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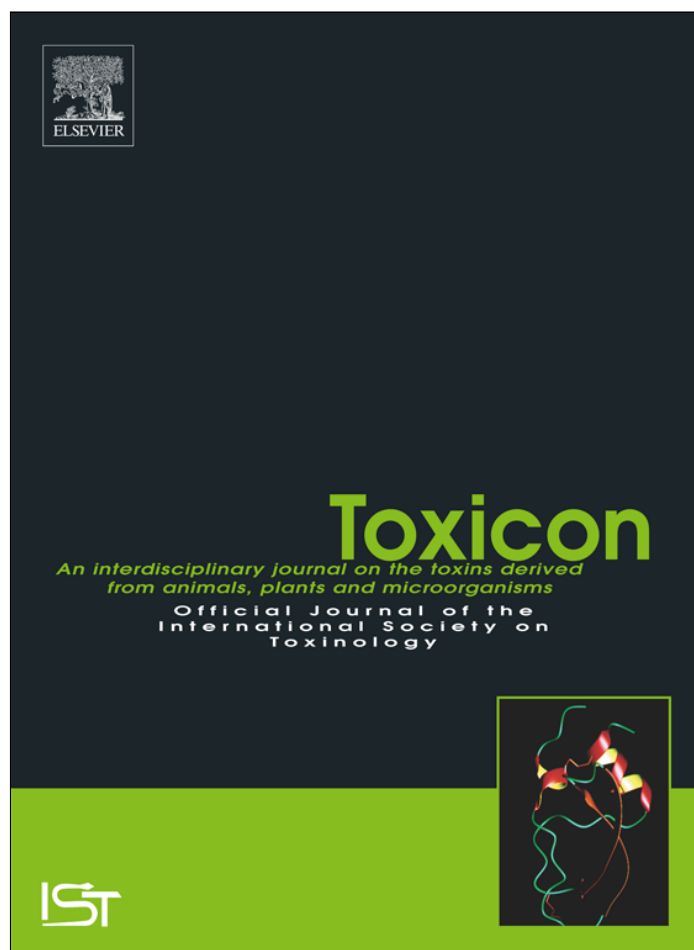

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The effects of hybridization on divergent venom phenotypes: Characterization of venom from *Crotalus scutulatus scutulatus* × *Crotalus oreganus helleri* hybrids

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Hybridization between divergent species can be analyzed to elucidate expression patterns of distinct parental characteristics, as well as to provide information about the extent of reproductive isolation between species. A known hybrid cross between two rattlesnakes with highly divergent venom phenotypes provided the opportunity to examine occurrence of parental venom characteristics in the F_1 hybrids as well as ontogenetic shifts in the expression of these characters as the hybrids aged. Although venom phenotypes of adult rattlesnake venoms are known for many species, the effect of hybridization on phenotype inheritance is not well understood, and effects of hybridization on venom ontogeny have not yet been investigated. The current study investigates both phenomena resulting from the hybridization of a male snake with type I degradative venom, *Crotalus oreganus helleri* (Southern Pacific Rattlesnake), and a female snake with type II highly toxic venom, *Crotalus scutulatus scutulatus* (Mojave Rattlesnake). SDS-PAGE, enzymology, Western blot and reversed phase HPLC (RP-HPLC) were used to characterize the venom of the *C. o. helleri* male, the *C. s. scutulatus* female and their two hybrid offspring as they aged. In general, *Crotalus o. helleri* × *C. s. scutulatus* hybrid venoms appeared to exhibit overlapping parental venom profiles, and several different enzyme activity patterns. Both hybrids expressed *C. o. helleri* father-specific myotoxins as well as *C. s. scutulatus* mother-specific Mojave toxin. Snake venom metalloprotease activity displayed apparent sex-influenced expression patterns, while hybrid serine protease activities were intermediate to parental activities. The *C. s. scutulatus* × *C. o. helleri* hybrid male's venom profile provided the strongest evidence that type I and type II venom characteristics are expressed simultaneously in hybrid venoms, as this snake contained distinctive characteristics of both parental species. However, the possibility of sex-influenced development of metalloprotease activity, as seen in the ontogenetic shifts of the hybrid female, may influence the levels of expression of both type I and type II characteristics in hybrid venoms. Ultimately, the chronological analysis of this known hybrid system reveals the most distinct characteristics that can be used in determining successful hybridization between snakes that follow the type I-type II trend in rattlesnake venom composition, namely the presence of metalloprotease activity and Mojave toxin.

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1. Introduction

The effectiveness of venom plays a critical role in the success of prey capture throughout a rattlesnake's lifetime. Ontogenetic shifts in venom composition are rooted in changes in diet preference as a snake ages (Andrade and Abe, 1999; Daltry et al., 1996; Gutiérrez et al., 1991; Mackessy, 1988; Mackessy et al., 2003), and venom composition has even been shown to undergo subtle changes as early as following the first natal shed, likely before a snake takes its first meal (Wray et al., 2015). Younger rattlesnakes are gape-limited to taking smaller prey items, primarily ectotherms including invertebrates, amphibians, and lizards, while adults tend to feed on larger endothermic prey (Mackessy, 1988). Prey availability also shifts as snakes get larger and are able to consume larger prey items. These dietary shifts often correlate with a shift in venom composition from a highly toxic venom with low metalloprotease activity (type II) to a largely proteolytic, lower toxicity venom (type

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I) (Durban et al., 2013; Mackessy, 1988, 2008, 2010a,b).

Type I venoms typically have lower toxicity and are thought to aid in predigestion of larger mammalian prey, while type II venoms are likely optimized for rapid prey incapacitation, facilitating capture and handling (Mackessy, 1988, 2010a; Mackessy et al., 2003). Although the type I and type II venom compositional trend is widespread among adults of many rattlesnake species (Mackessy, 2008), and a type II to type I ontogenetic shift has been observed most frequently, a modified type I to type II ontogenetic shift in venom composition has been noted in *C. v. viridis* (Prairie Rattlesnake). This opposite trend involved a decrease in metalloprotease activity and an increase in myotoxin concentration, likely correlating with an ontogenetic shift in diet (Saviola et al., 2015). Because of the clear differentiation in venom components and activities observed in venoms of adult rattlesnakes, type I and type II venom characteristics are thought to be largely mutually exclusive (Mackessy, 2010b).

Some snakes retain a highly toxic juvenile venom phenotype through adulthood, likely as a result of paedomorphosis (Calvete et al., 2009, 2012; Gutiérrez et al., 1991; Mackessy, 2010b; Mackessy et al., 2003). In rattlesnakes, this paedomorphic venom profile is typically characterized by the presence of multi-subunit PLA_2-based neurotoxins, all homologs of crotoxin, which are largely responsible for the high toxicity of these venoms (Calvete et al., 2012; Mackessy et al., 2003; Saravia et al., 2002). Interestingly, this highly toxic phenotype is maintained through adulthood despite an age-related shift in diet (e.g., Mackessy et al., 2003).

Crotalus scutulatus (Mojave Rattlesnake) is a snake typically found in deserts of the southwestern United States through central Mexico. Its venom contains a potently toxic PLA_2-based neurotoxin (Mojave toxin), and adult *C. s. scutulatus* venom has been shown to contain very low protease activity (Cate and Bieber, 1978; Glenn and Straight, 1978; Glenn et al., 1983; Ho and Lee, 1981; Mackessy, 1988). However, geographic variation in *C. scutulatus* venom has also been observed previously in a population in Arizona, USA (Glenn and Straight, 1983; 1990), and more recently in Mexico (Borja et al., 2014). Individuals that contained Mojave toxin and low metalloprotease activity were designated A venoms, and those that lacked Mojave toxin but had hemorrhagic venom were designated B venoms. Despite this geographic variation in venom composition, the type II venom characteristics of high neurotoxicity coupled with low metalloprotease activity is the most common and widespread venom phenotype observed in *C. scutulatus*.

