, and C-type lectins (CTLs). We chose these 3 toxin gene families because of their near-ubiquitous presence across our sampling and their generally high expression. For each species, all transcripts aligning with CRISPs, SVMPs, or CTLs were extracted, and a consensus transcriptome was assembled for each toxin family for each species in our dataset. Complexity calculations, using the k-mer method detailed above, were then calculated for these toxin family-specific sequences and were then compared to dietary complexity measures.

Lastly, we note that body size and age class of snakes might potentially alter toxin expression (Hogan et al. 2024). However, nearly all of our animals were mature adults, and we did not investigate any ontogenetic variation in expression complexity or transcriptomic complexity because of the low number of juvenile individuals.

Results

DVG transcriptomics

We sequenced DVG tissues from 51 *Thamnophis* individuals and produced high-quality reference transcriptomes for 16 species from the United States and Mexico (Supplementary Material 1, Figs. 1 and 2). After merging read pairs, we were left with between 6,070,570 and 29,551,060 (average: 13,754,479 ± 4,266,364.67 reads) reads per individual (Supplementary Material 1). The final, consensus transcriptomes comprised a range of distinct toxin transcripts representing 25 toxin protein families (Fig. 2). Total toxin expression (proportion of total DVG expression representing toxin families) varied widely across species, ranging from 0.34% of total expression to 74.47% of total expression (average: 26.28% ± 21.62%) (Fig. 2). Large amounts of variation in toxin profiles were present across the species.

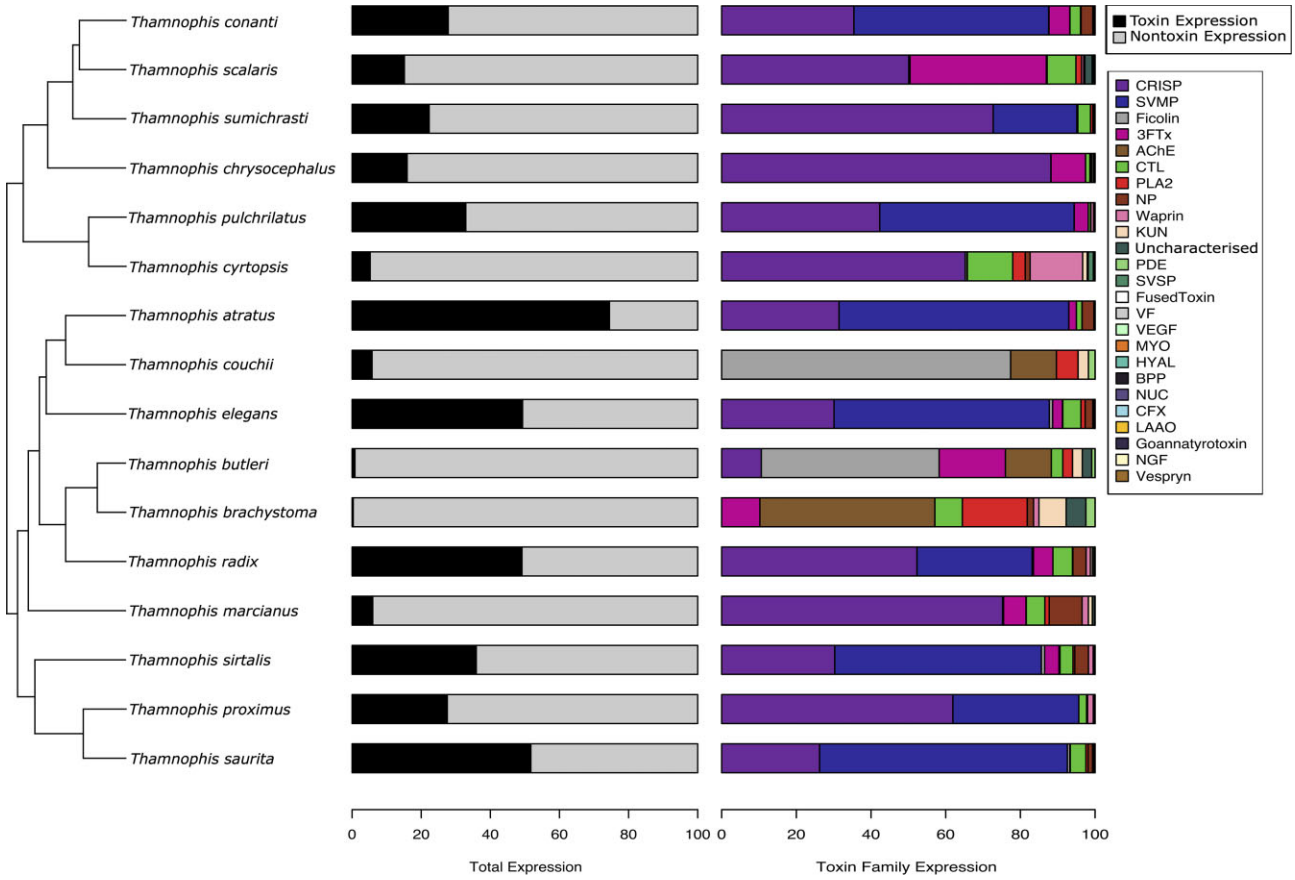

Fig. 2 DVG transcriptomic profiles used in our study of sixteen species of *Thamnophis*. Phylogeny adapted from Hallas et al. (2022) used to demonstrate the phylogenetic relationships among our sampled species. The middle bar chart represents the proportional expression levels of all transcripts present in the DVG. Darker bars represent the relative expression of genes that mapped to putative toxins, while lighter bars represent non-toxin (housekeeping gene) expression. The right bar plot represents the proportion of putative toxin expression by toxin family.

