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Predator-prey interactions and venom composition in a high elevation lizard specialist, *Crotalus pricei* (Twin-spotted Rattlesnake)

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ABSTRACT

The Twin-spotted Rattlesnake (*Crotalus pricei*) is a small lizard specialist restricted to higher montane habitat in the Sky Islands of Arizona and México. Though this species is restricted to high elevations and dispersal between mountaintops is impossible, few studies have investigated venom composition or the predator-prey relationship between *C. p. pricei* and its primary prey source, Yarrow's Spiny Lizard (*Sceloporus jarrovii*). Because of current isolation of populations in disjunct mountain ranges, it is possible that populations show local adaptation to prey and/or environmental conditions, as reflected by distinct venom phenotypes. Here we characterize venom composition of *C. pricei* from several different Sky Island mountain ranges and its relationship with *S. jarrovii* by using various analytical techniques and comparative toxicity tests. Results of venom analyses indicate that there is limited geographic variation in venom composition, occurring primarily in venoms of *C. pricei* from Durango, México, which had unusual PLA_2 isoforms that are lacking from venoms of U.S. populations. Toxicity assays reveal that *S. jarrovii* has not developed resistance specific to *C. p. pricei* venom but does display a general tolerance to venom of several snakes in the genus *Crotalus*. These results provide insight into the evolutionary relationship between a lizard specialist and its natural prey, in addition to novel information on the venom composition of a little-studied species with a narrow range in the United States.

1. Introduction

Crotalus pricei (Twin-spotted Rattlesnake) is a rarely-studied species endemic to México, with a restricted distribution in southeastern Arizona, U.S. (Campbell and Lamar, 2004). Relatively little is known about their natural history, population sizes, and venom composition, and a handful of studies have provided insight into demographics (Prival and Schroff, 2012) and venom composition (Mackessy, 2008; Minton and Weinstein, 1984). The nominate subspecies, *Crotalus pricei pricei*, is widely distributed in the Sierra Madre Occidental, from southeastern Arizona and northeastern Sonora/western Chihuahua, México, south to Aguascalientes and Durango, México (Campbell and Lamar, 2004). Within the United States, *C. p. pricei* is found primarily in montane ecosystems on Sky Islands of only four mountain ranges (Campbell and Lamar, 2004; Prival, 2016). A second subspecies, *Crotalus pricei miquihuanus* (Eastern Twin-spotted Rattlesnake), is found in higher montane regions of the Sierra Madre Oriental in the states of Coahuila, Nuevo Léon, Tamaulipas and San Luis Potosí, México (Armstrong and Murphy, 1979; Campbell and Lamar, 2004).

Crotalus pricei is a small-bodied pitviper, unique in both morphology and habitat requirements, and consequently it has been a target for wildlife trafficking since at least 1960 (Prival and Schroff, 2012). They are small snakes, reaching an average snout to vent length (SVL) of 388 mm and a mass of 40 g, most commonly observed near southeast-facing talus slopes, but they exhibit no obvious microhabitat site fidelity (Prival and Schroff, 2012). These snakes can tolerate a wide temperature range (13.0 °C–34.0 °C) and can be found basking in the open, moving across rocks, or coiled under rocks or vegetation (Campbell and Lamar, 2004; Prival and Schroff, 2012). Despite their apparent hardiness, *C. pricei* are limited to very specific, higher elevation habitat and are most commonly associated with talus.

As a consequence of its somewhat extreme habitat and morphological constraints (size and gape), *C. pricei* has a specialized diet composed primarily of lizards. Evaluation of fecal samples demonstrated that *Sceloporus sp.* comprise approximately 68%–87% of *C. p. pricei* diet in the Chiricahua Mountains of southeast Arizona, and mammals make up 13%–32% (depending on snake life stage). Adult snakes are more likely than juveniles to consume mammals, likely due to larger gape size that can accommodate larger prey. Approximately 18% of fecal samples were specifically identified as *Sceloporus jarrovii* (Yarrow's Spiny Lizard; Prival and Schroff, 2012). *Sceloporus jarrovii* is broadly distributed in Arizona and is one of five *Sceloporus* species (*S. cowlesi*, *S.*

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clarkia, S. jarrovii, S. magister and *S. slevini*) having a distribution overlap with *C. p. pricei* in Arizona (Jones and Lovich, 2009). *Sceloporus jarrovii* often occurs at high population densities and is most common at higher elevations, and its range in Arizona directly overlaps with that of *C. p. pricei.*

The montane regions referred to as Sky Islands range from Arizona through México, creating a fragmented landscape of higher elevation forests separated by lower elevation desert. A rapid change in ecosystem vegetation occurs as elevation increases, making the Sky Islands extremely species-rich and home to many endemic species (Peterson and Navarro-Sigü; enza, 1999). Desert scrub, chaparral, pine-oak scrub, montane scrub, and mixed deciduous forests can all be found in one mountain range (Arriaga et al., 2005). Based on packrat midden radiocarbon dating, Sky Island geographic formations did not exist until approximately 18,000 radiocarbon years BP (^{14}C years before present) (Thompson and Anderson, 2000). Before this, the southwestern landscape of North America was primarily forested, quite different from the current xeric ecosystem. As the Earth's climate changed, the lower elevation forests became deserts, isolating the more mesic forests on higher elevation Sky Islands (Thompson and Anderson, 2000). These unique ecosystems can present a number of challenges for native species, including geographically-limited genetic variation and highly restricted distributions due to a rapidly changing climate and vegetation gradient (Davis et al., 2015). Given the unique habitat requirements of *C. p. pricei* and dynamic nature of Sky Island habitats, *C. p. pricei* may face threats similar to other high elevations species, such as the Pika (*Ochotona princeps*), that have already experienced significant range decline due to climate change (Beever et al., 2003).

Coevolution as a driving force in an ecological system can be hard to identify conclusively (e.g., Brodie III and Brodie Jr., 1999), but evidence of prey-specific venom toxicity (Heyborne and Mackessy, 2013; Mackessy et al., 2006; Modahl et al., 2018; Pawlak et al., 2006, 2009) and venom resistance via multiple physiological mechanisms has been demonstrated in several systems (Arbuckle et al., 2017; Barlow et al., 2009; Poran et al., 1987). California Ground Squirrels (*Otospermophilus beecheyi*) exhibit venom resistance when sympatric with large populations of Northern Pacific Rattlesnakes (*Crotalus oreganus*), but they show less resistance when *C. oreganus* are absent or uncommon (Biardi et al., 2006; Poran et al., 1987). In these populations, *O. beecheyi* comprise up to 69% of *C. oreganus* diet (Poran et al., 1987), indicating that resistance in these squirrels has evolved in response to greater predation pressures by *C. oreganus.* Adaptive toxin resistance in *Otospermophilus beecheyi* exhibits a geographic mosaic pattern similar to other coevolutionary systems (Brodie Jr. et al., 2002; Poran et al., 1987). Given that toxin resistance as an adaptation in predator-prey relationships has been observed in multiple systems and considering *Sceloporus* comprise approximately 67.6% of *C. p. pricei* diet, there is a possibility that this dynamic is also driven by adaptive toxin resistance (Prival and Schroff, 2012).

Rattlesnake venoms can generally be classified into one of two groups based on toxins present and pathology of envenomation: type I or type II venom (Mackessy, 2008, 2010). These venoms can be differentiated by moderate to high metalloproteinase activity and lower toxicity (type I) versus a near lack of metalloproteinase activity and high lethal toxicity (type II). However, very few studies have analyzed *C. p. pricei* venom (but see Mackessy, 2008; Minton and Weinstein, 1984). Based on this preliminary work (Mackessy, 2008), *C. p. pricei* venom showed high enzymatic activity and high toxicity toward mice. We expected results to be similar between *C. p. pricei* from the various regions analyzed and *C. p. miquihuanus* because of their close phylogenetic relationship (Blair and Sánchez-Ramírez, 2016; Bryson et al., 2011a). In addition, we tested several hypotheses:

1. H_1: *Crotalus p. pricei* venom will be more toxic to a non-native species (House Geckos; *Hemidactylus frenatus*) than toward *S. jarrovii.*

H_0: There will be no significant difference in *C. p. pricei* venom toxicity towards *Hemidactylus frenatus* and *S. jarrovii.*

We predicted that *Crotalus p. pricei* venom would be more toxic to a reptile model organism, *Hemidactylus frenatus*, than to prey (*S. jarrovii*) found within their range.

2. H_2: *Crotalus p. pricei* venom will be more toxic to *S. jarrovii* that are allopatric than *S. jarrovii* that are sympatric with *C. p. pricei.*

H_0: There will be no difference between *C. p. pricei* venom toxicity to *Sceloporus jarrovii* that occur sympatrically and allopatrically.

