Biochimie 213 (2023) 176–189

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

The best of both worlds? Rattlesnake hybrid zones generate complex combinations of divergent venom phenotypes that retain high toxicity

Cara F. Smith [a], Zachary L. Nikolakis [b], Blair W. Perry [b], Drew R. Schield [b, 1], Jesse M. Meik [c], Anthony J. Saviola [d], Todd A. Castoe [b], Joshua Parker [e], Stephen P. Mackessy [a, *]

[a] School of Biological Sciences, 501 20th Street, University of Northern Colorado, Greeley, CO, 80639, USA
[b] Department of Biology, 501 S. Nedderman Drive, University of Texas at Arlington, Arlington, TX, 76019, USA
[c] Department of Biological Sciences, Tarleton State University, 1333 W. Washington Street, Stephenville, TX, 76402, USA
[d] Department of Biochemistry and Molecular Genetics, 12801 East 17th Avenue, University of Colorado Denver, Aurora, CO, 80045, USA
[e] Fresno City College, 1101 E. University Avenue, Fresno, CA, 93741, USA

ARTICLE INFO

Article history:
Received 28 January 2023
Received in revised form
27 June 2023
Accepted 10 July 2023
Available online 13 July 2023

Handling Editor: Dr B Friguet

Keywords:
Crotalus
Evolution
Hybridization
Phenotype
RADseq
Toxin

ABSTRACT

Studying the consequences of hybridization between closely related species with divergent traits can reveal patterns of evolution that shape and maintain extreme trophic adaptations. Snake venoms are an excellent model system for examining the evolutionary and ecological patterns that underlie highly selected polymorphic traits. Here we investigate hybrid venom phenotypes that result from natural introgression between two rattlesnake species that express highly divergent venom phenotypes: *Crotalus o. concolor* and *C. v. viridis*. Though not yet documented, interbreeding between these species may lead to novel venom phenotypes with unique activities that break the typical trends of venom composition in rattlesnakes. The characteristics of these unusual phenotypes could unveil the roles of introgression in maintaining patterns of venom composition and variation, including the near ubiquitous dichotomy between neurotoxic or degradative venoms observed across rattlesnakes. We use RADseq data to infer patterns of gene flow and hybrid ancestry between these diverged lineages and link these genetic data with analyses of venom composition, biological activity, and whole animal model toxicity tests to understand the impacts of introgression on venom composition. We find that introgressed populations express admixed venom phenotypes that do not sacrifice biological activity (lethal toxicity) or overall abundance of dominant toxins compared to parental venoms. These hybridized venoms therefore do not represent a trade-off in functionality between the typical phenotypic extremes but instead represent a unique combination of characters whose expression appears limited to the hybrid zone.

1. Introduction

Understanding the functional consequences of hybridization on extreme adaptations is a major goal of evolutionary biology. Snake venoms are an excellent model system for studying evolutionary and ecological patterns that underlie adaptive polymorphic traits, due to their compositional complexity, potent bioactive properties, and tight links to foraging fitness [1–4]. Venom composition and activity are variable across a wide range of scales in snakes, from phylogenetic to ontogenetic [5–7]. Despite the broad influences of phylogeny [8,9] on venom composition and the potential for genetic drift [2,10,11], venoms are understood to be under strong selection due to their roles in prey acquisition [1,2,4]. Selection for diet-specific venoms and prey-specific toxins, in addition to climatic and habitat influences, are therefore hypothesized to be major drivers of venom variation [5,12–17].

Hybrid zones are natural evolutionary experiments that can be used to understand potential reproductive barriers between species, adaptive qualities of introgressed genotypes, and factors that may select for or against hybridization [16,18–22]. Studying the distribution and consequences of hybridization between closely

* Corresponding author.
E-mail address: stephen.mackessy@unco.edu (S.P. Mackessy).
[1] Current address: Department of Ecology & Evolutionary Biology, 1900 Pleasant Street, University of Colorado, Boulder, CO 80309.

https://doi.org/10.1016/j.biochi.2023.07.008
0300-9084/

Abbreviation

CVOS	*Crotalus cerberus*, *C. viridis*, *C. oreganus* and C. *scutulatus*
SVMP	snake venom metalloproteinases
PLA2	Phospholipase A_2
SVSP	Snake venom serine protease
PDE	Phosphodiesterase
L-AAO	L-amino acid oxidase
ddRADseq	double digest restriction enzyme sequencing

related species with mutually exclusive traits can therefore lead to a new understanding of the evolutionary patterns and processes that govern divergent adaptations in hybridizing lineages, including major trophic adaptations such as venom composition [19,23].

In addition to morphological, geographic, and ecological differentiation, lineages within the Western Rattlesnake' clade, which include four recognized species (*Crotalus cerberus*, *C. viridis*, *C. oreganus* and *C. scutulatus*, hereafter referred to as CVOS), demonstrate considerable intra- and interspecific venom variation, which can be broadly classified into two mutually exclusive groups: Type 1 venoms and Type 2 venoms [6] (also referred to as Type B and A venoms respectively in *C. scutulatus* [24]). This dichotomy highlights divergent adaptations for prey incapacitation that derive from a trade-off between highly degradative (Type 1) and highly neurotoxic (Type 2) venoms. The major compositional axes that determine these opposing evolutionary trajectories are high levels of snake venom metalloproteinases (SVMP) expressed in Type 1 venoms and the presence of the two-subunit phospholipase A_2-based (PLA_2) neurotoxins in Type 2 venoms. Though this phenotypic dichotomy was delineated some time ago [6,25], the genetic architecture that may underlie this pattern was more recently described by Dowell et al. [23], who showed that venom phenotype was in part determined by the genomic presence or absence of metalloprotease and PLA_2 gene family members. It has been previously hypothesized that these divergent genotypes are maintained by disruptive selection against intermediate venoms [16,26], but evidence supporting lower functionality of hybrid venom phenotypes is lacking.

Patterns of this venom composition dichotomy in CVOS are not fully explained by phylogeny or species limits, leading to the hypotheses that phenotypes are driven to these extremes by broad-scale dietary constraints [6], or that population-level directional selection (based on dietary or environmental factors) has led to the fixation of either divergent phenotype across populations [16]. Investigating the unique biological functions and evolutionary trajectories of Type 1 and Type 2 venoms in CVOS, and examples that break this pattern, is valuable for understanding the complex patterns of selection, evolutionary history, and ecology of this clade and of venom composition in general. Within this lineage, there are examples of both venom phenotypic extremes, which are largely representative of venom variation observed at higher taxonomic levels (grossly, elapid vs. viperid venoms, or between various lineages of colubrid snakes [27,28]).

Unique venom phenotypes and unusual patterns of venom variation due to suspected or confirmed hybridization have been reported previously in rattlesnakes, some of which defy this otherwise fairly strict dichotomy observed across rattlesnake species [16,19,24,29,30] and other species of pitvipers [31]. Of particular interest, previous work in *C. scutulatus* has shown that different populations express Type 1 or Type 2 venom phenotypes [25,32–37], but instances of Type 3 venoms (i.e., A + B venoms) are very rare and only occur in narrow zones of inferred introgression [16,24,26,33]. The finding that Type 3 venoms are so rare in nature, together with the vast majority of populations that are fixed for either Type 1 or Type 2, suggests that trade-offs may exist between Type 1 and Type 2 venoms, that there may be negative consequences associated with having both (i.e., Type 3 venoms), and/or that Type 3 venoms may be favored only in rare and highly localized circumstances [16,19,23,38]. In Arizona, *C. scutulatus* venoms have been further characterized as falling into phenotypes A-F, defined on the presence/absence of SVMPs and Mojave toxin as above (Types 1–3) and also integrating the presence/absence of a short peptide toxin, myotoxin a [39]. Myotoxin a also shows significant regional variation in abundance in *C. viridis viridis* venoms [40], and like Mojave toxin and homologs, myotoxin a homologs are present in venoms of some species but absent from others [41].

In this study, we investigate the distributions of venom phenotypes within a hybrid zone between two divergent lineages of the North American 'Western Rattlesnake' clade (CVOS). The combined geographic range of this group encompasses the entire western United States and regions of southern Canada through central Mexico [42,43]. The history of this clade has been strongly influenced by allopatric divergence in isolation, followed by subsequent secondary contact and introgression of lineages, leading to numerous zones of hybridization between lineages [44]. We focus on a zone of introgression in western Colorado between two CVOS lineages, *Crotalus viridis viridis* (Prairie Rattlesnake) and *C. oreganus concolor* (Midget Faded Rattlesnake), which possess highly divergent morphological and venom phenotypic traits. The present study was prompted by reports of phenotypically intermediate snakes in these areas and the inference of genetic introgression between these lineages by Schield et al. [44].

Previous work demonstrated that *C. o. concolor* and *C. v. viridis* diverged in allopatry followed by range expansion, secondary contact in northwestern Colorado, and subsequent introgression [22,44–46]. Genomic patterns of this introgression indicate the presence of a zone where hybrid genotypes are shaped by seemingly opposing forces: genomic incompatibilities between parents at some loci and adaptive introgression of other regions [22]. This indicates that these species are on the trajectory of divergence due to intrinsic factors while simultaneously suggesting that admixture of specific loci is advantageous in this particular zone of overlap.

A generalist and a specialist, respectively, *C. v. viridis* and *C. o. concolor* fall into opposite poles of the Type 1/2 dichotomy of venom composition [6]. *Crotalus v. viridis* venom (Type 1) induces hemorrhage and myonecrosis, partly due to the abundance of SVMPs and peptide myotoxins [47–49]. It has also been shown to contain an abundance of larger enzymes characteristic of rattlesnake venoms, including snake venom serine proteases (SVSP), PLA_2, L-amino acid oxidases (L-AAO) and phosphodiesterases (PDE [6]). In contrast to the Type 1 venom of *C. v. viridis*, *C. o. concolor* has particularly toxic Type 2 venom due to the presence of concolor toxin, a presynaptic 2-subunit neurotoxin based on a phospholipase A_2 scaffold, and several small non-enzymatic myotoxins; metalloproteinase activity is exceptionally low [50–53].

Here we identify mixed venom phenotypes in a hybrid system, and we integrate genetic characterization of snakes in this zone of introgression with analyses of venom composition to investigate relationships between venom variation and introgression between these two lineages. We use RADseq data to define patterns of genetic introgression across this zone and to infer the hybrid ancestry of individuals and populations (see also [22]). We then compare these genetic patterns of introgression to patterns of venom composition by investigating the presence, abundance, and biological activity of major venom toxin families in parental and hybrid populations and individuals. Finally, we test the assumption that

hybridized venoms are rare because they are less fit and represent a trade-off in the advantages of expression of either Type 1 or Type 2 venom by exploring the functionality of hybrid venoms both *in vitro* and *in vivo* with a comparison of biological activities, toxin abundances, and lethal toxicities of venoms from parental and admixed individuals.

2. Methods and materials

2.1. Venom collection and storage

Venom from snakes collected in potential areas of introgression (n = 69; Fig. 1A) was manually extracted as previously described [5]. Samples were either dried in the field over calcium carbonate and placed on ice until storage at −20 °C or were placed in liquid nitrogen until lyophilization and then stored at −20 °C. Venom samples were also collected from *C. v. viridis* (n = 7) and *C. o. concolor* (n = 8) that occur in areas far from overlap zones, eliminating the possibility of contact between these parental reference species (Fig. 1A). Snakes were either field extracted or processed in the UNC Animal Resource Facility and returned to their location of capture within three days, or they were maintained in the UNC Animal Resource Facility per UNC IACUC approval (protocol #1302D-SM-S-16). All snake collections and field-collected samples were made under the auspices of permits from the Colorado Parks and Wildlife (scientific collecting permits #14HP974, 17HP0974, 20HP0974) and the Wyoming Game and Fish Department (scientific collecting permit #1544). All protocol and procedures employed were ethically reviewed and approved by the UNC Institutional Animal Care and Use Committee (protocols #1302D-SM-S-16, 1701D-SM-S-20, and 1905D-SM-SBirdsLM-22).

2.2. Population sampling, reduced-representation genome sequencing, and variant calling

We used a combination of previously published data from both Schield et al. ([44]; $n = 13$) and Nikolakis et al. ([22]; $n = 20$), which included sampling across both parental lineages and the identified hybrid zone between these species. To increase sampling and include greater matched sampling of genomic data and venom data for the same individuals, new reduced representation genomic libraries for an additional 26 samples were generated (Table S1). DNA was extracted from tissue that was either snap frozen or stored in ethanol or in RNAlater and kept at −80 °C, using phenol-chloroform-isoamyl DNA extraction protocols. Genomic libraries were generated using the double digest restriction enzyme (ddRADseq) approach (F with modifications outlined in Schield et al. [16,54]. Genomic DNA was fragmented using the restriction enzymes *SbfI* and *Sau3aI* at 37 °C, followed by purification of fragmented DNA using Serapure beads. We then normalized samples by concentration and individually barcoded each sample with Illumina DNA adapters that have unique 8bp molecular identifiers (UMIs) to allow for downstream filtering of PCR clones. Samples were then pooled into groups of six to eight and purified again using Serapure beads.

