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Intraspecific sequence and gene expression variation contribute little to venom diversity in sidewinder rattlesnakes (*Crotalus cerastes*)

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Traits can evolve rapidly through changes in gene expression or protein-coding sequences. However, these forms of genetic variation can be correlated and changes to one can influence the other. As a result, we might expect traits lacking differential expression to preferentially evolve through changes in protein sequences or morphological adaptation. Given the lack of differential expression across the distribution of sidewinder rattlesnakes (*Crotalus cerastes*), we tested this hypothesis by comparing the coding regions of genes expressed in the venom gland transcriptomes and fang morphology. We calculated Tajima's D and F_{ST} across four populations comparing toxin and nontoxin loci. Overall, we found little evidence of directional selection or differentiation between populations, suggesting that changes to protein sequences do not underlie the evolution of sidewinder venom or that toxins are under extremely variant selection pressures. Although low-expression toxins do not have higher sequence divergence between populations, they do have more standing variation on which selection can act. Additionally, we found significant differences in fang length among populations. The lack of differential expression and sequence divergence suggests sidewinders—given their generalist diet, moderate gene flow and environmental variation—are under stabilizing selection which functions to maintain a generalist phenotype. Overall, we demonstrate the importance of examining the relationship between gene expression and protein-coding changes to understand the evolution of complex traits.

1. Background

Traits involved in antagonistic coevolutionary interactions can evolve rapidly under strong selective pressures [1–3]. Selection can act through changes in gene expression or through mutational changes to the genes themselves, altering the structure and function of the proteins underlying the trait. These forms of genetic variation on which selection can act can be highly correlated: high-expression genes tend to show lower rates of coding sequence evolution due to their importance in producing the focal phenotype [4]. Therefore, to understand the evolution of traits at the heart of coevolutionary interactions, differences in both coding sequence evolution and gene expression should be examined.

Snake venom is a rapidly evolving polygenic trait in which significant changes to both gene expression and protein-coding sequences have been observed [5–7]. Snake venom exhibits considerable variation at both intra- and interspecific levels, which is largely believed to be the result of local adaptation to either prey or

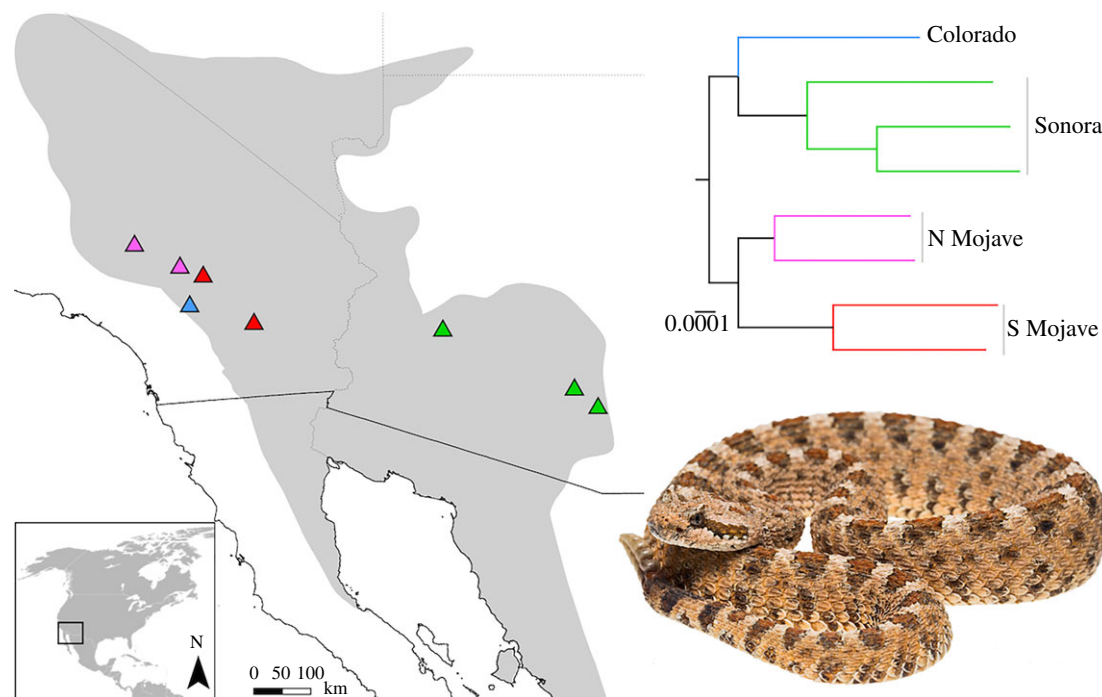


Figure 1. Distribution of sidewinder rattlesnakes (*Crotalus cerastes*), and localities of samples used herein. Sample colours correspond to lineage assignments based on phylogenetic analyses of 1508 nontoxin loci (approx. 1.4 million bp) [28] (upper right). Inset photo of *C. cerastes* by Travis Fisher. (Online version in colour.)