We predicted that *S. jarrovii* within *C. p. pricei* range will show greater tolerance to venom than those outside of the normal *C. p. pricei* range due to evolution of resistance mechanisms in prey (cf. Red Queen hypothesis; van Valen, 1973).

3. H_3: Venom from *C. p. pricei* will show taxon-specific toxicity – that is, lizard prey (*Hemidactylus frenatus* and *S. jarrovii*) will be differentially more sensitive to toxic effects of venom than will mammalian prey (a model species, *Mus musculus*).

H_0: *Crotalus p. pricei* venom will show similar levels of toxicity to mammalian prey models (*Mus musculus*) and lizard prey models (*Hemidactylus frenatus* and *S. jarrovii*).

We predicted that taxon-specific toxicity, as observed in several species of colubrid snakes (Heyborne and Mackessy, 2013; Modahl et al., 2018; Pawlak et al., 2009), would be observed in the venom of this lizard specialist rattlesnake. *Crotalus p. pricei* venom may also contain lizard-specific toxins that facilitate capture of this common natural prey.

2. Materials and methods

2.1. Supplies and reagents

Protein concentration reagents (Pierce® BCA Protein Assay kit, bovine γ-globulin) were obtained from ThermoFisher Scientific (Waltham, MA USA). NuPage gels, buffers and standards for protein electrophoresis were obtained from Life Technologies, Inc. (Grand Island, NY, USA). All reverse phase-high performance liquid chromatography equipment (515 HPLC Pump, Fraction Collector II, and 2487 Dual λ Absorbance Detector) were from Waters Corporation (Milford, MA, USA), and Jupiter 5 µm C_{18} 300 Å 250 × 4.60 mm reversed phase columns were purchased from Phenomenex, Inc (Torrance, CA, USA). All other reagents (analytical grade or higher) were supplied by Sigma Biochemical Corp. (St. Louis, MO, USA).

2.2. Animals and venoms

Crotalus p. pricei specimens or venoms were collected from three of the four Sky Island locations in the United States with known populations: Chiricahua Mountains, Santa Rita Mountains, and Pinaleño Mountains; we were unable to obtain venom samples of snakes from the Huachuca Mountains. All animals collected in Arizona were in accordance with guidelines provided by Arizona Game and Fish Department (AGFD) under AGFD Scientific Collection Permits SP745788 and SP591359 (to S.P. Mackessy). Venom samples from Durango, México were extracted from three snakes housed at the Chiricahua Desert Museum, and the two *C. p. pricei* venom samples from the Santa Rita Mountains were obtained from snakes housed at Sternberg Museum of Natural History. Venom samples of snakes from several locations in the Chiricahua Mountains were previously acquired under AGFD permit MCKSY000221 (1992 & 1993) and are included in this study. Three *Crotalus p. miquihuanus* venom samples were obtained from captive snakes originating from Nuevo Léon, México. All venom samples were manually extracted, lyophilized and stored at −20 °C

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until analyzed (Mackessy, 1988). This method retains consistent venom composition over repeated extractions of adult snakes (Rex and Mackessy, 2019).

Sceloporus jarrovii were collected from each of two locations: the Chiricahua Mountains, within *C. p. pricei* range, and the Dragoon Mountains (southeast of Tucson, AZ), outside of the known range of *C. p. pricei.* Live animals were held in the Animal Research Facility at the University of Northern Colorado. All experimental protocols were completed in accordance with the UNC Institutional Animal Care and Use Committee (IACUC protocols 1302D-SM-16 and 1701D-SM-S-20).

2.3. Protein concentration determination

Lyophilized venom samples were reconstituted at an apparent concentration of 4.0 mg/mL in Millipore-filtered water. Protein concentration of the crude venom samples was determined using the Thermo Scientific Pierce® BCA Protein Assay kit, with bovine γ-globulin as the standard, as described by the manufacturer. These standardized protein concentrations were used in all assays described below.

2.4. Gel electrophoresis

Crotalus p. pricei venom samples and RP-HPLC fractions (reduced) were electrophoresed on NuPAGE Novex bis-tris 12% acrylamide mini gels with MES running buffer to provide a "molecular fingerprint" of dominant venom components. Mark 12 standards were run on each gel to provide an estimate of molecular weight. Crude venom (20 µg) or RP-HPLC-fractionated proteins (approximately 5 µg) and Mark 12 protein standards (7 µL) were loaded into wells and gels were electrophoresed at 150 V, 125 mA for about 90 min. Gels were then stained in 0.1% Coomassie Brilliant Blue R-250 and placed on a gyrating shaker overnight. Excess stain was removed the next day with rapid destain (30% methanol, 7% glacial acetic acid in water) for approximately 2 h; gels were then imaged on an HP Scanjet. Typical protein families were labeled, verified by calibration with purified toxins and by following protocols outlined in previous studies (Mackessy, 2010a,b; Saviola et al., 2015; Jones et al., 2019).

2.5. Enzyme assays

Assays of enzyme activities common to rattlesnake venoms were conducted to compare enzymatic activity levels in *C. pricei* venoms from different locations (Smith and Mackessy, 2016). These assays included metalloproteinase (azocasein substrate), thrombin-like and kallikrein-like serine proteinases (Bz-PheValArg-pNA and Bz-ProPheArg-pNA substrates, respectively), phosphodiesterase (bis-nitrophenyl phosphate substrate) and phospholipase A_2 (4-nitro-3-(octanoyloxy) benzoic acid substrate) activities. L-amino acid oxidase activity was assayed via an alternative method.

2.5.1. L-amino acid oxidase assay

Venom samples were assayed for L-amino acid oxidase activity using the method of Kishimoto and Takahashi (2001); all samples and controls were run in duplicate. Ten-fold concentrated stock solutions of reagents were prepared: L-methionine (MET) substrate was dissolved at 7.46 mg/mL in buffer (50 mM borax, pH 8.5), o-phenylenediamine (OPD)-coupled substrate was resolubilized in buffer at 2.16 mg/mL, and horseradish peroxidase (HRP) was resolubilized at 8.1 U/mL. Crude venom samples (10.0 µL at 0.1 mg/mL) were added to each well on a 96-well plate. Ninety µL master mix (70% buffer, 10% MET, 10% OPD, and 10% HRP solutions, v/v) were then added to each well. The plate was then incubated at 37 °C for 30 min before returning to a cold surface and rapidly adding 50 µL termination solution (2.0 M sulfuric acid). Sample absorbance was read at 492 nm on a SpectraMax plate reader, and specific activity was expressed as $\triangle A_{492\,nm}$/min/mg protein after absorbance of buffer control was subtracted.

2.6. Reverse-phase high performance liquid chromatography (RP-HPLC)

Crotalus p. pricei venom samples were analyzed using an RP-HPLC method similar to that outlined in Smith and Mackessy (2016). Venom was resuspended in Millipore-filtered 18.2 MΩ water at 10 mg/mL. Samples were then centrifuged at 10,000 × *g* for 5 min and filtered through a 0.45 µm syringe tip filter before injection of 200 µL (2 mg) onto a Jupiter (5 µm C_{18} 300 Å) 250 × 4.60 mm RP-HPLC column. One minute fractions were collected at a rate of 1 mL/min for 104 min. Venoms were fractionated using a gradient of 0.1% trifluoroacetic acid in Millipore-filtered water (solution A) and 0.1% trifluoroacetic acid in acetonitrile (solution B). Venom was injected onto the column with starting conditions of 95% A and 5% B. The following gradient was used: 5–10% solution from minutes 0–10; 10–25% B from minutes 10–20; 25–45% B from minutes 20–80; 45–70% B from minutes 80–92; and 70–95% B from minutes 92–95. Eluting proteins and peptides were detected at 220 nm and 280 nm. One minute fractions were collected, and those fractions corresponding to protein/peptide peaks were placed in a −80 °C freezer overnight and then lyophilized.

2.7. LC-MS/MS analysis

Two RP-HPLC peaks from Durango *C. p. pricei* and from *C. p. miquihuanus* samples (45 & 48) were unique and were analyzed as reported previously (Jones et al., 2019). Approximately 10 µg protein (reduced and alkylated) was rehydrated in 20 µL of sequencing-grade trypsin (6.66 ng/µL in 25 mM ammonium bicarbonate), and samples were digested overnight at 37 °C. Following digestion, the samples were vortexed for 1 h at room temperature, sonicated in an ultrasonic bath for 3 min, and the tryptic peptides were desalted and then extracted in 20 µL of 50% ACN/0.1% TFA using Millipore C_{18} ZipTips. Digests were dried in a vacuum centrifuge and resuspended in 12 µL of 5% ACN containing 0.1% formic acid (FA) and submitted to liquid chromatography mass spectrometry (LC-MS/MS) analysis.