DNA fragments were next size selected for lengths of 575–655

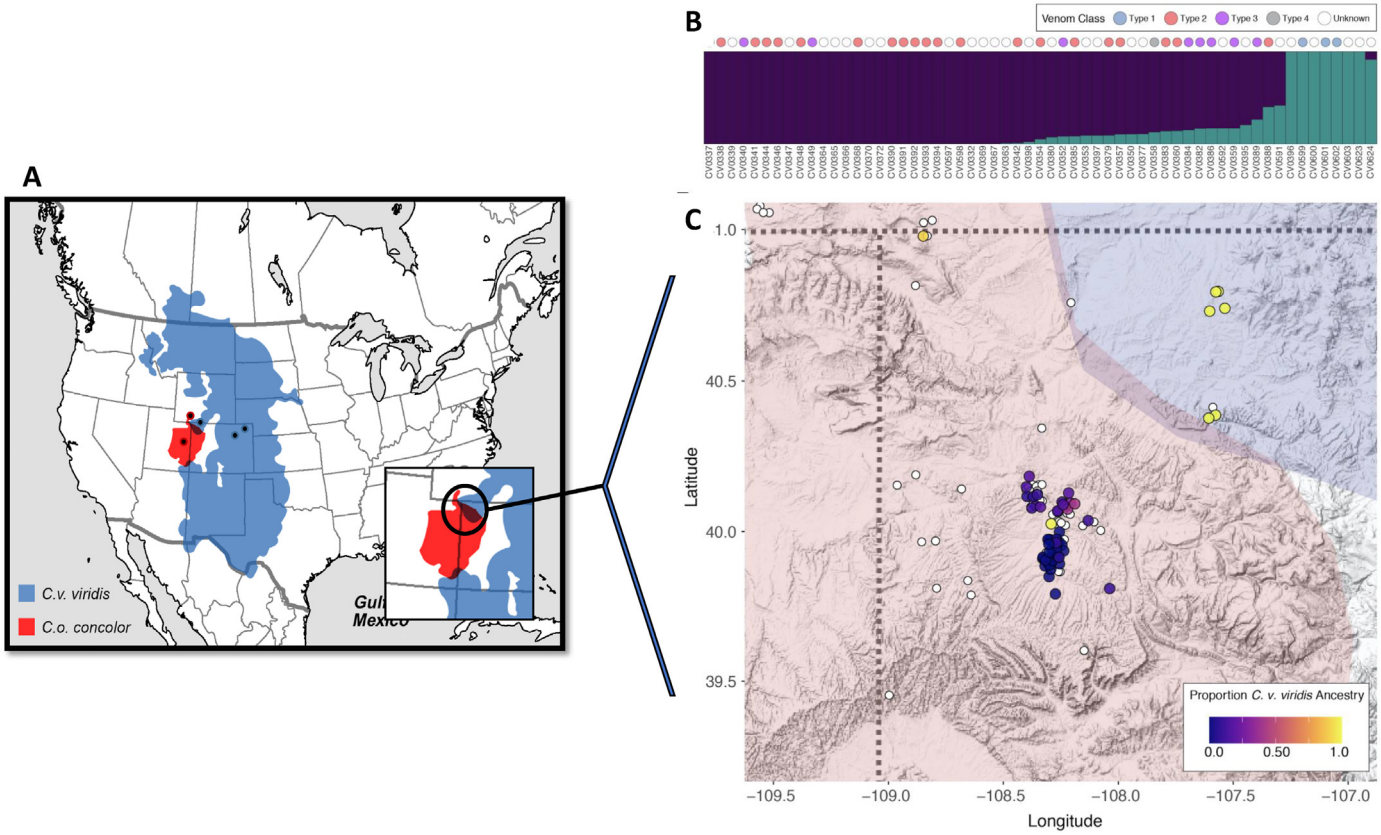


Fig. 1. Parental species and hybrid zone ranges. (A) Distribution of *C. v. viridis* (blue), *C. o. concolor* (red), areas of introgression in Colorado (insert, circle), and sampling locations of non-introgressed *C. v. viridis* and *C. o. concolor* populations (black dots). (B) Admixture plot showing population assignments (K = 2) based on RADseq, with venom phenotypes shown above. Purple = *C. o. concolor*, blue = *C. v. viridis*. Note the majority of Type 3 hybridized venoms come from introgressed snakes. (C) Map showing geographic distribution of inferred ancestry, shown here as the proportion of *C. v. viridis* ancestry. Points are jittered to avoid overplotting but remain close to the true sample locality. Overlaid are the IUCN range maps for *C. oreganus* (red) and *C. viridis* (blue). White points indicate samples for which genetic data was not collected.

bp using a Blue Pippin Prep (Sage Science). Fragment lengths were determined based upon the aim of targeting ~20,000 loci (based on predictions from *in silico* restriction digest of the Prairie Rattlesnake v2.0 (CroVir2.0) genome (NCBI BioSample SAMN07738522)) using recognition sites of the restriction enzymes used within this study. Libraries were then PCR amplified using pooled specific index primers, quantified with a Qubit fluorimeter, and combined in equimolar amounts for final sequencing library pooling. These libraries were then sequenced using 100 bp single-end reads on an Illumina HiSeq 2500.

The clone_filter module in the program Stacks v.1.42 [55] was used to filter PCR clones from raw sequencing data and then trimmed the 8 bp adapter sequences from all reads using the FastxToolkit [56]. Trimmed and filtered sequences were then demultiplexed according to their 6 bp individual barcode and checked for complete restriction cut sites using the Stacks process_radtags module. Full processed and demultiplexed data are available from the NCBI SRA (BioProject PRJNA548132). We used the program BWA v.0.7.10 [57] with default settings to map trimmed and filtered read data for all samples to the *Crotalus viridis* reference genome [58]. We then used SAMtools and BCFtools [59] to generate pileups of all sample alignments and call variants. We recoded raw variant calls for each individual with a read depth of less than five (DP < 5) as missing data using BCFtools. We filtered variants to only retain biallelic SNPs with a minor allele frequency greater than 0.05, quality score above 30, and had data from at least 50% of samples across the entire dataset using VCFtools [60].

2.3. Population structure and ancestry coefficient estimations

The program ADMIXTURE v.1.3.0 [61] was used to infer population structure and ancestry coefficients across individuals, which utilizes the likelihood model in the program STRUCTURE [62]. We ran 10 iterations for each *K* value from K = 2 to K = 5 and determined the best fit *K* value using the default cross-validation method in ADMIXTURE. Ancestry coefficients were visualized using the program Pophelper v.2.2.6 (https://github.com/royfrancis/pophelper) in R [63].

2.4. Venom protein concentration determination

Protein determination reagents were purchased from BioRad, Inc. (Hercules, CA, USA). Dried venom samples were reconstituted at an apparent concentration of 4.0 mg/mL in Millipore-filtered water, vortexed, and centrifuged for 5 min at 9500×*g*, and protein concentration of the supernatant was determined using the Bradford protocol [64] as modified by BioRad, Inc., with bovine γ globulin as a standard. Amount of material used in all subsequent assays was based on these determinations. Reconstituted samples were frozen at −20 °C until use and then thawed and centrifuged at 9500×*g* for 5 min to pellet cellular debris.

2.5. Venom type delineations

Because of the ontogenetic shifts in composition observed in *C. o. concolor* [53] and *C. v. viridis* [49] venoms, only venoms from adult potential hybrids were used in venom profiling to determine likely hybrid status. Venom type parameters were determined using methods previously described [6,16,49]. Venom profiles were assigned based upon metalloprotease levels (determined by enzyme activity assays as in Mackessy [6] and the presence of HPLC peaks as in Saviola et al. [49], and the presence of concolor toxin was determined by the occurrence of a unique RP-HPLC peak at 41 min (concolor toxin B subunit). Type 1 and 2 venoms were defined based on activity/toxin abundance levels of "pure" parental samples collected outside of the hybrid zone. For venom profile delineations, adult samples were categorized as follows: (A) Type 1 venoms displayed SVMP activity ≥0.25 U (within *C. v. viridis* range) and lacked concolor toxin; (B) Type 2 venoms displayed SVMP activity ≤0.15 U (within *C. o. concolor* range) and contained concolor toxin; (C) Type 3 venoms displayed SVMP activity above *C. o. concolor* levels (≥0.2 U) and contained concolor toxin; and (D) Type 4 venoms displayed very low SVMP activity (<0.02 U) and lacked concolor toxin (Table 1).

2.6. Protein gel electrophoresis

SDS-PAGE materials were obtained from Life Technologies, Inc. (Grand Island, NY, USA). Reduced venom (16–20 μg) or lyophilized protein (approximately 5 μg - RP-HPLC fractionated) was loaded into wells of a NuPAGE Novex Bis-Tris 12% acrylamide Mini Gel and electrophoresed in MES buffer under reducing conditions for 45 min at 175 V; 7 μl of Mark 12 standards were loaded for molecular weight estimates. Gels were stained overnight with gentle shaking in 0.1% Coomassie brilliant blue R-250 in 50% methanol and 20% acetic acid (v/v) in water and destained in 30% methanol, 7% glacial acetic acid (v/v) in water until background was sufficiently destained (approximately 2 h). Gels were then placed in storage solution (7% acetic acid, v/v) for several hours with gentle shaking at room temperature and imaged on an HP Scanjet 4570c scanner.

2.7. Enzymology

The azocasein metalloprotease assay procedure was performed as outlined in Aird and da Silva [65], and all samples and controls were run in triplicate. Briefly, 20 μg of venom was incubated with 1 mg of azocasein substrate in buffer (50 mM HEPES, 100 mM NaCl, pH 8.0; total volume 1.0 mL) for 30 min at 37 °C. The reaction was stopped with 250 μl of 0.5 M trichloroacetic acid, vortexed, brought to room temperature, and centrifuged at 2000 rpm for 10 min. Absorbance of the supernatant was read at 342 nm, and all values were expressed as $\Delta A_{342\ nm}$/min/mg venom protein.

L-amino acid oxidase assays were performed as outlined in Weissbach et al. [66]; all samples and controls were run in triplicate. L-kynurenine substrate was solubilized at 1.04 mg/mL buffer (50 mM HEPES, 100 mM NaCl, pH 8.0), and 75 μl was added to 20 μg of venom in 645 μl buffer. The reaction was incubated at 37 °C for 30 min and then terminated with 750 μl of 10% trichloroacetic acid. Samples were brought to room temperature, and absorbances were read at 331 nm. Specific activity was calculated as nanomoles product formed/min/mg venom protein from a standard curve of the reaction product, kynurenic acid.

Phosphodiesterase assays were performed based on Laskowski's [67] modification of Björk [68]. Twenty μg of crude venom (5 μL) was added to 220 μl of buffer (100 mM tris-HCl, 10 mM $MgCl_2$, pH 9.0). One hundred fifty μl of 1.0 mM bis-p-nitrophenylphosphate substrate was added, and the reaction was incubated at 37 °C for 30 min. The reaction was terminated with 375 μl of 100 mM NaOH containing 20 mM disodium-EDTA, vortexed, brought to room temperature and absorbance read at 400 nm. Activity was expressed as $\Delta A_{400\ nm}$/min/mg venom protein.

Thrombin-like serine protease assays were performed as described previously [69]. Eight μg of crude venom (2 μL) was added to 373 μl of buffer (50 mM HEPES, 100 mM NaCl, pH 8.0). Tubes were incubated at 37 °C for approximately 3 min 50 μl of 1.0 mM substrate (BzPheValArg-pNA; Sigma) was added and the tube was vortexed and placed back at 37 °C. Reactions were stopped after 5.0 min with 75 μL of 50% acetic acid. Tubes were read at 405 nm, and activity was calculated from a standard curve of p-nitroaniline and expressed as nanomoles product produced/min/mg venom.

Table 1
Definition of venom types found in the putative hybrid zone. Type 1 corresponds to the *C. v. viridis* phenotype, Type 2 to the *C. o. concolor* phenotype, and Types 3 and 4 are unique to the hybrid zone.

Venom type	1	2	3	4
SVMP Act. (U)	0.31 (0.26–0.4)	0.02 (0–0.13)	0.34 (0.2–0.7)	0.018 (0.018–0.019)
% CTx	0	5	5.7	0
LD_{50} (μg/g)	5.1 (2.55)	0.7 (0.17)	0.6 (0.48)	5.1 (2.54)

% CTx = % relative abundance of concolor toxin. LD_{50} = median lethal toxicity (IP) at 24 h in NSA mice. SVMP Act. = snake venom metalloproteinase activity. U = ΔA342 nm/min/mg protein; average (range). LD_{50} values are ± (95% confidence intervals).

Kallikrein-like serine protease assays were performed based on modifications of Mackessy [69]. Briefly, 0.8 μg of crude venom (2 μL) was added to 373 μl of buffer (50 mM HEPES, 100 mM NaCl, pH 8.0). Tubes were incubated at 37 °C for approximately 3 min. Fifty μl of 1.0 mM substrate (Bz-ProPheArg-pNA; Bachem) was then added, and tubes were vortexed and placed back at 37 °C. Reactions were stopped after 3.0 min with 75 μL of 50% acetic acid. Tubes were read at 405 nm, and activity was calculated from a standard curve of p-nitroaniline and expressed as nanomoles product produced/min/mg venom.

All enzymatic data for adult snakes were analyzed using a Kruskal-Wallis test of independent samples in SPSS® (Ver. 25) to determine if activities of each enzyme tested and percent peak areas varied between all venom phenotypes, with an $\alpha = 0.05$. Pairwise significance was determined using a pairwise post hoc test in SPSS®.

