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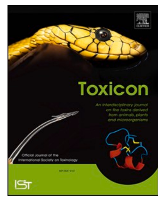

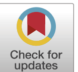

Ontogenetic and geographic venom variation in the Great Basin Rattlesnake, *Crotalus oreganus lutosus*

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ABSTRACT

Venom composition among the species of the Western Rattlesnake clade is often quite variable, depending on several factors such as geographic location and ontogeny. Venom composition not only affects the ability of a snake to acquire prey efficiently, but it can also significantly impact snakebite symptomology. Currently, there has been limited characterization of the venom from the Great Basin Rattlesnake (*Crotalus oreganus lutosus*), a lineage that is broadly distributed in the intermontane western United States. In this study we sample 67 individual Great Basin Rattlesnakes collected in Idaho, Utah, California, and Arizona. We find evidence for substantial ontogenetic and geographic variation in venom composition. Of the six toxin families assessed, all showed ontogenetic shifts to varying extents, with some trends differing from those observed in other rattlesnake species, suggesting species-specific ontogenetic patterns. Notably, the P-I snake venom metalloproteinases and disintegrins were absent or significantly reduced in neonates and juveniles yet abundant in adults. Geographic trends were also observed, with L-amino acid oxidase activity being higher in the California population, while thrombin-like serine proteinase activity and phospholipase A_2 activity was significantly different between Idaho and Utah populations – both trends may be related to local prey specificity. The observed variation in venom activities across populations suggests the presence of venom phenotypic metapopulations. This study shows that in this broadly distributed species, both ontogeny and geographic population structure contribute significantly to range-wide venom compositional variation, which has both ecological and clinical relevance.

1. Introduction

Snake venoms are composed of a complex mixture of toxic and pharmacologically active proteins and peptides (Calvete, 2009), including metalloproteinases and other enzymatic toxins and non-enzymatic toxins that interact with specific physiological targets (Mackessy, 2008, 2009, 2010). Characterizing venom composition within a species has broad ecological and clinical relevance, as venom phenotype influences prey capture efficiency and contributes to geographically variable patterns of snakebite symptomology. Accordingly, understanding how venom composition varies within and among populations is central to linking venom function with both ecological adaptation and medical outcomes.

Venom serves several biologically important roles for the snakes that produce it, most notably enhancing prey acquisition through biochemical rather than mechanical means (Kardong et al., 1997). Snake venoms exhibit substantial phenotypic plasticity and both inter- and intraspecific variation that can vary substantially by geographic location, sex, and ontogeny (Calvete, 2013; Chippaux et al., 1991). Such variation is widely interpreted as adaptive, reflecting local ecological conditions and prey assemblages (Balchan et al., 2024; Barlow et al., 2009; Gibbs and Mackessy, 2009). Recent studies have begun to elucidate regulatory and genetic mechanisms underlying venom phenotypes and their variation (Perry et al., 2022; Schield et al., 2022; Gopalan et al., 2024), as well as links between venom variation and environmental variables such as climate and habitat (Smith et al., 2023; Strickland et al., 2018; Zancolli et al., 2019).

Regional variation in venom composition is common among members of the Western Rattlesnake clade (*Crotalus cerberus, C. viridis, C. oreganus, C. scutulatus*; CVOS), and notable ontogenetic shifts in venom

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are also seen among some of these taxa (Mackessy, 1988, 2010; Saviola et al., 2015; Strickland et al., 2018; Zancolli et al., 2019). In addition to the presence or absence of specific toxins in a particular species, populations frequently differ in the relative abundance and activity of venom components, resulting in distinct venom phenotypes across populations (e.g., Smith et al., 2023). Although documenting this variation is straightforward with adequate sampling across populations, identifying the ecological and evolutionary drivers of this variation remains challenging. Rattlesnake venoms are typically dominated by several enzymatic protein families, including snake venom metalloproteinases (SVMPs), phospholipase A_2s (PLA_2s), phosphodiesterases (PDEs), snake venom serine proteases (SVSPs), and L-amino acid oxidases (L-AAOs) (Mackessy, 2008, 2009). These enzyme families account for much of the functional diversity of rattlesnake venoms and therefore provide a useful framework for assessing geographic and ontogenetic variation.

Despite its broad distribution, venom composition in the Great Basin Rattlesnake (*Crotalus oreganus lutosus*) remains poorly characterized. Because of its wide distribution, it is a good model species to further investigate phenotypic variation of snake venom toxins. This species occurs across much of the intermontane western United States (Fig. 1), including six states that span the Columbia Plateau and Great Basin Desert and encompass substantial environmental and ecological heterogeneity (Glaudas et al., 2008). Its wide geographic range makes *C. o. lutosus* an excellent system for examining how venom phenotype varies across space and environmental gradients. Characterizing venom variation across this range also has clinical relevance, as geographically structured differences in venom composition may contribute to regional variation in snakebite effects and treatment efficacy.

The venom of *C. o. lutosus* has been investigated to some extent, but with small sample sizes and with limited characterization (Adame et al., 1990; Aird, 1985, 2008; Aird et al., 1988; De Almeida et al., 2016). Although these studies suggested geographic differences in venom composition, the extent to which observed variation reflected true geographic structuring versus individual-level variation remained unclear (Adame et al., 1990; Aird, 1985; Aird et al., 1988). For example, prior limited comparisons of individuals from Idaho and Utah indicated that Idaho specimens possessed greater relative abundances of high molecular weight components and small peptides in their venom (Aird et al., 1988), although it has remained unclear if these differences represent true regional variation, or if this is an artifact of the limited individuals and localities sampled. There is also evidence of varying enzymatic activity, specifically among the serine proteases, even within the same population, making it difficult to discern whether patterns of differences between geographically distinct populations are indeed consistent, or instead represent variation observed across specific individuals sampled (Aird, 2008). Additionally, individuals of southern Utah and northern Arizona have been shown to have different venom chromatographic profiles compared to individuals of central and northern Utah (Adame et al., 1990), although these differences have not been associated with toxin activities or abundance.

