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# Lack of an ontogenetic shift in *Crotalus enyo cerralvensis* venom supports integration of cranial morphology and venom expression in rattlesnakes

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Abstract.—Island environments impose distinct ecological pressures that shape the evolution of complex traits. In snakes, body and head size are often related to diet, as gape limitation constrains the size of prey an individual can consume. Many venomous snakes exhibit ontogenetic venom expression variation that parallels shifts in prey selection over development, reflecting the integration of venom and morphology into a complex feeding phenotype. On Isla Cerralvo, the Baja California Rattlesnake (*Crotalus enyo cerralvensis*) has previously been shown to exhibit a significantly smaller head compared to its mainland counterpart (*Crotalus enyo enyo*), potentially constraining prey size. We hypothesized that reduced head size would correspond with a lack of ontogenetic shift in venom expression in *C. e. cerralvensis*, as smaller heads may limit ontogenetic prey shifts. We sampled venom from island and mainland populations of *C. enyo* and assessed venom expression patterns across different size classes in each population. Although island and mainland rattlesnakes significantly differed in venom expression, only mainland snakes exhibited size-dependent venom expression variation; island snakes expressed a single venom phenotype independent of size. The absence of ontogenetic venom variation in island snakes, coupled with their smaller head sizes, suggests the integration of venom expression and cranial morphology as part of an integrated feeding phenotype and highlights the parallel evolution of functionally linked traits in response to unique ecological conditions.

Resumen.—Los entornos insulares imponen presiones ecológicas particulares que moldean la evolución de rasgos complejos. En las serpientes, el tamaño del cuerpo y de la cabeza suele relacionarse con su dieta, debido a que la limitación de la apertura de la boca restringe el tamaño de las presas que un individuo puede consumir. Muchas serpientes venenosas muestran variaciones ontogenéticas en la expresión del veneno que acompañan los cambios en la selección de presas durante el desarrollo, lo que refleja la integración del veneno y la morfología en un fenotipo de alimentación complejo. En Isla Cerralvo, se ha demostrado previamente que la serpiente de cascabel de Baja California (*Crotalus enyo cerralvensis*) posee un tamaño de cabeza significativamente menor en comparación con su contraparte continental (*Crotalus enyo enyo*), característica que podría limitar el tamaño de las presas que consume. Planteamos la hipótesis de que la reducción en el tamaño de la cabeza se asociaría con la ausencia de un cambio ontogenético en la expresión del veneno en *C. e. cerralvensis*, ya que una cabeza más pequeña podría restringir las variaciones ontogenéticas en la dieta. Recolectamos muestras de veneno de poblaciones insulares y continentales de *C. enyo* y analizamos los patrones de expresión del veneno en diferentes clases de tamaño en cada población. Aunque las serpientes de cascabel insulares y continentales mostraron diferencias significativas en la expresión del veneno, solo las continentales presentaron variaciones dependientes del tamaño; las serpientes insulares expresaron un único fenotipo de veneno sin relación con el tamaño. La ausencia de variación ontogenética en el veneno de las serpientes insulares, junto con el menor tamaño de sus cabezas, sugiere una integración entre la expresión del veneno y la morfología craneal como parte de un fenotipo de alimentación integrado, y resalta la evolución paralela de rasgos funcionalmente vinculados en respuesta a condiciones ecológicas únicas.

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| SRH | orcid.org/0000-0002-3172-5210 | CMV | orcid.org/0009-0009-9244-8518 | HFC | orcid.org/0009-0000-5889-763X |
| VVC | orcid.org/0000-0001-5446-1855 | AKH | orcid.org/0009-0006-8850-0406 | RARG | orcid.org/0000-0002-0110-435X |
| GCG | orcid.org/0000-0002-1896-0937 | MB | orcid.org/0000-0003-4098-7279 | CLP | orcid.org/0000-0002-2020-6992 |
| JLS | orcid.org/0000-0002-1927-7259 | MJM | orcid.org/0000-0002-6153-6701 | | |

