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The effects of hybridization on divergent venom phenotypes: Characterization of venom from *Crotalus scutulatus scutulatus* × *Crotalus oreganus helleri* hybrids

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previously characterized, but the effects of hybridization on venom ontogeny have not been investigated. Because type I and type II venom characteristics are typically mutually exclusive, the venom phenotypes of hybrids between species that express these divergent venom phenotypes could reveal the mechanisms behind these potential incompatibilities. The current study outlines the venom phenotypes and ontogenetic shifts in venom composition resulting from the hybridization of a type I snake, C. o. helleri, and a type II snake, C. s. scutulatus. SDS-PAGE, venom enzymology, immunoblotting, MALDI-TOF MS and reversed phase HPLC were used to characterize the venom of a C. o. helleri male parent, a C. s. scutulatus female parent (both from southern California) and two of their offspring over a period of eight years (2007–2015). In addition, venoms from six adult C. o. helleri and six adult C. s. scutulatus individuals from southern California were used as parental reference samples to confirm that the venom activities and characteristics of the mother and father of the hybrids were distinctive and characteristic for each species. Based on the few published reports on hybridization and venom composition, we hypothesized that C. o. helleri × C. s. scutulatus hybrids would display both the type I venom characteristic of high metalloprotease activity and the type II venom characteristic of expression of neurotoxic PLA_2s.

2. Materials and methods

2.1. Supplies and reagents

Protein concentration reagents were purchased from BioRad, Inc. (Hercules, CA, USA). NuPage gels and Western blot materials were obtained from Life Technologies, Inc. (Grand Island, NY, USA). 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). All other reagents (analytical grade or higher) were purchased from Sigma Biochemical Corp. (St. Louis, MO, USA).

2.2. Venom collection and storage

Crotalus s. scutulatus × C. o. helleri hybrids and parents were obtained from Dan Grubb in 2007 when the two hybrid offspring (one male, one female) were approximately one year old. The male adult C. o. helleri and the female adult C. s. scutulatus originated from Los Angeles Co., California. All snakes were housed individu-

was vortexed and placed back at 37 C. Reactions were stopped after three minutes with 50% acetic acid. Tubes were read at 405 nm, and speci c activity was calculated from a standard curve of p-nitroaniline and expressed as nanomoles product produced/ min/mg venom.

2.5. Puri cation of Mojave toxin and concolor toxin

Puri cation of the type II neurotoxins Mojave toxin and concolor toxin was achieved as outlined in Aird et al. (1986)

~14 kDa), with a band at approximately 14 kDa, and to non-neurotoxic PLA_2s, at approximately 14 e 15 kDa (Fig. 4). Crotalus s. scutulatus venom had a B subunit band at approximately 14 kDa and a more prominent A subunit band at approximately 9 kD. Crotalus o. helleri venom had a prominent PLA_2 band at approxi-

C. s. scutulatus, C. o. helleri and hybrid venoms. However, SDS-PAGE demonstrated that similar protein components eluted at comparable times regardless of species. The low abundance of smaller peaks from RP-HPLC product did not adversely affect protein detection with SDS-PAGE, and amounts as low as 2 μg were visible. The RP-HPLC chromatograms of three individual C. s. scutulatus reference venoms revealed that 1) the dominant peak of C. s. scutulatus venom (Mojave toxin subunit B) eluted at approximately 41 min, 2) no myotoxins were present in these C. s. scutulatus venoms, and 3) metalloprotease peaks were minimal in all C. s. scutulatus chromatograms (Fig. 5A, Fig. 7A, and Supplemental Figs. 1 and 2).

Reversed phase HPLC fractionation of C. o. helleri venom revealed that myotoxins eluting at approximately 23 min were the dominant component (Supplemental Fig. 14). In addition, all three C. o. helleri individuals lacked a 41 min peak (Mojave toxin) and had sizeable clusters of metalloprotease peaks eluting from approximately 84 to 90 min (Figs. 5B and 7B, and Supplemental Figs. 3, 4, and 5).