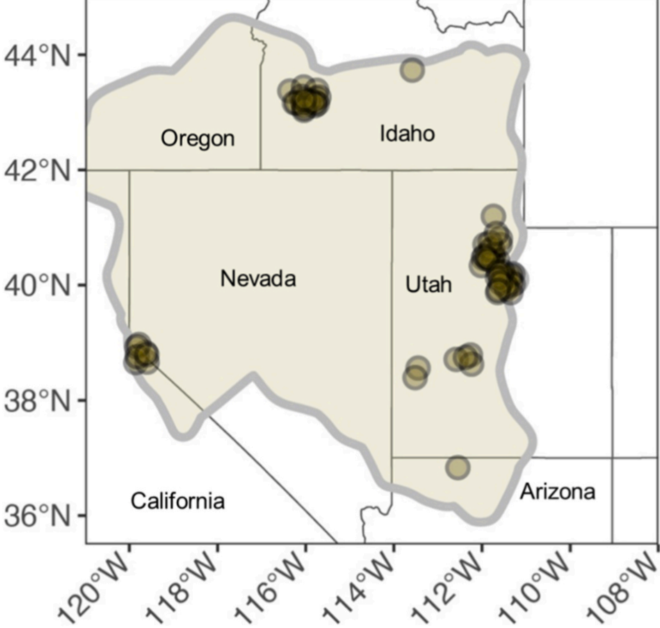

Fig. 1. Geographic distribution of venom samples collected for this study. A total of 67 individual samples were collected from locations in Idaho, Utah, California, and Arizona. Geographic positions were jittered to facilitate visualization. The light yellow area represents the distribution range of *C. o. lutosus*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Ontogenetic variation in venom composition also remains poorly characterized in *C. o. lutosus*, although there is evidence that this species shows substantial ontogenetic shifts in diet, with younger (smaller) snakes preying predominately on lizards and larger adults preying more frequently on mammals (Glaudas et al., 2008). In other rattlesnake species, comparable dietary transitions are often accompanied by pronounced ontogenetic shifts in venom composition and toxicity (Mackessy, 1988; Saviola et al., 2015; Schonour et al., 2020). These shifts may occur gradually or discretely, although such shifts, and their mechanistic and ecological drivers, remain almost entirely unknown (Durban et al., 2013; Schonour et al., 2020).

Given the limited characterization of venom composition variation and the absence of information on potential ontogenetic shifts, a comprehensive, range-wide investigation of venom variation in *C. o. lutosus* is warranted. We hypothesize that venom composition, as characterized in this study by toxin enzymatic activity, varies geographically across the species' range, reflecting a combination of geographic population structure, ecological differences, and environmental gradients. We further predict that ontogenetic shifts in venom phenotype occur in parallel with dietary transitions, as observed in other rattlesnake species. By integrating geographic and ontogenetic perspectives, this study establishes a range-wide framework for understanding venom phenotypic diversity in *C. o. lutosus*, with implications for ecology, evolution, and the clinical management of envenomation.

2. Materials and methods

2.1. Snakes and venom samples

All animal work was conducted under approved IACUC protocols (University of Northern Colorado, 2004D-SM-S-23). Venom was collected from a total of 67 *C. o. lutosus* individuals from locations in Idaho (n = 19), Utah (n = 40), California (n = 7), and Arizona (n = 1) (Fig. 1). There was a total of 42 adults, 6 juveniles, and 19 neonates, with 13 of the neonates being born to two different snakes of the same location in Utah. Age classes were defined as neonates (<400 mm SVL, young of the year), juveniles (<500 mm SVL, sexually immature), and adults (>600 mm SVL, sexually mature). GPS coordinates of capture location for each snake, as well as morphological data, were collected.

The distribution range of *C. o. lutosus* was obtained by downloading specimen coordinates available on GBIF with the R package rgbif (Chamberlain et al., 2026;GBIF.org, 2026). These coordinates were then converted to a polygon using the function concaveman (concaveman package; Gombin et al., 2025), which was then smoothed with the function smooth from smoothr (Strimas-Mackey, 2025).

Venom was manually extracted from the snakes following previous protocols (Mackessy, 1988). Capillary tubes were placed on each fang while the venom glands were massaged. The resulting venom was collected into microfuge tubes, centrifuged to pellet cellular debris, and immediately lyophilized for storage at −20 °C. Venoms were

reconstituted in ultrapure Millipore-filtered water at concentrations of 4 mg/mL and stored frozen at −20 °C between use. Protein concentrations were standardized for all assays below using a Nanodrop 2000 (Thermo Scientific) at 280 nm.

2.2. Gel electrophoresis

Overall venom composition was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using NuPage bis-tris 12% acrylamide gels (Cytiva). Approximately 20 μg of each venom in LDS buffer containing 50 mM DTT, or 10 μL of Mark 12 standard, were loaded per lane. Samples were run in SDS-MES (N-morpholino-ethane sulfonic acid) running buffer containing antioxidant (Cytiva) at 200 V for approximately 50 min and subsequently stained in 0.1% Coomassie brilliant blue R-250 in 50% methanol and 20% acetic acid (v/v) overnight with gentle shaking. After destaining in 30% methanol with 7% glacial acetic acid (v/v) in water, the gels were placed in a storage solution, 7% acetic acid (v/v). They were then imaged on an HP Scanjet 4570c scanner or Epson Perfection V39 scanner. Band densitometry was performed using ImageJ software with band densities normalized to the ladder band most closely correlating to the size of the bands analyzed (Schneider et al., 2012). Adults were classified as having a snout-vent length >600 mm, juveniles 400-600 mm, and neonates <400 mm.