Oceanic islands have long captivated biologists as natural laboratories for studying evolution (Darwin 1859, MacArthur and Wilson 1963, Foster 1964, Van Valen 1973, Case 1978). The geographic isolation of islands fosters unique ecological conditions, where limited resources, altered species interactions, and simplified communities create selective pressures that can produce distinct evolutionary patterns (Whittaker and Fernández-Palacios 2007, Whittaker et al. 2017). These conditions frequently lead to pronounced shifts in morphology, including changes in body size as described by the island rule (Van Valen 1973), though responses vary widely across taxa (Baur 1889, Van Denburgh 1914, Stock and Furlong 1928, Case 1978, Sondaar 1991, Barahona et al. 2000, Lomolino 2005, Raia and Meiri 2006, Jaffe et al. 2011, van der Geer et al. 2016, Benítez-López et al. 2021). Such trait evolution is typically shaped by the interplay of life history trade-offs and ecological pressures, including predation, competition, and resource availability (Dayan and Simberloff 1998, Millien 2006). Among these traits, those related to feeding ecology may be particularly responsive to island conditions, where shifts in prey availability or competition can strongly influence morphological evolution.

In snakes, body and head size are critical traits that influence feeding ecology and survival. As gape-limited predators, head morphology constrains the maximum prey size a snake can consume (Shine 1991, Greene and Wiseman 2023), and many species exhibit ontogenetic dietary shifts, with juveniles feeding on smaller prey and adults transitioning to larger prey as they grow (Forsman and Lindell 1993, Seigel and Collins 1993, Hampton 2018). Head size typically increases with body size during growth, reflecting an allometric relationship (King 2002, Patterson et al. 2022); however, this relationship is not always fixed. Dietary differences can drive population-level variation in head shape and size even among individuals of similar body size, likely through developmental plasticity and selection (Forsman 1996, Ryerson 2020). Such variation may be especially pronounced on islands, where altered prey diversity and/or competition shape feeding morphology in distinct ways. For example, *Boa constrictor* populations on offshore islands in Belize show tail elongation and reduced body size, likely in response to arboreal foraging on small birds (Boback 2005, 2006). Conversely, *Crotalus angelensis* endemic to Isla Ángel de la Guarda exhibits body size gigantism, potentially driven by the abundance of large prey such as chuckwallas (*Sauromalus hispidus*; Meik et al. 2010).

In venomous snakes, morphology and venom frequently evolve in parallel, forming an integrated feeding phenotype that enhances foraging efficiency (Margres et al. 2015b, 2021, Strickland et al. 2018, Schendel et al. 2019, Xie et al. 2022, Lakušić et al. 2025). Snake venom is a complex trait composed of 40–100 proteinaceous toxins that play essential roles in prey immobilization, digestion, and defense (Daltry et al. 1996, Barlow et al. 2009, Casewell et al. 2011, Mackessy 2021). Venom varies significantly across species (Casewell et al. 2014, Margres et al. 2015b, Jackson and Fry 2016, Jackson et al. 2016, Durban et al. 2017, Pla et al. 2019, Senji Laxme et al. 2019, Holding et al. 2021), populations (Massey et al. 2012, Margres et al. 2015b, 2019, Holding et al. 2018, Strickland et al. 2018, Smith et al. 2023, Hirst et al. 2025, Borja et al. 2025), and life stages (Andrade and Abe 1999, Alape-Girón et al. 2008, Barlow et al. 2009, Margres et al. 2015a, 2015b, Wray et al. 2015, Modahl et al. 2016, Cipriani et al. 2017, Durban et al. 2017, Rokyta et al. 2017, Borja et al. 2018, Schonour et al. 2020, Hirst et al. 2024, Borja et al. 2025), often reflecting differences in diet and potential coevolutionary interactions with prey (Holding et al. 2016, Margres et al. 2017). In vipers, ontogenetic shifts in venom expression correspond with changes in head and body size, likely reflecting dietary transitions across life stages. Juvenile venoms typically differ from adult venoms in toxin expression and function, with juveniles expressing lower levels of metabolically costly enzymes (e.g., snake venom metalloproteinases; Mackessy 2010, 2021, Margres et al. 2015b, Schonour et al. 2020). In contrast, adult venoms are often enriched with metabolically costly enzymes, which may facilitate the digestion of larger prey items, consistent with their expanded dietary breadth (Mackessy 2010, Margres et al. 2015b). Ontogenetic venom variation, often the primary axis of variation in many species (Durban et al. 2017, Hirst et al. 2024, Borja et al. 2025), underscores the strong evolutionary link between venom expression and morphology, both of which respond to shifting ecological pressures across life stages (Margres et al. 2015b, Moon et al. 2019, Schonour et