CRISPs were the most highly expressed toxin family in 7 species, and SVMPs were the highest expressed toxin family in 6 species. Our phylogenetic analyses of the CRISP and SVMP sequences show that there is a high likelihood of homology with sequences found in other venomous snakes, with *Thamnophis* CRISPs forming a monophyletic clade nested within CRISP venom sequences of other snakes (Supplementary Material 4), and *Thamnophis* SVMPs forming a paraphyletic clade across our SVMP tree (Supplementary Material 5). Ficolin was the highest expressed toxin family in 2 species, and acetylcholinesterase (AChE) was highest expressed in one species (Fig. 2). *Thamnophis butleri* and *T. couchii*, whose toxin expression was dominated by ficolin, represent the second and fourth lowest species regarding the total proportion of the transcriptome represented by toxins, respectively. In contrast, *T. brachystoma*, which displayed the single lowest total toxin expression, was dominated by AChEs.

Because of the unique composition within transcriptomic profiles, especially *T. butleri*, *T. brachytstoma*, and *T. couchii*, we felt it was necessary to validate that we had sequenced DVG rather than other, neighboring tissues. We tested for deviation from a significantly positive relationship in non-toxin gene expression between species and found no evidence of deviations between all species as all cross-species comparisons were significantly, positively correlated (P-value = $<2.2e^{-16}$), giving us confidence that all samples were indeed from DVGs (Supplementary Material 6).

Intraspecific differential expression of toxins

Our differential expression analyses yielded multiple differentially expressed toxins among subspecies of *T. sirtalis*, *T. elegans, T. saurita,* and *T. cyrtopsis*. *Thamnophis sirtalis* analyses yielded 12 differentially expressed toxins among the 3 subspecies, with *T. s. sirtalis* showing 10 differentially expressed toxins