2.3. Snake venom metalloproteinase assay

To assess metalloproteinase activity, an azocasein assay was performed using a previously developed protocol (Aird & da Silva, 1991). One mg of azocasein substrate in 495 μL buffer (50 mM HEPES, 100 mM NaCl, pH 8.0) was incubated with 20 μg of crude venom for 30 min at 37 °C; all samples were run in triplicate due to the variable nature of the substrate. The reaction was terminated with 250 μL of 0.5 M trichloroacetic acid. Samples were then centrifuged at 2000 rpm for 10 min to pellet unreacted substrate before reading the absorbance of the supernatant at 342 nm on an Agilent Cary 60 UV-Vis spectrophotometer. Specific activity was expressed as $\Delta A_{342\ nm}$/minute/mg venom protein.

2.4. Phospholipase A_2 assay

Phospholipase A_2 activity was assessed using an aqueous endpoint assay modified from a previously developed protocol (Holzer and Mackessy, 1996), and all samples were run in duplicate. Using a microplate, 20 μg of crude venom in 200 μL buffer (10 mM tris-HCl, pH 8.0 with 10 mM $CaCl_2$ and 100 mM NaCl) was incubated with 20 μL of 4-nitro-3-(octanoyloxy) benzoic acid substrate (3.0 mM, 0.96 mg/mL) in buffer for 40 min at 37 °C. The reaction was terminated with 20 μL of 2.5% Triton X-100 (v/v) and the absorbance was read at 425 nm using a SpectraMax plate reader. Specific activity was expressed as $\Delta A_{425\ nm}$/minute/mg venom protein.

2.5. Phosphodiesterase assay

Phosphodiesterase activity was assessed using a modified protocol that was previously described (Björk, 1963; Laskowski, 1980). Per sample, 150 μL of 1.0 mM bis-p-nitrophenylphosphate substrate in buffer (100 mM tris-HCl, 10 mM $MgCl_2$, pH 9.0) was incubated with 20 μg of crude venom in 225 μL buffer for 30 min at 37 °C. The reaction was stopped with 375 μL of 100 mM NaOH containing 20 mM disodium-EDTA. The absorbance at 400 nm was then read on a spectrophotometer. Specific activity was expressed as $\Delta A_{400\ nm}$/minute/mg venom protein.

2.6. Thrombin-like and kallikrein-like serine proteinase assays

To assess thrombin-like and kallikrein-like serine proteinase activity, a previously developed protocol was used (Mackessy, 1993). For this, 8 μg of crude venom in 373 μL of buffer (50 mM HEPES, pH 8.0 with 0.1M NaCl) was incubated with 50 μL of 1.0 mM pNA substrate (benzoyl-Phe-Val-Arg-paranitroanaline for thrombin-like and benzoyl-Pro-Phe-Arg-paranitroanaline for kallikrein-like in ddH_2O with 1% DMSO) for 5.0 min at 37 °C. The reaction was terminated with 75 μL of 50% acetic acid (v/v) and the absorbance at 405 nm was read on a spectrophotometer. Specific activities were expressed as nmol product/minute/mg venom protein.

2.7. L-amino acid oxidase assay

L-AAO activity was measured using a spectrophotometric microplate assay developed by Kishimoto and Takahashi (2001). A total of 1 μg of crude venom in 10 μL buffer (50 mM borax, pH 8.5) was incubated with 90 μL substrate master mix (700 μL borax buffer, 100 μL L-methionine at 5.0 mM, 100 μL o-phenylenediamine at 2.0 mM, and 100 μL horseradish peroxidase at 0.0316 mg/mL) at 37 °C for 30 min. The reaction was terminated with 50 μL of 2.0 M sulfuric acid and the absorbance was read on a spectrophotometer at 492 nm. Specific activity was expressed as $\Delta A_{492\ nm}$/minute/mg venom protein.

2.8. Statistical analyses

Normalized band density values were compared between age classes using a one-way ANOVA followed by a Tukey's multiple comparisons post hoc test in GraphPad Prism 8.4.3.

A simple linear regression was performed using R v4.5.2 (R Core Team, 2025) with the 'lm' function to assess the relationship between snout-vent length and toxin activity. Snout-vent length was used as a proxy for age of the snake to assess any ontogenetic shifts in toxin activities.

To assess geographic variation, cluster and outlier analyses were performed with principal component analysis (PCA) on scaled toxin activity data using R (R Core Team, 2025) and the 'prcomp' function. Resulting components were then mapped to both the State in which the animal was collected and the latitude to visually assess geographic clustering. To confirm this geographic clustering, we assess the relationship between population and toxin activity using a one-way ANOVA in R v4.5.2 (R Core Team, 2025) with the 'aov' function. This was followed by Tukey's multiple comparison's post hoc test with the 'TukeyHSD' function to assess the difference between population toxin activities. We formally assessed the assumptions of a one-way ANOVA using Q-Q plots, Shapiro-Wilk normality test, and Bartlett's test. All ANOVAs met assumptions, except for the normality assumption in one case (L-AAO). ANOVA is generally robust to slight deviations from normality, and we confirmed the significance of the population differences with the non-parametric Kruskal-Wallis test. Considering this, we report the parametric result. Ontogenetic analyses were conducted using venom from all 67 individuals. Most geographic analyses were conducted using data from only the 42 adult snakes to mitigate ontogenetic effects; the exception to this was in the one-way ANOVA between population and toxin activity, where a sample from Custer Co., Idaho was removed because it represented both a geographic and toxin activity outlier (see Fig. 5 for details).