predator resistance, potentially resulting in coevolutionary arms races [7–11]. Importantly, despite being polygenic, venom is the result of a relatively straightforward pathway connecting the expression of venom genes to the venom proteins and ultimately the venom phenotype [12,13]. This linear genotype to phenotype pathway allows variation at both levels (i.e. expression and sequence evolution) to directly affect venom functionality, making it simple to simultaneously study the effects of selection on both protein-coding sequences and expression of toxins. Positive selection is frequently detected in toxin genes in a variety of venomous snake species [5,6,14,15]. Frequent toxin gene duplication has facilitated neo- and sub-functionalization and led to the formation of large toxin gene families [5,15,16]. However, recent research at the intraspecific level suggests that snake venom evolution might be driven by changes in toxin gene expression (i.e. toxin quantity) rather than coding sequence evolution (i.e. toxin function) [7,17–20]. The architecture of venom allows expression variation to occur without significant deleterious effects to other genes (i.e. pleiotropy), allowing for rapid evolutionary adaptation to coevolutionary interactions [21–23]. Toxin expression differences are known in several venomous snake systems. For example, mainland and insular populations of eastern diamondback rattlesnakes (*Crotalus adamanteus*) display differential expression that results in local adaptation to the resistance phenotype of sympatric prey [7]. High-expression toxins are considered vital for venom functionality (i.e. general prey killing) and therefore evolve under strong purifying selection across populations [7,24,25]. Meanwhile, low-expression toxins can exhibit prey-specific functions over relatively short time scales, and therefore have higher expression variation and differentiation in response to changes in prey resistance [7,24,25].

Undoubtedly, selection on both toxin function and quantity have influenced the evolution of snake venom, as evidenced by the high variability exhibited by numerous species [26–28]. However, most tests for positive selection have examined

multiple species rather than populations of a single species. Similarly, some species have similar venom expression profiles across large geographical scales, displaying little evidence of differential expression [17,29]. The lack of differential expression suggests that the venoms of these species are under different selection pressures than those exhibiting high intraspecific expression variation. For example, eastern coral snakes (*Micrurus fulvius*) lack variation in toxin expression across their distribution, and although there is evidence of positive selection on toxin sequences when compared to other coral snake species, no study has examined intraspecific sequence variability [17,30]. Examining the coding sequences of toxins in species that lack differential expression patterns can highlight the role of selection on protein-coding regions to test if there is a relationship between toxin expression and sequence divergence, and how variation in one form of genetic variation (or lack thereof) can influence the other.

The sidewinder rattlesnakes (*Crotalus cerastes*) is a small (approx. 40 cm) rattlesnake native to warm deserts of the southwestern United States and northwestern Mexico (figure 1) [31,32]. Hofmann *et al.* [29] used venom-gland transcriptomics to test for evidence of differential expression between subspecies, mitochondrial lineages and lineages inferred from loci in the transcriptome. Unlike the extreme differences found in venom expression profiles of some sympatric rattlesnake species—including Mojave rattlesnakes, *C. scutulatus* [33,34] and southern Pacific Rattlesnakes, *C. helleri* [35,36]—few genes were differentially expressed between lineages of sidewinders, and overall venom expression was similar across their range [29]. The lack of differential expression among toxin genes could be a product of a relatively flat adaptive landscape and stabilizing selection functioning to maintain a generalist venom arsenal [37–39]. Sidewinders prey on a variety of small mammals and lizards, and there is no evidence of geographical variation in their diet, supporting this hypothesis [40,41]. Alternatively, if there is a relationship between toxin expression and sequence divergence, changes to sidewinder

venom could instead occur via changes to the protein sequences as they respond to an adaptive landscape. A single case of neurotoxicity in sidewinder venom [42], together with data showing that expression and sequence evolution are highly correlated [4], favour this hypothesis.

Here, we investigate the role sequence divergence has played in driving the evolution of sidewinder rattlesnake venom. Specifically, because sidewinder rattlesnakes lack considerable differential expression of toxin genes, we hypothesize that their venoms have diverged in protein sequences. Secondly, because high-expression genes are constrained by selection to ensure translational efficiency and production of the focal phenotype, they tend to have lower rates of sequence evolution [43]. Therefore, we hypothesize that sequence divergence occurs mainly in low-expression proteins. To test these hypotheses, we use recently characterized venom gland transcriptomes and compare several metrics of toxin sequence divergence to a larger set of nontoxin genes among lineages of sidewinder rattlesnakes. If positive selection and changes in toxin function underlie the evolution of sidewinder venom, then we expect toxins will have significantly higher rates of coding sequence evolution than nontoxins, with non-synonymous mutations being highly differentiated between populations. Alternatively, if sidewinders have a generalist venom phenotype evolving via stabilizing selection, then we expect toxins to have lower or similar rates of coding sequence evolution to nontoxins. In addition to examining the venom phenotype, we use museum specimens to test for differences in fang morphology between lineages to fully investigate the potential for local adaptation in this integrated feeding phenotype.

2. Methods

(a) Variant calling

To examine toxin sequence evolution in sidewinder rattlesnakes, we used samples and venom-gland transcriptomes generated by [29]. First, to assess the role of sequence variation on venom evolution, we called single-nucleotide polymorphisms (SNPs) using a combination of BWA-MEM, Picard and GATK (electronic supplementary material, S1).

(b) Sequence evolution

To summarize the variant data, we determined whether each SNP was synonymous or nonsynonymous, calculated the number of SNPs per kilobase, calculated nucleotide diversity (π), and tested for differences between toxins and nontoxin loci (electronic supplementary material, S1).

We tested for evidence of selection by calculating Tajima's D and Weir and Cockerham's F_{ST} across all individuals and populations, and then tested for differences in means estimates between toxins and nontoxins (electronic supplementary material, S1). To account for differences in sample sizes between toxins and nontoxins, we randomly sub-sampled nontoxins to toxin sample size and performed 1000 bootstrap replicates of the regression analyses. The proportion of the bootstraps (b) which detected significance ($p < 0.05$) is reported alongside the results from the full dataset.