2.8. Purification of concolor toxin

Purification of concolor toxin was performed as outlined in Aird et al. [70]. Seventy-five mg of crude *C. o. concolor* venom was dissolved in HEPES buffer and fractionated on a BioGel P-100 gel filtration column (2.6 × 89 cm) using 25 mM HEPES, 100 mM NaCl, 5 mM $CaCl_2$, pH 6.8, buffer at a flow rate of 6 mL/h at 4 °C. The toxin-containing peak III was concentrated and buffer was exchanged using a Millipore 3.0 kDa molecular weight cutoff centrifuge concentrator. Concolor toxin was purified on a Life Technologies FPLC using Unicorn software, a DEAE FF HiTrap 5 mL column in 50 mM tris buffer pH 8.3 and a linear gradient of 0–0.5 M NaCl in this buffer. Purified material was analyzed using a Bruker Ultraflex MALDI-TOF/TOF mass spectrometer at the Proteomics and Metabolomics Facility at Colorado State University (Fort Collins, CO) to confirm the expected mass. Concolor toxin identity was further confirmed by tryptic fragment analysis on an Orbitrap LC-MS/MS at the College of Medicine and Translational Science Core facility at Florida State University (Tallahassee, FL) as described in Modahl et al. [71]. Purified concolor toxin (Supplemental Figs. 1–5) was used as a standard to identify the RP-HPLC elution time of concolor toxin from potential hybrid venoms.

2.9. High-performance liquid chromatography

High-performance liquid chromatography equipment and materials were obtained from Waters Corporation (Milford, MA, USA), and reversed-phase columns were purchased from Phenomenex, Inc (Torrance, CA, USA). Venoms were subjected to reversed-phase HPLC using Empower software and a Phenomenex Jupiter C_{18} (250 × 4.6 mm, 5 μm, 300 Å pore size) column as outlined in Smith and Mackessy [72]. Protein/peptide was detected at 280 nm with a Waters 2487 Dual λ Absorbance Detector. Fractions corresponding to each peak were then frozen at −80 °C overnight, lyophilized and then analyzed along with 20 μg crude venom via SDS-PAGE (as described above) in order to determine peak complexity, mass and toxin families. The area under the curve at 280 nm was used to assign quantitative values (% peak area) for major toxin families (myotoxin, concolor toxin, SVMPs, SVSPs) using Waters Empower software, and these values were used to elucidate changes in abundance levels of that particular protein family between the different venoms [73]. Scatterplots and Spearman's correlations comparing all venom characteristics and regressions of proportion ancestry and venom characters were graphed in SPSS® (Ver. 25). A Mantel test was run using Euclidean distance measures for both matrices in PC-ORD (ver. 7) for each individual toxin to assess the association between venom components, geographic location, and potential hybrid status.

2.10. Lethal toxicity (LD_{50}) assays

Three samples representing each venom phenotype (types 1–4) were pooled and the four samples were tested for lethal toxicity towards NSA mice. Mice (ave. mass = 18.2 g, ±2.1 g) were given intraperitoneal injections on the right side of the body. Doses of 0.2, 0.5, 0.75, 1, 5, 7.5 and 10 μg/g were adjusted for body mass (3 mice per dose) and administered in a 100 μL bolus of sterile saline. 24 h survivorship was scored and LD_{50} values were calculated using the "Quest Graph™ LD50 Calculator" (AAT Bioquest, Inc., 1 Feb. 2023; https://www.aatbio.com/tools/ld50-calculator).

3. Results

3.1. Variant dataset, population structure, and ancestry coefficients

Our final analyses included 59 individuals and a total of 12,473 retained SNPs after filtering our dataset to retain high-quality (GQ > 30) biallelic SNPs with minor allele frequencies greater than 0.05. Results from our ADMIXTURE analyses showed that the best fitting *K* value was 2, using the cross-validation approach (Fig. S6). From this model, we find a continuum of ancestry coefficients across all samples, with most comprised primarily of *C. o. concolor* genotypes (Fig. 1B–C) and only eight samples having ancestry coefficients greater than 0.5 from the *C. v. viridis* cluster. Our results also show that measures of admixture seem to decrease drastically outside of a small geographic region (Fig. 1C), with most samples ($n = 39$) having admixture levels of greater than 0.9 from either inferred genetic cluster (Fig. 1B).

3.2. Hybrid individuals exhibit unique venom composition

Venoms of snakes collected far from the zone of contact in NW Colorado were used to define Type 1 (*C. v. viridis*) and Type 2 (*C. o. concolor*) venom phenotypes. Venoms collected from snakes in the putative hybrid zone showed variable phenotypes, including typical Types 1 and 2 and two additional phenotypes, termed Types 3 and 4 (Table 1, Fig. 2). Based on metalloprotease activity levels and the presence or absence of concolor toxin (see Methods and Table 1) in the 69 adult venom samples tested from the contact zone, 7% displayed Type 1 venom (n = 5), 64% displayed a Type 2 venom profile (n = 44), and 25% of venoms had a hybridized Type 3

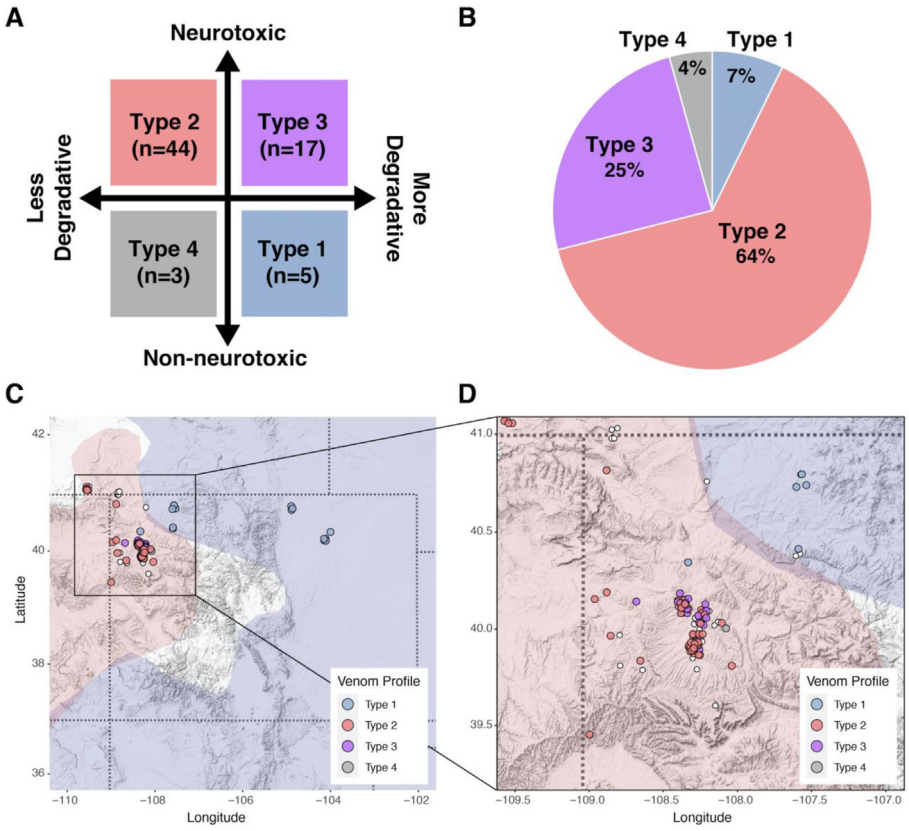


Fig. 2. Hybrid zone venom phenotypes. (A) Distribution of venom phenotypes from areas of introgression. (B) Percentages of each venom profile from adult snakes captured in northwestern intergrade zone. (n = 69). (C-D) Maps showing geographic distribution of venom types amongst sampled individuals. Note that Type 3 and Type 4 venom profiles are found primarily within the known region of introgression (center of map). Points are jittered to avoid overplotting but remain close to the true sample locality. Overlaid are the IUCN range maps for *C. oreganus* (red) and *C. viridis* (blue). White points indicate samples for which genetic data was not collected.

venom profile (n = 17); Type 4 venoms were observed in only 3 individuals (4.3%) from the contact zone (Table 1; Fig. 2A–D). In total, only 8 individuals (five Type 1, three Type 4) tested negative for the presence of concolor toxin. Except for one individual, all hybrid venom phenotypes are expressed in genetically introgressed snakes (Fig. 1B).

All crude venoms showed a high level of complexity (Fig. 3; Fig. S7) with 10–20 protein bands ranging from approximately 5 kDa–165 kDa in size; protein families commonly occurring in rattlesnake venoms are indicated at specific known masses. Based upon protein size, major protein families including L-amino acid oxidases (55–65 kDa), serine proteases (29–36 kDa), phospholipase A_2 (~14 kDa), and myotoxins (~4.8 kDa) were present in most venoms. P-III metalloproteinases (approx. 53 kDa) were present most abundantly in *C. v. viridis* venoms. Notably, the prominence of the P-III metalloprotease band varied more significantly between adults and non-adults than between adult samples with different activity levels (data not shown). However, Type 1 venoms displayed higher levels of P-III SVMPs and higher mass enzymes (nucleases, L-AAO) than Type 2 and Type 4 venoms, which displayed virtually no P-III SVMPs (Fig. 3). Type 3 venoms appeared to have intermediate levels of P-III SVMPs, nucleases, and L-AAO compared to Type 1 and Type 2 samples. PI-SVMPs were observed only in venoms from southern populations of *C. v. viridis*.

3.3. Variation in venom activity between parental and hybrid venom types

Pure *C. v. viridis* venom SVMP (azocasein-hydrolyzing) activity ranged from 0.26 to 0.4 U (n = 7, average = 0.31, median = 0.28; Fig. 4A); thrombin-like SVSP activity ranged from 708.5 to 868.2 nmol product/min/mg (n = 7, average = 775.6, median = 753.6), and kallikrein-like SVSP activity ranged from 427.2 to 791.6 nmol product/min/mg (n = 7, average = 603.9, median = 582.8; Fig. 4B and C, respectively). L-AAO activity ranged from 30.1 to 77.5 nmol product/min/mg (n = 7,

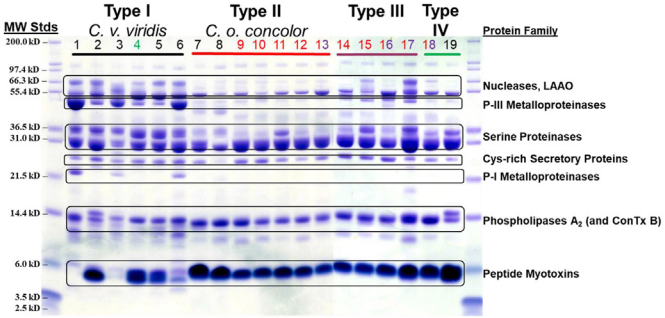


Fig. 3. Compositional differences between venom phenotypes. SDS-PAGE of crude venom (16 μg) from *C. v. viridis* (black bar) and *C. o. concolor* (red bar) and from a subset of introgression snakes (purple and green bars), with dominant protein families identified. Localities: 1, Lincoln Co., CO; 2, Weld Co., CO; 3, Montrose Co. CO; 4–5, Moffat Co, CO; 6, Lincoln Co., CO; 7, Garfield Co., UT; 8, Sweetwater Co., WY; 9–19, Rio Blanco Co., CO. Numbers marked with colors indicate samples also shown in the Structure plot (Fig. 2). Single colors – no sign of admixture, samples show the parental genotype; two colors – admixture detected; black – no genotype data. Con Tx B, concolor toxin B subunit; LAAO, L-amino acid oxidase; MW Stds., molecular weight standards.