Crotalus o. helleri (Southern Pacific Rattlesnake) is found from southwestern California to Baja California, Mexico. Its venom has previously been reported to disrupt clotting mechanisms and affect smooth and striated muscle, among other activities (Metsch et al., 1983; Ruiz et al., 1980). *Crotalus o. helleri* venom shifts from a more toxic type II phenotype to a more proteolytic type I phenotype as snakes age, and diet changes from primarily lizards to a diet that consists of mammalian prey (Mackessy, 1988). Proteolytic activity continues to increase as its capacity for larger mammalian prey also increases, suggesting a link between venom phenotype and prey taken. Though in most cases it fits a classical type I phenotype, *C. o. helleri* has been shown to have a moderate level of intraspecific diversity (Sunagar et al., 2014). Moreover, a highly localized population of *C. o. helleri* in southern California was determined to contain Mojave toxin and have reduced proteolytic activities. However, because these populations of Mojave toxin-positive *C. o. helleri* were geographically isolated from *C. s. scutulatus*, it was concluded that this novel venom phenotype was most likely not the result of recent introgression between *C. o. helleri* and *C. s. scutulatus* (French et al., 2004).

Hybridization in rattlesnakes has been documented in scientific literature as early as 1942, when Bailey identified a hybrid between *Crotalus horridus horridus* (Timber Rattlesnake) and *Sistrurus catenatus catenatus* (Eastern Massasauga) by conducting a thorough examination of scalation, body proportions and color patterns (Bailey, 1942). Instances of both intergeneric and interspecific hybridization between various species of rattlesnakes have been investigated via morphology, venom characteristics and genetics. Morphologically, laboratory bred *Crotalus atrox* (Western Diamondback Rattlesnake) × *C. s. scutulatus* (Mojave Rattlesnake) F_1 and F_2 hybrids appeared to display overlapping parental characteristics (Aird et al., 1989). Characters such as the presence of dark flecks on dorsal scales, the number of scales between supraocular scales, position of the postocular light stripe, dorsal blotch patterning and light and dark tail rings were used to investigate inheritance patterns of morphological characteristics in both F_1 and F_2 generations. *Crotalus atrox* × *C. horridus* hybridization was investigated by Meik et al. using scalation, color pattern and scanning electron microscopy of scale features (2008). A hybrid between *C. s. scutulatus* and *C. atrox* was genetically confirmed using allozyme data (Murphy and Crabtree, 1988). The hybrid individual presented with morphological characteristics intermediate to both parents, and allozyme data revealed that the hybrid contained species-specific markers of both *C. s. scutulatus* and *C. atrox*.

Hybridization between rattlesnake species with highly divergent adult venoms provides the opportunity to characterize the venom variation that results from hybridization, to contextualize this variation within the type I-type II dichotomy of venom composition, and to study the venom gene inheritance patterns that result in a hybridized venom phenotype. Previously, only four studies have investigated rattlesnake hybridization using venom profiles. Glenn and Straight determined that hybridization was occurring between two species based on the presence of a type II neurotoxin in a type I species (1990). Snakes captured from an intergrade zone in southwestern New Mexico involving *Crotalus viridis viridis* (Prairie Rattlesnake) and *C. s. scutulatus* showed a typical *C. v. viridis* morphology, but venom contained Mojave toxin, a distinct component of *C. s. scutulatus* that is not found in the venom of *C. v. viridis*. The authors concluded this was the result of historical hybridization and backcrossing of hybrids into *C. v. viridis* populations. However, this study assumed hybridization based on the presence of Mojave toxin in *C. v. viridis* populations without investigating metalloprotease activities as well. As such, it was not confirmed if hybrids displayed both type I and type II characteristics. Further characterizing hybridization between *C. v. viridis* and *C. s. scutulatus* in southwestern New Mexico, Zancolli et al. used both venomic and genomic data to determine that in this case, hybridization between snakes with type I and type II venoms did not result in the adaptive radiation of Mojave toxin into *C. v. viridis* populations, and ultimately these hybridized venoms remained confined to the hybrid zone (2016).

Another study investigated hybrid morphology and venom profiles in a known hybrid system between one *C. atrox* and one *C. s. scutulatus* (Aird et al., 1989). Hybrids retained characteristics of both parents, but the *C. s. scutulatus* parent was described as having a type B venom (corresponding to type I venom), and once again, the presence of type I and type II characteristics resulting from hybridization was not confirmed. Aird et al. (2015) used venom gland transcriptomics and venomics to determine that *Protobothrops flavoviridis* × *Protobothrops elegans* hybrids, the result of species invasion (by *P. elegans*) due to human action on the Ryukya Islands of Japan, express overlapping parental venom profiles; however, *P. flavoviridis* and *P. elegans* have similar venom components and do not follow the type I-type II trend in venom composition seen in American rattlesnakes.

Venom ontogeny in a number of rattlesnake species has been

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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 individually in the University of Northern Colorado (Greeley, CO) Animal Resource Facility. Venom was extracted from all snakes as previously described (Mackessy, 1988) at least once per year beginning in 2007, with the exception of 2012; the *C. s. scutulatus* mother died in 2010 and only three yearly samples were collected. All venoms were centrifuged at 9500×*g* for 5 min, lyophilized and stored at −20 °C.

2.3. Protein concentration determination

Dried venom samples were reconstituted at an apparent concentration of 4.0 mg/ml in Millipore-filtered water, vortexed, centrifuged for 5 min at 9500×*g*, and protein concentration of the supernatant was determined using the Bradford protocol (1976) as modified by BioRad, Inc. and using bovine globulin as standard. Amount of material used in all subsequent assays was based on these determinations. Reconstituted samples were frozen at −20 °C until used and then thawed and centrifuged at 9500×*g* for 5 min to pellet cellular debris.

2.4. Protein gel electrophoresis

Crude venom (20 μg) or lyophilized protein (approximately 5 μg - RP-HPLC) was loaded into wells of a NuPAGE Novex bis-tris 12% acrylamide mini gel and electrophoresed in MES buffer for 45 min at 175 V; 7 μl of Mark 12 standards were loaded for molecular weight estimates. Gels were stained with 0.1% Coomassie brilliant blue R-250 in 50% methanol and 20% acetic acid (v/v) overnight with gentle shaking 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) overnight with gentle shaking at room temperature and imaged on an HP Scanjet 4570c scanner.

2.4.1. Azocasein metalloprotease assay

The azocasein metalloprotease assay procedure was performed as outlined in Aird and da Silva (1991), 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) 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 $\Delta_{342\ nm}$/min/mg venom protein.

2.4.2. L-Amino acid oxidase

L-amino acid oxidase assays were performed as outlined in Weissbach et al. (1960); 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.

2.4.3. Phosphodiesterase assay

Phosphodiesterase assays were performed based on Laskowski (1980) modification of Björk (1963). 20 μg of crude venom was added to 200 μl of buffer (100 mM tris-HCl, 10 mM $MgCl_2$, pH 9.0). 150 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. Specific activity was expressed as $\Delta_{400\ nm}$/min/mg venom protein.

2.4.4. Thrombin-like serine protease assay

Thrombin-like serine protease assays were performed as outlined in Mackessy (1993). Eight μg of crude venom 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 substrate (BzPheValArg-pNA; Sigma) was added and the tube was vortexed and placed back at 37 °C. Reactions were stopped after five minutes with 50% acetic acid. Tubes were read at 405 nm, and specific activity was calculated from a standard curve of p-nitroaniline and expressed as nanomoles product produced/min/mg of venom.

2.4.5. Kallikrein-like serine protease assay

Kallikrein-like serine protease assays were performed based on modifications of Mackessy (1993). Briefly, 0.8 μg of crude venom 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 substrate (Bz-ProPheArg-pNA; Bachem) was added, and the tube

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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 specific activity was calculated from a standard curve of p-nitroaniline and expressed as nanomoles product produced/min/mg venom.