To look for specific toxins under selection, we generated a null distribution from the nontoxin transcripts and looked for toxin outliers outside the 95th percentile. Additionally, to test if low-expression toxins were under stronger positive or balancing selection pressures, we used linear regression to correlate average

toxin expression [29] to estimates of Tajima's D and F_{ST} . We tested positive and negative values of Tajima's D separately to account for potential differences in expression related to alternate selection pressures.

Lastly, we tested for differentiation between lineages using pairwise F_{ST} calculated for each locus between the four phylogeographic lineages (i.e. Colorado, Sonora, North Mojave, South Mojave), and analyses were performed as above (electronic supplementary material, S1). To maximize our ability to detect evidence of sequence differentiation, we combined lineages into larger clades and re-performed F_{ST} analyses. Larger clades were based on the phylogeny recovered by [29] or on geography (electronic supplementary material, S1).

(c) Fang morphology

To characterize variation in fang length among sidewinder lineages, we measured formalin-preserved specimens housed at the Museum of Vertebrate Zoology at Berkeley (MVZ), the California Academy of Sciences (CAS) and the University of Illinois Museum of Natural History (UIMNH). Specimens were assigned to lineages based on geography, with geographically ambiguous samples removed prior to analysis. Sampling included specimens from the North Mojave ($n = 9$), South Mojave ($n = 12$), Colorado ($n = 19$) and Sonora ($n = 14$) lineages. The left and right fangs were measured from tip to base using digital callipers, and the average of the left and right measurements was used for statistical analysis. To control for the effect of individual snake body size on fang length, we also measured the head length of each snake. We measured head length using the 'straight' tool in IMAGEJ v. 1.52a [44] using dorsal photographs of the head taken over a 0.5 cm grid. By dividing fang length by head length, we obtained a ratio describing fang size relative to total head size. We tested for an effect of sidewinder lineage on the relative fang lengths using a one-way ANOVA, and compared individual lineages with Tukey's HSD test.

3. Results

(a) Sequence variation

The majority of toxin and nontoxin transcripts had one or more SNPs (table 1). However, toxins maintained a significantly higher number of SNPs per kb ($R^2 = 0.01$, $p < 0.01$, $b = 0.93$) and there was a significant association between the type of mutation and whether the transcript was a toxin or nontoxin; toxins showed significantly more nonsynonymous variants ($X^2 = 175.31$, d.f. = 1, $p < 0.01$; table 1) and maintained significantly higher values of nucleotide diversity (π ; $R^2 < 0.01$, $p = 0.04$, $b = 0.20$; table 1) than nontoxins.

(b) Signatures of selection

To test for signatures of selection, we first calculated Tajima's D across all eight individuals. On average, estimates of Tajima's D for nontoxin transcripts were found to be significantly different from zero ($\bar{x} = -0.42 \pm 0.82$, $t = -21.53$, d.f. = 1770, $p < 0.01$; figure 2a). However, estimates of Tajima's D for toxins were not significantly different from zero ($\bar{x} = -0.11 \pm 0.96$, $t = -0.87$, d.f. = 57, $p = 0.39$), suggesting toxins are evolving along the mutation-drift equilibrium with little evidence of selection or that toxins undergo highly variable selection pressures (figure 2a). Estimates of Tajima's D between toxins and nontoxins were significantly different ($R^2 < 0.01$, $p < 0.01$, $b = 0.39$), providing further evidence that toxins and nontoxins are evolving differently with

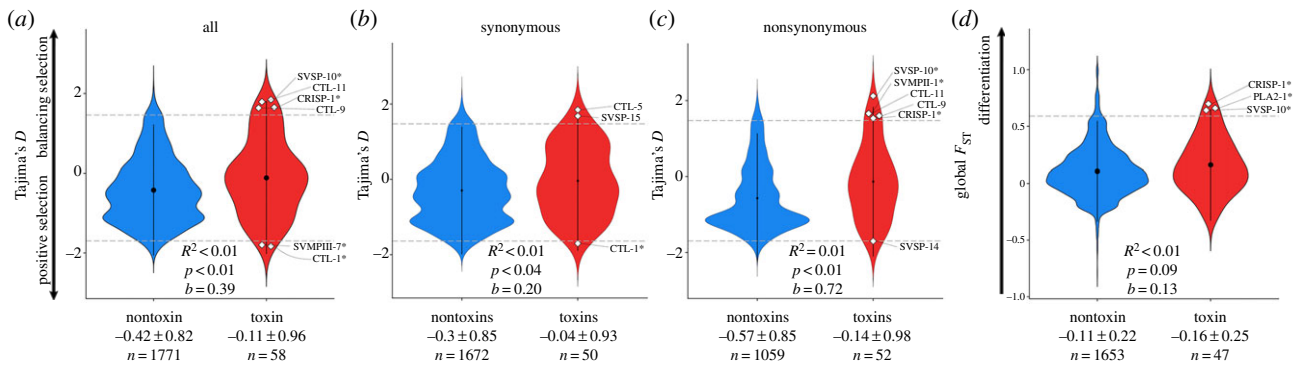


Figure 2. Violin plots of Tajima's D for (a) all mutations, and separately for (b) synonymous mutations and (c) nonsynonymous mutations. (d) Weir and Cockerham's F_{ST} estimates from nontoxin and toxin transcripts of all eight sidewinders (*Crotalus cerastes*). Average nontoxin estimates were significantly different than toxins for estimates of Tajima's D , but were not significantly different for estimates of F_{ST} . Both metrics had low bootstrap support for significance, with the majority of the signal for Tajima's D coming from nonsynonymous mutations. Nontoxins were used as a null distribution to identify toxin transcripts that might be evolving under strong positive or balancing selection. Toxins were under significant selection if they were located outside the 95th percentile of nontoxin transcripts indicated by the dotted grey line and white diamonds. Asterisks next to toxins indicate that the toxin was proteomically verified by [28]. (Online version in colour.)