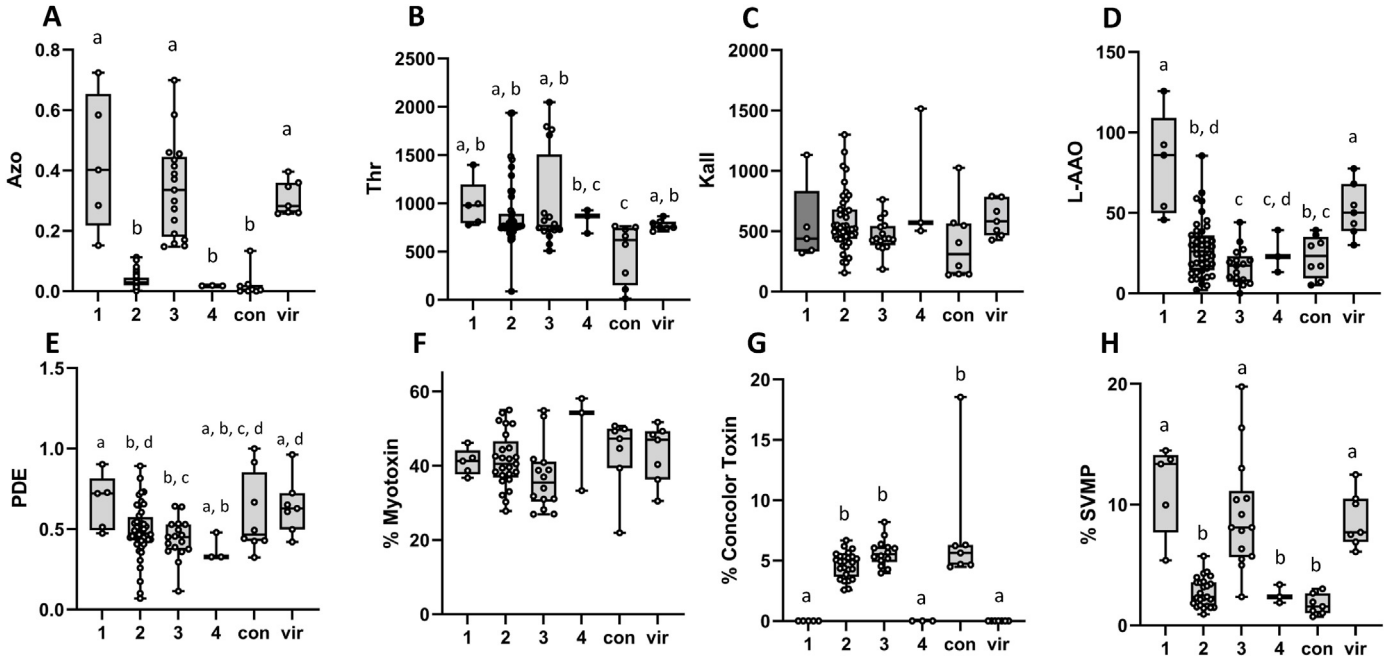


Fig. 4. Comparisons of toxin abundance and activity between parental and introgression zone venom phenotypes. Specific enzyme activities and toxin levels for all venom types (hybrid zone, 1–4) and pure parent species (con, vir). Letters above bars indicates significance at an alpha of 0.05. A, snake venom metalloprotease activity (Azo). B, Thrombin-like serine protease activity (Thr). C, Kallikrein like serine protease activity (Kall). D, L-amino acid oxidase activity (L-AAO). E, Phosphodiesterase activity (PDE). F, % myotoxin. G. % concolor toxin (ConTox). H, % snake venom metalloprotease (SVMP). Note that type 3 venoms are not significantly different from *C. v. viridis* in SVMP (Azo) activity or level and are not significantly different from *C. o. concolor* in level of concolor toxin.

average = 51.8, median = 50.25; Fig. 4D), and PDE activity ranged from 0.42 to 0.96 U (n = 7, average = 0.64, median = 0.63; Fig. 4E).

Pure *C. o. concolor* venom SVMP activity ranged from 0.0 to 0.13 U (n = 8, average = 0.02, median = 0.01; Fig. 4A), thrombin-like SVSP activity ranged from 14 to 763 nmol product/min/mg (n = 8, average = 485.8, median = 618.9; Fig. 4B), and kallikrein-like SVSP activity ranged from 138.6 to 1025.8 nmol product/min/mg (n = 8, average = 398.9, median = 310.5; Fig. 4C). L-AAO activity ranged from 5.2 to 39.3 nmol product/min/mg (n = 8, average = 22.8, median = 23.31; Fig. 4D), and PDE activity ranged from 0.32 to 1.0 U (n = 8, average = 0.59, median = 0.47; Fig. 4E).

Average adult *C. v. viridis* venom SVMP activities were significantly higher than in *C. o. concolor* venom (χ^2 = 53.14, df = 5, p < 0.000; Fig. 4 and Table 2). Except for one individual from Flaming Gorge National Recreation Area that had an activity of 0.13 U, all *C. o. concolor* adult venoms had metalloprotease activities at or below 0.02 U. Thrombin-like SVSP (χ^2 = 24.14, df = 5, p = 0.05) and L-amino acid oxidase (χ^2 = 33.1, df = 5, p = 0.008) varied significantly between parent species, with *C. v. viridis* being higher than *C. o. concolor* for both. Neither kallikrein-like SVSP (χ^2 = 7.93, df = 5, p = 0.16) nor phosphodiesterase activity (χ^2 = 14.14, df = 5, p = 0.257) was significantly different between *C. v. viridis* and *C. o. concolor* venoms (Fig. 4, Table 2).

The Type 1-Type 2 venom dichotomy that is found within the *Crotalus viridis/oreganus* complex [6] is consistent with the activities of *C. v. viridis* and *C. o. concolor* venoms assayed in the present study. However, these venoms also differed significantly in the abundance of other major toxin families (L-AAO and thrombin-like SVSP) that were noted previously [6] but not investigated statistically until now.

Snakes from the introgressed area displayed a wide range of SVMP activities, from nearly undetectable (0.001 U) to high levels (0.72 U; Fig. 4A). Type 3 SVMP activity ranged from 0.148 to 0.699 U (n = 14, average = 0.339, median = 0.335). Thrombin-like SVSP activity ranged from 509.2 to 2047.2 U (n = 16, average = 1004.8, median = 766.8; Fig. 4B), and kallikrein-like SVSP activity ranged from 185.58 to 763.1 U (n = 16, average = 466.8, median = 439.2; Fig. 4C). Type 3 venoms had L-AAO activity that ranged from 0 to 44.2 U (n = 17, average = 16.7, median = 17.2; Fig. 4D), and PDE activity that ranged from 0.11 to 0.64 U (n = 17, average = 0.44, median = 0.45; Fig. 4E).

Type 4 venom SVMP activity ranged from 0.018 to 0.019 U (n = 3, average = 0.018, median = 0.018; Fig. 4A), thrombin-like SVSP activity ranged from 690.3 to 931.1 U (n = 3, average = 830.2, median = 869.2; Fig. 4B), and kallikrein-like SVSP activity from 504.65 to 1515.5 (n = 3, average = 863.48, median = 570.3; Fig. 4C). L-AAO activity in Type 4 venoms ranged from 13.34 to 39.32 U (n = 3, average = 25.2, median = 22.96; Fig. 4D), and PDE activity ranged from 0.327 to 0.479 U (n = 3, average = 0.378, median = 0.327; Fig. 4E).

Type 3 venoms did not vary significantly from pure *C. o. concolor* venoms or Type 2 venoms from admixed individuals in PDE activity (χ^2 = -13,24, df = 5, p = 0.200; χ^2 = 9.21, df = 5, p = 0.181, respectively; Table 2), and they did not vary significantly from only pure *C. o. concolor* venom in L-AAO activity (χ^2 = -9.33, df = 5, p = 0.37). Type 3 venoms were not significantly different from pure *C. v. viridis* or Type 1 admixed venoms in levels of SVMP activity (χ^2 = 1.62, df = 5, p = 0.881; χ^2 = 3.64, df = 5, p = 0.767, respectively) or thrombin-like serine protease activity (χ^2 = 3.67, df = 5, p = 0.734; χ^2 = 19.79, df = 5, p = 0.105, respectively). However, Type 4 venoms were significantly lower in levels of SVMP activity compared to Type 3 venoms (χ^2 = 52.24, df = 5, p = 0.004), admixed Type 1 venoms (χ^2 = 55.9, df = 5, p = 0.006), and pure *C. v. viridis* Type 1 venoms (χ^2 = -50.64, df = 5, p = 0.009). Type 4 venoms did not differ from Type 3 venoms in PDE (χ^2 = 6.27, df = 5, p = 0.728), L-AAO (χ^2 = -3.2, df = 5, p = 0.859), or thrombin-like SVSP activity (χ^2 = 4.8, df = 5, p = 0.788). No venom profiles

Table 2
Results of Kruskal-Wallis test with post-hoc pairwise comparisons of significantly different toxin abundances across venom types.

	Azo						Thr						L-AAO					
	1	2	3	4	Cvv	Coc	1	2	3	4	Cvv	Coc	1	2	3	4	Cvv	Coc
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	**0.000**	-	-	-	-	-	0.098	-	-	-	-	-	**0.001**	-	-	-	-	-
3	0.767	**0.000**	-	-	-	-	0.105	0.867	-	-	-	-	**0.000**	**0.027**	-	-	-	-
4	**0.006**	0.423	**0.004**	-	-	-	0.217	0.728	0.788	-	-	-	**0.017**	0.492	0.859	-	-	-
Cvv	0.71	**0.000**	0.881	**0.009**	-	-	0.093	0.618	0.734	0.952	-	-	0.52	**0.005**	**0.000**	**0.042**	-	-
Coc	**0.000**	0.076	**0.000**	0.896	**0.000**	-	**0.000**	**0.002**	**0.007**	0.222	**0.05**	-	**0.002**	0.528	0.367	0.748	**0.008**	-
	PDE						SVMP						ConTox					
	1	2	3	4	Cvv	Coc	1	2	3	4	Cvv	Coc	1	2	3	4	Cvv	Coc
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	**0.047**	-	-	-	-	-	**0.00**	-	-	-	-	-	**0.003**	-	-	-	-	-
3	**0.009**	0.181	-	-	-	-	0.526	**0.00**	-	-	-	-	**0.000**	0.065	-	-	-	-
4	0.059	0.374	0.728	-	-	-	**0.017**	0.763	**0.027**	-	-	-	1.00	**0.049**	**0.006**	-	-	-
Cvv	0.752	0.064	**0.011**	0.082	-	-	0.641	**0.000**	0.902	**0.031**	-	-	1.00	**0.001**	**0.000**	1.00	-	-
Coc	0.176	0.664	0.2	0.306	0.257	-	**0.000**	0.226	**0.000**	0.711	**0.00**	-	**0.000**	0.144	0.986	**0.01**	**0.000**	-

$\alpha = 0.05$; significant values are **bolded**. Venom types are listed as 1–4. Azo, metalloproteinase activity; Coc = *Crotalus oreganus concolor*; ConTox, concolor toxin % abundance; Cvv = *Crotalus viridis viridis*; L-AAO, L-amino acid oxidase activity; PDE, phosphodiesterase activity; SVMP, metalloproteinase % abundance; Thr, thrombin-like serine proteinase activity.

differed significantly in levels of kallikrein-like SVSP activity (($\chi^2 = 7.93$, df = 5, p = 0.16). Both Type 3 and Type 4 venoms were significantly lower in L-AAO activity compared to pure *C. v. viridis* venom ($\chi^2 = -42.42$, df = 5, p = 0.000; $\chi^2 = -39.21$, df = 5, p = 0.042, respectively).

3.4. High-performance liquid chromatography analyses of venom composition

RP-HPLC of defined species and potential hybrid venoms showed high complexity of protein components (Figs. 5 and 6; Supplemental Figs. 6 and 7). SDS-PAGE revealed that similar protein components eluted at consistent times during the gradient regardless of sample or species (Supplemental Figs. 6 and 7). Myotoxins eluted at 20–25 min, and several isoforms appeared to be present, likely those characterized as myotoxins a, 2 and 3 [49,74–76]. The B subunit of concolor toxin eluted at approximately 41 min (see also Supplemental Figs. 4 and 5). Serine proteases eluted from 56 to 67 min, L-amino acid oxidases eluted at approximately 81 min and metalloproteases eluted at 85–87 min.

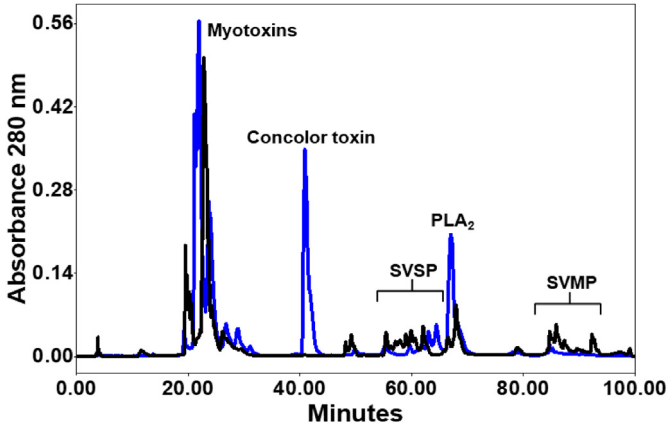


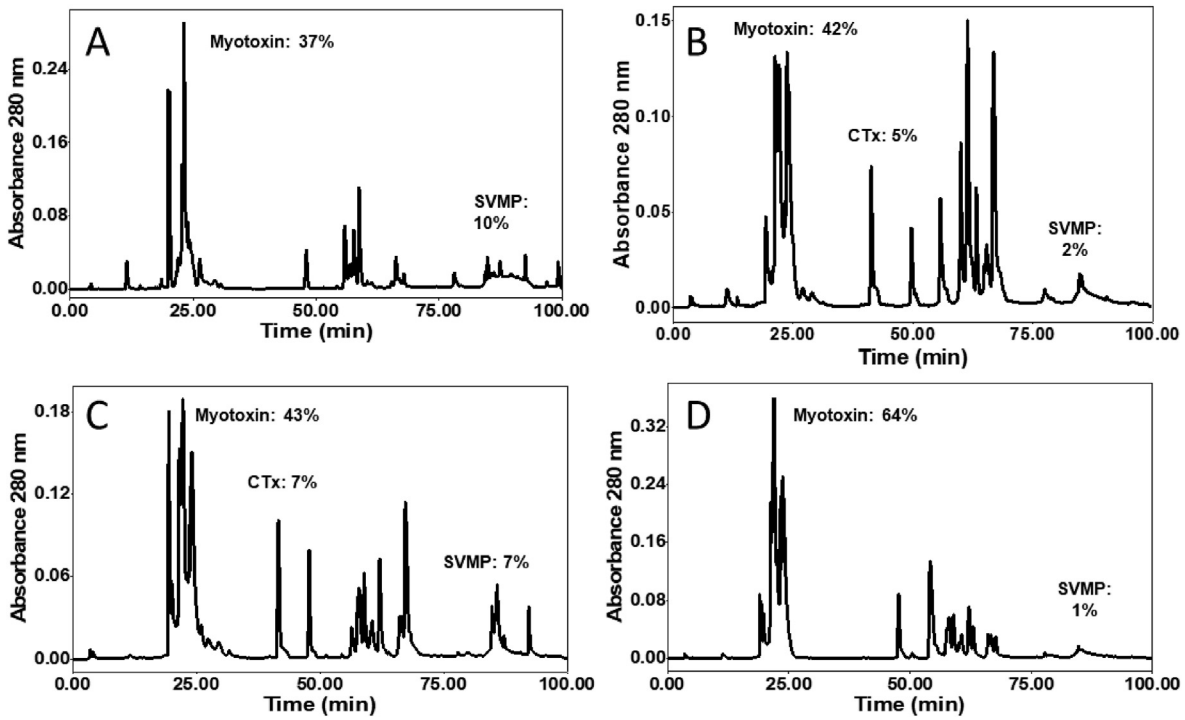
Fig. 5. Parental species venom profile comparison. Overlay of reverse phase HPLC chromatograms of *C. o. concolor* crude venom (2 mg; blue) from Flaming Gorge Recreation Area and *C. v. viridis* crude venom (2 mg; black) from Weld Co. Note the presence of concolor toxin and lack of metalloproteases (SVMP) in *C. o. concolor* venom and the lack of concolor toxin in *C. v. viridis* venom. PLA_2, phospholipases A_2; SVMP, snake venom metalloprotease; SVSP, snake venom serine protease.