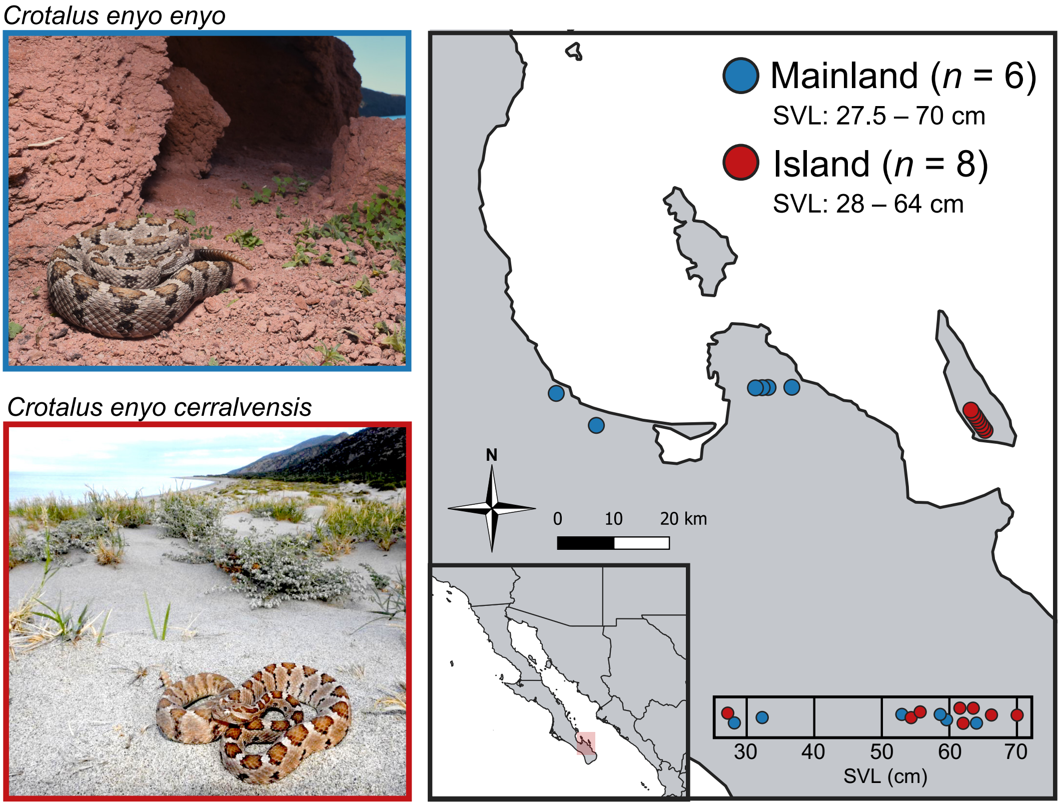

Fig. 1. Sampling locations of the 14 individual rattlesnakes used in this study. Red points (jittered for visual clarity) represent 8 individuals from Isla Cerralvo, while blue points indicate 6 individuals from the nearby mainland. Size distributions of each population (snout–vent length [SVL] in cm) are indicated next to the map inset. On the left, the top photo shows *Crotalus enyo enyo* from the mainland, and the bottom photo shows *Crotalus enyo cerralvensis* from Isla Cerralvo. Snake photo credits: Ricardo Ramírez Chaparro (top) and Jason L. Strickland (bottom).

al. 2020). In the Eastern Diamondback Rattlesnake, (*Crotalus adamanteus*), for example, ontogeny explains more variation in toxin expression than geographic effects, emphasizing the dominant role of life history in shaping venom expression (Margres et al. 2015a, 2015b).