3. Results

3.1. Gel electrophoresis

A clear difference between neonates/juveniles and adults, in terms of relative toxin abundances based on the intensity and presence or absence of bands, can be seen in the SDS-PAGE gels (Fig. 2A, Fig. S1). Notably, the SVMP P-III bands are more prominent in adults, while the SVMP P-I band is absent in neonates. Additionally, the PLA_2 bands have a higher band intensity in general compared to adults. Neonates appear to either have a greatly reduced or negligible quantity of low molecular

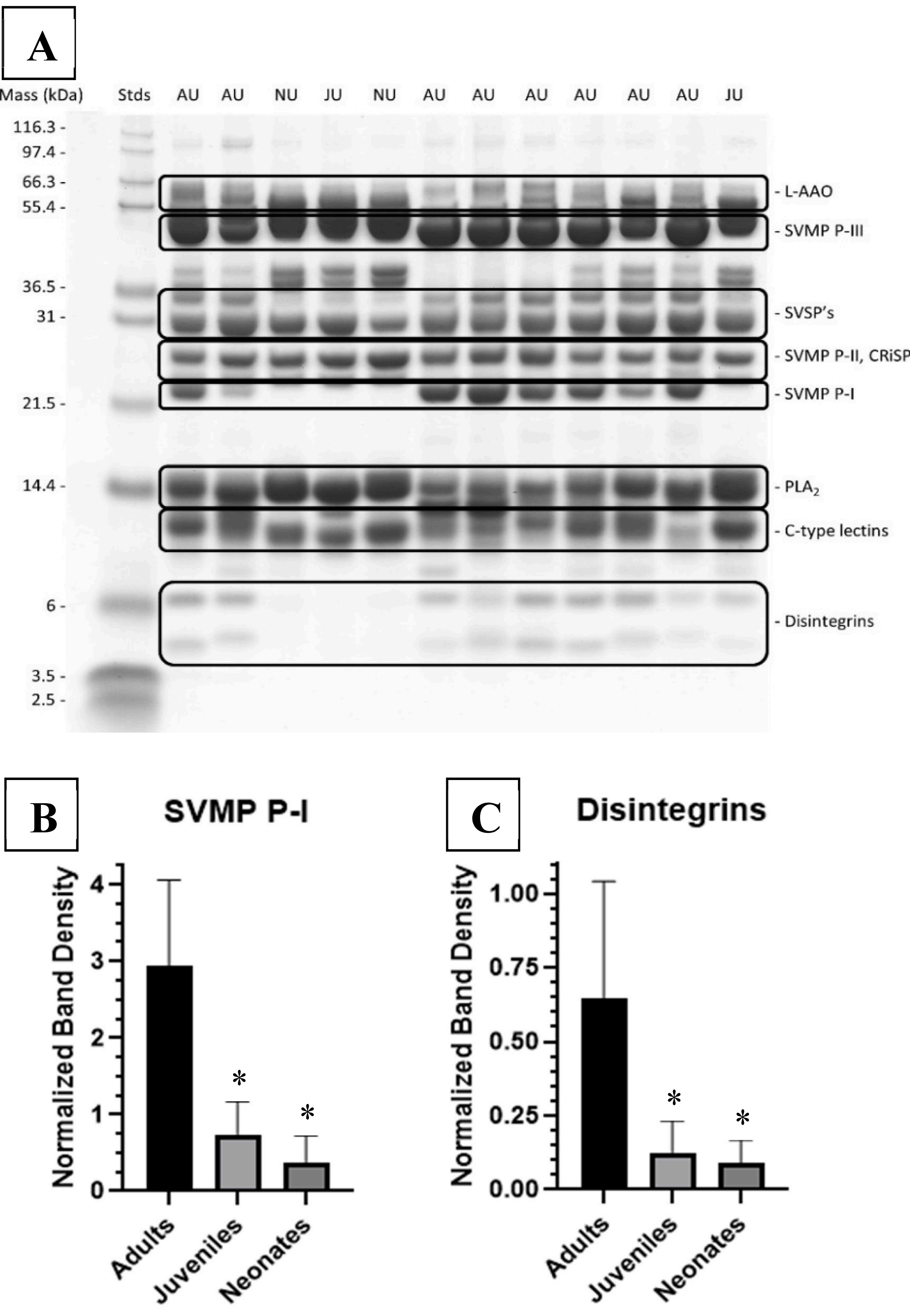

Fig. 2. Gel electrophoretic venom patterns of three age classes. (A) Comparison of venoms for a subset of individuals used in this study; for all samples, see Supplemental file 1. For individual lanes, the first letter corresponds to age class, where N = neonate, J = juvenile and A = adult. The second letter corresponds to the state in which the snake was found: U = Utah, A = Arizona, C = California and I = Idaho. Toxin families are outlined and labeled. (B, C) Band densitometry analyses for SVMP P-I and disintegrin bands from all samples comparing band densities between adults (n = 42), juveniles (n = 6), and neonates (n = 19). There are statistically significant differences between the adults and the neonates/juveniles as indicated by the asterisk (*), but no significance between juveniles and neonates for either toxin family.

weight components at approximately 4 and 6 kDa, putatively disintegrins. Myotoxin a, observed in some members of the *Crotalus oreganus* clade (such as *C. o. concolor*; Mackessy et al., 2003), is not known from this subspecies; initial probes with a myotoxin a-specific antibody were negative following Western blot analyses (unpub. obs.)

3.2. Ontogenetic shift in toxin activities

With the 67 snakes total, a simple linear regression showed a significant relationship between snout-vent length (SVL, the best estimate of size and age) and the enzymatic activity of SVMPs ($p = 0.0257$), PDEs ($p < 0.0001$), L-AAOs ($p < 0.0001$), thrombin-like SVSPs ($p = 0.0097$), and kallikrein-like SVSPs ($p < 0.0001$). Of the six toxin classes assessed, only the PLA_2s showed no significant relationship ($p = 0.1758$). However, without including the 13 neonates born from two females of the Salt Lake City, Utah region, there is a significant relationship observed between snout-vent length and PLA_2 activity with a p-value of <0.0001. SVMP, PDE, and thrombin-like SVSP activity increased with respect to length, while PLA_2 (with and without the 13 neonates), L-AAO, and kallikrein-like SVSP activity decreased with respect to length (Fig. 3).