Table 1. Sequence variation results between toxins and nontoxins including the number of transcripts found to have SNPs, the number of SNPs by mutation type, SNPs per kilobase (kb) and nucleotide diversity (π).

	transcripts	SNPs	synonymous	nonsynonymous	SNPs/kb	π
nontoxin	1777 (90%)	9423	6893 (73%)	2530 (27%)	5.47 ± 3.43	0.26 ± 0.09
toxin	58 (94%)	392	166 (42%)	226 (58%)	7.69 ± 4.20	0.28 ± 0.10

nontoxins—unexpectedly—evolving under stronger positive selection (figure 2a).

We then calculated Tajima's D separately for nonsynonymous and synonymous mutations. We found that, as expected, nonsynonymous mutations are under stronger positive selection than synonymous mutations (figure 2b,c). However, given that toxins have a mean estimate closer to zero for both types of mutations, toxins are either not evolving under strong selection pressures or are under more variable selection pressures which neutralizes the signal in the mean value. Additionally, nonsynonymous mutations appear to be driving the signal of outliers detected in the analysis across all SNPs.

Although we found no evidence of selection across all toxin loci, we did not expect all toxins to be evolving equally. Therefore, we used the nontoxins as a null distribution to identify individual toxin outliers that might be evolving under positive or balancing selection. Based on the sample size of toxins used in our outlier analyses, we would expect to identify between two and three outliers by chance (5%). In this analysis of Tajima's D , we identified six toxin transcripts that fell outside the 95th percentile generated from the nontoxin distribution (figure 2a). Only CTL-1 and SVMPIII-7 were found to be under significant positive selection, while CRISP-1, CTL-9, CTL-11 and SVSP-10 were found to be under significant balancing selection (electronic supplementary material, table S1).

Next, we calculated F_{ST} across all lineages. Differentiation in toxins relative to nontoxins was used to represent signatures of divergent selection among lineages. On average, estimates of F_{ST} for nontoxins were not significantly different than toxins ($R^2 < 0.01$, $p = 0.09$, $b = 0.13$) with toxins maintaining a slightly higher average (0.16 ± 0.25) than nontoxins (0.10 ± 0.22 ; figure 2d). Using the nontoxins as a

null distribution, we identified three toxin transcripts that fell outside the 95th percentile: CRISP-1, PLA2-1 and SVSP-10 (figure 2d; electronic supplementary material, table S1). Only CRISP-1 and SVSP-10 were shared between Tajima's D and F_{ST} analyses (electronic supplementary material, table S1).

(c) Effect of toxin expression on sequence evolution

To test our prediction that toxin sequence divergence was inversely related to expression, we used average expression level of toxins estimated by transcripts per million reads (TPM) [29]. First, we performed a linear regression of average TPM and Tajima's D and found that low-expression toxins are more likely to be evolving via balancing selection ($n = 58$, $R^2 = 0.08$, $p = 0.03$; figure 3a). Given that positive and negative values of Tajima's D suggest alternate forms of selection (balancing and positive selection, respectively), we tested positive and negative values of Tajima's D separately for trends with expression. The trend toward balancing selection for low-expression transcripts remained significant when analysing only positive values of Tajima's D ($n = 26$, $R^2 = 0.16$, $p = 0.04$; figure 3a). However, expression level did not predict the strength of positive selection as there was not a significant trend when analysing only negative values of Tajima's D ($n = 32$, $R^2 = 0.03$, $p = 0.34$; figure 3a). Similarly, toxins found to be differentially expressed by Hofmann *et al.* [29] did not have significantly different values of Tajima's D than toxins not differentially expressed ($n = 58$, $R^2 < 0.01$, $p = 0.46$). High-expression toxins are constrained by selection to ensure translational efficiency and produce the focal phenotype [43]. Our results support that low-expression toxins have higher sequence variation and evolve via balancing selection.