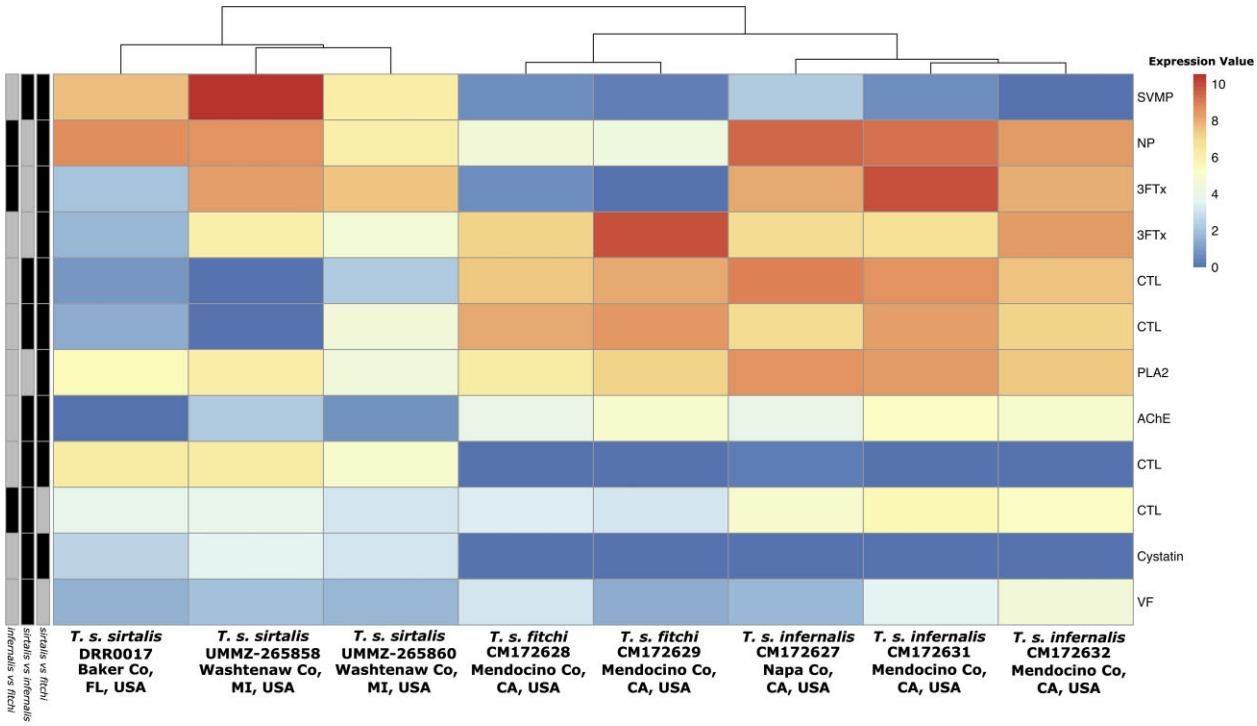

Fig. 3 Heatmap displaying differentially expressed toxin transcripts between 3 subspecies of *Thamnophis sirtalis*. Warm colors indicate higher expressed toxins, while cool colors represent lower expression. On the left side of the figure, a dark box indicates significant differences in expression between the populations being compared, while a lighter box represents non-significant expression differences.

when compared to *T. s. fitchi* and 8 differentially expressed toxins when compared to *T. s. infernalis* (Fig. 3). *Thamnophis s. fitchi* and *T. s. infernalis* only had 3 differentially expressed toxins (Fig. 3). Comparisons of *T. elegans* resulted in 11 differentially expressed toxins: 8 between *T. e. elegans* and *T. e. terrestris*, 5 between *T. e. elegans* and *T. e. vagrans*, and 4 between *T. e. vagrans* and *T. e. terrestris* (Fig. 4). Results of our analyses of *T. saurita* yielded 13 differentially expressed toxins between *T. s. saurita* and *T. s. septentrionalis* (Supplementary Material 7). Analyses of *T. c. cyrtopsis* and *T. c. ocellatus* yielded 8 differentially expressed toxins, while there were 12 between *T. c. collaris* and *T. c. cyrtopsis* and 13 between *T. c. collaris* and *T. c. ocellatus* (Supplementary Material 8).

Toxin and dietary complexity

Thamnophis species varied in both toxin and dietary complexity metrics. Transcriptomic complexity ranged from 24,236.77 to 92,128.49 effective k-mers (average: 57,215.41 ± 20,713.84) (Fig. 5, Supplementary Material 9). Expression complexity ranged from 1.60 to 3.50 effective expressed toxin families (average: 2.64 ± 0.51) (Fig. 5, Supplementary Material 9). Dietary complexity also showed wide-ranging results. MPD at the prey genus level yielded a range of 222–992 million years (average: 495.08 ± 248.34), while Shannon's H index calculated at the genus level yielded a range of 1.96–8.48 effective genera (average: 4.01 ± 2.10) (Fig. 5, Supplementary Material 9). Our investigations of phylogenetic signal and venom complexity showed a moderate-to-low effect of phylogeny (Expression complexity—$K = 0.738$; $\lambda = 7.33137e^{-5}$; Transcriptomic complexity—$K = 0.861$; $\lambda = 0.287$).

Our linear regressions displayed contrasting results when comparing the results of transcriptomic and expression complexity models. We found non-significant, negative correlations between transcriptomic sequence complexity at all k-mer count levels and both MPD and Shannon's H index calculations regardless of prey taxonomic classifications (Fig. 5, Supplementary Material 10). Relationships comparing toxin expression complexity were positively correlated with all measures of dietary complexity, with a statistically significant correlation found with MPD at all prey taxonomic levels and Shannon's H index calculated at the prey "Class" taxonomic level (Fig. 5, Supplementary Material 10). Our expression and transcriptomic complexity comparisons yielded a non-significant, negative relationship (P-value = 0.106; adjusted R-squared = 0.135).