LC-MS/MS experiments were performed with an Easy nLC 1000 instrument coupled to a LTQ Orbitrap Velos mass spectrometer (both from ThermoFisher Scientific). Tryptic peptides were loaded on a C_{18} column (75 µM inner diameter x 15 cm) packed in-house with Aqua® 3 µm C_{18} 125 Å resin (Phenomenex Inc.). The flow rate was set to 0.6 µL/min and the column was developed with a linear gradient of 5% ACN, in 0.1% formic acid (FA) in ddH_2O (solution A) and 0.1% FA in ACN (solution B) at 1% B for 1 min, followed by 1–12% B for 1 min, 12–40% B for 15 min, and 40–85% B for 2 min. MS/MS was performed using a data-dependent acquisition (DDA) top 10 method, with the instrument operating in positive mode nanoelectrospray with a spray voltage of +2.3 kV. Precursor ion scans were executed in the Orbitrap mass analyzer at 60 K resolving power, and monoisotopic precursor selection (MIPS) was enabled for charge states ≥ 2 (+1 charge state was enabled for RP-HPLC peak 26 which contained small peptides). Fragment ion scans were performed in the linear ion trap mass analyzer using a collision-induced dissociation at 35% normalized collision energy. Dynamic exclusion was set to 60 s at 10 ppm tolerance. Fragmentation spectra were interpreted using Integrated Proteomics Pipeline (IP2, version 6.0.2; www.integratedproteomics.com) against the NCBI non-redundant database downloaded on 30 September 2018. MS/MS mass tolerance was set to ± 0.6 Da and carbamidomethyl cysteine and oxidation of methionine were selected as fixed and variable modifications, respectively. An FDR (false discovery rate) of 1% (protein level) was used.

2.8. Lethal toxicity (LD_{50}) assays

LD_{50} assays of *C. p. pricei* venoms were performed on *S. jarrovii* and *Hemidactylus frenatus* to determine acute (lethal) toxicity in a native prey and a model lizard species, respectively. *Sceloporus jarrovii* were wild-caught in Cochise County, Arizona. Two experimental groups were

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used - one from high elevation habitat in the Chiricahua Mountains within known *C. p. pricei* range, and one group from lower elevation habitat in the Dragoon Mountains outside of known *C. p. pricei* range. *Hemidactylus frenatus* (House Geckos; Indonesia) were purchased from Bushmaster Reptile (Boulder, CO, USA). These results were compared to LD_{50} assays previously performed on Non-Swiss Albino (NSA) mice (Mackessy, 2008).

Venom toxicity methods were adapted from Mackessy (2008). Three subadult lizards (3.0–6.0 g body mass, *S. jarrovi*; 2.0–4.0 g, *Hemidactylus*) were used at each dose level, and six adult *C. p. pricei* venom samples from the Chiricahua Mountains were combined for use in order to obtain an average lethal toxicity measure for this population. Lyophilized venom was reconstituted in Millipore-filtered water at a concentration of 1.0 mg/mL. Doses of 0.5, 1.0, and 5.0 μg venom/g body weight were administered initially, with intervening doses as needed to determine LD_{50}. Doses appropriately adjusted to individual lizard mass were injected intraperitoneally anterior to the right hind leg using a 28G x ½ in. needle and 0.5 mL syringe; all lizards were maintained at 26 °C, and a 24-h time frame was used to determine lethal toxicity.

2.9. Statistical analyses

Significance was calculated for enzymatic activities using a 2-way Analysis of Variance (ANOVA). A Tukey HSD test was completed post-hoc to determine differences between all combinations of means. P-values < 0.05 were considered statistically significant. However, a very limited number of samples ($n = 2$) were available for several locations, and due to violation of certain assumptions of ANOVA tests relating to sample size, statistical results involving these venoms should not be considered as statistically rigorous.

3. Results

3.1. Gel electrophoresis of crude venom

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of venoms showed the presence of 10–12 bands for most individuals analyzed (Fig. 1). These bands corresponded to toxins typical of known *Crotalus* venom toxins that have previously been identified (cf. Mackessy, 2010a). Toxins typical of type I venoms, PIII and PI snake venom metalloproteinases (SVMP), were visible at ~53 kDa and 23 kDa, respectively (Fig. 1). The greatest variation was present in the SVMP PI band (~23 kDa) and the PLA_2 bands (~14 kDa; Fig. 1); these differences appeared to vary randomly and there was no clear correlation with geographic origin. However, all Durango *C. p. pricei* samples showed very prominent double PLA_2 bands, with masses slightly lower than most other samples, and PI SVMPs were absent from almost half of all venom samples.

3.2. Enzyme activities

Average specific activities were calculated for six different enzyme assays. Venom samples included 14 *C. p. pricei* from the Chiricahua Mountains, two from the Pinaleño Mountains, two from the Santa Rita Mountains, three from Durango, México, and two *C. p. miquihuanus* from Nuevo León, México (Table 1). Because of the larger sample size of the Chiricahua region, the range of specific activities likely represents snakes within this mountain range more accurately than populations in other regions (Fig. 2). In general, snakes from México of both subspecies showed the highest values of snake venom serine proteases (SVSP) and phosphodiesterase (PDE) activities.

High activity was observed for SVSPs compared to other species in the genus *Crotalus*, as previously reported by Mackessy (2008). Chiricahua snakes also displayed the widest range of specific activity values for both thrombin-like and kallikrein-like SVSPs. Durango snakes had the highest overall SVSP activity and the Pinaleño snakes had the lowest (Table 1; Fig. 2). Kallikrein-like SVSP activities from *Crotalus p. miquihuanus* and samples from the Durango regions were significantly higher than samples from the Chiricahuas and Pinaleños ($p < 0.05$), and Durango samples were also significantly higher than Santa Ritas samples ($p < 0.05$).

Overall, SVMP activity was also relatively high compared to other *Crotalus* species (Mackessy, 2008) and varied slightly between individuals from the Chiricahuas, with a range from 1.19 to 1.62. Lower specific activity was apparent in *C. p. miquihuanus* individuals and *C.p. pricei* individuals from Durango (Table 1; Fig. 2) and these values were significantly lower than individuals from the Pinaleños ($p < 0.05$).

Phospholipase A_2 (PLA_2) activity was relatively high as well, and results were similar to previous studies (Mackessy, 2008). Activities of individuals from the Chiricahuas ranged from 43.1 to 68.5 nmol/min/mg. *Crotalus p. miquihuanus* samples were generally higher than *C. p. pricei* samples, and the Santa Rita samples had the greatest range in values (17.2–89 nmol/min/mg; Table 1, Fig. 2). Despite the apparent differences, PLA_2 activity levels were not statistically different between regions or subspecies.

Phosphodiesterase (PDE) activity was variable and relatively low for all individuals when compared to other *Crotalus* sp. (Mackessy, 2008). Individuals from the Chiricahuas ranged from 0.012 to 0.072 $\Delta A_{400\ nm}$/min/mg. *Crotalus p. miquihuanus* activity was considerably higher at 0.15–0.32 $\Delta A_{400\ nm}$/min/mg, and snakes from the Pinaleños appeared to have the lowest amount of activity (Table 1; Fig. 2). Samples from Durango (*C. p. pricei*) and Nuevo León (*C. p. miquihuanus*) were significantly higher than all regions in AZ ($p < 0.01$).

L-amino acid oxidase (LAAO) activity was moderate compared to other *Crotalus* species (Mackessy, 2008). Individuals from Durango had the highest activity, while those from the Chiricahuas had the lowest activity (14.4), with *C. miquihuanus* samples displaying slightly higher value (16.8: Table 1; Fig. 2). Samples from the Chiricahuas were significantly lower than those from the Santa Ritas and Durango ($p < 0.05$) and samples from Durango were significantly higher than Nuevo León ($p < 0.05$).

3.3. Reverse-phase high performance liquid chromatography (RP-HPLC)

Based on five individuals, venom samples from the Chiricahua Mountains showed nearly identical RP-HPLC profiles (Figs. 3 and 4). Arizona populations of *C. p. pricei* (Chiricahua, Santa Rita, and Pinaleño Mountains) showed similar RP-HPLC profiles, with only minor variation in peak area and elution time of toxin peaks (Fig. 4B). Samples were analyzed by SDS-PAGE for two venom samples with different RP-HPLC profiles: one Chiricahua sample and one Durango sample. Overall, *C. p. pricei* venoms from the Chiricahua Mountains and from Durango displayed similar venom profiles, but two distinct peaks in the Durango samples (minutes 44–48) did not occur in *C. p. pricei* venoms from U.S. localities (Fig. 5). These two peaks contain primarily PLA_2 toxins (Fig. 5B), while all PLA_2 proteins for Chiricahua Mountain venom samples elute in the peaks between minutes 59–65 (Figs. 3 and 4).