3.3. Geographic venom variation

A PCA run using adult data for toxin activities demonstrates evidence of geographically-defined variation in venom phenotype, associated with differences in *C. o. lutosus* population and latitude (Fig. 4A). An outlier (Custer Co., Idaho) is evident, which is characterized by higher thrombin, PDE, and L-AAO as well as lower kallikrein activity compared to other snakes at similar latitudes (Fig. 4B). Most venom families display evidence of geographic structuring. PLA_2, PDE, and thrombin activity is generally higher in northern populations compared to southern sites, with moderate expression in central and southern locations. L-AAO activity displayed a graded activity profile across populations, slightly higher in western populations compared to other locations. Overall, we find evidence that venom composition demonstrates clear geographic clustering.

A one-way ANOVA showed several significant differences between L-AAO, thrombin-like SVSP, and PLA_2 activities when split by geographic population group (Fig. 5). The single individual from northern Arizona was included with the Utah population group due to proximity. There are significant differences in L-AAO activity between California and Idaho populations ($p = 0.0062$), as well as between California and Utah/Arizona populations ($p = 0.0073$). We see the inverse difference with thrombin-like SVSP and PLA_2 activities, where only the Idaho and Utah/Arizona populations show a significant difference between populations ($p = 0.000028$ and $p = 0.0079$, respectively). We confirmed the normality assumption of ANOVA was met with Q-Q plots and the Shapiro-Wilk normality test (Fig. S2) and the equal variance assumption with Bartlett's test (Fig. S3). The normality assumption was violated in the case of the L-AAO ANOVA, so we confirmed the significance of the population differences with Kruskal-Wallis test ($p = 0.0011$). As the ANOVA is robust to deviations from normality and the Kruskal-Wallis test was significant, we report the parametric result (Fig. 5).

4. Discussion

4.1. Ontogenetic shifts in venom phenotype

Our findings indicate that there are significant ontogenetic effects on venom phenotype in *C. o. lutosus*, and these ontogenetic shifts in venom composition correlate with dietary shifts and preferences, which have been observed in this species (Glaudas et al., 2008). In general, shifts

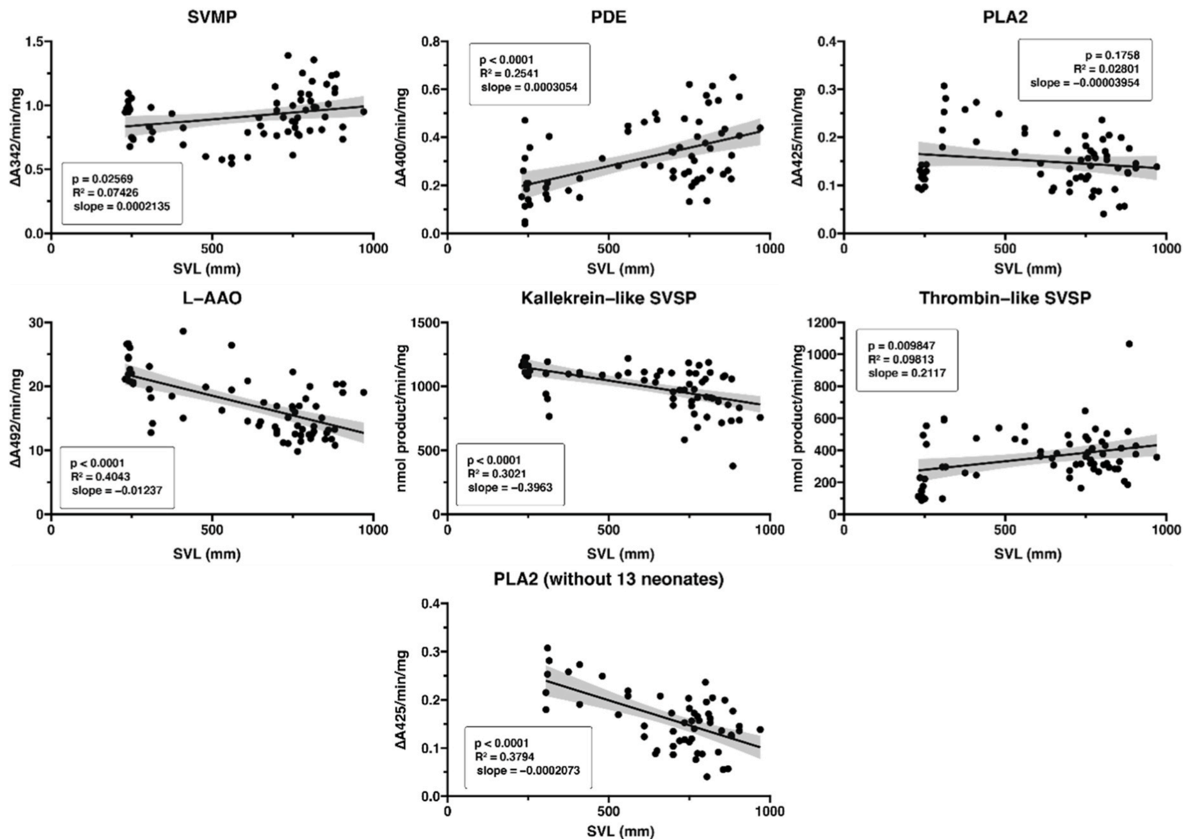

Fig. 3. Simple linear regressions of toxin activities as a function of snout-vent lengths. All slopes are significantly non-zero, with the exception of length versus PLA_2 activity. The last plot has the 13 neonate samples removed. SVMP = snake venom metalloproteinase, PDE = phosphodiesterase, PLA2 = phospholipase A_2, L-AAO = L-amino acid oxidase, and SVSP = snake venom serine proteinase.