The Baja California Rattlesnake (*Crotalus enyo*) provides a model system for investigating how insular environments shape the parallel evolution of venom and morphology. Endemic to the Baja California Peninsula and several adjacent islands in the Gulf of California, *C. enyo* inhabits arid and semiarid environments, including deserts, rocky hillsides, and scrublands, where it is commonly associated with cacti and thorny vegetation (Klauber 1997, Grismer 2002). Its diet primarily consists of small mammals and lizards, and adults can reach a snout–vent length (SVL) of ~82 cm (Klauber 1997, Taylor 2001, Grismer 2002). Notably, the subspecies *Crotalus enyo cerralvensis*, found exclusively on Isla Cerralvo, exhibits a smaller head relative to body length than the mainland *Crotalus enyo enyo* (Cliff and Cliff 1954). Because venom and head morphology often evolve together as components of an integrated feeding phenotype (Margres et al. 2015b, Moon et al. 2019, Holding et al. 2022), reduced head size of *C. e. cerralvensis* may reflect parallel changes in venom expression, with smaller heads potentially constraining dietary shifts and reducing the selective pressure for ontogenetic variation in venom (Mackessy 2010, Margres et al. 2015b).

In this study, we investigated (1) whether *C. e. cerralvensis* exhibited a distinct venom phenotype compared to mainland *C. e. enyo*, and

(2) whether ontogeny and morphology evolved concordantly in the island population. Given the proportionally smaller head size of *C. e. cerralvensis*, we hypothesized that island snakes would lack ontogenetic shifts in venom expression, resulting in a pseudopaedomorphic venom phenotype.

## Methods

### Sampling

We sampled *C. e. cerralvensis* (*n* = 8) and *C. e. enyo* from the adjacent Baja California Peninsula (*n* = 6; Fig. 1). Snakes were captured via visual encounter surveys or on roadways. Upon capture of each snake, sampling locality, snout–vent length (SVL), and sex were recorded. Venom was collected by allowing the snake to bite through a parafilm-covered sample collection cup. Venom was immediately dried upon collection. All island snakes were released at their site of capture following sample collection. For mainland snakes, 2 were released at their site of capture and 4 were euthanized, dissected, vouchered, and deposited at La Colección Herpetologica de la Facultad de Ciencias Biologicas de la Universidad Juárez del Estado de Durango in Gómez Palacio, Durango, Mexico. Snakes were collected under the following permits: Secretaría de Medio Ambiente y Recursos Naturales Oficio N SGPA/DGVS/06166/20, SGPA /DGVS/01178/20, SGPA/DGVS/04856/21, and SGPA/DGVS/04999/22. The procedures outlined were approved by the University of South Florida Institutional Animal Care and Use Committee (IACUC) under protocol IS00011949 and Clemson University IACUC protocol 2017-067.

### Venom Proteomics

Venom samples were analyzed using reversed-phase high-performance liquid chromatography (RP-HPLC) on either a Dionex ultimate 3000 UHPLC DAD (Thermo Fisher Scientific) or a Shimadzu Prominence HPLC (Shimadzu) with a Jupiter® 5 µm C18 300 Å, LC Column 250 × 2 mm, Ea column. Dried venom was resuspended in HPLC-grade water, and 50 g of total venom protein was injected onto the column using a solvent system of A = 0.1% trifluoroacetic acid (TFA) in water and B = 0.075% TFA in acetonitrile. After 5 min at 5% B, a 1% per minute linear gradient of A and B was run to 25% B, followed by a 0.25% per minute gradient from 25% to 65% B at a flow rate of 0.6 mL per min (Margres et al. 2014). Column effluent was monitored at 220 nm. RP-HPLC peaks were quantified in the Chromeleon™ software (Thermo Fisher Scientific, Waltham, MA). We estimated the relative abundance of each protein peak by measuring the area under the peak relative to the total area of all peaks identified (Supplementary Material 1; Gibbs and Rossiter 2008) as previously described (Margres et al. 2014, 2017).