Chromatograms of venoms from the Durango, MX population of *C. p. pricei* and the *C. p. miquihuanus* population from Nuevo Léon showed similarities to each other (Fig. 6B). Likewise, no differences in major peaks were identified when crude *C. p. pricei* venom samples were compared between and within populations located in Arizona (Fig. 4B). Differences in peak height was apparent between some samples, indicating differences in concentrations of specific components, and these differences were also reflected in differences observed in enzyme activity assays (Table 1). However, the presence of two distinct peaks at 45 and 48 min were noted for venoms of *C. p. pricei* from Durango and *C. p. miquihuanus* (Fig. 6A and B); SDS-PAGE analysis indicated the presence of protein(s) in the mass range typical for PLA_2s or C-type lectins (fraction 45) and PLA_2s and SVMP fragments (fraction 48) (Fig. 5B). Because these peaks were not noted in other venom samples,

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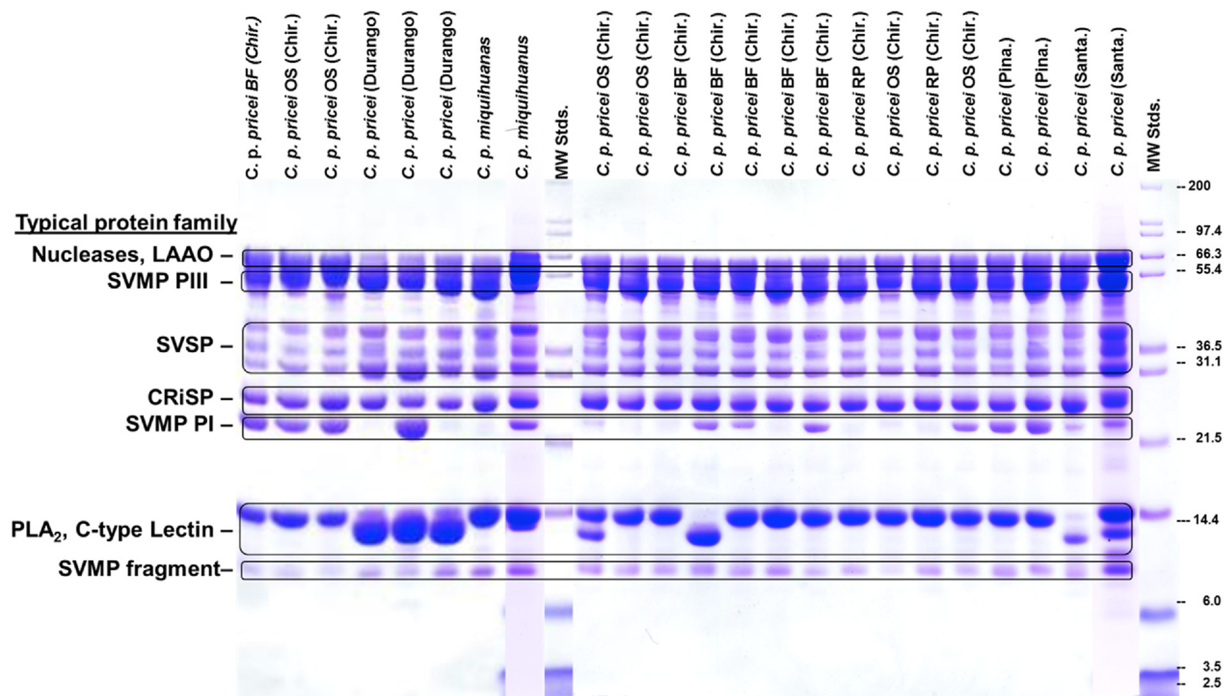

Fig. 1. SDS-PAGE gel of *Crotalus pricei pricei* (and two *C. p. miquihuanus*) venom samples from different populations and geographic locations: Chiricahua Mountains (Chir.), Pinaleño Mountains (Pina.), Santa Rita Mountains (Santa.), and Durango, México. Venom samples from the Chiricahua Mountains were collected from multiple locations including Onion Saddle (OS), Rustler's Park (RP), and Barfoot (BF). Approximate molecular weight is displayed to the right (in kDa), and typical protein families (based on published results, mass and previous purifications of rattlesnake venom toxins) are shown on the left.

they were subjected to tryptic digest followed by LC-MS/MS analysis. Fraction 45 matched several basic PLA_2 homologs that lack enzymatic activity (P86975 – 401 spectral counts; P0C616 - 235 spectral counts), and fraction 48 matched snake venom vascular endothelial growth factor (B0VXV4 – 260 spectral counts) and the same PLA_2 homolog (P86975 – 176 spectral counts).

3.4. Lethal toxicity (LD_{50}) assays

There was a slight difference in toxicity of *C. p. pricei* venom toward sympatric *S. jarrovii* and allopatric *S. jarrovii,* with values of 6.9 µg/g and 7.2 µg/g, respectively (Fig. 7); however, in terms of envenomation potential by *C. pricei*, these differences were not biologically significant. In comparison, the lethal dose of *C. p. pricei* venom was substantially lower when tested on NSA lab mice (1.25 µg/g; Mackessy, 2008) and *Hemidactylus frenatus* geckos (0.8 µg/g). Additional LD_{50} assays performed on Chiricahua Mountain *S. jarrovii* using *Crotalus lepidus klauberi* venom (also from the Chiricahuas) resulted in a similar LD_{50} value of 7.9 µg/g (Fig. 7). The assay of *C. l. klauberi* venom allowed for comparison of *C. p. pricei* results to those of a second rattlesnake that is sympatric with *S. jarrovii* throughout most of its range.

4. Discussion

The Sky Island mountain ranges and the Mexican highlands are unique habitats due to their isolated nature and stratification of climate conditions and vegetation components as elevation increases. These distinctive geographic regions are considered biodiversity hotspots, and the study of species endemic to these regions has provided essential information regarding species divergences and origins in México (Bryson et al., 2011a, b; Coblentz and Riitters, 2005; Gottfried and Hodges, 2005; Mastretta-Yanes et al., 2015; Peterson and Navarro-Sigü;enza, 1999). Due to their current isolation, and the dramatic climatic shifts that occurred during the last glacial maximum (LGM)

Table 1
Averaged enzyme activities of *Crotalus pricei* venoms ($\bar{x}$ ± SD) originating from five different localities.

Species	Location	Thr (nmol/min/mg)	Kal (nmol/min/mg)	MPr (ΔA_{342} nm/min/mg)
Crotalus p. pricei	Chiricahuas n = 14	1859 ± 719	1996 ± 1202	1.40 ± 0.12
	Pinaleños n = 2	1704 ± 339	1426 ± 218	1.56 ± 0.16
	Santa Ritas n = 2	2231 ± 1018	2374 ± 1256	1.43 ± 0.09
	Durango, MX n = 3	3333 ± 44	5419 ± 107	1.19 ± 0.15
C. p. miquihuanus	Nuevo León, MX n = 2	3236 ± 29	5201 ± 196	1.19 ± 0.06
Species	**Location**	**PLA_2 (nmol/min/mg)**	**PDE (ΔA_{400} nm/min/mg)**	**LAAO ($\triangle A_{492}$ nm/min/mg)**
Crotalus p. pricei	Chiricahuas n = 14	58.2 ± 8.7	0.033 ± 0.017	14.37 ± 2.61
	Pinaleños n = 2	53.2 ± 1.9	0.013 ± 0.009	17.92 ± 0.42
	Santa Ritas n = 2	53.2 ± 50.9	0.044 ± 0.003	22.87 ± 2.52
	Durango, MX n = 3	57.9 ± 9.4	0.184 ± 0.066	24.11 ± 3.16
C. p. miquihuanus	Nuevo León, MX n = 2	79.6 ± 11.4	0.232 ± 0.124	16.78 ± 3.34

Abbreviations: thrombin-like (Thr), kallikrein-like (Kal), metalloproteinase (MPr), phospholipase A_2 (PLA_2), phosphodiesterase (PDE), and L-amino acid oxidase (LAAO).