All adult *C. o. concolor* venoms had similar HLPC chromatograms, most notably including a peak at approximately 41 min (concolor toxin subunit B) and small or nearly absent metalloprotease peaks (Fig. 5). All *C. v. viridis* adult venoms lacked a 41-min peak and had a larger cluster of metalloprotease peaks (Fig. 5). Though *C. o. concolor* and *C. v. viridis* chromatograms typically shared the presence of myotoxins and many serine proteinase isoforms, the main distinguishing characteristics between the venoms were the presence or absence of a 41-min concolor toxin peak and the relative abundance of metalloprotease peaks (Fig. 5).

Examining changes in protein family abundance levels between the different venoms, adult pure *C. v. viridis* individuals had SVMP percentages ranging from 6.1 to 12.5% (n = 7, average = 8.8, median = 7.1; Fig. 4H) and myotoxin levels ranging from 30.5 to 51.7% (n = 7, average = 43.3, median = 46.9; Fig. 4F). Pure *C. o. concolor* had venom SVMP percentages ranging from 0.73 to 3.03% (n = 7, average = 1.73, median = 1.56), concolor toxin levels ranging from 4.49 to 18.54% (n = 7, average = 7.2, median = 5.6; Fig. 4G), and myotoxin percentages ranging from 22 to 50.7% (n = 7, average = 43.3, median = 47.3). Adult *C. v. viridis* venoms had significantly higher SVMP percentages than *C. o. concolor* ($\chi^2 = 35.36$, df = 5, p < 0.000), and *C. o. concolor* had significantly higher levels of concolor toxin ($\chi^2 = 35.93$, df = 5, p < 0.000, Table 2). However, the percentage of myotoxin ($\chi^2 = 10.4$, df = 5, p = 0.065) was not significantly different between the two venoms.

Snakes from the zone of introgression displayed wide variation in venom component presence and abundances (Fig. 6A–D). Similar to chromatograms from known *C. v. viridis* and *C. o. concolor*, a single concolor toxin peak at approximately 41 min was either clearly present or absent in all samples. Metalloprotease enzyme activity correlated with the size of the metalloprotease reverse phase HPLC peaks (Table 3). Type 3 venoms showed myotoxin levels that ranged from 27.0 to 54.8% (n = 14, average = 36.8, median = 35.4; Fig. 4F), and Type 3 venom SVMP percentages ranged from 2.4 to 19.8% (n = 14, average = 9.15, median = 8.1; Fig. 4H). Percentage of concolor toxin ranged from 4.0 to 8.2% in Type 3 venoms (n = 14, average = 5.66, median = 5.54; Fig. 4G). Type 4 venom percentage of myotoxin ranged from 33.3 to 58.1% (n = 3, average = 48.54, median = 54.25) and percentage of SVMP ranged from 1.9 to 3.4% (n = 3, average = 2.53, median = 2.35).

Type 3 venoms did not vary significantly from pure *C. o. concolor*



E

Phenotype	Myotoxin	CTx	SVMPs
1	✓		✓
2	✓	✓	
3	✓	✓	✓
4	✓✓		

Fig. 6. Venom profile comparisons of snakes from zone of introgression. Reverse phase HPLC chromatograms of potential hybrids with Type 1 venom (A), Type 2 venom (B), Type 3 venom (C), and Type 4 venom (D); CTx = concolor toxin, SVMP = metalloproteases. Note the presence of both concolor toxin and SVMPs in Type 3 venom and the high abundance of myotoxin and lack of both concolor toxin and SVMPs in Type 4 venom. (E) summary table of major components in each phenotype.

Table 3
Spearman's correlation coefficients (ρ) and two-tailed significance comparing each toxin component. Significantly correlated toxins are bolded. Abbreviations follow Table 2.

		% Myo	% ConTx	% SVMP	Azo	Thr	Kall	L-AAO	PDE
% Myo	ρ	-	−0.288	**−0.522****	**−0.350***	−0.039	**0.297***	0.105	−0.074
	Sig.	-	0.050	0.000	0.016	0.796	0.047	0.483	0.621
% ConTx	ρ	-	-	0.042	0.089	−0.106	−0.093	−0.246	−0.039
	Sig.	-	-	0.777	0.550	0.484	0.543	0.096	0.796
% SVMP	ρ	-	-	-	**0.853****	0.217	**−0.403****	−0.007	0.262
	Sig.	-	-	-	0.000	0.147	0.006	0.960	0.076
Azo	ρ	-	-	-	-	0.081	−0.086	−0.049	0.003
	Sig.	-	-	-	-	0.511	0.491	0.692	0.982
Thr	ρ	-	-	-	-	-	**0.402****	0.025	0.065
	Sig.	-	-	-	-	-	0.001	0.840	0.599
Kall	ρ	-	-	-	-	-	-	0.150	−0.098
	Sig.	-	-	-	-	-	-	0.226	0.432
L-AAO	ρ	-	-	-	-	-	-	-	**0.246***
	Sig.	-	-	-	-	-	-	-	0.042
PDE	ρ	-	-	-	-	-	-	-	-
	Sig.	-	-	-	-	-	-	-	-

venoms or Type 2 (hybrid zone) venoms in the percentage of concolor toxin (χ^2 = -0.143, df = 5, p = 986; χ^2 = -10.69, df = 5, p = 0.065, respectively; Table 2). Further, hybrid Type 3 venoms were not significantly different from pure *C. v. viridis* or Type 1 venom from hybrid zone individuals in levels of SVMPs (χ^2 = -1.0, df = 5, p = 0.902; χ^2 = 5.77, df = 5, p = 0.526, respectively). In contrast, Type 4 venoms, which lacked concolor toxin and expressed virtually no metalloprotease activity, were significantly

lower in levels of concolor toxin ($\chi^2 = 35.77$, df = 5, p = 0.006), and SVMPs ($\chi^2 = 29.18$, df = 5, p = 0.027) compared to Type 3 venoms. No hybrid venom profiles differed significantly in levels of myotoxin ($\chi^2 = 10.4$, df = 5, p = 0.065). It is important to highlight that the percentages calculated here are based on the chromatographic profile monitored at 280 nm (aromatic side chains) and not at the absorption wavelength of a peptide bond 214–220 nm. Therefore, while these percentages provide a reliable proxy for comparing protein abundance changes between the different venoms within the current study, we caution against using these values for cross-protein family comparisons or for comparing to the percentages calculated in other studies that used 214–220 nm.

3.5. Variation in toxicity among venom types

Crotalus v. viridis (Type 1) and *C. o. concolor* (Type 2) venoms showed very different toxicities in NSA mice (Table 1), as expected from these venom phenotypes. However, Type 3 and 4 venoms also showed divergent toxicities, and both Type 2 and 3 venoms had substantially lower LD_{50} values (0.7 μg/g and 0.6 μg/g, respectively) than Type 1 and Type 4 venoms (both 5.1 μg/g).

3.6. Correlations between major toxins

Spearman's correlations revealed significant relationships in expression between percentage of myotoxin and percentage SVMP ($\rho = -0.522$, p = 0.000; Table 3), azocasein activity ($\rho = -0.297$, p = 0.016), and kallikrein-like SVSP ($\rho = 0.297$, p = 0.047) in hybrid zone snakes. Percentage of SVMPs had a significant positive relationship with SVMP activity ($\rho = 0.853$, p = 0.000), and a significantly negative relationship with kallikrein-like SVSP activity ($\rho = -0.403$, p = 0.006). Thrombin-like and kallikrein-like serine proteases were significantly positively correlated ($\rho = 0.402$, p = 0.001), and L-AAO activity was positively correlated with PDE activity ($\rho = 0.246$, p = 0.042). Notably, the inverse relationship between percentage of concolor toxin (and by proxy overall toxicity) and percent abundance of SVMP or SVMP enzyme activity, as displayed in Type 1 and Type 2 venoms [6], was absent in the area of introgression ($\rho = 0.042$, p = 0.777; $\rho = 0.089$, p = 0.55, respectively).

3.7. Correlation between venom composition and geographic location

SVMP activity (r = 0.185, p = 0.026), L-amino acid oxidase activity (Mantel Test; r = 0.16, p = 0.049), and concolor toxin percentage (r = 0.42, p = 0.001) were found to be significantly associated with geographic location. All other venom characters did not vary significantly with geographic location.

3.8. Relationships between genetic ancestry and venom composition

Positive relationships between ancestry and venom characteristics were seen between the proportion of *C. v. viridis* ancestry and percentage of concolor toxin (R = −0.72; Fig. 7A), SVMP percentage (R = 0.57; Fig. 7B), SVMP activity (R = 0.5; Fig. 7D), L-AAO activity (R = 0.56; Fig. 7F), and PDE activity (R = 0.45; Fig. 7G). Regressions of ancestry and venom characteristics showed no relationship between ancestry and abundance of myotoxin (R = −0.07; Fig. 7C), kallikrein-like SVSP (R = 0.021; Fig. 7E), and thrombin-like SVSP (R = 0.24; Fig. 7H).

4. Discussion

From an adaptive perspective, Type 1 and Type 2 venoms represent a trade-off between rapid prey incapacitation via flaccid paralysis induced by the action of potent neurotoxins [77,78] versus less toxic but pre-digestive and prey-relocation capacity due to the abundance of SVMPs and disintegrins, respectively [5,6,79,80]. Though the co-occurrence of both suites of properties in a single venom might seem advantageous, this strict dichotomy is commonly observed in nature [6,25], suggesting that these mechanisms rely on antagonistic or mutually exclusive adaptive actions, that is, rapid prey incapacitation that occurs within seconds or more prolonged incapacitation followed by prey relocation. Thus, there may be negative costs to expressing these divergent phenotypes simultaneously; this is supported by the rarity of combined (Type 3) venoms. While novel but inefficient venoms may prevent toxin radiation and expression outside of hybrid zones and into parental lineages [19], our data on toxicity do not support the inefficiency of hybrid venoms. Instead, we find that admixed venoms do not exhibit lower toxin abundances or overall toxicity compared to parental venoms, and therefore do not appear to compromise the adaptive function of either venom phenotype. These admixed venom phenotypes could confer unique advantages in particular habitats or specific prey populations (i.e., those that occur in ecotones associated with hybrid zones), allowing for exploitation of new or more varied prey sources, or even exploitation of multiple prey types using a single venom.

This region of secondary contact in Colorado contains a diversity of venom phenotypes within a small geographic area. The majority of snakes expressed Type 2 venoms, indicative of the depth of *C. o. concolor* ancestry in this region, but snakes with Type 3 hybrid venoms expressed statistically equivalent levels of the majority of toxins tested, including SVMPs, concolor toxin, and myotoxins, and were slightly more toxic than Type 2 venoms in this region (though this difference is unlikely to be biologically relevant in terms of prey acquisition). The uncommon Type 4 venoms lacked both concolor toxin and metalloprotease activity and instead contained high levels of myotoxins. High expression levels of myotoxins apparently compensate for the lack of other dominant toxin families, but the very low abundance of Type 4 venoms suggests that this phenotype may still be maladaptive and associated with lower fitness in hybrids.

There are some reasons to expect that admixed venoms may be favored specifically in hybrid zones due to unique characteristics associated with these zones, including the ecotonal nature and diversity of prey species in these transitional habitats. In support of this hypothesis, previous studies have shown that venom compositional variation correlates with preferred prey [2,5,81–83], and that higher phylogenetic diversity of prey species may favor a wider variety of venom components and activities [2,84,85] [2,84]. The 'best of both worlds' composition found in Type 3 venoms (the most abundant form of "hybrid venom" in our study) may represent an example of heterosis, allowing snakes in the hybrid zone to capitalize on a broader diversity of available prey items in an ecotone with unique (or uniquely diverse) prey availability. While other data from across pitviper lineages suggest that there is likely a negative fitness cost of having Type 3 or 4 venoms (given their rarity), it is plausible that these negative fitness costs may be overcome by other secondary fitness gains of such venoms in hybrid zones specifically.