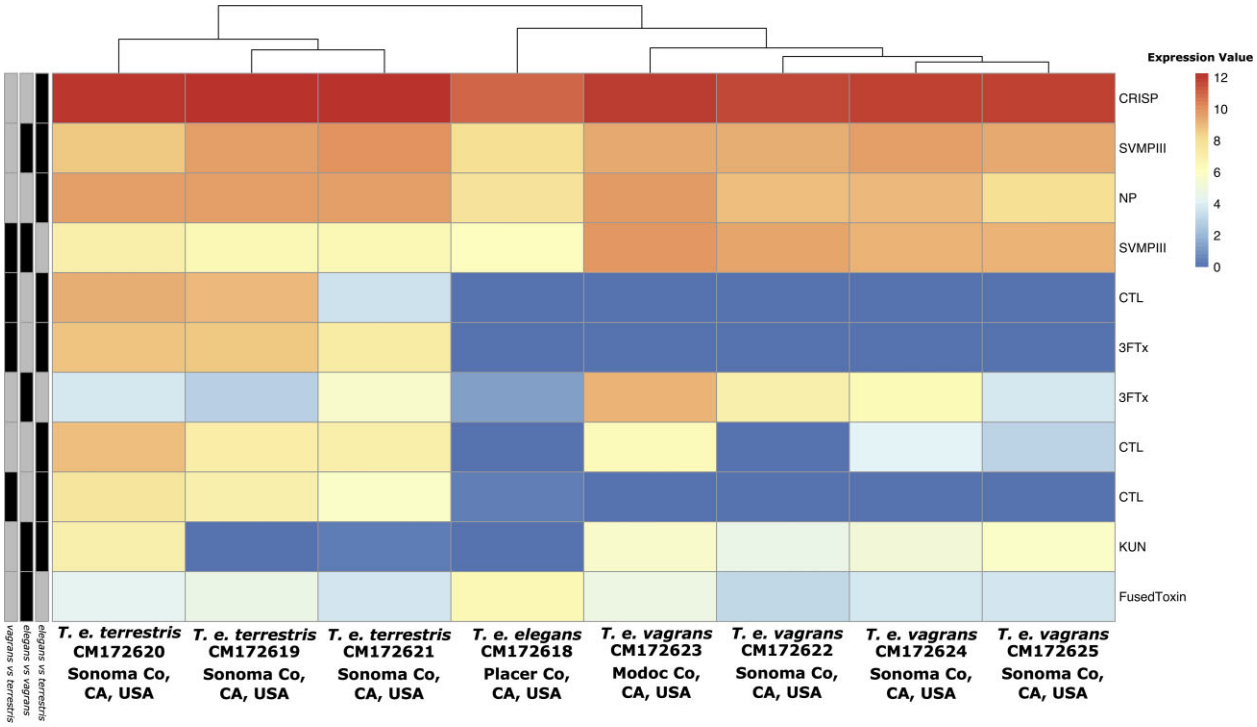

Fig. 4 Heatmap displaying differentially expressed toxin transcripts between 3 subspecies of *Thamnophis elegans*. Warm colors indicate higher expressed toxins, while cool colors represent lower expression. On the left side of the figure, a dark box indicates significant differences in expression between the populations being compared, while a lighter box represents non-significant expression differences.

Using the Hill Diversity metric with a varied "l" exponent yielded no significant results with any measure of toxin complexity. However, the trend of negative correlations between MPD and dietary values and positive correlations between expression complexity and dietary values held true (Supplementary Material 11). k-mer complexity showed stronger relationships with lower exponent values, which emphasizes the importance of common prey items, while expression complexity correlated most strongly with an exponent value of 0, which represents a balance between emphasizing common and rarer species (Supplementary Material 11).

Thamnophis butleri and *T. brachystoma* were not included in analyses investigating the relationships between venom complexity and dietary breadth or the diversity profile as they represented extreme venom complexity outliers. *Thamnophis butleri* displayed a transcriptomic complexity value of 132,670.74 and an expression complexity value of 4.21, while *T. brachystoma* displayed a transcriptomic complexity value of 172,950 and an expression complexity value of 4.73—all of which fall outside 3 standard deviations of the mean.

Our investigation into the importance of transcriptomic complexity of specific toxin families yielded no significant results; however, CTL transcriptomic complexities showed the strongest relationship with both MPD and Shannon's H index (Supplementary Material 12).

Discussion

Venom complexity is a key axis of venom variation in taxa such as snakes, spiders, and snails, where a more complex venom allows for the efficient processing of a wide range of prey items (Daltry et al. 1996; Phuong et al. 2016; Pekár et al. 2018; Davies and Arbuckle 2019; Holding et al. 2021; Schaeffer et al. 2023). Nevertheless, non-front fanged snakes remain understudied. We provide the most in-depth characterization of *Thamnophis* venom to date, unveiling both inter- and intraspecific variation in expression associated with phylogenetic diet diversity.

DVG transcriptomics

Thamnophis toxin expression was most commonly dominated by SVMPs and CRISPs, which are also reported to be expressed at high levels in other species of non-front-fanged snakes (Junqueira-de-Azevedo et al. 2016). SVMPs have been found in both front-fanged and non-front-fanged snakes in high levels, and are thought to cause local tissue damage and increased hemorrhaging due to anticoagulatory behavior at the