2.5. Purification of Mojave toxin and concolor toxin

Purification of the type II neurotoxins Mojave toxin and concolor toxin was achieved as outlined in Aird et al. (1986). 75 mg of crude *C. s. scutulatus* or *C. o. concolor* venom was dissolved in HEPES buffer and fractionated on a BioGel P-100 size exclusion column (2.6 × 89 cm) using buffer (25 mM HEPES, 100 mM NaCl, 5 mM $CaCl_2$, pH 6.8) at a flow rate of 6 mL/h at 4 °C. The toxin-containing peak was concentrated using a Millipore 3.0 kDa molecular weight cutoff centrifuge concentrator and further purified using a DEAE FF HiTrap 5 mL column in 50 mM tris buffer pH 8.3 (0–1.0 M NaCl gradient) on a Life Technologies FPLC using Unicorn software. Concolor toxin was used as a positive control in Western blots and as a standard to identify the elution time of closely-related Mojave toxin on RP-HPLC (see below). The A subunit of Mojave toxin was purified and used to generate polyclonal antibodies in rabbits (GenScript, Piscataway, NJ, USA). Purified material was analyzed using a Bruker Microflex LRF MALDI-TOF mass spectrometer at the Proteomics and Metabolomics Facility at Colorado State University (Fort Collins, CO) to confirm 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. (2016).

2.6. Western blots

To test for the presence of Mojave toxin subunit A, 2 μg of purified concolor toxin or 20 μg of crude venom were loaded onto a NuPAGE Novex bis-tris 12% acrylamide gel and run in MES buffer at 175 V under reducing conditions for approximately 45 min as described above (mass standards - 7 μl of Novex Sharp pre-stained protein standard). Gels were blotted onto Novex 0.2 μm pore nitrocellulose membranes in a Novex Xcell II Blot Module in transfer buffer (25 mM bicine, 25 mM bis/tris, 1.0 mM EDTA and 0.05 mM chlorobutanol, pH 7.2 in 20% methanol) for approximately 1 h at ~175 mA in an ice bath. After blocking with 3% BSA in PBS and washing with PBS (3x), membranes were incubated with a 1:1500 dilution of rabbit anti-Mojave toxin subunit A polyclonal antibodies in 15 mL of 3% BSA in PBS with gentle shaking at room temperature overnight. Membranes were washed 3x in tris-buffered saline (TBS) and then incubated for 1 h with alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody (1:3000 dilution) in TBS. Membranes were washed again in TBS (3x), and antibody binding was detected with Sigma *FAST* BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) tabs dissolved in 10 mL of Millipore-filtered water. The detection reaction was stopped with 20 mL of 20 mM disodium EDTA in PBS with gentle shaking for 10 min, rinsed in Millipore-filtered water and dried, and membranes were imaged on an Epson Perfection 4870 photo scanner.

2.7. High performance liquid chromatography

One mg of crude venom was resolubilized in 100 μl of Millipore-filtered water and centrifuged at 9500×*g* for 5 min to pellet cellular debris; supernatant was filtered with a 0.45 μm syringe tip filter. Venom was subjected to reverse phase HPLC using a Phenomenex Jupiter C_{18} (250 × 4.6 mm, 5 μm, 300 Å pore size) column, and one minute fractions were collected at a flow rate of 1 mL/min for a total run time of 100 min. To elute proteins, a gradient of 95% solution A (0.1% trifluoroacetic acid in Millipore-filtered water) to 95% solution B (0.1% TFA in 100% acetonitrile) was used (5% solution B for 10 min; 10–25% B over 10 min; 25–45% B over 60 min; 45–70% over 10 min; 70–90% over 2 min; 90–95% over 3 min; and returning to 5% B over 5 min). Protein/peptide was detected at 220 nm and 280 nm with a Waters 2487 Dual λ Absorbance Detector. Fractions corresponding to each peak were then frozen overnight at −80 °C and lyophilized. These fractions were then analyzed along with 20 μg crude venom using SDS-PAGE as described above in order to determine mass and designate probable toxin families. Myotoxin, Mojave toxin and PLA_2 peaks were also analyzed using a Bruker Microflex LRF MALDI-TOF mass spectrometer at the Proteomics and Metabolomics Facility at Colorado State University (Fort Collins, CO) to confirm protein identity. Approximately 1.0 μg protein was dissolved in 1.0 μL 50% acetonitrile containing 0.1% TFA, mixed with 1.0 μL sinapinic acid matrix (10 mg/mL, dissolved in the same solvent), and spotted onto target plates.

2.8. Principal coordinates analysis

HPLC peaks for 3 *C. o helleri*, 3 *C. s. scutulatus* and both hybrids were scored as present (1) or absent (0) and analyzed with GenAlEx (version 6.5 (http://www. http://biology-assets.anu.edu.au/GenAlEx); Peakall and Smouse 2006, 2012). Genetic distance was calculated for all 51 variable loci as a haploid binary system. Principal coordinate analysis was computed from genetic distance as a tri distance matrix using the standardized covariance method.

2.9. Lethal toxicity (LD_{50}) assays

Crotalus s. scutulatus mother, *C. o. helleri* father and hybrid venoms were tested for lethal toxicity towards NSA mice (20–24 g). Mice were given intraperitoneal injections on the right side of the body. Doses of 0, 0.1, 0.25, 0.5, 1.0, 1.2 and 1.5 μg/g were adjusted for body mass and administered in a 100 μl bolus of 0.9% saline, and 24 h survivorship was scored.

2.10. Statistics

To test for significance between samples for enzymatic activities and reverse phase HPLC chromatogram peak areas, a two sample, two-tailed *t*-test assuming unequal variance was run. Significance level was set at $p < 0.05$.

3. Results

3.1. Gel electrophoresis

SDS-PAGE revealed a high level of complexity in both parents and adults, and each individual had a consistent electrophoretic profile from year to year, with the exception of one band at approximately 10 kD that appeared between 2008 and 2009 in both hybrids and was retained through adulthood (Fig. 1). Both hybrids venoms contained components specific to each parent, and they showed overlapping profiles compared to parents. Notably, both hybrids contained father-specific myotoxin bands at approximately 4 kD, though in lower abundance. The dominant protein in *C. s. scutulatus* venom was a protein at approximately 14 kD (Mojave toxin subunit B), which was also present at lower abundances in both hybrid venoms, but absent in the male *C. o. helleri.*

3.1.1. Azocasein metalloprotease activity

Crude venoms were assayed for five enzymes commonly found in rattlesnake venoms. Metalloprotease activity was significantly

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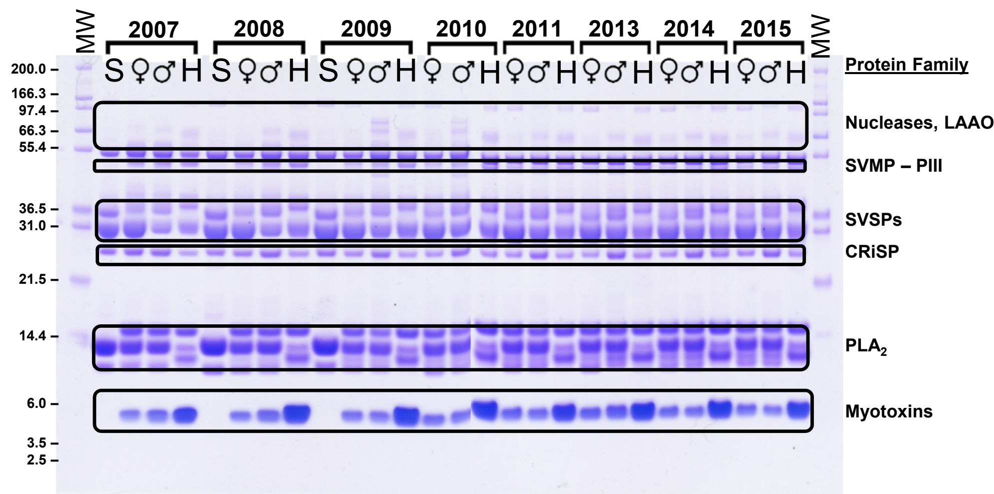

Fig. 1. Reducing SDS-PAGE of *C. s. scutulatus* female parent (S), *C. o. helleri* male parent (H), female hybrid (♀) and male hybrid (♂) venoms from 2007 to 2015 (except 2012). Protein families typically found at specific molecular masses are indicated by ovals. Note that the *C. s. scutulatus* female parent samples were only available through 2009. MW = Molecular weight standards (in kDa).

different ($p = 0.004$) between adult *C. s. scutulatus* venom and adult *C. o. helleri* venom (Fig. 2A). The type I-type II dichotomy that encompasses the *C. viridis/oreganus* complex (Mackessy, 2010a,b) is also mirrored in the venom phenotypes of *C. o. helleri* and *C. s. scutulatus* in the present study. *Crotalus o. helleri* venom metalloprotease activity was typically orders of magnitude higher than the nearly undetectable metalloprotease activity of *C. s. scutulatus* venom (see also Mackessy, 1988). In addition, although *Crotalus o. helleri* adults consistently showed higher metalloprotease activity, they also displayed overall higher variation in activity.