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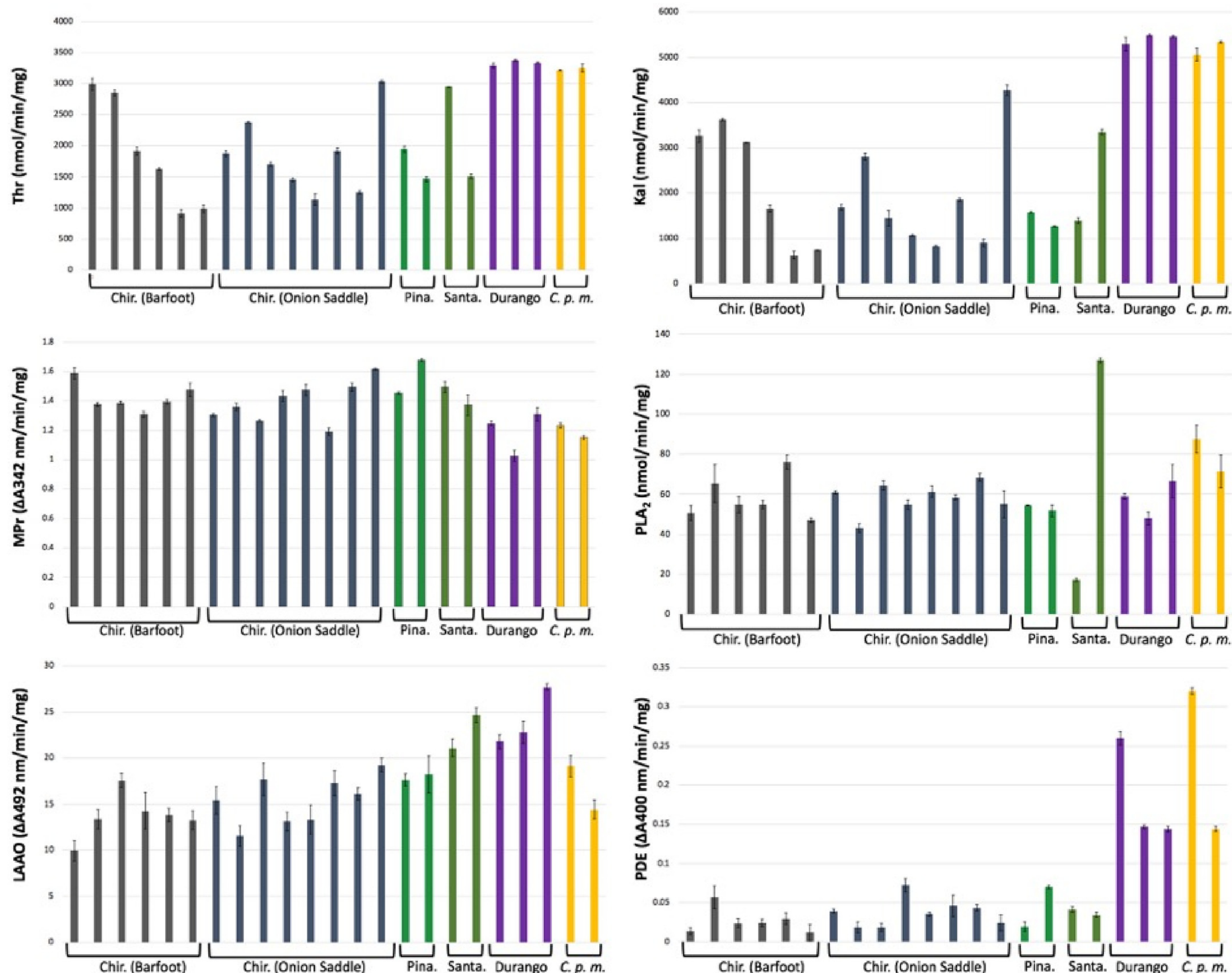

Fig. 2. Enzyme activities of *C. p. pricei* crude venoms from the Chiricahuas (Chir. (Barfoot), Chir. (Onion Saddle)), Pinaleño (Pina.), Santa Ritas (Santa.), Durango, and *C. p. miquihuanus* (C. p. m.). A. thrombin-like serine protease (Thr); B. kallikrein-like serine protease (Kal); C. azocasein metalloprotease (MPr); D. phospholipase A_2 (PLA_2); E. L-amino acid oxidase (LAAO); F. phosphodiesterase (PDE). Each bar represents an individual venom with error bars representing ± 1 SD.

(approximately 23,000–10,000 yr BP; Bryson et al., 2011a; Metcalfe et al., 2000), rapid radiation apparently occurred in various taxonomic groups, including *Sistrurus* and *Crotalus* (Blair and Sánchez-Ramírez, 2016; Castoe and Parkinson, 2006; Gottfried and Hodges., 2005; Mastretta-Yanes et al., 2015). The xerification of México and the southwestern United States led to the unique, stacked biotic communities and fauna with physiologically adapted traits present within and between Sky Islands (Arriaga et al., 2005; Thompson and Anderson, 2000). As a canonical member of these communities, *Crotalus pricei* has likely been isolated on these Sky Islands since the LGM (Bryson et al., 2011a), and venom differentiation seemed likely. Other species of high elevation rattlesnakes, such as those in the *C. lepidus* and *C. willardi* species groups, showed differential levels of certain toxins (SVSP and SVMP), but not others (PLA_2), as different subspecies/regions were compared (Saviola et al., 2017).

For some enzyme activities (thrombin-like and kallikrein-like SVSP, SVMP, PLA_2), there appears to be a geographic pattern of specific activity differences, and *C. p. pricei* venoms from Durango and *C. p. miquihuanus* are more similar in their activity levels than their Arizona counterparts. Thrombin- and kallikrein-like SVSPs, SVMP toxins, and enzymatic PLA_2 toxins are primarily lytic and hemorrhagic compounds that, together, result in incapacitation of prey via circulatory collapse, hemorrhage and myonecrosis, and they also may aid in prey predigestion (Mackessy, 2010a). All venoms analyzed show high levels of metalloproteinase activity, consistent with the hypothesis that *C. pricei* will display properties of type I venoms. In an ecological context, higher enzymatic activity may be required to immobilize prey in certain regions based on presumed ability to escape in complex environments (for example, three dimensional talus rock habitat in which *S. jarrovii* can quickly escape) or prey tolerance of venom toxicity. Due to a lack of natural history information regarding regional variation in ecology, it can only be predicted that *C. pricei* prefers to prey on *Sceloporus* lizards throughout their range, based on snakes in the Chiricahua Mountains of Arizona (Prival and Schroff, 2012). The regional differences in Mexican *C. p. pricei* and *C. p. miquihuanus* venoms, in comparison to the Arizona populations, could also be attributed to glacial patterns leading to formation of distinct populations and genetic isolation, resulting in variation between populations due to random mutations or selective pressures, and/or climate patterns favoring higher enzymatic protein activity. However, small sample sizes from several populations sampled make broad generalizations hypothetical.

Reverse phase HPLC (and SDS-PAGE) allowed for identification of differences in toxin abundance and presence between venoms of *C. p. pricei* from Arizona populations and *C. p. pricei* from Durango, México,

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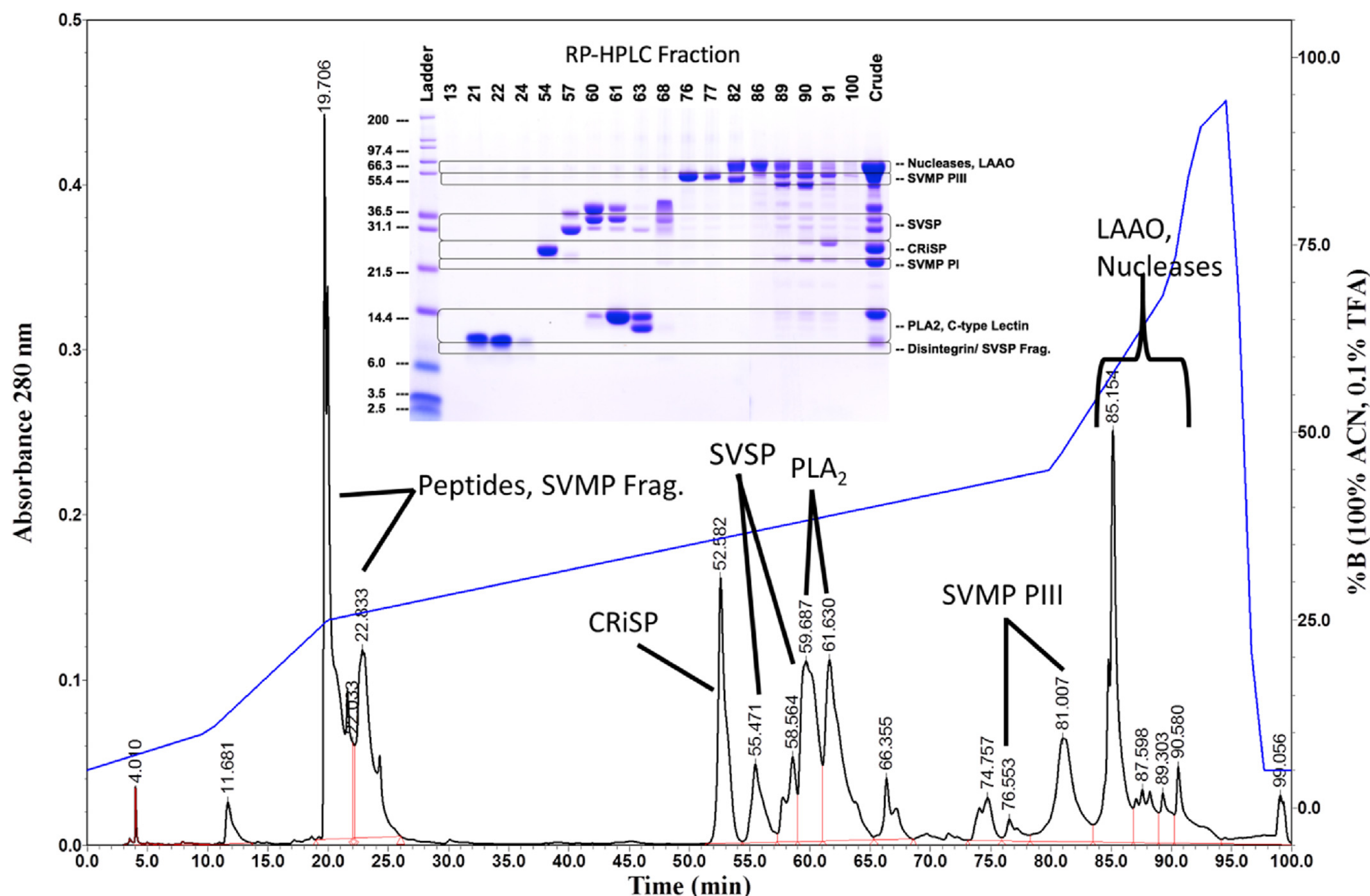