Distinct lineages in CVOS have been shown to introgress fairly commonly in zones where they come into contact, regardless of the level of divergence, due to weak reproductive barriers, yet parental species remain highly distinct throughout the vast majority of the rest of their respective ranges [44]. Ecotones that are characterized

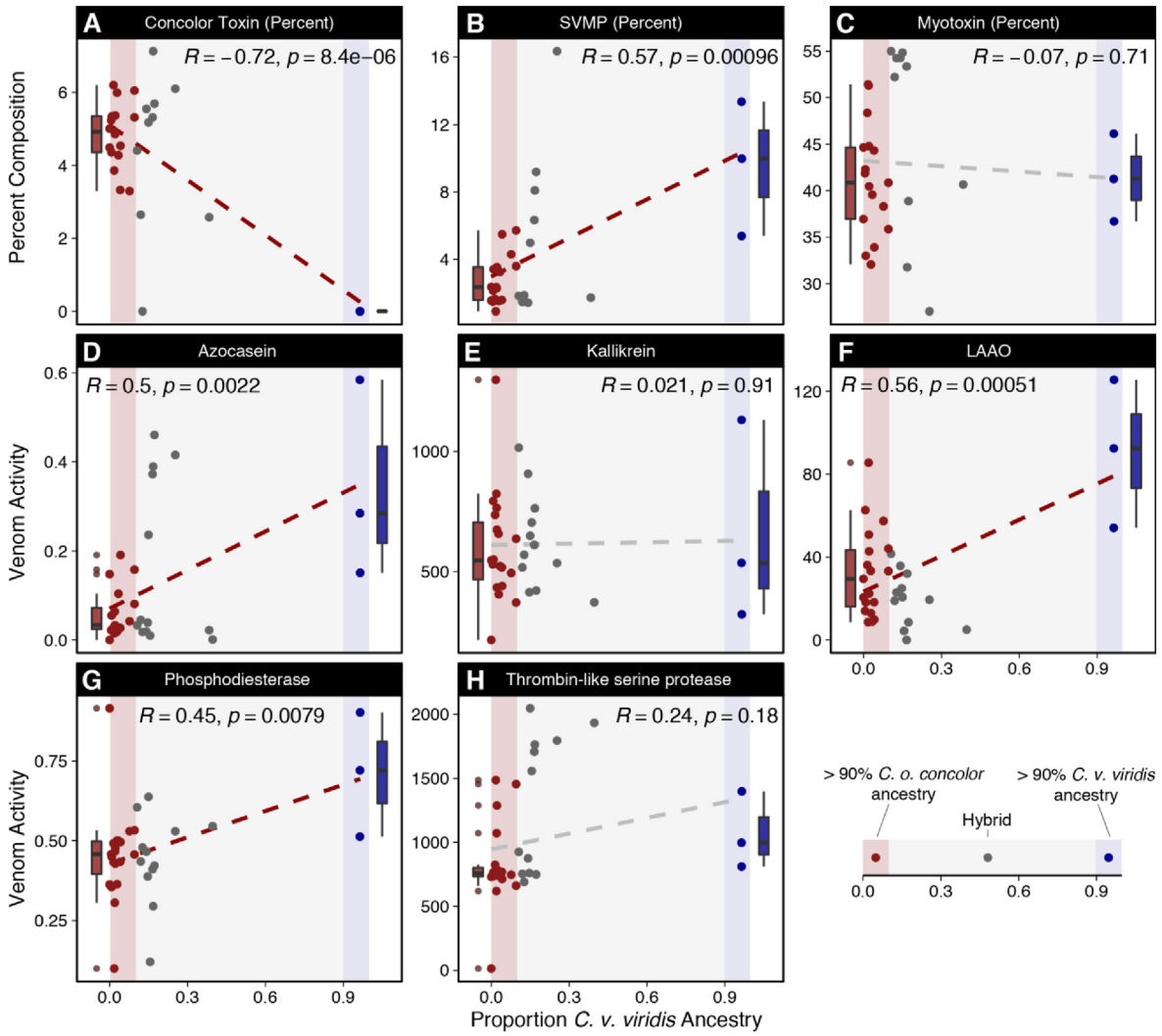


Fig. 7. Regressions between genetic ancestry (Proportion *C. v. viridis* ancestry) and measures of toxin abundance or activity. Red dotted lines indicating significant relationships ($p < 0.05$). Red points indicate individuals with >90% *C. o. concolor* ancestry, blue points indicate individuals with >90% *C. v. viridis* ancestry, and grey points indicate hybrid individuals. Boxplots indicate the measures of parental (>90% *concolor* or *viridis*) individuals. Note that A) concolor toxin percentage, B) SVMP percentage, D) azocasein activity, F) LAAO activity, and G) phosphodiesterase activity are all associated with level of *C. v. viridis* ancestry.

by intermediate environments may be most suitable for admixed phenotypes but suboptimal for parental species [18,86]. The hybrid zone studied here appears to represent an ecotone on the border of suitable habitat for both a canyon crevice specialist (*C.o. concolor* [87]) and a grassland generalist (*C. v. viridis*; [42], resulting in a bounded hybrid zone between parental forms that limits gene flow into parental populations [88]. Previous characterization of this hybrid zone indicates that selection favors admixture of some genomic regions [22]; hybridized venoms may therefore represent a transgressive phenotype only within this unique ecotone. Shifts in environmental factors across even small geographic scales are associated with rapid switches in venom composition [16,26], indicative of strong selective pressures for or against one phenotype. Therefore, hybridized venoms simply may not represent enough of an advantage outside of introgressed areas for strong positive selection to lead to expansion of either toxin family into parental ranges [19].

The geographically limited distribution of hybrid zones and venom phenotypes that break the dichotomy [6,19] may also result from fitness reductions in hybrid venoms due to a dosage-dependent reduction in toxin performance. That is, if levels of neurotoxin are reduced, prey will not be rapidly incapacitated, and if degradative enzyme function is also reduced, the snake will also not benefit from the pre-digestive action of these enzymes and may also have difficulty relocating prey. Indeed, lab-bred F_1 hybrids between Type 1 and Type 2 snakes expressed roughly half as much myotoxin and neurotoxin as parent species; however, overall toxicity was equivalent to the more toxic parent [72]. Hybrids in the current study also do not demonstrate decreased toxicity or enzymatic activity relative to parental lineages, therefore there may be other incompatibilities responsible for the disruptive selection hypothesized to maintain the Type 1 vs. Type 2 dichotomy [26].

Our inferences of relative toxicity based on assays in lab mice, *in vitro* assays, and expression levels of toxin families predicted that Type 3 hybrid venoms would demonstrate *in vivo* toxicity similar to *C. o. concolor* venom. However, we acknowledge that these proxies for fitness (venom lethality) are limited by the lack of local prey toxicity (or resistance) data, which could elucidate more complex coevolutionary interactions between venom and prey resistance that explain the abundance of Type 3 venoms in hybrids yet enforce the containment of hybrid venoms to this narrow hybrid zone. Ultimately, integrated studies that account for local prey resistance

to hybrid and parental venoms (cf [89–92]) may provide greater clarification of the processes that govern the Type 1 vs 2 dichotomy, yet appear to maintain hybrid (e.g., Type 3) venoms in narrow zones of admixture.

5. Conclusions

Venom phenotypic and genomic data confirm the existence of a hybrid zone between *C. v. viridis* and *C. o. concolor* in northwestern Colorado. Snakes with hybrid ancestry from this zone are associated with unique patterns of venom composition and activity, including higher metalloprotease activity characteristic of Type 1 venoms, and the neurotoxic concolor toxin characteristic of Type 2 venoms. These hybridized venoms represent admixed venom profiles that do not appear to sacrifice the advantages of either Type 1 or Type 2 venoms. This hybrid population is one of a very small number of examples in venomous snakes in which a population 'breaks the dichotomy' of having either Type 1 or Type 2 venom. Available evidence indicates the overall rarity of hybrid (e.g., Type 3) venoms in nature, yet our findings that these venoms don't appear to be obviously maladaptive in terms of toxicity suggest there may be other tradeoffs associated with having hybrid (Type 3 or 4) venom, and that hybrid zones may be uniquely suited to favor such venoms due to distinctive ecological or prey-community conditions associated with these transitional zones. Future studies investigating the influences of introgression, environmental factors, and prey availability on venom composition can help elucidate how hybridization affects the selective forces that shape extreme and divergent phenotypes. This can ultimately shed light on the unique patterns of evolution in hybrid zones.

Author contributions

CFS, SPM, TAC and JP designed the study. CFS, JP, TAC, SPM, DRS, BWP, JMM and ZLN collected samples, CFS, DRS, BWP, ZLN and SPM generated data. CFS, DRS, BWP, AJS, JMM and ZLN analyzed data. SPM and TAC provided materials, reagents, computational resources and lab space. CFS, SPM, ZLN, BWP, AJS and TAC wrote the manuscript. All authors edited, reviewed, and approved the final manuscript.

Funding

This work was supported by a grant from the NSF (DEB Phylogenetic Systematics; DEB-1655571) to TAC and SPM. CFS received further financial support from the UNC Graduate Student Association and the College of Natural and Health Sciences.

Data accessibility

All raw sequencing data is publicly available on NCBI under the BioProject ID PRJNA548132.

Declaration of competing interest

The authors have no competing interests to declare.

Acknowledgements

The assistance of Graham Dawson in the field is gratefully acknowledged, as is the assistance in the lab (SPM) of Keira Lopez and Castilleja Olmsted.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2023.07.008.

References

[1] A. Barlow, C.E. Pook, R.A. Harrison, W. Wüster, Coevolution of diet and prey-specific venom activity supports the role of selection in snake venom evolution, Proc. R. Soc. B Biol. Sci. 276 (2009) 2443–2449, https://doi.org/10.1098/rspb.2009.0048.

[2] J.C. Daltry, W. Wüster, R.S. Thorpe, Diet and snake venom evolution, Nature 379 (1996) 537–542, https://doi.org/10.1038/379537a0.

[3] F.J. Vonk, N.R. Casewell, C.V. Henkel, A.M. Heimberg, H.J. Jansen, R.J.R. McCleary, H.M.E. Kerkkamp, R.A. Vos, I. Guerreiro, J.J. Calvete, W. Wüster, A.E. Woods, J.M. Logan, R.A. Harrison, T.A. Castoe, J. De Koning, D.D. Pollock, M. Yandell, D. Calderon, C. Renjifo, R.B. Currier, D. Salgado, D. Pla, L. Sanz, A.S. Hyder, J.M.C. Ribeiro, J.W. Arntzen, G.E.E.J.M. Van Den Thillart, M. Boetzer, W. Pirovano, R.P. Dirks, H.P. Spaink, D. Duboule, E. McGlinn, R.M. Kini, M.K. Richardson, The king cobra genome reveals dynamic gene evolution and adaptation in the snake venom system, Proc. Natl. Acad. Sci. U.S.A. 110 (2013) 20651–20656, https://doi.org/10.1073/pnas.1314702110.

[4] D.R. Schield, B.W. Perry, R.H. Adams, M.L. Holding, Z.L. Nikolakis, S.S. Gopalan, C.F. Smith, J.M. Parker, J.M. Meik, M. DeGiorgio, S.P. Mackessy, T.A. Castoe, The roles of balancing selection and recombination in the evolution of rattlesnake venom, Nat. Ecol. Evol. 6 (2022) 1367–1380, https://doi.org/10.1038/s41559-022-01829-5.

[5] S.P. Mackessy, Venom ontogeny in the Pacific rattlesnakes *Crotalus viridis helleri* and *C. v. oreganus*, Copeia 92 (1988), https://doi.org/10.2307/1445927, 1988.

[6] S.P. Mackessy, Evolutionary trends in venom composition in the Western Rattlesnakes (*Crotalus viridis* sensu lato): toxicity vs. tenderizers, Toxicon 55 (2010) 1463–1474, https://doi.org/10.1016/j.toxicon.2010.02.028.

[7] J.P. Chippaux, V. Williams, J. White, Snake venom variability: methods of study, results and interpretation, Toxicon 29 (1991) 1279–1303, https://doi.org/10.1016/0041-0101(91)90116-9.

[8] A. Barua, A.S. Mikheyev, Toxin expression in snake venom evolves rapidly with constant shifts in evolutionary rates, Proc. R. Soc. B Biol. Sci. 287 (2020) 20200613, https://doi.org/10.1098/rspb.2020.0613.

[9] B.G. Fry, W. Wüster, Assembling an arsenal: origin and evolution of the snake venom proteome inferred from phylogenetic analysis of toxin sequences, Mol. Biol. Evol. 21 (2004) 870–883, https://doi.org/10.1093/molbev/msh091.

[10] D. Mebs, Toxicity in animals. Trends in evolution? Toxicon 39 (2001) 87–96, https://doi.org/10.1016/S0041-0101(00)00155-0.

[11] M. Sasa, Diet and snake venom evolution: can local selection alone explain intraspecific venom variation? Toxicon 37 (1999) 249–252, https://doi.org/10.1016/S0041-0101(98)00121-4.

[12] M. Li, B.G. Fry, R.M. Kini, Eggs-only diet: its implications for the toxin profile changes and ecology of the marbled sea snake (*Aipysurus eydouxii*), J. Mol. Evol. 60 (2005) 81–89, https://doi.org/10.1007/s00239-004-0138-0.