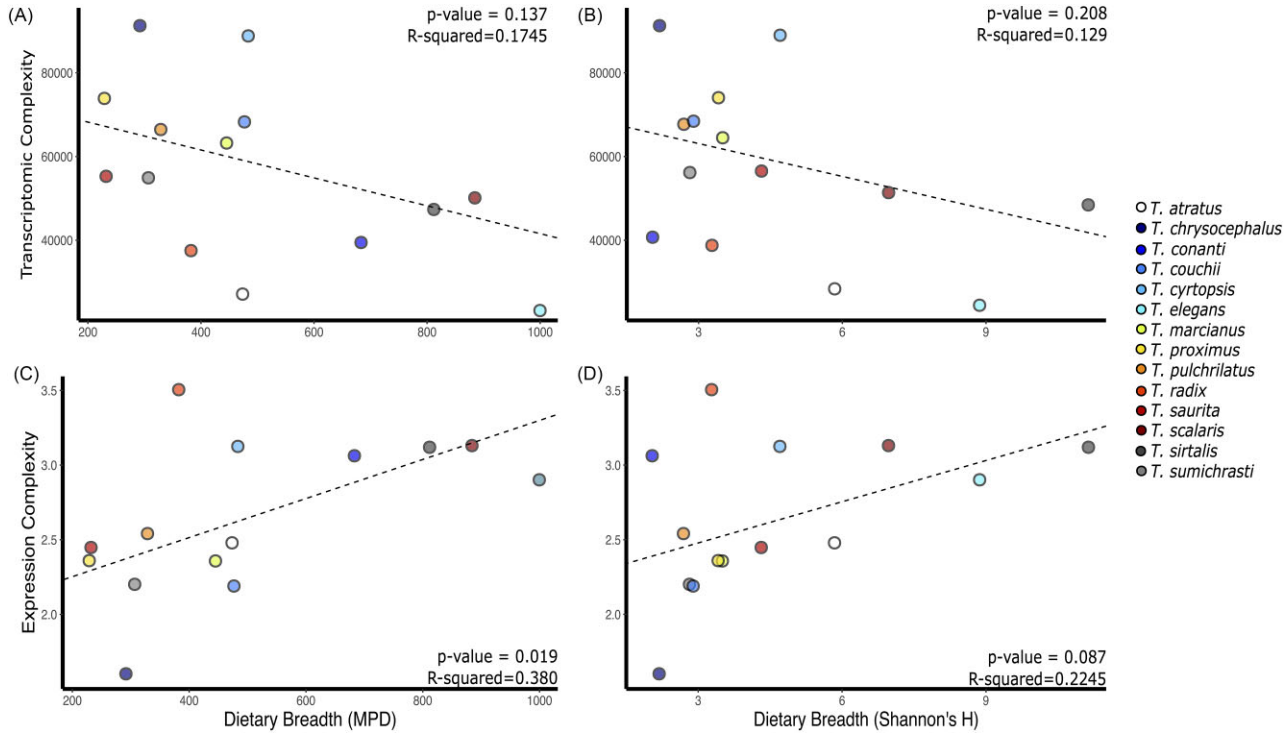

Fig. 5 Relationships between toxin and dietary complexity measures. (A) Relationship of the negative correlation between transcriptomic complexity (60-mer) and dietary breadth in terms of MPD. (B) Relationship of the negative correlation between transcriptomic complexity (60-mer) and dietary breadth in terms of Shannon's H index at the genus level. (C) Statistically significant relationship of the positive correlation between expression complexity and dietary breadth in terms of MPD. (D) Relationship of the positive correlation between expression complexity and dietary breadth in terms of Shannon's H index at the genus level. Phylogenetic linear regressions were used for each comparison, and suggest complexity of toxin expression, rather than sequence complexity, are under selection by dietary breadth.

location of a bite (Modahl et al. 2016). CRISPs are also widespread in reptiles; however, their function is less certain than that of SVMPs (Modahl et al. 2016). A CRISP isolated from *Helicops angulatus* exhibited neurotoxic-like effects and prompted respiratory paralysis in mice (Estrella et al. 2011), while another CRISP from *Philodryas patagoniensis* exhibited hemotoxic-like effects and caused muscular damage (Peichoto et al. 2009). Thus, the function of the CRISPs found in *Thamnophis* transcriptomes requires further investigation, but their phylogenetic relationships to sequences from other venomous snakes lends support to their toxic potential. CTLs, which have been shown to disrupt blood flow to the brain (Tian et al. 2020), and 3-finger toxins, which display a wide-range of biological activities (Kini and Doley 2010) were also found in many of our *Thamnophis* transcriptomes but at lower expression levels. Overall, it appears *Thamnophis* species have relatively simple venoms (mainly comprised of CRISPs and SVMPs) to aid in subjugating and processing prey items.

Transcriptome profiles were largely dominated by the SVMP and CRISP toxin families across our species, apart from *T. butleri*, *T. brachystoma*, and *T. couchii*, which displayed transcriptome profiles similar to each other but quite distinct from the remaining 13 species. *Thamnophis couchii* displayed the fourth lowest total toxin expression (5.8%), while *T. butleri* (0.83%) and *T. brachystoma* (0.34%) were the second lowest and lowest, respectively. *Thamnophis couchii* and *T. butleri* toxin expression was primarily dominated by the ficolin protein family, which was first characterized in the venom and transcriptome of *Cereberus rynchops*, from the family Homalopsidae (OmPraba et al. 2010). While related ficolin transcripts are commonly found in several colubrid species, they are usually found lowly expressed, such as found here (OmPraba et al. 2010; Modahl and Mackessy 2019). *Thamnophis brachystoma* toxin gene profile was dominated by AChEs, and AChE activity has been documented in several snake species, but is most prominent in the venom of *Boiga* species, where they display prey-specific toxicity (Mackessy et al. 2006). While ficolins and AChEs have been documented as the dominant toxin gene families in a few non-front-fanged snakes, they were most commonly found with low expression levels.