The C. o. helleri male parent's metalloprotease peaks accounted for approximately 5% of the total venom composition, while the C. scutulatus female parent's metalloprotease peak made up less than 1% of the venom (Fig. 5A and B). Both hybrid venoms also had metalloprotease peaks in the two years that were analyzed; however, the female hybrid's peaks were a smaller percentage of the total venom as a juvenile (in 2007; Table 1; Fig. 6A) and an adult (in 2015; Fig. 5C) than the hybrid male (Fig. 6B and D, respectively).

The female hybrid's percentage of metalloproteases dropped slightly from 1.5% to 1.3% between 2008 and 2015 (Table 1; Fig. 6A and C). This is consistent with the ontogenetic decrease seen in the female hybrid's azocasein metalloprotease activity. Conversely, the male hybrid's percentage of metalloproteases increased from 5.1% to 7.9%, which also mirrors the increase seen in the azocasein metalloprotease assay (Table 6

venom of two other C. s. scutulatus venoms (Supplemental Figs. 1 and 2). Both hybrid chromatograms showed a 41 min Mojave toxin subunit B peak; however, this was not the dominant component as observed in the C. s. scutulatus individuals (Figs. 6 and 7C and D). Mass spectrometry revealed that both hybrid Mojave toxin peak components had masses consistent with the subunit B of Mojave toxin present in the C. s. scutulatus female parent (14,186 Da; Fig. 7A, C, and D). The percentage of Mojave toxin increased slightly for both the female and male hybrids as they aged, from 6.8% to 9.8% and 8.3% to 10.3%, respectively (Table 1). This peak was absent from the male parent's venom; the only PLA_2 observed (RP-HPLC peak 62 min) had a mass of 13, 665 Da (Fig. 7B).

3.5. Principal coordinate analysis (PCoA)

Principal coordinate analysis of reverse phase HPLC chromatograms of three C. s. scutulatus individuals, three C. o. helleri individuals and both hybrid venoms revealed three distinct clusters (Fig. 8). All C. s. scutulatus and C. o. helleri individuals clustered tightly together by species, and hybrids from all years analyzed clustered together, in between C. o. helleri and C. s. scutulatus with regards to Coordinate 1.

3.6. Lethal toxicity

The male parent C. o. helleri venom had a substantially higher LD_{50} value than C. s. scutulatus and both hybrid venoms (Fig. 9). The female parent Crotalus s. scutulatus and the female and male hybrid venoms had LD_{50} values of 0.14, 0.14 and 0.18 mg/g, respectively.

4. Discussion

The type I-type II dichotomy of venom composition seen in the majority of rattlesnakes represents a tradeoff between highly toxic venom, resulting from the presence of PLA_2-derived neurotoxins, and degradative venom, characterized primarily by high metalloprotease activity (Mackessy, 2010a). This dichotomy in venom composition is based on the observation that high metalloprotease activity and neurotoxicity appear to be mutually exclusive characteristics of many species, and these typically are

adapted to a particular localized prey type, and ultimately undermine the molecular mechanisms used for prey capture by snakes in these areas.

The venom phenotypes and ontogenetic shifts that occur in

and translational regulation, because they may inherit differing quantities of various toxin genes.

Venom pro les of hybrids may also be further complicated by

posttranscriptional regulation of crotoxin B-subunit and PIII metalloprotease expression during the ontogenetic shift from a type II to a type I venom in C. s. simus (Durban et al., 2013). As such, it is possible that some inherited toxin genes are transcribed in hybrids,

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Understanding the inheritance and expression patterns of venom toxin genes as well as the regulatory mechanisms responsible for the venom pro les observed in known hybrid systems (particularly those that span the type I-type II dichotomy) can help to clarify the diversity of venom phenotypes that occur in an ecological context. Similar evolutionary mechanisms may underpin the venom variation observed in the intergrade zones between rattlesnakes with divergent venoms, allowing for the application of these venom pro ling methods to new or cryptic hybrid systems. Moreover, knowledge of prey availability and abundance at sites of high venom variation may help elucidate the adaptive roles of highly divergent venom types and the evolutionary forces at work between snakes and available prey species. This often elusive component of snake natural history can expand our understanding of not only the phenotypic consequences of hybridization but also the effects of hybridization on venom markers under selection. Hybridization events between species with divergent venom pro- les may be a source of novel venom phenotypes and could also further clarify the evolutionary and functional consequences of intergrade zones between rattlesnake species.

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).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at

Linking the transcriptome and proteome to characterize the venom of the