Fig. 3. Reverse-phase HPLC chromatogram and peak elution times of 2.0 mg crude *C. p. pricei* venom from the Chiricahua Mountains, AZ (Onion Saddle, snake 493). Elution gradient is displayed to the right of chromatogram. Inset: SDS-PAGE gel of each fraction peak. Protein families are displayed to the right of the gel and fraction numbers displayed at the top of the gel represent each fraction peak.

primarily in the presence of unique PLA_2 toxins in Mexican snakes (Figs. 3 and 5). The biological activities and functional significance of these PLA_2 toxins have yet to be determined, but these differences may be related to phylogeographic distribution patterns of *C. pricei.* Sky Island habitats allow for little, if any, current gene exchange between mountain ranges (Favé et al., 2015; Lomolino et al., 1989; Thompson and Anderson, 2000), and based on predicted historical distribution patterns, fragmentation of *C. pricei* populations likely occurred after the LGM, when pine-oak corridors connecting the Mexican Plateau were present (Metcalfe et al., 2000). During this time, it is estimated that dominant vegetation communities were approximately 1000 m lower than present day, associated with the cooler, wetter montane climate and more aligned with apparent physiological needs of *C. p. pricei* and other high elevation herpetofauna (Bryson et al., 2011a; McDonald, 1993; Prival and Schroff, 2012; Thompson and Anderson, 2000). Bryson et al. (2011a) found that *C. p. pricei* from Durango and *C. p. miquihuanus* from the northern Sierra Madre Occidental (Nuevo Léon) were genetically more similar, based on a mixed-model Bayesian approach. This apparent genetic similarity, inconsistent with current taxonomy, could account for the venom similarities between *C. p. pricei* from Durango and *C. p. miquihuanus* and differences between these groups and *C. p. pricei* from Arizona. Additional samples from the Pinaleño, Santa Rita, and Huachuca Mountain ranges are needed to provide substantial support for levels of toxin variation and differentiation within Arizona populations of *C. p. pricei,* though no consistent differences were apparent from the samples analyzed in the present study. Furthermore, many of the venom samples collected from snakes originating in the Chiricahua Mountains were located in only two distinct areas, and sampling multiple areas within each mountain range could reveal local variation in venom composition. However, multiple sampling visits to other Arizona Sky Island habitats for *C. p. pricei* in two distinct seasons in 2018 yielded no specimens, so the vicariant nature of field sampling can limit broader interpretation of results.

Lethal toxicity results indicate that some level of resistance to venom exists among populations of *Sceloporus jarrovii,* the dominant prey of *C. p. pricei.* These results are consistent with hypothesis 2, that *C. p. pricei* venom would be more toxic to a model prey organism (*Hemidactylus frenatus*) than natural prey (*S. jarrovii*). Results were similar between venom tested on allopatric *S. jarrovii* from the Dragoon Mountains, outside of the known range of *C. p. pricei,* and *S. jarrovii* from the Chiricahua Mountains that are sympatric with *C. p. pricei,* contrary to predictions of hypothesis 3, indicating that local adaptation (resistance to *C. p. pricei* venom) has not occurred. However, toxicity of *C. l. klauberi* venom (Chiricahuas) toward *S. jarrovii* from the Chiricahuas was also similar to that observed for *C. pricei* (6.9 µg/g vs 7.9 µg/g). We hypothesize that *S. jarrovii* may not be specifically adapted to resist *C. p. pricei* venom, but instead a more generalized resistance to venom of snakes from the genus *Crotalus* has evolved. These data also indicate that these lizards may be “evolutionarily ahead” of high elevation rattlesnake species, evolving a general adaptive resistance to toxins present in venoms of species that target them most frequently, namely *C. p. pricei* and *C. l. klauberi*. This phenomenon of toxin resistance has been reported in several taxonomic groups to varying degrees (Arbuckle et al., 2017). Specifically, the correlation between high toxicity towards model lizards (*Hemidactylus frenatus*) and a diet composed primarily of lizard prey is also apparent in the Desert Massasauga (*Sistrurus catenatus edwardsii*) (Gibbs and Mackessy,

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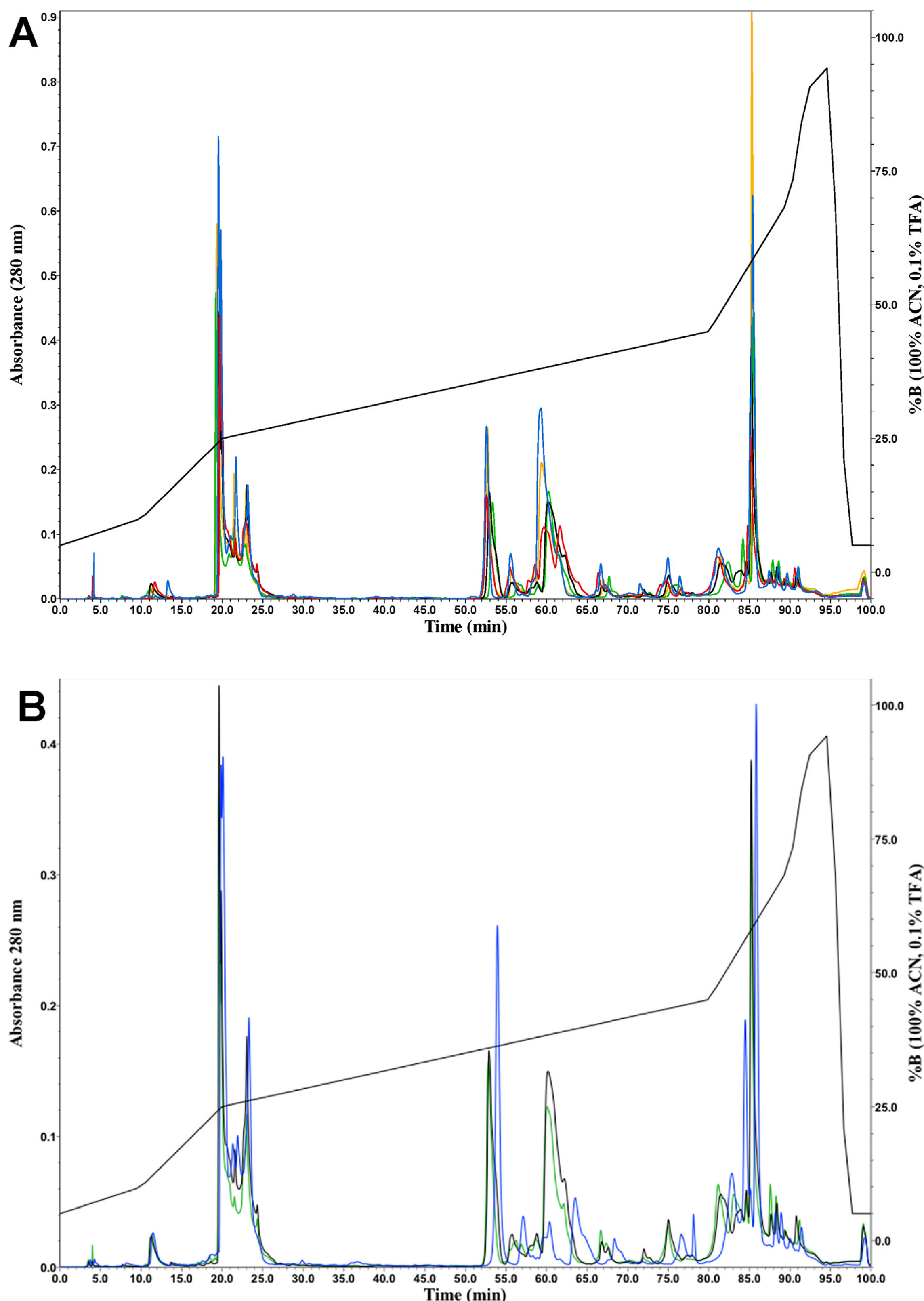