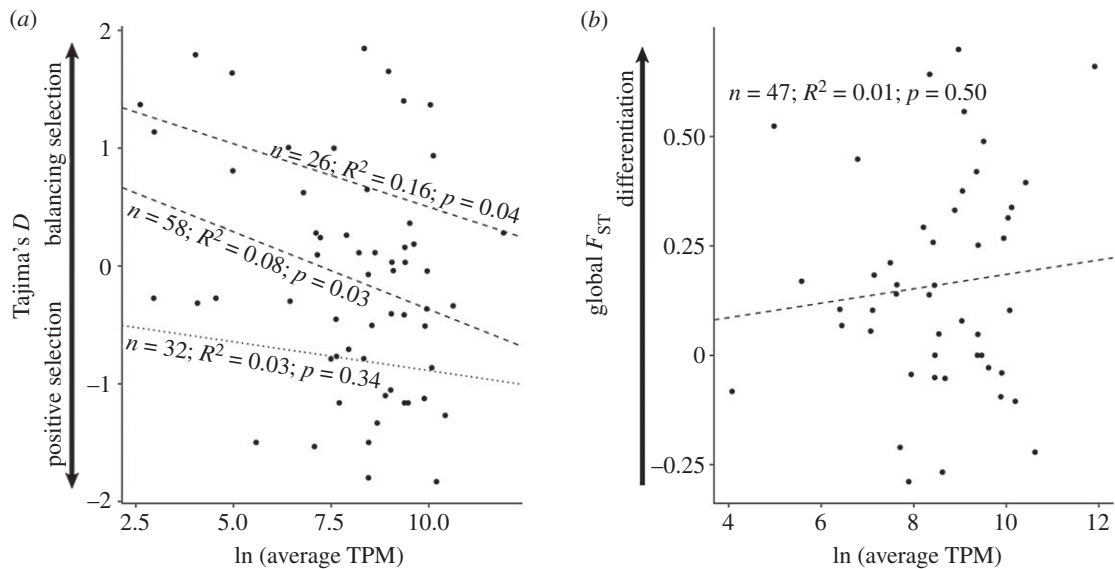


Figure 3. Relationship of (a) Tajima's D and (b) Weir and Cockerham's F_{ST} with the average toxin expression calculated by log-transformed transcripts per million (TPM) in [28]. Tajima's D was analysed in three ways: we analysed all toxins together (middle line), and then separately analysed values greater than (upper line) and less than (lower line) zero. Tajima's D shows a significant correlation when considering all toxins or toxins found to have a positive Tajima's D value.

We then performed regressions of average TPM with global F_{ST} and found that this approximation of sequence evolution was not correlated to expression (F_{ST} : $n = 47$, $R^2 = 0.01$, $p = 0.50$; figure 3b). Similarly, toxins found to be differentially expressed by Hofmann *et al.* [29] did not have significantly different values of F_{ST} ($n = 47$, $R^2 = 0.05$, $p = 0.15$). These results suggest that toxin expression and sequence divergence are not correlated, rejecting our hypothesis that low-expression toxins have higher sequence divergence.

(d) Population and toxin differentiation

We estimated F_{ST} between phylogeographic lineages using the nontoxin transcripts to assess population differentiation, and calculated F_{ST} between toxin transcripts to determine if toxins maintained higher levels of sequence divergence. Overall, there is little genetic differentiation within *Crotalus cerastes* even between the most geographically distant lineages (i.e. North Mojave versus Sonora; figures 1 and 4). The mean nontoxin F_{ST} estimate between the North Mojave and Sonoran lineages ($F_{ST} = 0.13$) indicates only moderate differentiation with all other pairwise comparisons having little to no genetic differentiation (figure 4). Mean estimates of F_{ST} between phylogeographic lineages indicate that toxins and nontoxins exhibit similar patterns of divergence with geographically distant populations maintaining higher differentiation of both toxins and nontoxins (figure 4). However, Mantel tests based on the average geographical point of our samples for each population found no correlation between geographical distance and F_{ST} for either toxins ($r = -0.04$, $p = 0.46$) or nontoxins ($r = 0.63$, $p = 0.12$). Mantel tests based on the average branch length between populations similarly found nonsignificance (nontoxins: $r = 0.45$, $p = 0.17$; toxins: $r = 0.15$, $p = 0.33$). Overall, toxins tended to have higher genetic differentiation than nontoxins (figure 4), but this difference was only significant when comparing the North Mojave and Sonora lineages, the North Mojave and Colorado lineages, and South Mojave and Sonora lineages ($p < 0.05$; figure 4).

We used nontoxins as a null distribution in each population comparison to determine which toxins had significant sequence divergence; toxins outside of the 95th percentile were

considered significantly divergent. No toxin transcripts exceeded the 95th percentile of the null distribution between the Colorado lineage and either Mojave lineage because the 95th percentile encompassed the maximum value ($F_{ST} = 1$) (figure 4). Nonetheless, a total of nine toxin alleles were found to be fixed between these populations (electronic supplementary material, table S1). The remaining four comparisons recovered several significantly differentiated toxins (figure 4). A total of six unique toxins were found to be significantly divergent in multiple lineage comparisons (figure 4). 3FTx-1 and CTL-9 were the only two toxins found to be significantly divergent in multiple lineage comparisons (figure 4). The comparison of the South Mojave and Sonoran lineage individuals recovered the largest number of significant toxin differentiation with four toxins: 3FTx-1, CRISP-1, CTL-9 and SVSP-1 (figure 4).

Lastly, to maximize our ability to detect evidence of sequence differentiation, we combined populations based on the phylogeny recovered by Hofmann *et al.* [29] and based on geography. Based on both phylogeny and geography, respectively, we found that toxin and nontoxin estimates were significantly differentiated ($R^2 = 0.01$, $p < 0.01$, $b = 0.85$; $R^2 < 0.01$, $p = 0.01$, $b = 0.21$) with toxins maintaining a higher differentiation ($F_{ST} = 0.18$; $F_{ST} = 0.15$) than nontoxins ($F_{ST} = 0.08$; $F_{ST} = 0.09$; figure 5). Based on the phylogeny, five toxins exhibited significant differentiation (figure 5a), while geography resulted in only four toxins (figure 5b). 3FTx-1, 3FTx-2 and SVSP-10 were shared between these two analyses, but CRISP-1 was unique to the phylogenetic grouping, and SVSP-7 was unique to the geographical grouping.