### Analyzing Venom Expression Variation Across Populations and Size

To determine whether venom expression was significantly different between the island (*n* = 8) and mainland (*n* = 6) populations and/or across SVL, we performed a permutational multivariate analysis of variance (PERMANOVA). Raw peak quantification data were first transformed in R using the isometric log-ratio (ILR) transformation from the ‘*robCompositions*’ package (Templ et al. 2023). We then performed PERMANOVA via the *adonis2* function (Oksanen et al. 2020) from the R package ‘*vegan*’ (Oksanen et al. 2020) on the ILR-transformed venom data as previously described (Margres et al. 2014, 2019, Wray et al. 2015).

To assess whether venom expression was more conserved in the island population, we compared the variance in venom expression between island and mainland populations. First, we calculated multivariate dispersions using the *betadisper* function in the R package ‘*vegan*’ (Oksanen et al. 2020). Dispersion values, representing the distance of each sample to its group centroid, were calculated based on a Euclidean distance matrix from the venom protein data. Differences in dispersion between populations were tested using ANOVA and a permutation test (*n* = 999 permutations) to assess the statistical significance of variance differences.

To further identify patterns of expression differentiation across our samples, we conducted principal coordinate analysis (PCoA) on the ILR-transformed venom protein data (*n* = 14) in R using the *prcomp* function from the stats package. We then plotted PCo1 with PCo2 to visualize overall variation in venom expression and identify patterns within and between populations and size classes. Finally, we performed simple regressions (*lm* function in R) comparing PCo1 and PCo2 with SVL for the island and