Fig. 4. Reverse-phase HPLC chromatogram overlays and peak elution times of 2.0 mg crude *C. pricei* venom samples. **A.** Samples from five individuals from the Chiricahua Mountains; note similarities in elution profiles. **B**. Samples from the Pinaleño Mountains (green), Chiricahua Mountains (black), and Santa Rita Mountains (blue). Elution gradient is indicated by the black line, with concentrations displayed on the right side of the chromatograms. (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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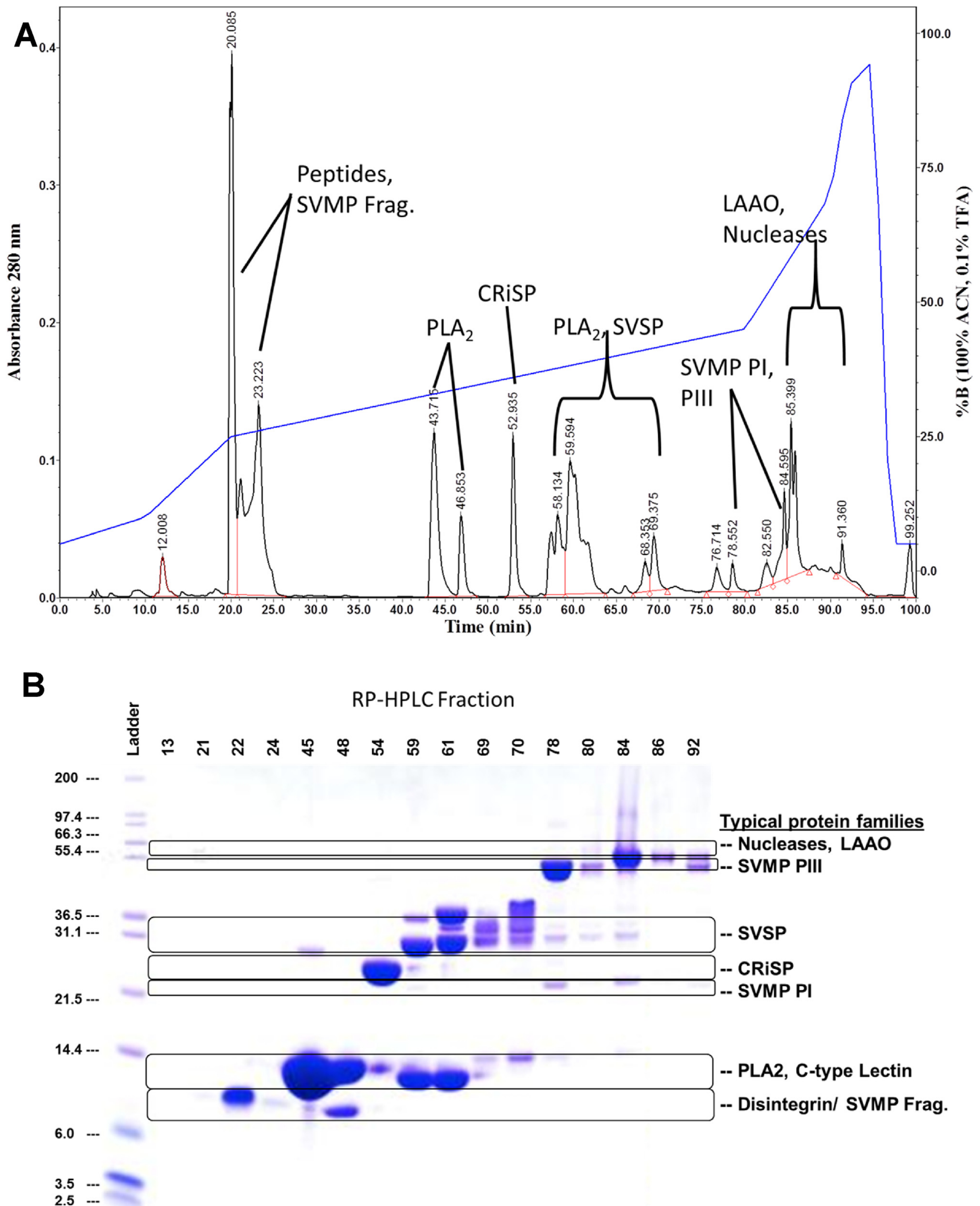

Fig. 5. A. Reverse-phase HPLC chromatogram and peak elution times of 2.0 mg crude C. p. pricei venom from Durango, México (#2) and **B.** SDS-PAGE of each fraction peak. Elution gradient is displayed to the right of chromatogram. Typical protein families are displayed to the right of the gel and numbers displayed at the top represent peak fractions.

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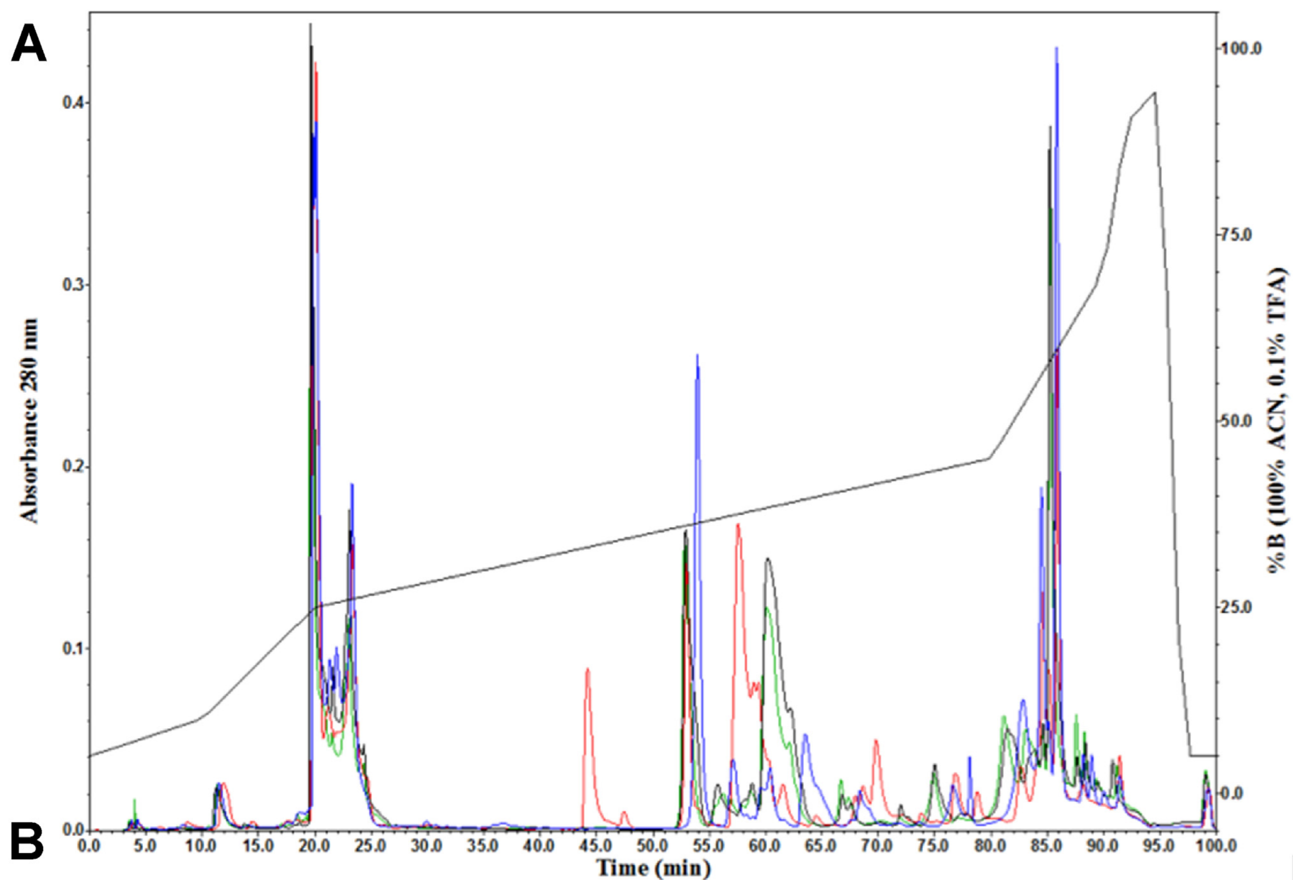