[13] J. Pawlak, S.P. Mackessy, B.G. Fry, M. Bhatia, G. Mourier, C. Fruchart-Gaillard, D. Servent, R. Ménez, E. Stura, A. Ménez, R.M. Kini, Denmotoxin, a three-finger toxin from the colubrid snake *Boiga dendrophila* (mangrove catsnake) with bird-specific activity, J. Biol. Chem. 281 (2006) 29030–29041, https://doi.org/10.1074/jbc.M605850200.

[14] J. Pawlak, S.P. Mackessy, N.M. Sixberry, E.A. Stura, M.H. Le Du, R. Ménez, C.S. Foo, A. Ménez, S. Nirthanan, R.M. Kini, Irditoxin, a novel covalently linked heterodimeric three-finger toxin with high taxon-specific neurotoxicity, Faseb. J. 23 (2009) 534–545, https://doi.org/10.1096/fj.08-113555.

[15] L.F. Sousa, J.A. Portes-Junior, C.A. Nicolau, J.L. Bernardoni, M.Y. Nishiyama-Jr, D.R. Amazonas, L.A. Freitas-de-Sousa, R.H. Mourão, H.M. Chalkidis, R.H. Valente, A.M. Moura-da-Silva, Functional proteomic analyses of *Bothrops atrox* venom reveals phenotypes associated with habitat variation in the Amazon, J. Proteonomics 159 (2017) 32–46, https://doi.org/10.1016/j.jprot.2017.03.003.

[16] J.L. Strickland, C.F. Smith, A.J. Mason, D.R. Schield, M. Borja, G. Castañeda-Gaytán, C.L. Spencer, L.L. Smith, A. Trápaga, N.M. Bouzid, G. Campillo-García, O.A. Flores-Villela, D. Antonio-Rangel, S.P. Mackessy, T.A. Castoe, D.R. Rokyta, C.L. Parkinson, Evidence for divergent patterns of local selection driving venom variation in Mojave Rattlesnakes (*Crotalus scutulatus*), Sci. Rep. 8 (2018), https://doi.org/10.1038/s41598-018-35810-9.

[17] C.M. Modahl, Mrinalini, S. Frietze, S.P. Mackessy, Adaptive evolution of distinct prey-specific toxin genes in rear-fanged snake venom, Proc. R. Soc. B Biol. Sci. 285 (2018), https://doi.org/10.1098/rspb.2018.1003.

[18] P. Tarroso, R.J. Pereira, F. Martínez-Freiría, R. Godinho, J.C. Brito, Hybridization at an ecotone: ecological and genetic barriers between three Iberian vipers, Mol. Ecol. 23 (2014) 1108–1123, https://doi.org/10.1111/mec.12671.

[19] G. Zancolli, T.G. Baker, A. Barlow, R.K. Bradley, J.J. Calvete, K.C. Carter, K. De Jager, J.B. Owens, J.F. Price, L. Sanz, A. Scholes-Higham, L. Shier, L. Wood, C.E. Wüster, W. Wüster, Is hybridization a source of adaptive venom variation in rattlesnakes? A test, using a *Crotalus scutulatus* × *viridis* hybrid zone in Southwestern New Mexico, Toxins 8 (2016) 188, https://doi.org/10.3390/toxins8060188.

[20] D.R. Schield, R.H. Adams, D.C. Card, B.W. Perry, G.M. Pasquesi, T. Jezkova, D.M. Portik, A.L. Andrew, C.L. Spencer, E.E. Sanchez, M.K. Fujita, S.P. Mackessy, T.A. Castoe, Insight into the roles of selection in speciation from genomic patterns of divergence and introgression in secondary contact in venomous rattlesnakes, Ecol. Evol. 7 (2017) 3951–3966, https://doi.org/10.1002/ece3.2996.
[21] B.T. Martin, M.R. Douglas, T.K. Chafin, J.S. Placyk, S.P. Mackessy, J.T. Briggler, M.E. Douglas, D. St, Relictual Hybridization and Biogeography of Massasauga Rattlesnakes, Sistrurus spp.), BioRxiv, 2022, https://doi.org/10.1101/2022.08.29.505772, 2022.08.29.505772.
[22] Z.L. Nikolakis, D.R. Schield, A.K. Westfall, B.W. Perry, K.N. Ivey, R.W. Orton, N.R. Hales, R.H. Adams, J.M. Meik, J.M. Parker, C.F. Smith, Z. Gompert, S.P. Mackessy, T.A. Castoe, Evidence that genomic incompatibilities and other multilocus processes impact hybrid fitness in a rattlesnake hybrid zone, Evolution 76 (2022) 2513–2530, https://doi.org/10.1111/EVO.14612.
[23] N.L. Dowell, M.W. Giorgianni, S. Griffin, V.A. Kassner, J.E. Selegue, E.E. Sanchez, S.B. Carroll, Extremely divergent haplotypes in two toxin gene complexes encode alternative venom types within rattlesnake species, Curr. Biol. 28 (2018) 1016–1026.e4, https://doi.org/10.1016/j.cub.2018.02.031.
[24] J.L. Glenn, R.C. Straight, Intergradation of two different venom populations of the Mojave rattlesnake (*Crotalus scutulatus scutulatus*) in Arizona, Toxicon 27 (1989) 411–418, https://doi.org/10.1016/0041-0101(89)90203-1.
[25] S.P. Mackessy, Venom composition in rattlesnakes: trends and biological significance, in: The Biology of Rattlesnakes Edited by, Loma Linda Univ. Press. Loma Linda, 2008.
[26] G. Zancolli, J.J. Calvete, M.D. Cardwell, H.W. Greene, W.K. Hayes, M.J. Hegarty, H.W. Herrmann, A.T. Holycross, D.I. Lannutti, J.F. Mulley, L. Sanz, Z.D. Travis, J.R. Whorley, C.E. Wüster, W. Wüster, When one phenotype is not enough: divergent evolutionary trajectories govern venom variation in a widespread rattlesnake species, Proc. R. Soc. B Biol. Sci. 286 (2019), https://doi.org/10.1098/rspb.2018.2735.
[27] S.P. Mackessy, Reptile venoms and toxins, in: Handb. Venoms Toxins Reptil., 2021, pp. 3–18, https://doi.org/10.1201/9780429054204-2.
[28] T. Tasoulis, G.K. Isbister, A review and database of snake venom proteomes, Toxins 9 (2017), https://doi.org/10.3390/toxins9090290.
[29] J.L. Glenn, R.C. Straight, Venom characteristics as an indicator of hybridization between *Crotalus viridis viridis* and *Crotalus scutulatus scutulatus* in New Mexico, Toxicon 28 (1990) 857–862, https://doi.org/10.1016/S0041-0101(09)80008-1.
[30] S.D. Aird, L.J. Thirkhill, C.S. Seebart, I.I. Kaiser, Venoms and morphology of western diamondback/mojave rattlesnake hybrids, J. Herpetol. 23 (1989) 131, https://doi.org/10.2307/1564018.
[31] S.D. Aird, S. Aggarwal, A. Villar-Briones, M.M.Y. Tin, K. Terada, A.S. Mikheyev, Snake venoms are integrated systems, but abundant venom proteins evolve more rapidly, BMC Genom. 16 (2015), https://doi.org/10.1186/s12864-015-1832-6.
[32] J.L. Glenn, R.C. Straight, Mojave rattlesnake *Crotalus scutulatus scutulatus* venom: variation in toxicity with geographical origin, Toxicon 16 (1978) 81–84, https://doi.org/10.1016/0041-0101(78)90065-X.
[33] J.L. Glenn, R.C. Straight, M.C. Wolfe, D.L. Hardy, Geographical variation in *Crotalus scutulatus scutulatus* (Mojave rattlesnake) venom properties, Toxicon 21 (1983) 119–130, https://doi.org/10.1016/0041-0101(83)90055-7.
[34] M. Borja, E. Neri-Castro, G. Castañeda-Gaytán, J.L. Strickland, C.L. Parkinson, J. Castañeda-Gaytán, R. Ponce-López, B. Lomonte, A. Olvera-Rodríguez, A. Alagón, R. Pérez-Morales, Biological and proteolytic variation in the venom of *Crotalus scutulatus scutulatus* from Mexico, Toxins 10 (2018) 35, https://doi.org/10.3390/toxins10010035.
[35] J. Dobson, D.C. Yang, B. op den Brouw, C. Cochran, T. Huynh, S. Kurrupu, E.E. Sánchez, D.J. Massey, K. Baumann, T.N.W. Jackson, A. Nouwens, P. Josh, E. Neri-Castro, A. Alagón, W.C. Hodgson, B.G. Fry, Rattling the border wall: pathophysiological implications of functional and proteomic venom variation between Mexican and US subspecies of the desert rattlesnake *Crotalus scutulatus*, Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol. 205 (2018) 62–69, https://doi.org/10.1016/j.cbpc.2017.10.008.
[36] J.L. Strickland, A.J. Mason, D.R. Rokyta, C.L. Parkinson, Phenotypic variation in mojave rattlesnake (*Crotalus scutulatus*) venom is driven by four toxin families, Toxins 10 (2018), https://doi.org/10.3390/toxins10040135.
[37] J.A. Wilkinson, J.L. Glenn, R.C. Straight, J.W. Sites, Distribution and genetic variation in venom A and B populations of the Mojave rattlesnake (*Crotalus scutulatus scutulatus*) in Arizona, Herpetologica 47 (1991) 54–68.
[38] W.J. French, W.K. Hayes, S.P. Bush, M.D. Cardwell, J.O. Bader, E.D. Rael, Mojave toxin in venom of *Crotalus helleri* (southern pacific rattlesnake): molecular and geographic characterization, Toxicon 44 (2004) 781–791, https://doi.org/10.1016/j.toxicon.2004.08.008.
[39] D.J. Massey, J.J. Calvete, E.E. Sánchez, L. Sanz, K. Richards, R. Curtis, K. Boesen, Venom variability and envenoming severity outcomes of the *Crotalus scutulatus scutulatus* (Mojave rattlesnake) from Southern Arizona, J. Proteonomics 75 (2012) 2576–2587, https://doi.org/10.1016/j.jprot.2012.02.035.
[40] C.F. Smith, Z.L. Nikolakis, K. Ivey, B.W. Perry, D.R. Schield, N.R. Balchan, J. Parker, K.C. Hansen, A.J. Saviola, T.A. Castoe, S.P. Mackessy, Snakes on a plain: biotic and abiotic factors determine venom compositional variation in a wide-ranging generalist rattlesnake, BMC Biol. 21 (2023) 136, https://doi.org/10.1186/S12915-023-01626-X.
[41] M.A. Bober, J.L. Glenn, R.C. Straight, C.L. Ownby, Detection of myotoxin a-like proteins in various snake venoms, Toxicon 26 (1988) 665–673, https://doi.org/10.1016/0041-0101(88)90248-6.
[42] J.A. Campbell, W.W. Lamar, The Venomous Reptiles of the Western Hemisphere, Comstock Pub. Associates., Ithaca, NY, 2004, https://doi.org/10.1580/1080-6032(2005)16[e13a:br]2.0.co;2.
[43] B.I. Crother (Ed.), Scientific and Standard English Names of Amphibians and Reptiles of North America North of Mexico, with Comments Regarding Confidence in Our Understanding, vol. 43, Society for the Study of Amphibians and Reptiles Herpetological Circular No, 2017.
[44] D.R. Schield, B.W. Perry, R.H. Adams, D.C. Card, T. Jezkova, G.I.M. Pasquesi, Z.L. Nikolakis, K. Row, J.M. Meik, C.F. Smith, S.P. Mackessy, T.A. Castoe, Allopatric divergence and secondary contact with gene flow: a recurring theme in rattlesnake speciation, Biol. J. Linn. Soc. 128 (2019) 149–169, https://doi.org/10.1093/biolinnean/blz077.
[45] C.E. Pook, W. Wüster, R.S. Thorpe, Historical biogeography of the Western Rattlesnake (Serpentes: viperidae: *Crotalus viridis*), inferred from mitochondrial DNA sequence information, Mol. Phylogenet. Evol. 15 (2000) 269–282, https://doi.org/10.1006/mpev.1999.0756.
[46] K.G. Ashton, A. De Queiroz, Molecular systematics of the western rattlesnake, *Crotalus viridis* (Viperidae), with comments on the utility of the D-loop in phylogenetic studies of snakes, Mol. Phylogenet. Evol. 21 (2001) 176–189, https://doi.org/10.1006/mpev.2001.1013.
[47] D. Iddon, R.D.G. Theakston, C.L. Ownby, A study of the pathogenesis of local skin necrosis induced by *Naja nigricollis* (spitting cobra) venom using simple histological staining techniques, Toxicon 25 (1987) 665–672, https://doi.org/10.1016/0041-0101(87)90113-9.
[48] C.L. Ownby, T.R. Colberg, Classification of myonecrosis induced by snake venoms: venoms from the Prairie rattlesnake (*Crotalus viridis viridis*), Western diamondback rattlesnake (*Crotalus atrox*) and the Indian cobra (*Naja naja naja*), Toxicon 26 (1988) 459–474, https://doi.org/10.1016/0041-0101(88)90185-7.
[49] A.J. Saviola, D. Pla, L. Sanz, T.A. Castoe, J.J. Calvete, S.P. Mackessy, Comparative venomics of the Prairie Rattlesnake (*Crotalus viridis viridis*) from Colorado: identification of a novel pattern of ontogenetic changes in venom composition and assessment of the immunoreactivity of the commercial antivenom Crotalidae Polyvalent Immunofab, J. Proteonomics 121 (2015) 28–43, https://doi.org/10.1016/j.jprot.2015.03.015.
[50] S.D. Aird, A quantitative assessment of variation in venom constituents within and between three nominal rattlesnake subspecies, Toxicon 23 (1985) 1000–1004, https://doi.org/10.1016/0041-0101(85)90394-0.
[51] A.L. Bieber, R.H. McParland, R.R. Becker, Amino acid sequences of myotoxins from *Crotalus viridis concolor* venom, Toxicon 25 (1987) 677–680, https://doi.org/10.1016/0041-0101(87)90115-2.
[52] J.L. Glenn, R. Straight, The midget faded rattlesnake (*Crotalus viridis concolor*) venom: lethal toxicity and individual variability, Toxicon 15 (1977) 129–132, https://doi.org/10.1016/0041-0101(77)90031-9.
[53] S.P. Mackessy, K. Williams, K.G. Ashton, Ontogenetic variation in venom composition and diet of *Crotalus oreganus concolor*. A case of venom paedomorphosis? Copeia (2003) 769–782, https://doi.org/10.1643/HA03-037.1.
[54] D.R. Schield, D.C. Card, R.H. Adams, T. Jezkova, J. Reyes-Velasco, F.N. Proctor, C.L. Spencer, H.W. Herrmann, S.P. Mackessy, T.A. Castoe, Incipient speciation with biased gene flow between two lineages of the Western Diamondback Rattlesnake (*Crotalus atrox*), Mol. Phylogenet. Evol. 83 (2015) 213–223, https://doi.org/10.1016/j.ympev.2014.12.006.
[55] J. Catchen, P.A. Hohenlohe, S. Bassham, A. Amores, W.A. Cresko, Stacks: an analysis tool set for population genomics, Mol. Ecol. 22 (2013) 3124–3140, https://doi.org/10.1111/mec.12354.
[56] A. Gordon, G.J. Hannon, Gordon, FASTX-toolkit, 2014. Hannonlab. Cshl. Edu/Fastx_ Toolkit, Http://Hannonlab. Cshl. Edu/Fastx_ Toolkit.
[57] H. Li, R. Durbin, Fast and accurate short read alignment with Burrows-Wheeler transform, Bioinformatics 25 (2009) 1754–1760, https://doi.org/10.1093/bioinformatics/btp324.
[58] D.R. Schield, D.C. Card, N.R. Hales, B.W. Perry, G.M. Pasquesi, H. Blackmon, R.H. Adams, A.B. Corbin, C.F. Smith, B. Ramesh, J.P. Demuth, E. Betrán, M. Tollis, J.M. Meik, S.P. Mackessy, T.A. Castoe, The origins and evolution of chromosomes, dosage compensation, and mechanisms underlying venom regulation in snakes, Genome Res. 29 (2019) 590–601, https://doi.org/10.1101/gr.240952.118.
[59] H. Li, B. Handsaker, A. Wysoker, T. Fennell, J. Ruan, N. Homer, G. Marth, G. Abecasis, R. Durbin, The sequence alignment/map format and SAMtools, Bioinformatics 25 (2009) 2078–2079, https://doi.org/10.1093/bioinformatics/btp352.
[60] P. Danecek, A. Auton, G. Abecasis, C.A. Albers, E. Banks, M.A. DePristo, R.E. Handsaker, G. Lunter, G.T. Marth, S.T. Sherry, G. McVean, R. Durbin, The variant call format and VCFtools, Bioinformatics 27 (2011) 2156–2158, https://doi.org/10.1093/bioinformatics/btr330.
[61] D.H. Alexander, J. Novembre, K. Lange, Fast model-based estimation of ancestry in unrelated individuals, Genome Res. 19 (2009) 1655–1664, https://doi.org/10.1101/gr.094052.109.
[62] J.K. Pritchard, M. Stephens, P. Donnelly, Inference of population structure using multilocus genotype data, Genetics 155 (2000) 945–959, https://doi.org/10.1093/genetics/155.2.945.
[63] C. R Team, R Core Team, R: A Language and Environment for Statistical Computing, R Found. Stat. Comput, Vienna, Austria, 2017. Www. R-Project. Org/., Page R Found. Stat. Comput. (2019) 2018.
[64] M.M. Bradford, A rapid and sensitive method for the quantitation of

microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254, https://doi.org/10.1016/0003-2697(76)90527-3.
[65] S.D. Aird, N.J. da Silva, Comparative enzymatic composition of brazilian coral snake (Micrurus) venoms, Comp. Biochem. Physiol. Part B Biochem. 99 (1991) 287–294, https://doi.org/10.1016/0305-0491(91)90043-D.
[66] H. Weissbach, A.V. Robertson, B. Witkop, S. Udenfriend, Rapid spectrophotometric assays for snake venom l-amino acid oxidase based on the oxidation of l-kynurenine or 3,4-dehydro-l-proline, Anal. Biochem. 1 (1960) 286–290, https://doi.org/10.1016/0003-2697(60)90026-9.
[67] M. Laskowski, Purification and properties of venom phosphodiesterase, Methods Enzymol. 65 (1980) 276–284, https://doi.org/10.1016/S0076-6879(80)65037-X.
[68] W. Bjork, Purification of phosphodiesterase from *Bothrops atrox* venom, with special consideration of the elimination of monophosphatases, J. Biol. Chem. 238 (1963) 2487–2490, https://doi.org/10.1016/s0021-9258(19)67998-6.
[69] S.P. Mackessy, Kallikrein-like and thrombin-like proteases from the venom of the northern Pacific rattlesnake (*Crotalus viridis oreganus*), J. Nat. Toxins 2 (1993) 223–239, https://doi.org/10.1016/0041-0101(90)90211-o.
[70] S.D. Aird, I.I. Kaiser, R.V. Lewis, W.G. Kruggel, A complete amino acid sequence for the basic subunit of crotoxin, Arch. Biochem. Biophys. 249 (1986) 296–300, https://doi.org/10.1016/0003-9861(86)90005-6.
[71] C.M. Modahl, A.K. Mukherjee, S.P. Mackessy, An analysis of venom ontogeny and prey-specific toxicity in the Monocled Cobra (*Naja kaouthia*), Toxicon 119 (2016) 8–20, https://doi.org/10.1016/j.toxicon.2016.04.049.
[72] C.F. Smith, S.P. Mackessy, The effects of hybridization on divergent venom phenotypes: characterization of venom from *Crotalus scutulatus scutulatus* × *Crotalus oreganus helleri* hybrids, Toxicon 120 (2016) 110–123, https://doi.org/10.1016/j.toxicon.2016.08.001.
[73] J.J. Calvete, L. Sanz, Y. Angulo, B. Lomonte, J.M. Gutiérrez, Venoms, venomics, antivenomics, FEBS Lett. 583 (2009) 1736–1743, https://doi.org/10.1016/j.febslet.2009.03.029.
[74] A.L. Bieber, D. Nedelkov, Structural, biological and biochemical studies of myotoxin a and homologous myotoxins, J. Toxicol. - Toxin Rev. 16 (1997) 33–52, https://doi.org/10.3109/15569549709064092.
[75] D.L. Cameron, A.T. Tu, Characterization of myotoxin a from the venom of Prairie rattlesnake (*Crotalus viridis viridis*), Biochemistry 16 (1977) 2546–2553, https://doi.org/10.1021/bi00630a035.
[76] P.R. Griffin, S.D. Aird, A new small myotoxin from the venom of the Prairie rattlesnake (*Crotalus viridis viridis*), FEBS Lett. 274 (1990) 43–47, https://doi.org/10.1016/0014-5793(90)81325-I.
[77] C. Bon, J.P. Changeux, T.W. Jeng, H. Fraenkel-Conrat, Postsynaptic effects of crotoxin and of its isolated subunits, Eur. J. Biochem. 99 (1979) 471–482, https://doi.org/10.1111/j.1432-1033.1979.tb13278.x.
[78] C.L. Ho, C.Y. Lee, Presynaptic actions of Mojave toxin isolated from Mojave rattlesnake (*Crotalus scutulatus*) venom, Toxicon 19 (1981) 889–892, https://doi.org/10.1016/0041-0101(81)90086-6.
[79] A.J. Saviola, D. Chiszar, C. Busch, S.P. MacKessy, Molecular basis for prey relocation in viperid snakes, BMC Biol. 11 (2013), https://doi.org/10.1186/1741-7007-11-20.
[80] R.G. Thomas, F.H. Pough, The effect of rattlesnake venom on digestion of prey, Toxicon 17 (1979) 221–228, https://doi.org/10.1016/0041-0101(79)90211-3.
[81] T. Chijiwa, M. Deshimaru, I. Nobuhisa, M. Nakai, T. Ogawa, N. Oda, K.I. Nakashima, Y. Fukumaki, Y. Shimohigashi, S. Hattori, M. Ohno, Regional evolution of venom-gland phospholipase A2 isoenzymes of *Trimeresurus flavoviridis* snakes in the southwestern islands of Japan, Biochem. J. 347 (2000) 491–499, https://doi.org/10.1042/0264-6021:3470491.
[82] N. Jorge Da Silva, S.D. Aird, Prey specificity, comparative lethality and compositional differences of coral snake venoms, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 128 (2001) 425–456, https://doi.org/10.1016/s1532-0456(00)00215-5.
[83] H.L. Gibbs, S.P. Mackessy, Functional basis of a molecular adaptation: prey-specific toxic effects of venom from *Sistrurus* rattlesnakes, Toxicon 53 (2009) 672–679, https://doi.org/10.1016/j.toxicon.2009.01.034.
[84] N.R. Casewell, W. Wüster, F.J. Vonk, R.A. Harrison, B.G. Fry, Complex cocktails: the evolutionary novelty of venoms, Trends Ecol. Evol. 28 (2013) 219–229, https://doi.org/10.1016/j.tree.2012.10.020.
[85] M.L. Holding, J.L. Strickland, R.M. Rautsaw, E.P. Hofmann, A.J. Mason, M.P. Hogan, G.S. Nystrom, S.A. Ellsworth, T.J. Colston, M. Borja, G. Castañeda-Gaytán, C.I. Grünwald, J.M. Jones, L.A. Freitas-De-Sousa, V.L. Viala, M.J. Margres, E. Hingst-Zaher, I.L.M. Junqueira-De-Azevedo, A.M. Moura-Da-Silva, F.G. Grazziotin, H. Lisle Gibbs, D.R. Rokyta, C.L. Parkinson, Phylogenetically diverse diets favor more complex venoms in North American pitvipers, Proc. Natl. Acad. Sci. U.S.A. 118 (2021), https://doi.org/10.1073/pnas.2015579118.
[86] R. Abbott, D. Albach, S. Ansell, J.W. Arntzen, S.J.E. Baird, N. Bierne, J. Boughman, A. Brelsford, C.A. Buerkle, R. Buggs, R.K. Butlin, U. Dieckmann, F. Eroukhmanoff, A. Grill, S.H. Cahan, J.S. Hermansen, G. Hewitt, A.G. Hudson, C. Jiggins, J. Jones, B. Keller, T. Marczewski, J. Mallet, P. Martinez-Rodriguez, M. Möst, S. Mullen, R. Nichols, A.W. Nolte, C. Parisod, K. Pfennig, A.M. Rice, M.G. Ritchie, B. Seifert, C.M. Smadja, R. Stelkens, J.M. Szymura, R. Väinölä, J.B.W. Wolf, D. Zinner, Hybridization and speciation, J. Evol. Biol. 26 (2013) 229–246, https://doi.org/10.1111/j.1420-9101.2012.02599.x.
[87] J.M. Parker, S.H. Anderson, Ecology and behavior of the Midget faded rattlesnake (*Crotalus oreganus concolor*) in Wyoming, J. Herpetol. 41 (2007) 41–51, https://doi.org/10.1670/0022-1511(2007)41[41:EABOTM]2.0.CO;2.
[88] N.H. Barton, G.M. Hewitt, Analysis of hybrid zones, Annu. Rev. Ecol. Systemat. 16 (1985) 113–148, https://doi.org/10.1146/annurev.es.16.110185.000553, 16.
[89] S.A. Smiley-Walters, T.M. Farrell, H.L. Gibbs, The importance of species: pygmy rattlesnake venom toxicity differs between native prey and related non-native species, Toxicon 144 (2018) 42–47, https://doi.org/10.1016/j.toxicon.2018.01.022.
[90] M.L. Holding, J.E. Biardi, H.L. Gibbs, Coevolution of venom function and venom resistance in a rattlesnake predator and its squirrel prey, Proc. R. Soc. B Biol. Sci. 283 (2016), https://doi.org/10.1098/rspb.2015.2841.
[91] K.E. Robinson, M.L. Holding, M.D. Whitford, A.J. Saviola, J.R. Yates, R.W. Clark, Phenotypic and functional variation in venom and venom resistance of two sympatric rattlesnakes and their prey, J. Evol. Biol. 34 (2021) 1447–1465, https://doi.org/10.1111/jeb.13907.
[92] T.M. Farrell, S.A. Smiley-Walters, D.E. McColl, Prey species influences foraging behaviors: rattlesnake (*Sistrurus miliarius*) predation on little Brown skinks (*Scincella lateralis*) and giant centipedes (*Scolopendra viridis*), J. Herpetol. 52 (2018) 156–161, https://doi.org/10.1670/16-094.