The combination of low total toxin expression and unusual dominant toxin families led to the transcriptomic profiles of *T. couchii*, *T. butleri*, and *T. brachystoma* to stand out from the remainder of the genus. There are multiple potential reasons behind this unusual variation, including (1) an almost complete loss of toxin production, (2) potential novel toxins not found elsewhere that went unannotated in our transcriptomes, (3) human error in the preparation of the transcriptomes, or (4) a difference in metabolic rates led to a difference in mRNA production. Human error is unlikely given that multiple samples of each species displayed similar results, and no deviation was found in non-toxin expression between species (Supplementary Material 6). While we cannot fully rule out human error, we will focus the remainder of our discussion on other hypotheses like (1) a loss of toxins in the transcriptome, (2) metabolic differences led to a difference in mRNA production, or (3) the potential recruitment of novel venom components. The 2 highest expressed transcripts from *T. butleri*, the 3 highest expressed transcripts from *T. brachystoma*, and the fourth highest expressed transcript of *T. couchii* all most closely align with the *T. elegans* minor allergen Can f 2-like transcript from the NCBI nucleotide database (Reference Sequence: XM_032,232,387.1). This transcript is classified under the lipocalin protein family known for enabling small molecule binding; however, recently, lipocalins have been identified as a potential venom component within non-front-fanged snakes (Junqueira-de-Azevedo et al. 2016). In *Oxyrhopus guibei*, lipocalins account for 29% of the sequencing reads in the transcriptome, suggesting an important role in olfactory-mediated behavior or even as a novel toxin; however, its function is impossible to determine without proteomic analysis and functional assays (Junqueira-de-Azevedo et al. 2016). Because of the lack of current evidence for lipocalins acting as a toxin, we kept their classification as non-toxins for our analyses, but it is worth noting the high expression values found here for future studies.

Our next hypothesis is that differences in metabolic rate are potentially leading to differences in timing of peak mRNA production between the species of *Thamnophis*. There is evidence of changes in metabolic rate within *Thamnophis* associated with current environmental characteristics and with the environment during development, allowing for potential variation within our samples (Gangloff et al. 2015). Additionally, there is evidence of metabolic adaptation for dietary and behavioral specialization within *T. elegans* and *T. butleri*, so the possibility of varied metabolic rates influencing mRNA production across our sampling cannot be ignored (Kamel and Gatten 1983; Britt et al. 2006). The dietary and behavioral specialization of *T. butleri* and *T. brachystoma* could potentially have a large effect on mRNA production and, in turn, production of toxins. However, *T. couchii* is largely a dietary generalist and lacks the smaller body size of *T. butleri* and *T. brachystoma* (Heptinstall et al. 2024). Thus, further work into the relationship between metabolic rate and toxin production needs to be conducted to provide evidence for this hypothesis.

Our last explanation for the unexpected transcriptome profiles of *T. couchii, T. butleri*, and *T. brachstoma* is a loss of toxin transcript production due to a fitness cost associated with producing these toxins. The production of venom is a metabolically expensive process (McCue 2006), so if venom is no longer significantly improving prey capture and processing for a species, venom expression may be selected against. It is possible that this functional tradeoff has driven changes in the venom profiles found in *T. butleri*, *T. brachystoma*, and *T. couchii*. *Thamnophis butleri* and *T. brachystoma* are specialist predators, feeding almost exclusively upon earthworms (Heptinstall et al. 2024). Similarly, *Aipysurus eydouxii* and *Emydocephalus annulatus* are sea snakes with specialized diets consisting of fish eggs and have evolved reduced fangs, venom glands, and an almost complete loss of venom (Li et al. 2005; Shine et al. 2022). Like fish eggs, earthworms pose minimal threat to the snakes during prey capture and are relatively easy to subdue. However, there is evidence for highly toxic venom evolution in *Toxicocalamus longissimus*, which also feeds heavily on earthworms (Calvete et al. 2012). Nonetheless, we hypothesize that, like in the sea snakes, natural selection favored a reduction in the production of the energetically costly toxins in *T. butleri* and *T. brachystoma*, which may have led to expression declines within their toxin profile via epigenomic changes or perhaps subsequent gene loss.

The situation with *T. couchii* is less clear, as these are sizeable generalist predators, not smaller gartersnakes taking innocuous invertebrate prey. The species is highly aquatic and displays adaptations such as more curved teeth that increase their ability to hold on to aquatic prey, such as fish and amphibians (Britt et al. 2009; Feldman et al. 2016; Heptinstall et al. 2024). It is possible that *T. couchii* preferentially hunts relatively small prey that are easily overpowered and swallowed, relying on other adaptations and reducing the need for venom. Greene and Wiseman (2023) found evidence of a positive association between venom use and an increase in relative prey mass in pitvipers, and it is reasonable to assume the inverse process could be taking place in non-front-fanged snakes like *Thamnophis*, leading to the reduction of toxin use in species such as *T. butleri*, *T. brachystoma*, and possibly *T. couchii*.