(e) Selection on individual toxins

We identified several toxins under significant selection pressures, yet few were shared across tests (electronic supplementary material, table S1). Additionally, further examination of the alleles for these loci found little evidence of allele fixation for a given population (electronic supplementary material, table S2). Toxins 3FTx-1, CRISP-1, CTL-9 and SVSP-10 were significant across multiple tests (electronic supplementary material, table S1). However, it is important to

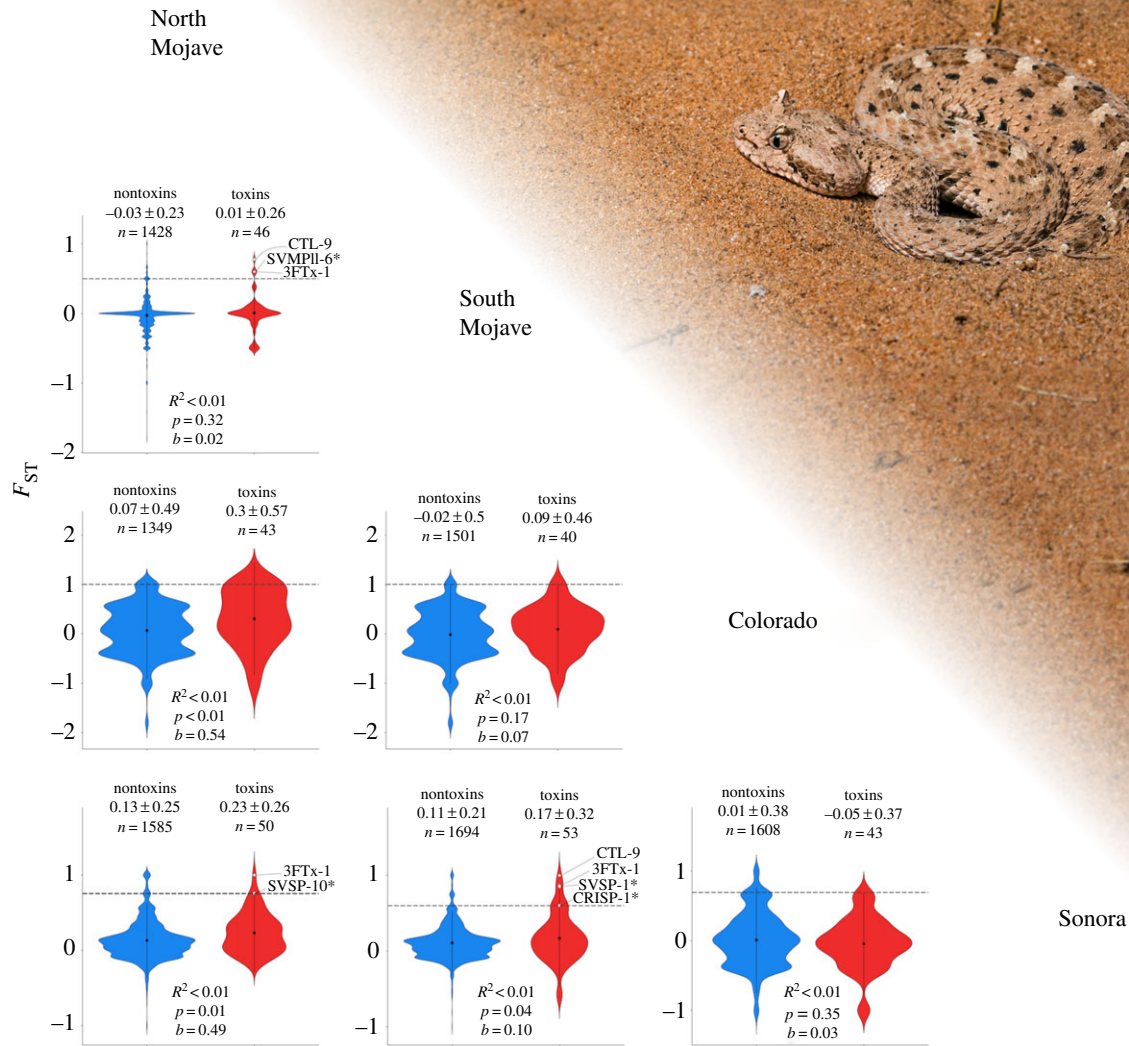


Figure 4. Violin plots showing the pairwise Weir and Cockerham's F_{ST} estimates for toxins and nontoxins between the four lineages identified by [28]. Nontoxins were used as a null distribution to identify toxin transcripts that might be evolving under strong selection. Toxins were under significant selection if they were located outside the 95th percentile of nontoxin transcripts indicated by the dotted grey line and white diamonds. Asterisks next to toxins indicate that the toxin was proteomically verified by [28]. Photo of *C. cerastes* by Travis Fisher. (Online version in colour.)