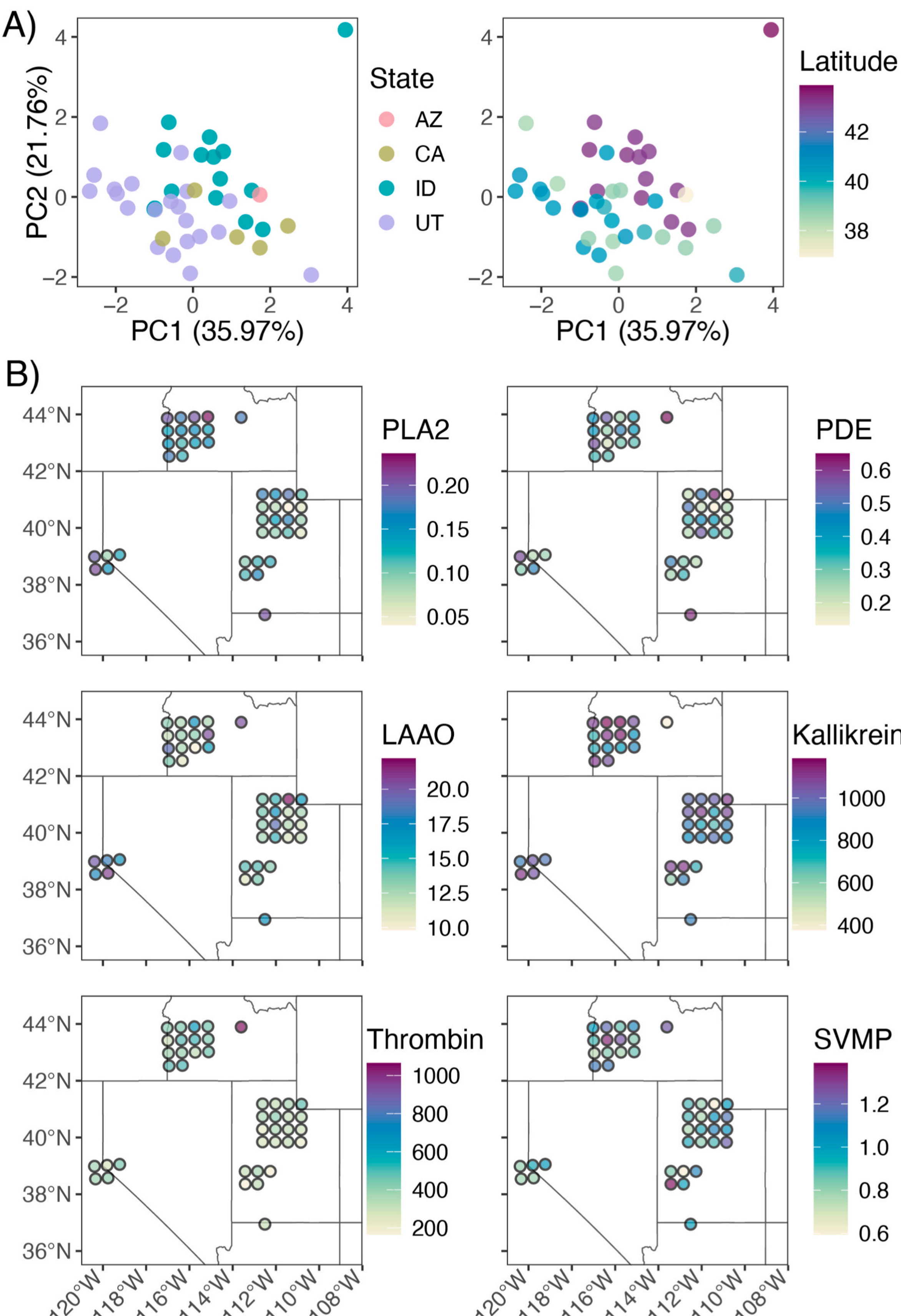

Fig. 4. Venom population structure. (A) PCA of toxin activities, with points colored by state (left) and latitude (right). Variance explained by PC1 and PC2 is shown in parentheses. (B) Geographic variation in venom toxin activities. Each point represents an individual, with color indicating measured toxin activity. Individuals are arranged in population-based grids for visualization. Only adult individuals were included in both sets of panels. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

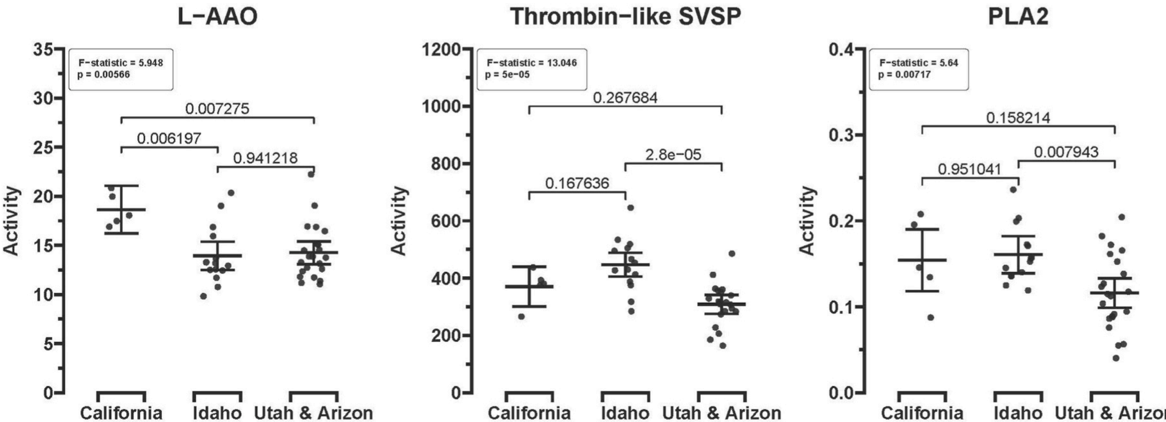

Fig. 5. Enzymatic activity variation by population. Different populations of *C. o. lutosus* can have significantly different enzymatic activities. Significance codes are shown above each pair and the mean for each group is shown with the center bar. The error-bar around the mean bar shows the confidence interval for the mean for each group. The ANOVA excluded non-adults and an outlier Idaho sample (n = 41).

from low SVMP activity and high toxicity in neonates, to high SVMP and low toxicity in adults, are often observed in rattlesnakes (Mackessy, 1988, 2008). These ontogenetic shifts in venom correspond with shifts in the consumption of larger mammalian prey, such as rodents, where the tissue degradation capabilities of SVMPs may be advantageous. Indeed, in several viperids (Borja et al., 2023; Durban et al., 2013, 2017; Mackessy et al., 2018), increasing SVMP prevalence and activity corresponds with increasing size and age of the snake. This could explain the utility of an additional SVMP class, P-I, in adults, which is absent or greatly reduced in neonates and juveniles (Fig. 2A and B).