The *C. s. scutulatus* female parent had consistently low azocasein metalloprotease activity that did not change over time, while the *Crotalus oreganus helleri* male parent had metalloprotease activity that steadily increased from 2007 to 2015 (Fig. 3A). Azocasein metalloprotease activity of *C. s. scutulatus* × *C. o. helleri* female hybrid venom dropped sharply from 2007 to 2008, and remained low through adulthood, the typical trend seen in type II snake venoms. The *C. s. scutulatus* × *C. o. helleri* hybrid male's venom had higher activity as a neonate than the hybrid female, and it followed the typical type I venom trend of increasing metalloprotease activity over time.

3.1.2. Thrombin-like serine protease activity

Average thrombin-like serine protease activities of all *C. s. scutulatus* and *C. o. helleri* adult venoms did not differ significantly ($p = 0.168$; Fig. 2B). Venom of the *C. s. scutulatus* female parent had consistently high thrombin-like serine protease activity from 2007 through 2009 that was slightly variable from year to year (Fig. 3B). These values were concordant with the activities determined from the six other *C. s. scutulatus* adults. Venom from the male parent *Crotalus o. helleri* had thrombin-like serine protease activity that was lower than the *C. s. scutulatus* female parent, but it gradually increased between 2007 and 2015. Thrombin-like serine protease activities of both hybrids fell below the male parent's activity, but gradually increased over time to fall intermediate to both parents by the time they reached adulthood.

3.1.3. Kallikrein-like serine protease activity

Kallikrein-like activities of *C. s. scutulatus* and *C. o. helleri* reference venoms varied significantly ($p = 0.0002$; Fig. 2C). Adult *C. s. scutulatus* venoms had a higher average activity than all adult *C. o. helleri*, but *C. o. helleri* venoms had a wider range of activities. The *C. s. scutulatus* female parent had consistently high kallikrein-like serine protease activity (Fig. 3C), consistent with the activities determined from the six other *C. s. scutulatus* adult venoms. *Crotalus oreganus helleri* venom had lower kallikrein-like serine protease activity than the *C. s. scutulatus* female parent that increased slightly over time. Activities of both hybrid venoms fell intermediate between parent activities: the hybrid female decreased slightly and the hybrid male increased slightly between 2007 and 2015.

3.1.4. L-Amino acid oxidase activity

Average activities of *C. s. scutulatus* and *C. o. helleri* reference venoms differed significantly ($p = 0.008$; Fig. 2D). Activities between both parents and hybrids remained relatively consistent across all years, with all individuals increasing slightly overtime (Fig. 3D). All individuals had comparable activities and no clear intermediate trend in activity was apparent.

3.1.5. Phosphodiesterase activity

Average activities of *C. s. scutulatus* and *C. o. helleri* reference venoms did not vary significantly ($p = 0.45$; Fig. 2E). All individuals had phosphodiesterase activity that increased over time; however, venom from the *C. s. scutulatus* female parent had consistently higher activity than the C. *o. helleri* male parent (Fig. 3E).

3.2. Mojave toxin and concolor toxin purification

Concolor toxin (*C. o. concolor* venom) and Mojave toxin (*C. s. scutulatus* venom) were purified on a low pressure size exclusion BioGel P-100 column and an FPLC DEAE FF HiTrap anion exchange column (data not shown). Identities of Mojave toxin and concolor toxin and subunits were confirmed using MALDI-TOF MS and Orbitrap LC-MS/MS, and this material was used in subsequent assays as a positive control (concolor toxin) and in antibody generation (Mojave toxin subunit A: see below).

3.3. Western blots

Rabbit antibodies raised against the A subunit of Mojave toxin (produced by GenScript, Inc.) were not monospecific and bound to the A subunit (a modified PLA_2; 9.4 kDa actual mass), with a diffuse band at approximately 9kD, the B subunit (a PLA_2; actual mass

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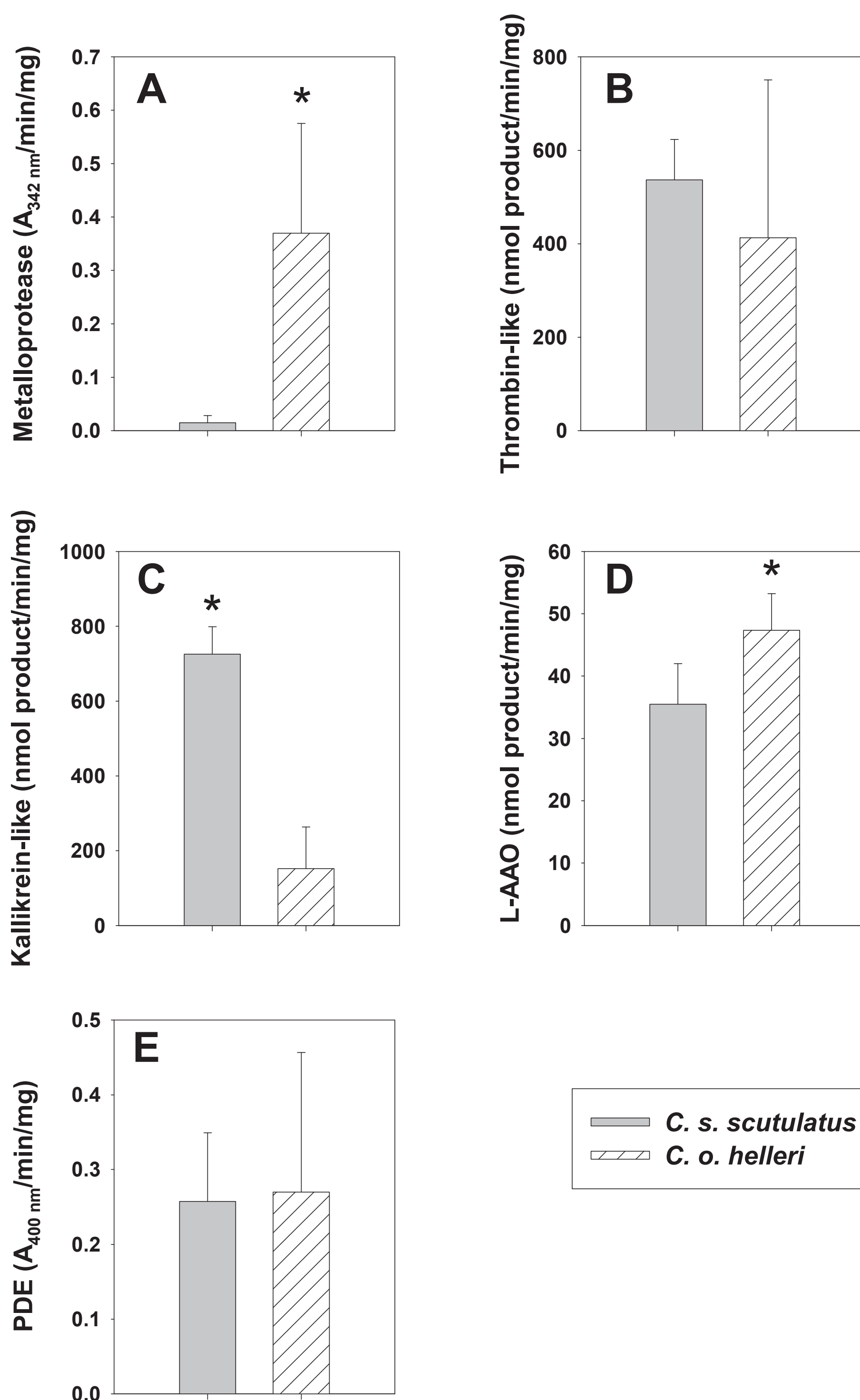

Fig. 2. Average azocasein metalloprotease (A), thrombin-like serine protease (B), kallikrein-like serine protease (C), L-amino acid oxidase (D), and phosphodiesterase activities of reference *C. s. scutulatus* (n = 6) and *C. o. helleri* (n = 6) venom. *, statistically different; $p < 0.01$.