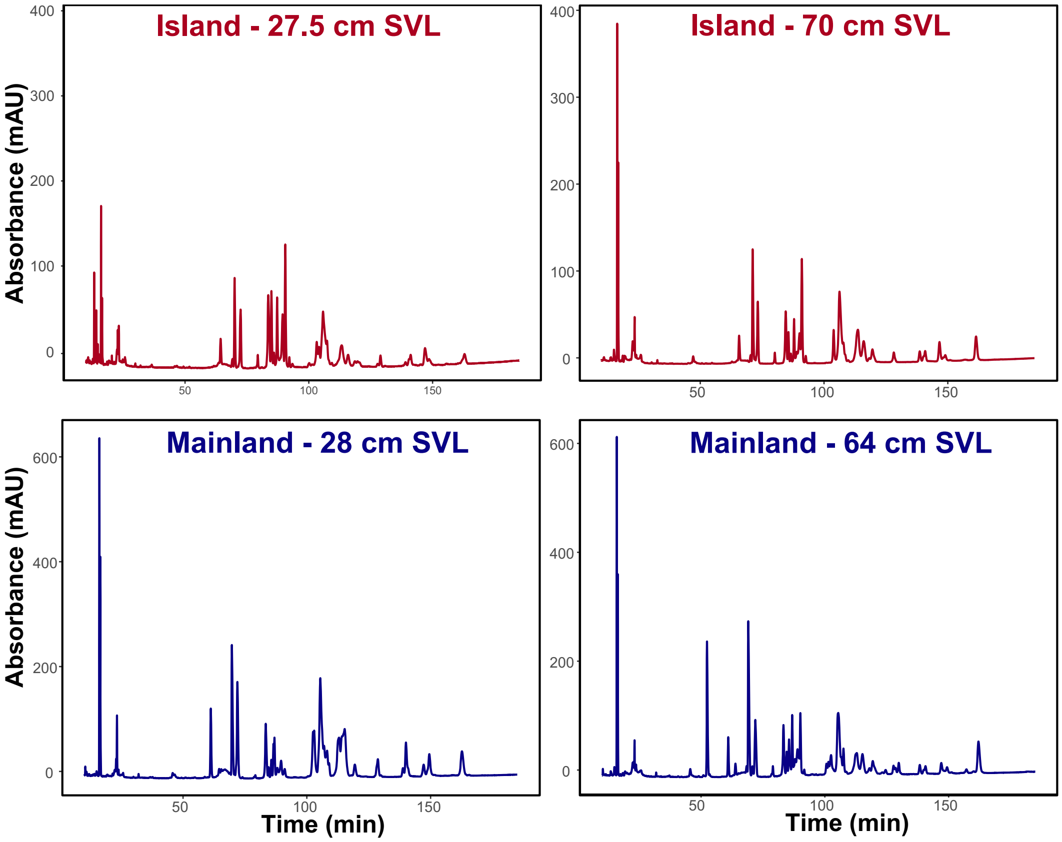

Fig. 2. Reversed-phase high-performance liquid chromatography (RP-HPLC) chromatograms for representative juvenile and adult snakes from each population. Snout–vent length (SVL) is listed for each individual. Each chromatogram illustrates venom components separated based on hydrophobicity, with retention time (minutes) on the *x*-axis and absorbance at 220 nm on the *y*-axis. Peaks correspond to venom components, with their relative intensities reflecting abundance.

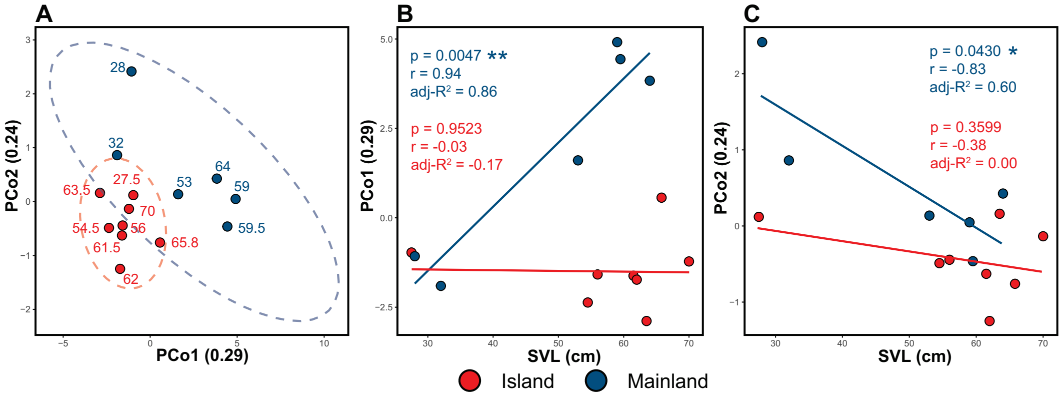

Fig. 3. Principal coordinate analysis (PCoA) and regression analyses of venom protein expression data. ***A,*** PCoA plot (PCo1 and PCo2) showing individuals from the island (red) and mainland (blue) populations, with clustering based on venom protein expression. Dashed ellipses represent 95% confidence regions based on the distribution of individuals in each population. Variation explained by PCo1 and PCo2 is 0.29 and 0.24, respectively; ***B,*** regression of PCo1 and SVL; and ***C,*** regression of PCo2 and SVL for island and mainland populations. Asterisks indicate statistical significance (**$P < 0.01$; *$P < 0.05$).

mainland populations individually to assess whether size predicted the largest 2 axes of variation in venom expression.

## Results

### Venom Expression Varies Significantly by Population and Snake Size

Venom protein expression differed significantly between island and mainland populations ($R^2 = 0.380$, $P < 0.001$) and across snake size ($R^2 = 0.187$, $P = 0.007$). The interaction between population and size was also significant ($R^2 = 0.190$, $P = 0.004$), indicating that the degree of venom differentiation between island and mainland populations varied with body size. Variance in venom expression was significantly reduced in island snakes compared to mainland individuals (ANOVA: $P = 0.0097$; permutation test: $P = 0.004$), indicating less intrapopulation venom variation in the insular population (Fig. 2, Supplemental Material 2).