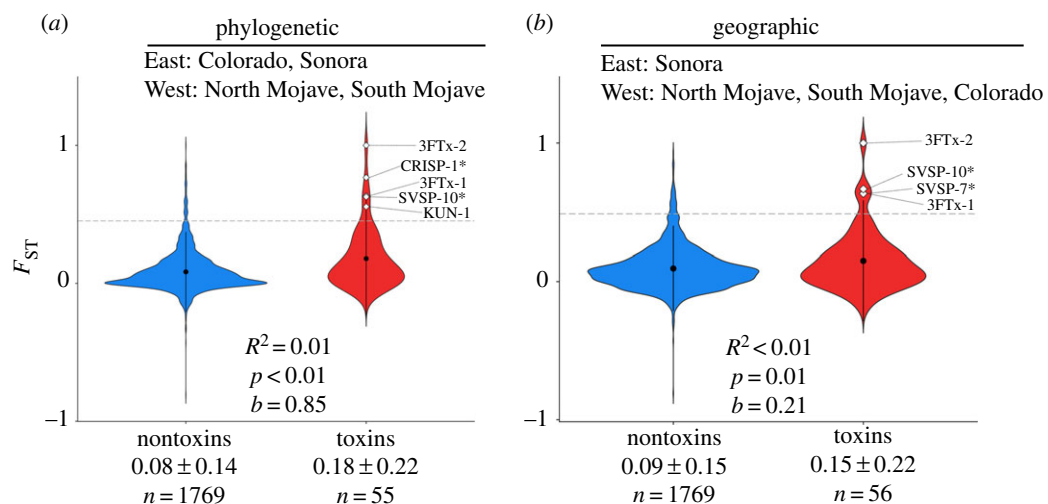


Figure 5. Violin plots and mean estimates of pairwise Weir and Cockerham's F_{ST} between phylogenetic and geographical East and West clades. Nontoxins were used as a null distribution to identify toxin transcripts that might be evolving under strong selection. Toxins were under significant selection if they were located outside the 95th percentile of nontoxin transcripts indicated by the dotted grey line and white diamonds. Asterisks next to toxins indicate that the toxin was proteomically verified by [28]. (Online version in colour.)

note that neither 3FTx-1 nor CTL-9 were verified proteomically [29], and SNPs in 3FTx-1 induced nonsense mutations (electronic supplementary material, table S2). CRISP-1 and SVSP-10 are only moderately expressed in the transcriptome, but displayed some phylogenetic patterns with regard to the SNPs present (electronic supplementary material, table S2). For example, CRISP-1 contained two fixed differences—one synonymous and one nonsynonymous—between the East and West clades (electronic supplementary material, table S2). Although the former probably does not affect venom function, the latter substitution should. The CRISP proteins are expressed widely across venomous snakes, but their function is not well-understood, as they have demonstrated a variety of biological effects [45,46]. Although CRISP-1 is only moderately expressed in the venom, the nonsynonymous SNP might impact the folding of the protein and function of the venom in each population's respective environment. Beyond these few examples, however, we found little evidence of sequence divergence or changes in toxin function involved in the evolution of sidewinder rattlesnake venom.

(f) Fang morphology

We used head-size-adjusted relative fang lengths to test for variation in morphology of the venom delivery system among sidewinder lineages. Relative fang length varied significantly among lineages ($F_{3,50} = 5.0, p = 0.004$, electronic supplementary material, figure S1A). The North Mojave lineage had significantly smaller fangs than both the Colorado ($p = 0.01$) and Sonora lineages ($p < 0.01$), while all other pairwise comparisons of mean relative fang length were not significantly different ($p > 0.1$; electronic supplementary material, figure S1A). Notably, lineages that differed significantly in their fang morphology were also those with the highest F_{ST} values for toxin sequences (electronic supplementary material, figure S1B). In fact, toxin sequence differentiation and fang morphology appear to be positively correlated (electronic supplementary material, figure S1B). Although this trend is not significant ($R^2 = 0.57, p = 0.08$), the correlation is significantly different than that of nontoxins (Anova: $p = 0.02$; electronic supplementary material, figure S1B).

4. Discussion

Venom is often involved in antagonistic interactions, leading to rapid evolutionary responses via changes in gene expression (i.e. toxin quantity) or through mutational changes to the protein sequences themselves (i.e. toxin function). Both toxin quantity and sequence evolution influence the overall evolution of venom in snakes, but these forms of genetic variation might be correlated [4,47]. In this study, we used sidewinder rattlesnakes (*Crotalus cerastes*) as a model to investigate the role sequence divergence and changes in toxin function play in the evolution of a venom with little differential expression. We hypothesized that toxin expression and sequence divergence are inversely related, expecting to find high sequence divergence across populations.

Instead, we found little evidence of positive or balancing selection acting on toxin loci when compared to nontoxins in the venom-gland transcriptomes of sidewinders. On average, toxins displayed weaker signals of positive selection than nontoxins, and estimates of Tajima's D were not significantly different from zero suggesting either a lack of selection or diverse selection pressures acting on toxins. Similarly, the

global toxin F_{ST} estimate was not significantly different than the nontoxin F_{ST} . Importantly, although significance was detected ($p < 0.05$) in some analyses of average values, standard deviations between toxins and nontoxins overlapped heavily and the effect size (R^2) was ubiquitously low. These results suggest weak differences in Tajima's D and F_{ST} metrics between toxins and nontoxins. Additionally, detection of significance was probably a result of the discrepancy in sample size between toxins and nontoxins since bootstrap resampling generally showed low support (figures 2 and 4).

As we did not expect all toxins to evolve similarly, we tested for outliers based on the 95th percentile of nontoxins. Given our sample size of toxins, we expected to find two to three outliers by chance (5%). We detected three or more toxin outliers under positive and balancing selection suggesting that toxins are under diverse selection pressures. In our analysis of average differences between toxins and nontoxins, these outliers probably provided strong conflicting signals of selection which might have influenced our results—particularly Tajima's D . Therefore, using average values might not have provided informative differences between toxins and nontoxins in some tests. Looking for outliers at the tails of the distributions probably provided more informative results for detecting selection. Nonetheless, few toxin outliers were shared across tests which further supports that toxins are under diverse selection pressures. Better statistical analyses could be done with a larger sample size to examine venom variation across the entire distribution.