~14 kDa), with a band at approximately 14 kDa, and to non-neurotoxic PLA_2s, at approximately 14–15 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 approximately 15 kDa, slightly higher than the *C. s. scutulatus* B subunit band and lacked an A subunit band. Both hybrid venoms contained overlapping profiles relative to parents with bands at approximately 15 kDa, 14 kDa and 9 kDa. Neither negative control venoms (*C. v. viridis, C. o. cerberus*) showed a band at 9 kDa.

3.4. High performance liquid chromatography

Reversed phase HPLC revealed a high level of complexity for all

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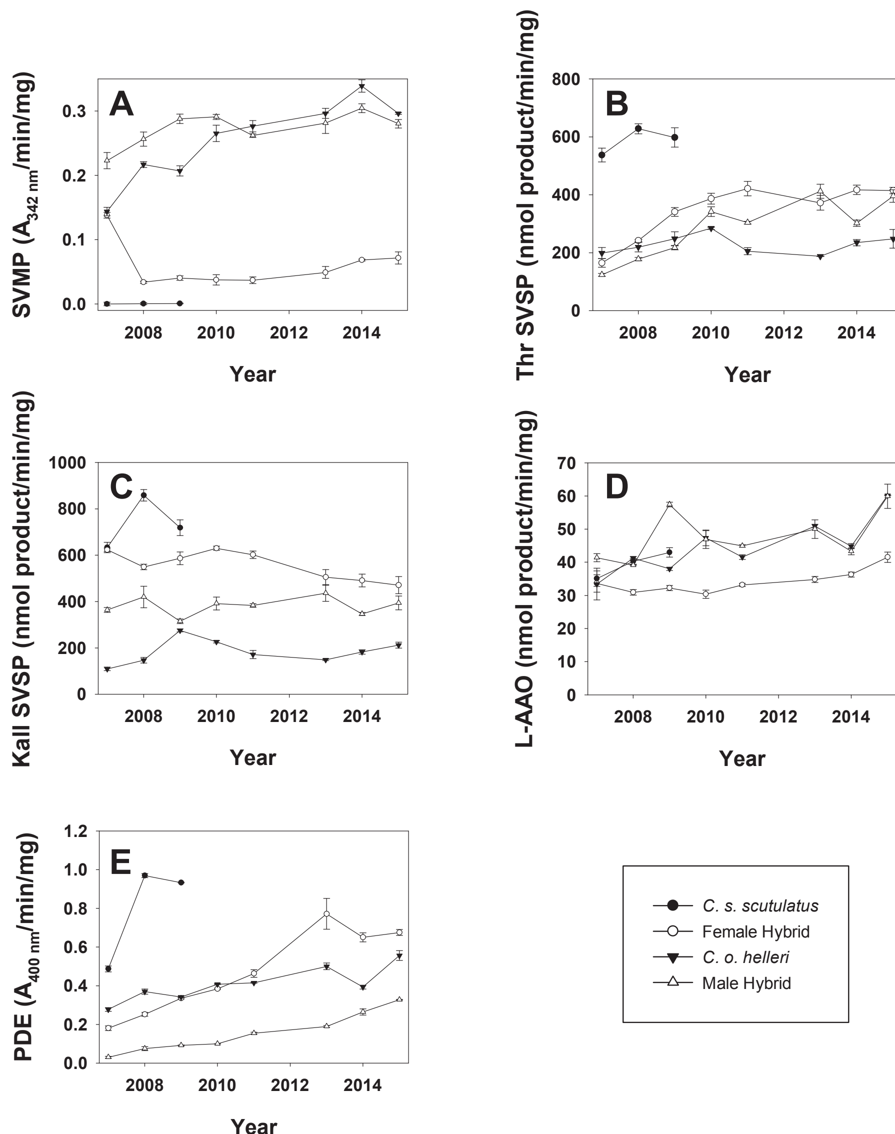

Fig. 3. Enzyme activities of parental and hybrid venoms. A) Azocasein metalloprotease, B) thrombin-like serine protease, C) kallikrein-like serine protease,D) L-amino acid oxidase, and E) phosphodiesterase activities of *C. s. scutulatus* (♀ parent), *C. o. helleri* (♂ parent) and male and female hybrids from 2007 through 2015.

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 1; Fig. 6B and D).

The Mojave toxin B peak (41 min) comprised approximately 18% of the *C. s. scutulatus* female parent's total venom (Fig. 5A). However, Mojave toxin comprised approximately 25% of the total

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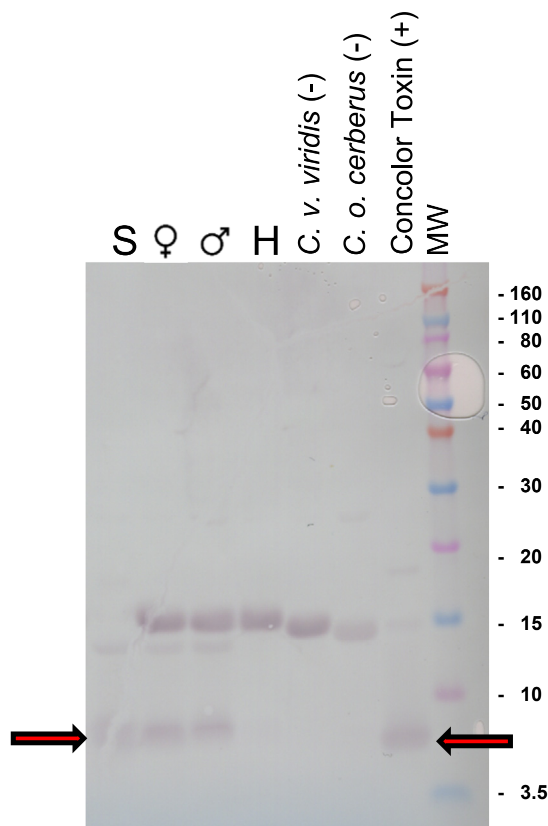

Fig. 4. Western blot of *C. s. scutulatus* (S), *C. o. helleri* (H), hybrids (♀, ♂), *C. v. viridis* and *C. o. cerberus* venoms (negative controls), and purified concolor toxin (positive control), developed with anti-Mojave toxin subunit A antibodies, showing binding to the A subunit (~9 kD; red arrows) in *C. s. scutulatus* and hybrid venoms and purified concolor toxin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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%–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 μg/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 consistent features of an entire species (Mackessy, 2010a). However, in a small number of instances, both neurotoxic and proteolytic activities have been shown to be expressed concurrently. For example, some populations of *C. s. scutulatus* were reported to express both the hemorrhagic components of B venom (Type I venom) and the neurotoxic Mojave toxin of A venom (Type II venom). These A + B venom profiles were determined to be the result of an intergrade zone between A and B venom type populations in southeastern Arizona (Glenn and Straight, 1989). This concept was further elaborated on by Massey et al. (2012), who identified six (A-F) *C. s. scutulatus* venom phenotypes, in the same southeastern region of Arizona, which formed a gradient of venom profiles from predominantly degradative to predominantly neurotoxic. A similar trend was observed in the distribution of Canebrake toxin among populations of *C. horridus* (Glenn et al., 1994). While the majority of venoms clustered into type A and B designations, with type B venom being the most common phenotype observed throughout the entire range of this species, a few individuals from eastern South Carolina, southwestern Arkansas, and northern Louisiana contained A + B venoms, indicating the presence of an intergrade zone between snakes with A and B venoms in these regions.

The expression of Mojave toxin and high metalloprotease activity seen in the venoms of *C. s. scutulatus* × *C. o. helleri* hybrids is another exception to the rule that type I and type II venom characteristics are usually mutually exclusive (Mackessy, 2010a). Moreover, the concurrent expression of Mojave toxin and high metalloprotease activity most clearly demonstrated in the venom profile of the hybrid male refutes French et al. (2004) assertion that expression of metalloprotease genes may be suppressed by the expression of Mojave toxin genes. However, the hybrids bred in captivity for this study are free from the ecological, trophic and dietary selective pressures that likely contribute to the reinforcement of a type I-type II trade-off.