In contrast, PLA_2 prevalence and activity appears to decrease as size and age of the snake increases. This decrease may be associated with a decrease in toxicity and is consistent with the general trend of higher toxicity in neonates compared to adults, as observed in other rattlesnakes (Mackessy, 1988). Neonates are limited in the amount of venom they can inject into prey, and so higher toxicity can help compensate and allow them to incapacitate their prey more quickly. Additionally, the smaller size of their prey suggests a lesser need for the degradative SVMPs, allowing for compensation by other toxins, notably the PLA_2s. However, 13 of the 19 total neonates, born from two females of one location, did not follow this trend. In fact, all of these 13 neonates had lower PLA_2 activity than their respective mother. This could be an artifact of individuals from that specific location but needs further investigation. Additionally, lethal toxicity was not assessed in this study so this, and further proteomic analyses, would provide better insight into the nature of the ontogenetic shift observed among the PLA_2s.

The low molecular weight components with sizes of approximately 4 and 6 kDa, found in the adults and faintly or not at all in neonates (Fig. 2A–C), are likely disintegrins based on their size. Disintegrins arise from either post-translational processing of SVMP P-II's or from translation of short messenger RNAs that do not have the sequence for the metalloproteinase domain, and thus both free metalloproteinases and disintegrins can be found in venoms (Almeida et al., 2023; Kini and Evans, 1992). Disintegrins interfere with the binding of adhesive proteins to cell surface molecules (integrins), thus inhibiting processes such as platelet aggregation, which may facilitate the spread of toxins throughout the prey (Scarborough et al., 1993). Beyond their role of disrupting cell-cell and cell-extracellular matrix interactions, these small non-enzymatic proteins have been found to aid in prey relocation after envenomation (Saviola et al., 2013). Since larger rattlesnakes are likely to be taking on larger prey items and are typically strike-and-release predators, thus having more of a need to track their prey, these molecules become increasingly important to larger snakes. In contrast, neonates and juveniles with more toxic venoms and smaller prey items may have less of a need for such molecules because neonate snakes often utilize a strike-and-hold mode of feeding.

In addition to shifts in SVMP and PLA_2 abundance and activities, the other four toxin families assessed displayed significant shifts as well. PDEs and thrombin-like SVSPs showed increasing activities with size, while L-AAO and kallikrein-like SVSP activities decreased with size (Fig. 3). PDEs may aid in prey immobilization by promoting hypotension as a result of the generation of purine nucleosides (Aird, 2002). Thrombin-like SVSPs are known to cleave and thus deplete fibrinogen via the production of non-functional microclots, perhaps acting in concert with hemorrhagic metalloproteinases by interfering with clot formation, and with kallikrein-like SVSPs to promote hypotension via the liberation of bradykinin from circulating kininogen (Komori and Nikai, 1998; Mackessy, 1993, 2010). Thrombin-like SVSP activity did not show a strong positive correlation and may have been skewed slightly by one outlier adult that had significantly higher activity than all other snakes. However, kallikrein-like SVSP activity showed a marked negative correlation with size. This venom component may be more important in the neonates and juveniles to facilitate rapid immobilization via hypotension. L-AAOs catalyze stereospecific oxidative deamination reactions of L-amino acids, and L-AAO activity showed a negative correlation with size. This reaction generates hydrogen peroxide, which is a highly toxic reactive oxygen species capable of altering cell membrane permeability and contributing to inhibition of platelet aggregation, as well as leading to necrosis or apoptosis (Izidoro et al., 2014; Machado-Neto and Traina, 2016; Mukherjee et al., 2015; Samel et al., 2006). While the utility of higher L-AAO activity in neonates and juveniles is currently unclear, all of these venom components likely act synergistically to incapacitate prey, so there may be further toxin interactions to explore.

Ontogenetic shifts in venom composition often correlate with diet-related shifts, and viperids commonly exhibit a major transition in prey type depending on the life stage of the snake, with neonates typically feeding on ectotherms and small endotherms, while adults feed primarily on larger endotherms (Glaudas et al., 2008; Saviola et al., 2012). This dietary shift has also been previously observed for the Great Basin Rattlesnake (Glaudas et al., 2008), but the exact mechanism causing the shift in venom toxin levels remains unknown. However, prior studies on the Central American rattlesnakes in the *C. simus* clade have indicated that microRNAs – small non-coding RNAs which can post-transcriptionally regulate gene expression – may be responsible for mechanistically modulating such ontogenetic shifts in venom composition (Durban et al., 2013, 2017). In particular, microRNAs (miRNAs) appear to mediate the transition from PLA_2 crotoxin rich neurotoxic type-II venom in neonates to SVMP rich hemotoxic type-I venom in adults via their dual activity of translational repression and degradation of crotoxin transcripts and the promotion of translation for SVMPs in adults (Durban et al., 2013, 2017). Additionally, recent studies (Hogan et al., 2024) showed differentially expressed transcription factors (TFs)

and differentially accessible cis-regulatory regions that correlate with ontogenetic shifts in venom composition in *C. adamanteus*. Together, these prior studies suggest that the observed ontogenetic shifts in *C. lutosus* may be driven by both pre- and post-transcriptional regulatory mechanisms, although this remains to be investigated specifically in this species.