### Venom Expression Varies Ontogenetically in Mainland but Not Island Snakes

To further investigate the significant interaction between population and snake size, we performed a PCoA on the venom protein expression data (Fig. 3). Along PCo1 (29%), individuals clustered primarily by population, with size-dependent separation occurring only in the mainland population. Along PCo2 (24%), juvenile mainland snakes clustered separately from both adult mainland and all island individuals.

To assess the relationship between venom expression and snake size, we conducted simple regressions of PCo1 and PCo2 against SVL for each population independently. For the mainland population, the relationship between snake size and both PCo1 and PCo2 was significant (adj-$R^2 = 0.86$, $P = 0.0047$; adj-$R^2 = 0.60$, $P = 0.0430$, respectively), while for the island population, no significant relationships were observed (adj-$R^2 = -0.17$, $P = 0.9523$; adj-$R^2 = 0.00$, $P = 0.3599$, respectively; Fig. 3B, C).

## Discussion

We tested whether island *C. e. cerralvensis* exhibited a venom phenotype distinct from mainland *C. e. enyo* and whether venom expression and morphology evolved concordantly in the insular population. We found significant differences in venom expression between island *C. e. cerralvensis* and mainland *C. e. enyo* populations, with island snakes exhibiting reduced variance and a conserved venom phenotype across life stages. In contrast, mainland populations displayed size-dependent shifts in venom expression, which are consistent with ontogenetic changes commonly observed in venomous snakes (Andrade and Abe 1999, Alape-Girón et al. 2008, Barlow et al. 2009, Margres et al. 2015a, 2015b, Wray et al. 2015, Modahl et al. 2016, Cipriani et al. 2017, Durban et al. 2017, Rokyta et al. 2017, Borja et al. 2018, Schonour et al. 2020, Hirst et al. 2024). These findings supported our hypothesis that the smaller head size of *C. e. cerralvensis* would correspond with juvenile-like venom expression throughout life history.

Because head size constrains prey size in snakes due to gape limitation, ontogenetic venom expression variation often coincides with increasing head and body size, allowing adults to consume larger prey (Mackessy 2010, Margres et al. 2015b, Moon et al. 2019, Holding et al. 2022, Lakušić et al. 2025). In *C. e. cerralvensis*, however, a proportionally smaller head may restrict this dietary shift, reducing or eliminating the selective pressures that typically favor ontogenetic venom expression changes. Indeed, we found that island snakes exhibited a stable, pseudopaedomorphic venom profile across life stages, a pattern that may reflect their constrained feeding ecology. The term *pseudopaedomorphic* is used here because although ontogenetic change was absent, venom expression in island adult snakes did not fully mirror the juvenile phenotype observed in mainland individuals, as indicated by separation along PCo2 between mainland juvenile snakes and all island snakes (Fig. 3A).

Reduced prey diversity on Isla Cerralvo may constrain dietary breadth as individuals mature, weakening selection for ontogenetic venom shifts. Additionally, putative competition with the Speckled Rattlesnake (*Crotalus mitchellii*), which also inhabits the island, may further restrict prey availability. Interspecific interactions among congeneric rattlesnakes are a major driver of venom variation in island systems (Hirst et al. 2025), suggesting that competition may contribute to the conserved phenotype observed here. Although venom and morphological divergence between island and mainland *C. mitchellii* individuals remain uncharacterized, future comparative analyses could clarify whether similar insular conditions drive analogous patterns in this species. Alternatively, founder effects, reduced gene flow, and genetic drift may have resulted in limited standing genetic variation in venom genes and their regulatory elements, contributing to the observed loss of ontogenetic expression during divergence from the ancestral mainland population (Barton et al. 1997, Jordan and Snell 2008, Stuessy et al. 2014, Schlaepfer et al. 2018). Regardless of mechanism, our findings were consistent with previous work suggesting parallel evolution of morphology and venom expression (Margres et al. 2015b, Strickland et al. 2018, Margres et al. 2021, Holding et al. 2022). In *C. e. cerralvensis*, a reduction in head size is accompanied by the loss of ontogenetic venom variation, supporting the role of functional integration in shaping complex feeding traits in venomous snakes.