In an ecological context, it is unclear how selection would act on venom phenotypes that overcome the type I-type II dichotomy. Depending upon the success of snakes with hybridized venoms in these areas, selection could play a critical role in the maintenance of a hybrid zone, in the reinforcement of reproductive isolation and ultimately species divergence, or in the exchange of specific toxin genes between species. Despite the assumption that type II neurotoxins are inherently more effective at prey capture and that hybridization will result in the radiation of these toxins into parental populations, Zancolli et al. (2016) reported that Mojave toxin found in the venom of *C. s. scutulatus* × *viridis* hybrids in southwestern New Mexico did not permeate into *C. v. viridis* populations and thus did not confer a selective advantage for snakes in these areas. However, in another context, the expression of type I and type II venom characteristics in conjunction with one another could broaden the range of prey items available, helping hybrids to capitalize on a more diverse array of prey items. On the other hand, hybridization could interfere with a venom phenotype specifically

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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 venom composition resulting from the hybridization of a type I snake, *C. o. helleri,* and a type II snake, *C. s. scutulatus*, suggest a

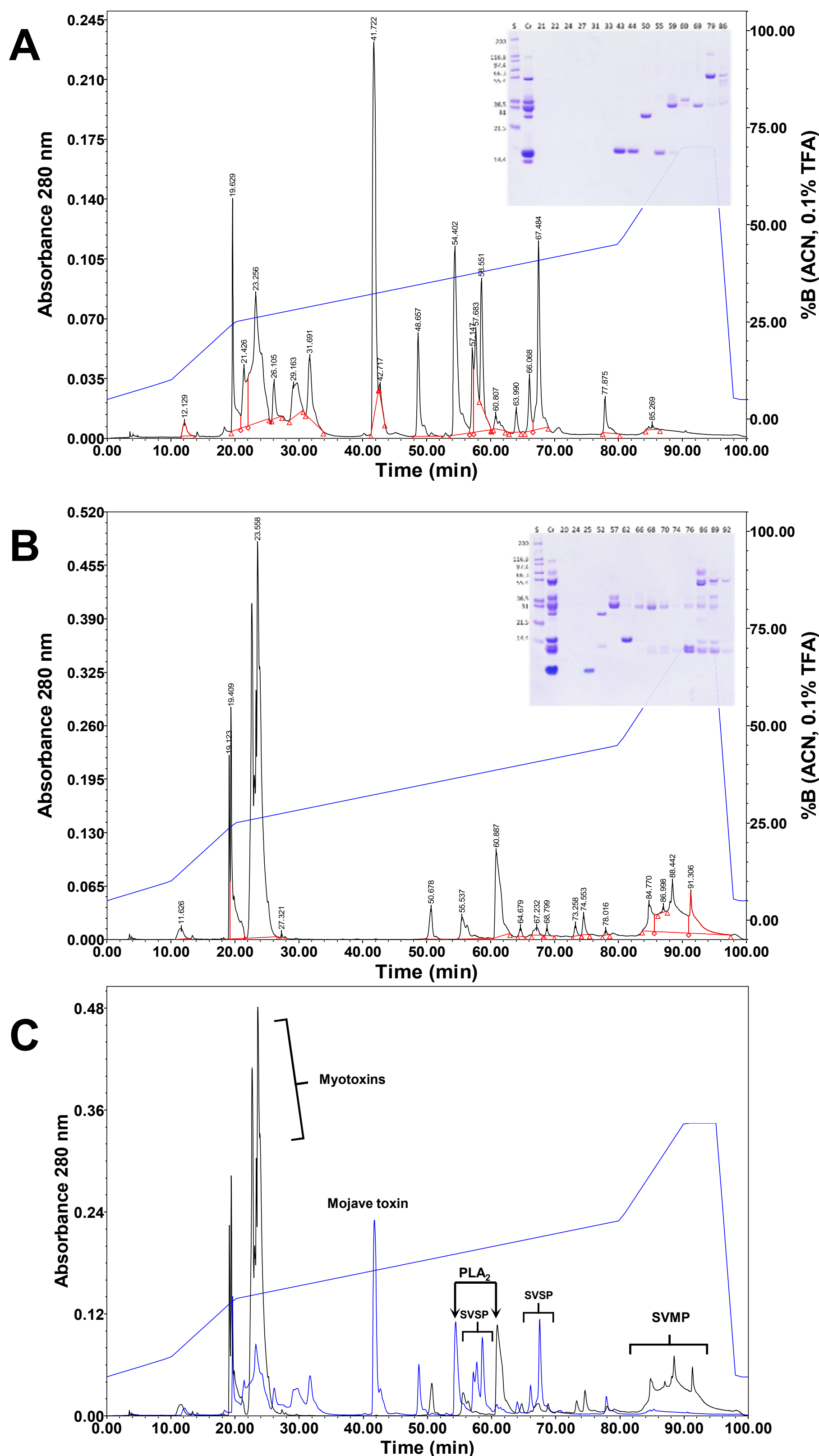

Fig. 5. Reverse phase HPLC chromatograms, peak elution time tables, and gel of eluted peaks of *C. s. scutulatus* female parent (A), *C. o. helleri* male parent (B) and overlay of the two chromatograms (C); *C. s. scutulatus* (blue) and *C. o. helleri* (black). Note the high abundance of myotoxins in *C. o. helleri*, the presence of Mojave toxin in *C. s. scutulatus* and the lack of metalloproteases (SVMP) in *C. s. scutulatus*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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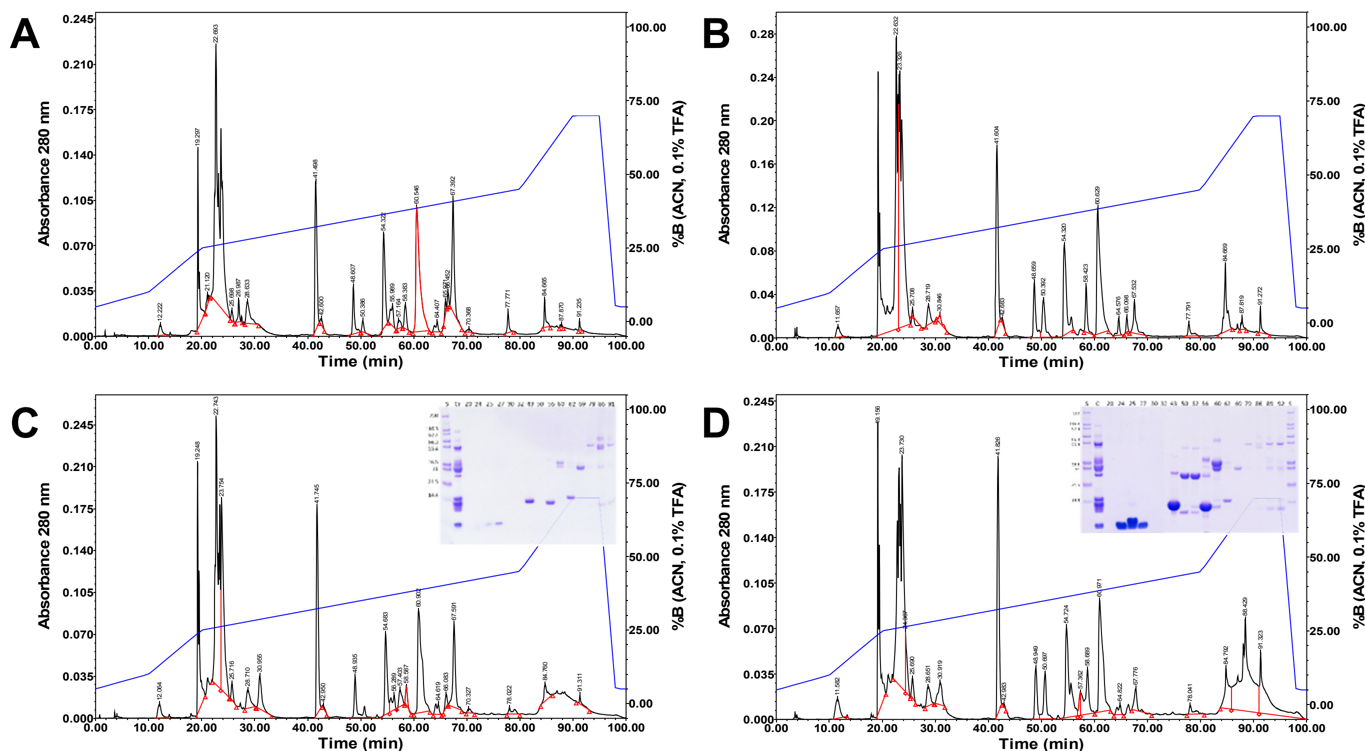