However, *T. couchii* is known to occasionally tackle large fish (Rossman et al. 1996) and other large-bodied gartersnakes did not show a reduction in venom production, and toxin expression and complexity values of *T. couchii* aligned more closely with other *Thamnophis* species that displayed more typical transcriptomic profiles. Thus, we recommend detailed ecological work to determine relative prey size of *T. couchii* (see Greene and Wiseman 2023) and further investigation into the venom profile of this species to verify our findings. Because of the outlying expression and complexity values of *T. butleri* and *T. brachystoma*, we predict it is less likely total toxin expression levels below 1% contribute to toxin production of ecological significance when compared to higher toxin expression levels and would thus not be under the same selective pressures as species with higher total toxin expression. However, further investigation of the transcriptomics, genomics, and proteomics, including LD50 assays, of venom of non-front-fanged snakes, and specifically *T. butleri*, *T. brachystoma,* and *T. couchii*, is required to verify our predictions.

Intraspecific differential expression of toxins

In addition to the interspecific variation of toxin expression, we discovered intraspecific toxin expression variation within the widespread and ecologically diverse species, *T. sirtalis* and *T. elegans*. The toxin expression variation found within *T. sirtalis* aligns with the genetically distinct populations of *T. sirtalis* found by Jones et al. (2023) in North America's central, southeastern, and western regions. When compared, the western *T. s. fitchi* and *T. s. infernalis* displayed a few significant and differentially expressed toxins, but when compared to *T. s. sirtalis* in the east/central portions of North America, a much larger number of differentially expressed toxins were discovered. These data further support the genetically distinct populations of *T. sirtalis* as described by Jones et al. (2023), regardless of the subspecies classifications presently recognized. These results suggest the venom phenotype is potentially adapting on a local scale to prey items or is significantly impacted by inherited genes; however, further investigation of these hypotheses is required to fully understand the implications of this intraspecific variation. Intraspecific variation within *T. elegans* aligns with the 3 well-documented distinct populations of *T. e. elegans*, *T. e. vagrans*, and *T. e. terrestris* (Hallas et al. 2021). Not only do these species vary in geography, but they also differ in ecology, which could be driving the variation in toxin expression. Some populations of *T. e. terrestris* are specialist slug predators, while *T. e. vagrans* and *T. e. elegans* are primarily generalist predators (Arnold 1977; Britt et al. 2006), potentially facilitating the evolution of divergent toxin profiles. Trends of differential expression among subspecies were also found within the *T. saurita* and *T. cyrtopsis* species complexes, further supporting the hypothesis of local adaptation in venom expression. However, further exploration of intraspecific toxin characteristics will lead to a more fine-scale view of the diversity of toxins found within the genus and expose how toxins evolve on a landscape scale.

Venom and dietary complexity

The variation of toxin genes and their expression found within *Thamnophis* is likely being selected on by dietary ecology, as we found toxin expression complexity to increase with phylogenetic diversity of diet. While we did find phylogenetic signal, the moderate-to-low values suggest this is not the primary driver of venom complexity in *Thamnophis* (Münkemüller et al. 2012). Thus, these results offer interesting comparisons to front-fanged snake venoms and the evolutionary pressures shaping their venom composition. While diet is shown to drive venom evolution in many species, the specific characteristic of venom being acted upon may change among species, across geographic scales, and among ecological communities. In North American pitvipers, an increase in phylogenetic diversity of diet drives an increase in transcriptomic complexity of venom (Holding et al. 2021). When investigating this relationship with a broad but sparse taxonomic sampling, Schaeffer et al. (2023) showed that dietary evenness rather than richness drives the evolution of relative abundance of toxins. We found that the phylogenetic diversity of diet drives the complexity of the relative abundance of toxin expression. Our findings partially agree with Schaeffer et al.'s (2023) conclusions about the importance of relative abundance (in our case, transcriptomic expression) of toxins and Holding et al.'s (2021) conclusions about the importance of phylogenetic distance. However, the exact relationships we uncovered in gartersnakes are novel. Additionally, we found a significant, positive relationship between expression complexity and dietary richness at the taxonomic class level, supporting the hypothesis that higher levels of taxonomic classifications could capture some of the phylogenetic distance of dietary groups. While the relationship between taxonomic class and expression complexity was weaker than relationships found when investigating MPD at the same class level, taxonomic class could potentially serve as a proxy if a phylogenetic tree is unavailable.

So, why might selection be acting on toxin family expression complexity in non-front-fanged snakes rather than transcriptomic complexity, as seen in front-fanged snakes? One hypothesis may be that non-front-fanged snakes lack the underlying genetic variation of toxins for selection to act upon. Another possibility is that