A key assumption of our interpretation was that island snakes maintain proportionally smaller heads throughout development. Although this pattern has been previously suggested (Cliff and Cliff 1954), those observations were based on a limited number of specimens, and we were unable to obtain precise cranial measurements from our sampled individuals. As such, statistical comparisons among SVL, cranial morphology, and venom expression remain a critical direction for future confirmatory work. Nevertheless, these limitations did not affect our conclusion of conserved venom expression in the island population but rather limit interpretation of its functional relationship with cranial morphology.

Future work should also investigate how prey availability and competition shape the evolution of integrated phenotypes in island systems. Comparative studies incorporating functional assays of venom efficacy in sympatric versus allopatric prey (Margres et al. 2017), genomic analyses of gene regulation (Hogan et al. 2024), broader sampling across insular and mainland populations, and comparisons across other species will help elucidate the roles of selection, competition, and genetic constraint. Additional work is also needed to clarify the molecular basis of the conserved venom phenotype. Given the pseudopaedomorphic venom profile in island snakes, transcriptomic or proteomic analyses could determine whether specific toxin genes or protein families exhibit consistent expression across life stages, including whether adult island snakes retain juvenile-like expression profiles at the level of particular loci or venom components (Mackessy 2010, Rokyta et al. 2012, Margres et al. 2014, Brahma et al. 2015, Margres et al. 2015b, Schonour et al. 2020, Mackessy 2021, Calvete et al. 2024, Hirst et al. 2024). Lastly, our dataset lacked individuals between 32 and 53 cm SVL and included only 3 snakes ≤32 cm SVL ($n = 2$ mainland; $n = 1$ island), limiting our ability to resolve early developmental transitions (Wray et al. 2015). Increasing representation across body size classes will help determine whether venom ontogeny proceeds along a continuous or discrete trajectory and evaluate how venom and morphology covary across life history.

Although the ecological drivers of morphological evolution on islands are well documented (Pafilis et al. 2009, Diniz-Filho et al. 2021), their effects on molecular traits remain less understood. Our findings supported that venom expression variation and cranial morphology are functionally integrated as parts of a complex feeding phenotype. In *C. e. cerralvensis*, smaller head size was paralleled by stable venom expression across life stages, reinforcing the role of phenotypic integration in shaping adaptive traits. Understanding how ecological pressures influence the evolution of integrated traits will be critical for further uncovering the mechanisms driving complex trait evolution.

## SUPPLEMENTARY MATERIAL

Three online-only supplementary files accompany this article (https://scholarsarchive.byu.edu/wnan/vol86/iss1/8).

SUPPLEMENTARY MATERIAL 1. Reversed-phase high-performance liquid chromatography (RP-HPLC) chromatograms for all sampled adult and juvenile snakes from each population; snout-vent length (SVL) is listed for each individual. Each chromatogram illustrates venom components separated based on hydrophobicity, with retention time (minutes) on the *x*-axis and absorbance at 220 nm on the *y*-axis. Peaks correspond to venom components, with their relative intensities reflecting abundance.

SUPPLEMENTARY MATERIAL 2. Reversed-phase high-performance liquid chromatography (RP-HPLC) chromatograms for all sampled adult and juvenile snakes from each population.

SUPPLEMENTARY MATERIAL 3. R code (R v4.5.0) used for all statistical analyses presented in the study.

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*Author contributions*. SRH, JLS, and MJM conceived and designed the study. All authors contributed to data collection or generation. SRH analyzed the data. SRH and MJM led writing with input from all coauthors.

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