Fig. 6. Reverse phase HPLC chromatogram and peak elution time table of 1.0 mg venom protein from (A) hybrid female venom extracted in 2008, (B) hybrid male venom extracted in 2007, (C) hybrid female venom extracted in 2015, and (D) hybrid male venom extracted in 2015.

complex pattern of inheritance of venom characteristics. Both hybrids expressed *C. o. helleri* (father-specific) myotoxins as well as *C. s. scutulatus* (mother-specific) Mojave toxin. This could indicate a dominant pattern of inheritance; however, SDS-PAGE band size and intensity were reduced relative to parents, and percent area of RP-HPLC Mojave toxin peaks in both adult hybrids were slightly higher than half (~10% of total venom protein) of the *C. s. scutulatus* female parent's percentage of Mojave toxin (~18% of total venom). Adult hybrid myotoxin peaks made up 38–45% of the total venom profile of hybrids, while the *C. o. helleri* male parent's myotoxin peaks accounted for 62% of the total venom. This suggests a pattern of codominant expression, as each hybrid would inherit myotoxin and Mojave toxin A subunit and B subunit genes from only one parent, and express less of each toxin than a parent with two alleles. Interestingly, hybrids had an LD_{50} value not significantly different from the *C. s. scutulatus* mother, indicating that activity of Mojave toxin plus myotoxin is so potent that even a reduced expression of the proteins is sufficient to induce a lethal dose.

Both thrombin-like and kallikrein-like serine protease activities were intermediate to parent activities, indicating possible codominant or additive expression of these compounds. Thrombin-like activity also increased over time in both hybrids, while kallikrein-like serine protease activity slightly decreased over time. Both of these trends in serine protease activity have been previously noted in type II ontogenetic shifts in venom composition (Mackessy et al., 2003). However, thrombin-like serine protease activity was not significantly different between the six *C. o. helleri* and six *C. s. scutulatus* adults used as representative samples and showed wide variation in activity, particularly between *C. o. helleri* individuals. As such, thrombin-like activity is likely not a good candidate for making inferences about hybrid status based on venom profiles.

Kallikrein-like serine protease activity was significantly higher in *C. s. scutulatus* than in *C. o. helleri*, with low variation in activity levels between individuals of both species. Kallikrein-like serine protease activity may make a better marker for hybridization, as parent activities are drastically different and expression appears to be codominant, so hybrids could fall intermediate between parent activities. However, serine protease activities have been shown to vary widely among *Crotalus* species (Mackessy, 2008, 2010a), and they are not associated with the type I-type II dichotomy as strongly as metalloprotease activity. Use of kallikrein-like activity to indicate hybridization may therefore be dependent on the species pair involved.

L-amino acid oxidase activity, while significantly different between the two species, did not show any significant differences in hybrid parents or definitive patterns in ontogenetic shifts in either hybrid, though activity in all individual venoms increased gradually over time. Phosphodiesterase activity was not significantly different between representative parent species, but it also gradually increased over time for both parents and hybrids. Because both L-amino acid oxidase and phosphodiesterase activities are not key components that consistently characterize the differences between type I or type II venoms and are not toxic compounds that contribute substantially to the lethality of a venom, they are not particularly useful in characterizing the venom of *C. s. scutulatus* × *C. o. helleri* hybrids in the current study.

Inheritance patterns of venom genes could be convoluted due to the large number of gene duplication events of functionally important toxins and the effects of random assortment and genetic recombination (Casewell et al., 2011; Kondrashov, 2012; Vonk et al., 2013). The extensive levels of gene duplication events and neofunctionalization result in large multigene families with high numbers of similar toxin sequences that have the potential for highly variable activities (Chang and Duda, 2012; Vonk et al., 2013). Thus, because of the randomizing effects of assortment and genetic recombination on the multitude of repeatedly duplicated toxin genes, hybrids will likely display varying levels of activities, regardless of further alterations to expression via transcriptional

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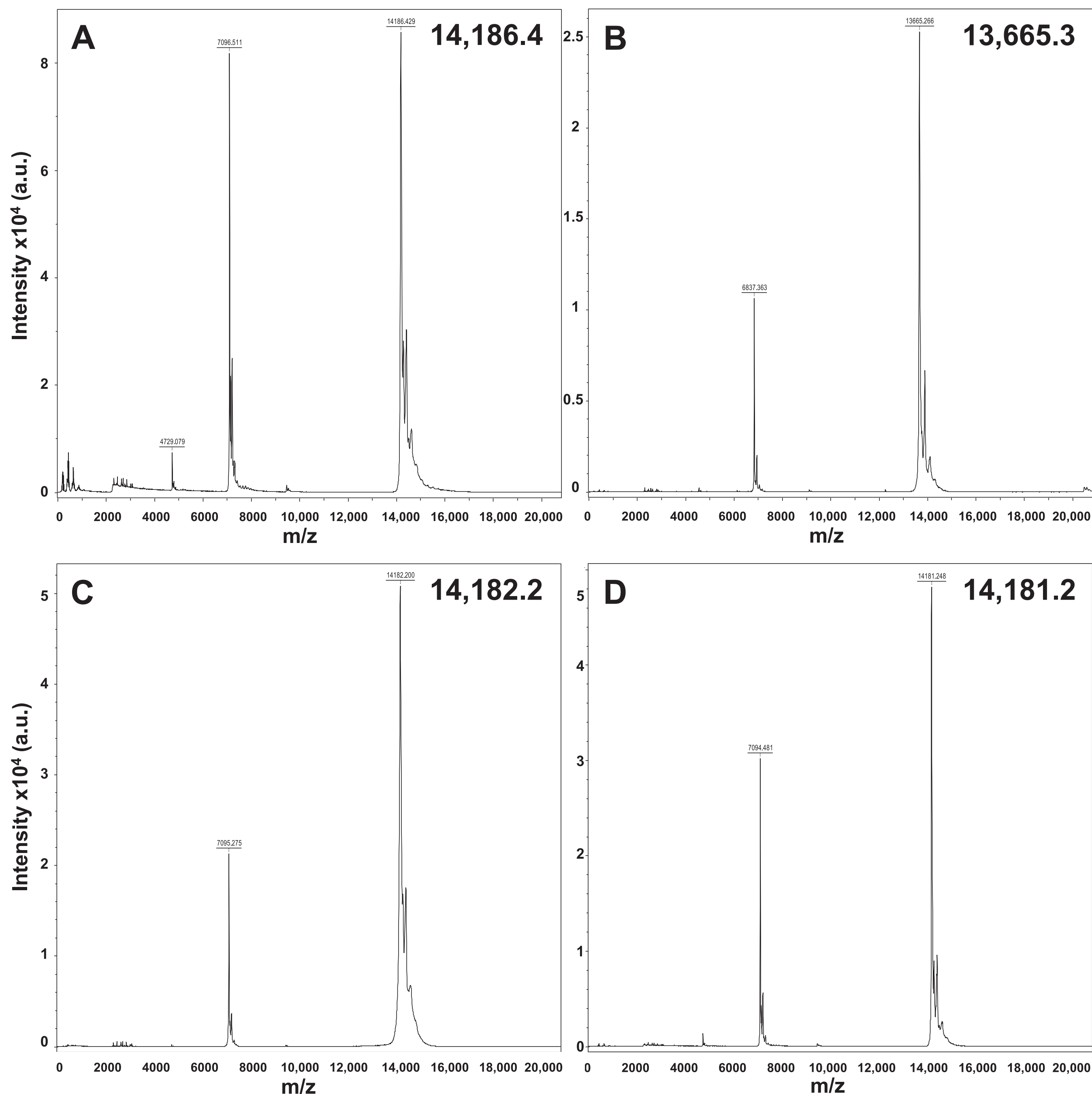

Fig. 7. MALDI-TOF mass spectra of reverse phase HPLC fractions of (A) *C. s. scutulatus* female parent Mojave toxin subunit B, (B) *C. o. helleri* male parent PLA_2, (C) hybrid female Mojave toxin subunit B, and (D) hybrid male Mojave toxin subunit B. Spectra are derived from HPLC fraction 41 for all samples except the male parent (fraction 62).