We hypothesized that low-expression toxins would have greater sequence divergence as a result of positive selection or relaxed purifying selection. Although we did not find evidence of a relationship between expression and positive selection, we did find a significant relationship between expression level and balancing selection. Low-expression toxins displayed evidence of balancing selection, suggesting higher standing sequence variation. Margres *et al.* [25] found that low-expression toxins have more standing expression variation between populations than high-expression toxins because high-expression toxins are constrained by selection to provide generic killing functions. Together, our results suggest that low-expression toxins tend to be more variable at the population level. This variation, on which selection can now act, probably contributes more heavily to the evolution of venom than high-expression toxins, at least intraspecifically.

Our investigation of relative fang length among Sidewinder lineages revealed variation in fang size concordant with the highest amounts of divergence in toxin sequences. These results conform to previous analyses in Mojave rattlesnakes (*C. scutulatus*) and eastern diamondback rattlesnakes (*C. adamanteus*) that demonstrated fang length variation to be associated with population-level variation in venom composition [18,28]. Thus, our work adds to a growing body of evidence suggesting that fang length and venom composition are part of a functionally integrated phenotype for capturing prey. Margres *et al.* [18] hypothesized that longer fang length might be associated with higher expression of myotoxins. As myotoxins cause hind-limb paralysis in mammals, longer fangs might increase envenomation efficiency. In support of this hypothesis, the population of sidewinders with the longest fangs (Sonora) also exhibited highest myotoxin expression [29].

Overall, our results strongly suggest that sidewinder rattlesnake toxins are not under strong positive selection pressures and are not highly differentiated between populations.

Therefore, changes in toxin function do not appear to underlie the evolution of sidewinder venom, contrary to our primary hypothesis. Instead, nontoxins and toxins appear to evolve at similar rates, suggesting that sidewinders have evolved a generalist venom arsenal as a result of stabilizing selection. In support of this result, Margres *et al.* [39] found that—for generalist species—venom only diverges in the absence of gene flow and that spatial environmental variation favours generalist phenotypes via stabilizing selection, consistent with ecological specialization theory [37].

Sidewinders prey on lizards and small mammals, and dietary studies have shown a cosmopolitan diet lacking geographical variation across the species range [40,41]. Sidewinders also have a large distribution across multiple deserts or environmental conditions further supporting ecological specialization theory [37,39]. Additionally, sidewinders exhibit moderate to high gene flow between populations and across the variable environments they inhabit (figure 4). Gene flow across environments can swamp signals of adaptation to local conditions and might increase the complexity of venom as populations share alleles, particularly for low-expression toxins. Sidewinder venom is composed of 62 putative toxins, the majority of which are moderately to highly expressed across its distribution [29]. This complexity might have evolved in response to a wide variety of ecological conditions and to facilitate a generalist diet.

Taken together, our results support the maintenance of a generalist venom arsenal in sidewinder rattlesnakes. Unlike sympatric species such as Mojave rattlesnakes, which appear to evolve under strong diversifying selection [28,33], the generalist venom of sidewinder rattlesnakes probably evolves along a relatively flat adaptive landscape void of significant differential expression and sequence divergence. In this case, the mild individual variation we observe in both expression and sequence divergence is probably the result of balancing selection and this variation is favoured by stabilizing selection to maintain a generalist venom phenotype [37–39]. Selection to maintain a generalist diet—rather than specialize—probably stabilizes sidewinder venom composition, despite populations diverging an estimated 1.5 Myr ago [48]. In comparison, the Mojave and Sonoran populations of Mojave rattlesnakes which show significantly different venom profiles are estimated to have diverged approximately 0.5–1 Myr ago [28,33,49]. Most venomous snakes demonstrate evidence of strong positive selection in the form of changes to gene expression or to the underlying

coding sequence [5–7]. However, we have found no evidence of strong differentiation in either expression or sequence divergence in sidewinder rattlesnakes. Although snake venom often evolves rapidly due to antagonistic coevolutionary interactions with prey resistance, stabilizing selection and gene flow can alternatively function to produce generalist phenotypes that allow species to span a variety of environmental conditions and feed on a similarly generalist diet.

5. Conclusion

Evidence of positive selection is nearly ubiquitous in venom, and few systems exist where significant changes to either the coding sequence or gene expression are not occurring. Here we investigated sequence divergence in the venom of sidewinder rattlesnakes (*Crotalus cerastes*). Surprisingly, coding sequence and gene expression divergence appear to contribute very little to the evolution of sidewinder venom. Overall, the venom appears remarkably similar across the distribution sampled in the United States and—given its generalist diet, moderate to high gene flow and environmental variation—is probably under the influence of stabilizing selection which functions to maintain a generalist phenotype, rather than involved in strong antagonistic coevolutionary interactions.

Data accessibility. Raw Illumina sequences and transcriptome assemblies are available in Genbank under accession numbers SRA: SRR6768682–SRR6768689; TSA: GGMJ00000000.

Authors' contributions. Conceptualization: R.M.R., E.P.H., D.R.R., C.L.P.; sampling: J.L.S., R.M.R.; sequencing: J.L.S., A.J.M.; data processing and analysis: R.M.R., E.P.H., J.L.S., A.J.M., M.L.H., D.R.R.; writing: R.M.R., E.P.H., M.J.M., M.L.H.; review and editing: all authors.

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