However, it appears that trends of ontogenetic shifts in toxin activities are not necessarily consistent between rattlesnake species. For example, *C. o. lutosus* in this study showed opposite patterns compared to *C. polystictus* for L-AAO and kallikrein-like SVSP activities and for L-AAO activity in *C. o. helleri* and *C. o. oreganus* (Mackessy, 1988; Mackessy et al., 2018). This may be related to different prey specificities, as species taken in central Mexico (*C. polystictus*), coastal California (*C. o. helleri* and *C. o. oreganus*), and the Great Basin Desert will have different assemblages of prey species available to them. However, the same trend of higher toxicity in the neonates and juveniles, and shifting to a more proteolytic venom with age, occurs in all three species. Additional sampling of *C. o. lutosus*, particularly with regards to the juveniles and neonates, would be useful to further clarify range-wide ontogenetic shifts.

4.2. Geographic venom variation

General trends of variation with geography were observed, with *C. o. lutosus* populations primarily being defined by variation in L-AAO, thrombin-like SVSP, and PLA_2 activity. Further range-wide sampling, especially among the southern portion of their range, could help clarify these trends. There is currently not a clear explanation for the observed clustering patterns; however, it could simply be an artifact of genetic distance as well as genetic barriers to gene flow among this species, particularly between individuals of the Columbia Plateau and the Great Basin Desert. The diet of *C. o. lutosus* has been shown to differ among individuals located in these two geographic regions, with individuals from the Columbia Plateau feeding almost entirely on mammals, while those in the Great Basin Desert have a higher proportion of lizards in their diet (Glaudas et al., 2008). Among the mammals consumed, there were significant differences in prey species, with individuals of the Columbia Plateau feeding predominately on sciurid rodents, while those of the Great Basin Desert feed primarily on murid rodents. Investigation in this study showed significantly higher activities of thrombin-like SVSPs and PLA_2s between Idaho and Utah/Arizona populations, which may be associated with geographic regions and thus correlate with the variation in prey consumption observed for this species. Additional studies have found selection, both local and regional, and inter-population genetic distances, to best account for venom variation (Holding et al., 2018; Margres et al., 2019; Smith et al., 2023). A combination of these factors, in addition to past population structure, is likely contributing to the variation observed in this study, as the range of *C. o. lutosus* covers a large and diverse geographic region.

Another possible driver of venom variation is co-evolution between snake venom and prey venom resistance, which has been shown to be highly geographically localized (Holding et al., 2016; Gibbs et al., 2020). Because such interactions can alter the relative efficacy of specific venom components for a local population of prey, they may also contribute to geographic variation in venom composition. These co-evolutionary arms races can subject venom genes to balancing selection – a process in which differing selective regimes maintain multiple alleles at intermediate frequencies within populations, thereby increasing genetic variation relative to neutral expectations (Llaurens et al., 2017; Schield et al., 2022). Consistent with this expectation, strong signatures of balancing selection on venom genes have been identified in *Crotalus o. oreganus* and *Crotalus v. viridis*, particularly within SVMP and SVSP gene regions (Schield et al., 2022), suggesting that similar processes may contribute to elevated venom variation, especially within populations, in *C. o. lutosus*. In addition to balancing selection, divergent patterns of local directional selection can also drive venom differentiation among populations (Alape-Girón et al., 2008; Strickland et al., 2018). For example, geographically structured venom variation in *C. scutulatus* is thought to reflect variable directional local selection operating at fine geographic scales (Strickland et al., 2018). However, despite the apparent venom phenotypic metapopulations, defined by clustering patterns of toxin activities observed in this study, the mechanisms and direct molecular underpinnings driving these patterns remains to be explored.

In addition to biotic drivers such as co-evolution and local selection, environmental variables such as temperature and precipitation can also affect snake ecology and prey diversity, which in turn may drive variation in venom composition (Amazonas et al., 2019; Strickland et al., 2018; Zancolli et al., 2019; Smith et al., 2023). For example, ectotherm species richness significantly decreases with declining temperatures associated with higher latitudes (Darlington, 1948), which has been shown to correlate with broad shifts in venom composition including increases in mammal-specific toxins with increasing latitude (Smith et al., 2023).

This study demonstrates that venom composition in *C. o. lutosus* is highly dynamic, exhibiting substantial variation across individual, geographic, and ontogenetic dimensions. Such variation has important ecological consequences, as venom phenotypes directly influence prey acquisition, while also carrying clear clinical implications through their relevance for envenomation outcomes. Clinically, antivenom efficacy can depend largely on the toxins present, and understanding variation in composition within a species can allow for clinicians to adjust patient care accordingly. Our results show that ontogenetic stage and geographic population variation are both major contributors to venom diversity in this species, underscoring the need to consider multiple spatial and life-history scales when evaluating venom variation. More broadly, these findings indicate that multiple distinct ontogenetic trajectories can occur among rattlesnakes, and that complex patterns of geographic differentiation may exist across populations. Together, these results suggest that the determinants of venom composition are multifactorial, reflecting the interplay of ecological, evolutionary, and developmental processes. Integrating life history and geographic perspectives will therefore be essential for fully understanding the origins and consequences of venom diversity.

Ethical statement

The authors hereby state that all procedures involving animals were conducted in a humane and ethical manner. All protocols were evaluated and approved (prior to initiating research) by the University of Northern Colorado Institutional Animal Care and Use Committee (UNC-IACUC).

CRediT authorship contribution statement

Eric Januszkiewicz: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Kaas Ballard:** Data curation, Formal analysis, Software, Writing – review & editing. **Siddharth S. Gopalan:** Formal analysis, Methodology, Software, Writing – review & editing. **Yannick Z. Francioli:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Todd A. Castoe:** Data curation, Formal analysis, Funding acquisition, Investigation, Resources, Writing – review & editing. **Stephen P. Mackessy:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Stephen P. Mackessy reports financial support was provided by National Science Foundation. Todd A. Castoe reports a relationship with National Science Foundation that includes: funding grants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2026.109169.

Data availability

Data will be made available on request.

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