Table 1
Reverse phase HPLC chromatogram percent peak areas of myotoxins, Mojave toxin and SVMPs for *C. s. scutulatus* female parent and two reference *C. s. scutulatus*, *C. o. helleri* male parent and two reference *C. o. helleri*, and the male and female hybrids from 2007 (♂), 2008 (♀) and 2015 (both).

Sample	Myotoxins	Mojave toxin	SVMPs
C. s. scutulatus (n = 3)	0	18–25	0.5–2
C. o. helleri (n = 3)	49–62	0	6–20
Hybrid ♀ (2008)	39.9	6.8	1.5
Hybrid ♂ (2007)	42.3	8.3	5.1
Hybrid ♀ (2015)	44.7	9.8	1.3
Hybrid ♂ (2015)	37.6	10.3	7.9

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

Venom profiles of hybrids may also be further complicated by epigenetic, transcriptional, or translational regulation that could alter the expression levels of inherited toxin genes (Casewell et al., 2014; Gibbs et al., 2009; Margres et al., 2014). For example, transcripts encoding three-finger toxins were sequenced from viperids, but were not expressed in the venom, though the particular mechanism of translational repression was not elucidated (Junqueira-de-Azevedo et al., 2006; Pahari et al., 2007). More recently, miRNAs have been shown to play a role in the

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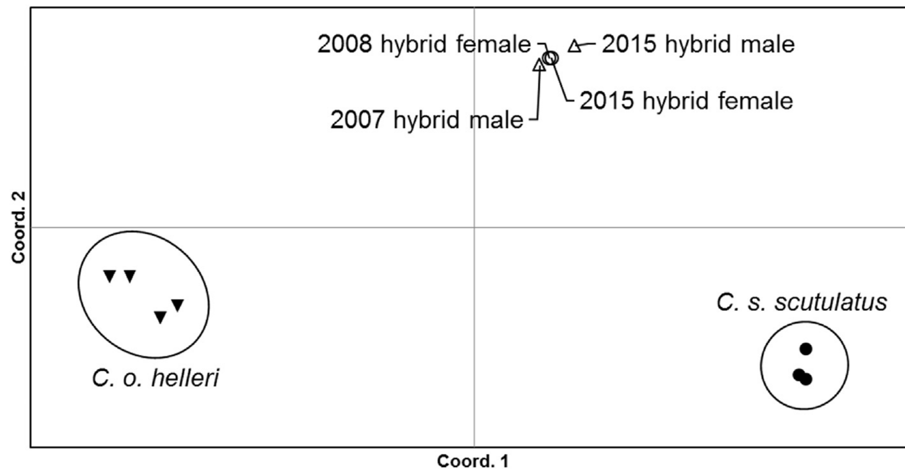

Fig. 8. Principal coordinate analysis of RP-HPLC results for three *C. s. scutulatus*, three *C. o. helleri*, and hybrids. Note the distinct clusters based on species, and that hybrids fall intermediate to *C. o. helleri* and *C. s. scutulatus* along coordinate 1.

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, but are ultimately not expressed in the venom due to translational regulation. Thus, the unique ‘hybridized’ venom profiles that result from hybridization between snakes with divergent venom phenotypes are likely the result of complex patterns in gene inheritance and the additive effects of transcriptional and translational regulation. However, despite these possible complications with inferring genotype from venom phenotype, there appears to be a genotype-phenotype linkage in the concurrent expression of Mojave toxin subunits A and B, simplifying hybrid status delineations of type I-type II hybrids based on venom profiles (Zancolli et al., 2016).

The ontogenetic shifts in azocasein metalloprotease activity of the hybrid male mirrored the increase in the *C. o. helleri* male parent's activity and the typical increase in metalloprotease activity over time seen in type I snakes. Following the trend seen in type II snakes, the hybrid female's activity decreased from moderate to low activity over time. However, metalloprotease activity seen in the female hybrid was not as low as the *C. s. scutulatus* mother (or other reference *C. s. scutulatus*), which had nearly nondetectable activity. Because ontogenetic shifts in metalloprotease activity of the female and male hybrid mirrored the trend of the *C. s. scutulatus* mother and *C. o. helleri* father, respectively, this could indicate sex-influenced inheritance or expression of metalloprotease genes. Therefore, in the event that hybridization is unknown but suspected, sex may play a role in the ontogenetic shifts in venom composition that occur and ultimately in the levels of metalloprotease activity observed in hybrids. We acknowledge that the apparent sex-linked ontogenetic changes are based on a highly limited sample size and therefore are speculative at this point. However, crosses of the F_1 hybrids and studies of the offspring produced by a *C. o. helleri* female and *C. s. scutulatus* male could further elucidate the sex-influenced ontogenetic trends suggested in this study. Because sex determination is genetic in snakes, further hybrid crosses could reveal a link between inheritance of sex chromosomes and the expression of metalloprotease genes.

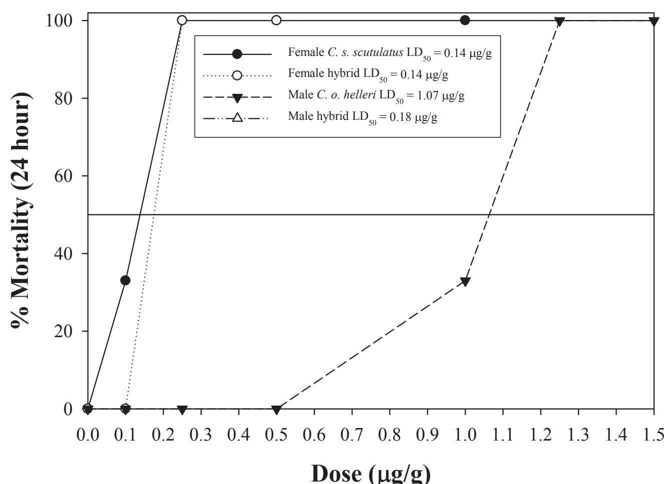

Fig. 9. Lethal toxicity (µg/g) of *C. o. helleri* male parent, *C. s. scutulatus* female parent and female and male hybrid neonate venoms to NSA mice.

5. Conclusions

Crotalus o. helleri × *C. s. scutulatus* hybrids appeared to exhibit a number of different venom component expression patterns. This study presents an analysis of the ontogenetic shifts of type I-type II hybrid venoms and confirms that the venom components that are most helpful in determining hybrid status using venom profiles are the compounds that most clearly illustrate the type I-type II dichotomy in venom composition. Thus, this known hybrid system can be used as a model to generate venom profiles of potential type I-type II hybrids and allow for hybrid status delineations based upon only two major venom components, despite the (likely) complexity of parent and potential hybrid venoms. The *C. s. scutulatus* × *C. o. helleri* hybrid male's venom profile provided the strongest evidence that venom profiles can be used to identify rattlesnake hybrids, as this snake contains identifying characteristics of both parental species. However, the possible influence of sex on metalloprotease activity development (as seen in the ontogenetic shifts of the hybrid female) may lead to a wide range in activities of hybrid venoms. This should be taken into account in the characterization and delineation of hybrid venoms due to the possibility that sex influence may obfuscate the confirmation of hybridization between rattlesnakes based on venom phenotypes alone.

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Understanding the inheritance and expression patterns of venom toxin genes as well as the regulatory mechanisms responsible for the venom profiles 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 profiling 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 profiles 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 http://dx.doi.org/10.1016/j.toxicon.2016.08.001.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.toxicon.2016.08.001.

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