prey are not coevolving with *Thamnophis* venom, minimizing the importance of paralogs within the transcriptome. In pitvipers, who are often ambush predators that employ a rapid bite, release, and relocation hunting strategy, venom is often used to neutralize relatively large prey that pose potentially significant threats to the snake itself if there are increased handling times (Greene 1997). However, gartersnakes are active foragers, and often select to prey on smaller and more innocuous species they can catch and physically overpower (Rossman et al. 1996). This potentially leads to the discrepancy of selection for increased toxin paralogs (and in turn, complexity) between many front-fanged snakes and *Thamnophis*. For example, in the venom gland transcriptome of *Crotalus cerastes*, a range of 33–44 toxin transcripts were identified across 8 individuals, with 62 unique toxin transcripts present in the consensus transcriptome (Hofmann et al. 2018). Meanwhile, *T. radix* displayed the highest expression complexity measurements of any species in our study, but with only 26 unique toxin transcripts in our consensus transcriptome across 3 individuals (ranging from 23–25 per individual). *Thamnophis* species, and potentially other non-front-fanged snakes, could overcome this lack of transcriptomic diversity via variation in relative toxin expression. Further evidence supporting the elevation of expression importance was shown with the negative correlations between transcriptomic complexity and all levels of dietary complexity, showing transcriptomic complexity could potentially be evolving inversely with dietary complexity. The negative relationship between transcriptomic and expression complexities across the genus further supports this. However, these relationships were not statistically significant, limiting our inferences. Additionally, the lack of relationships between the transcriptomic complexity of specific toxin families and dietary complexity also support the importance of expression complexity in non-front-fanged snakes. Transcriptomic complexity of toxin families has also been investigated in North American pitvipers, where SVMPs, snake venom serine proteases, and phospholipase A2 (PLA2s) all displayed positive correlations with the phylogenetic diversity of prey (Holding et al. 2021). However, we found no relationship when investigating transcriptomic complexity of SVMPs. CRISPs, or CTLs here, suggesting transcriptomic complexity does not significantly impact the toxin characteristics of *Thamnophis* at any level. Conversely, it is possible that some *Thamnophis* species with a specialized diet and their prey are in evolutionary "arms-races" (Brodie et al. 2002; Reimche et al. 2020) similar to relationships found in some western rattlesnakes and their mammalian prey (Holding et al. 2016; Robinson et al. 2021). This coevolution could potentially drive more diverse venoms in species with less diverse diets and lead to the non-significant trends we found between increased transcriptomic complexity and wider dietary breadth. More focused work within species may be required to understand how venom complexity varies with local diet in *Thamnophis* and other non-front fanged snakes.

Conclusion

Using transcriptomic approaches, we have provided an in-depth view of venom diversity and evolution within the widespread and ecologically important genus *Thamnophis*. We produced DVG transcriptomes representing 16 species, identifying putative toxins and quantifying expression for each. We found that most species exhibited SVMP and CRISP-dominated venoms, suggesting a primary role of increased hemorrhaging and muscle digestion in prey. In addition, we provided tentative evidence of secondary loss of homologous toxin gene expression in *T. butleri*, *T. brachystoma*, and potentially *T. couchii*; however, further investigation is needed to support these conclusions. In our investigation of venom evolution, we found no relationships between transcriptomic complexity with dietary complexity or dietary phylogenetic diversity, in contrast to similar investigations of front-fanged snakes (see Holding et al. 2021; Mason et al. 2022; Schaeffer et al. 2023). However, we found significant increases in toxin expression complexities in response to increased phylogenetic diversity of snake diet, suggesting the selective pressures imposed by diet are acting upon toxin expression rather than toxin allelic variation itself in non-front-fanged snakes. Broadly, we have linked venom evolution in non-front-fanged snakes to ecologically based selective pressures. Additionally, our study provides an example of how selection may act on the expression of genes rather than variation in the gene sequences themselves to produce phenotypic patterns in nature. Primarily, our results showed how the gene expression of a homologous phenotype can be shaped by selection in varying ways across a group of closely related species, contributing to the phenotypic diversity observed today.

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Supplementary data

Supplementary Data available at *IOB* online.

Conflict of interest

The authors declare no competing interests.

Author contributions

T.C.H, R.A.R.G., E.A.M., R.M.R., M.L.H., and C.L.P. conceived the ideas for the study. T.C.H, R.A.R.G., E.A.M., R.M.R., M.L.H., D.R.R., A.J.M., E.P.H., T.D.S., and C.L.P. designed the study. T.C.H., R.A.R.G., R.M.R., M.L.H., D.R.R., M.B., G.C.G., C.R.F., T.D.S., E.P.H., M.P.H., and C.L.P. assisted with permitting and sample collection. T.C.H., R.A.R.G., and R.M.R. conducted analyses. T.C.H. wrote the initial draft of the manuscript with R.A.R.G., R.M.R., E.A.M., M.L.H., C.R.F., and C.L.P. significantly contributing to manuscript writing and review. All authors have given final approval for publication.

Data availability

The data underlying this article are available in the article and in its online supplementary material. Transcriptomic sequences are deposited at DDBJ/EMBL/GenBank under the accessions GKWJ00000000, GKVS00000000, GKVT00000000, GKVU00000000, GKVV00000000, GKVW00000000, GKVX00000000, GKVY00000000, GKVZ00000000, GKWA00000000, GKWB00000000, GKWC00000000, GKWG00000000, GKWF00000000, GKWE00000000, and GKWD00000000. Annotated transcriptomes are available at https://github.com/theptin/Thamnophis_Venom.

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