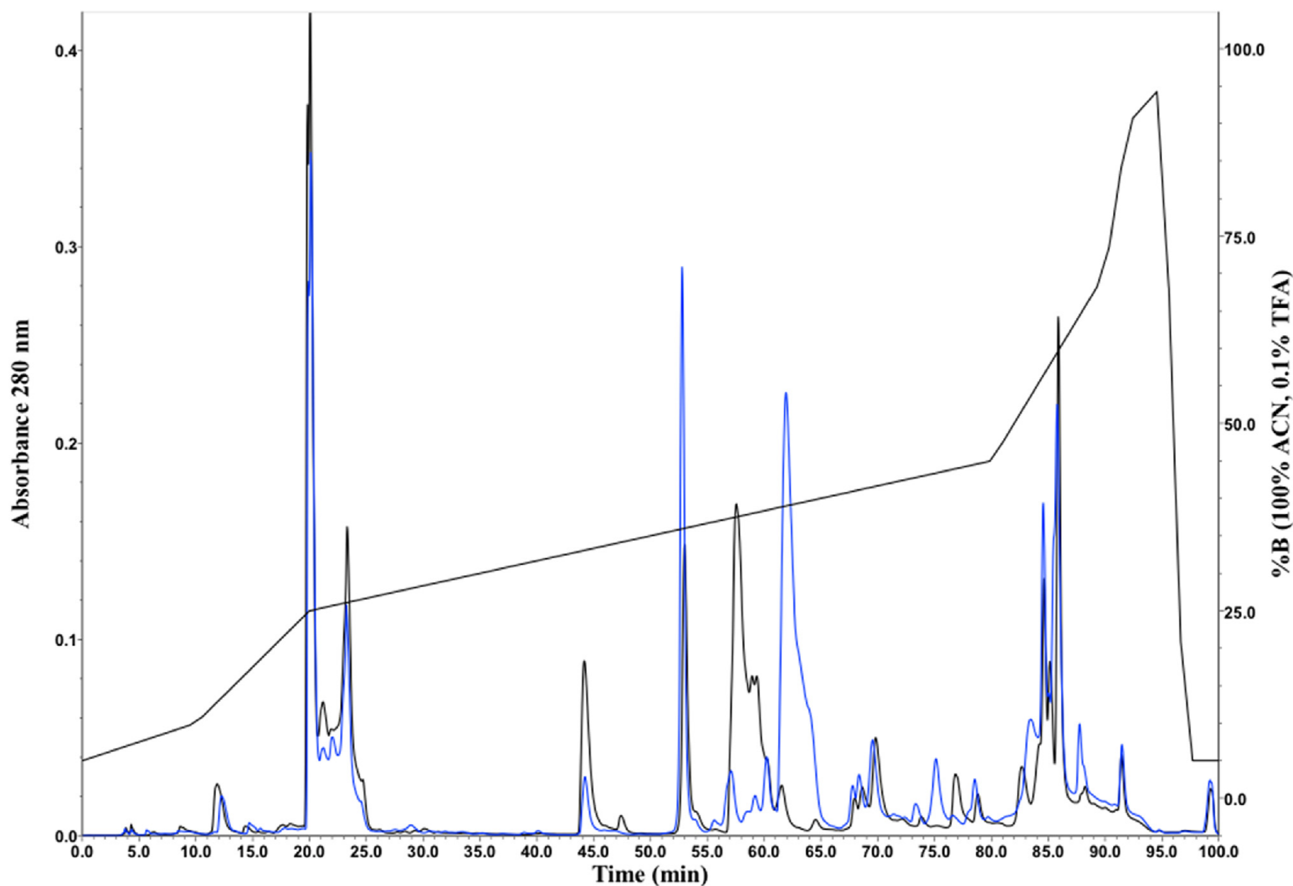

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Fig. 6. Reverse-phase HPLC chromatogram overlays and peak elution times of 2.0 mg crude *C. pricei* venom. **A.** Samples (*C. p. pricei*) from the Pinaleño Mountains (green), Chiricahua Mountains (black), Santa Rita Mountains (blue), and Durango, MX (red). **B.** Samples from Durango, MX (black) and *C. p. miquihuanus* from Nuevo León, MX (blue). Note the unique peaks at approx. 45 and 48 min in samples from México. Elution gradient is indicated by the black line, with concentrations displayed on the right side of the chromatogram. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

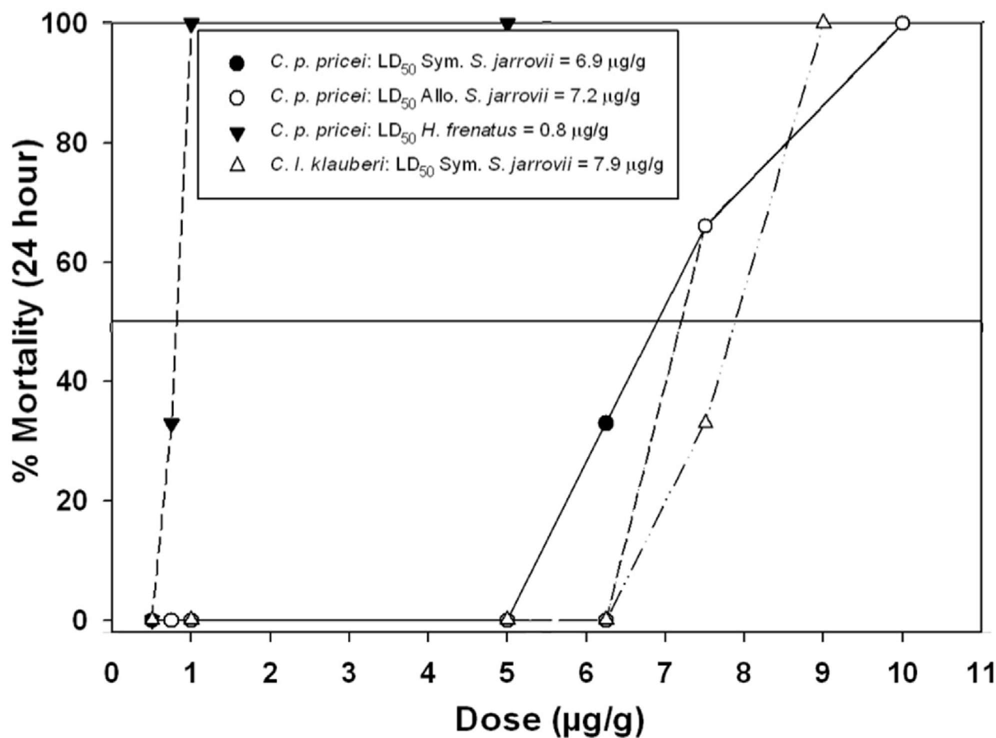

Fig. 7. Lethal toxicity (μg/g) of *C. p. pricei* and *C. l. klauberi* venom from the Chiricahua Mountains to sympatric (Sym.) and allopatric (Allo.) *Sceloporus jarrovii* and toward *Hemidactylus frenatus* geckos.

2009; Holycross and Mackessy, 2002), a species with a similar diet and body size to that of *C. p. pricei*.

4.1. Conclusions

Overall, there are well-defined similarities in venom composition between the five *C. pricei* populations surveyed, including the distribution of protein families common to all samples, as demonstrated by SDS-PAGE, enzyme assays, and comparative HPLC, but there are also several obvious differences that should be explored further. These venoms show type I venom characteristics with relatively high SVMP, SVSP, and enzymatic PLA_2 activities present, but the venom is quite toxic to mammal and lizard model species. However, native lizards (*S. jarrovii*) are an order of magnitude less sensitive to the venoms, consistent with an emerging pattern of general resistance in native prey species relative to model species (Smiley-Walters et al., 2018). The few regional differences noted in venom composition could be explained by the long period of time that these populations have been isolated and/or slight differences in natural history characteristics of each region that have yet to be explored. The differences seen between Arizona/U.S. *C. pricei* and México *C. pricei* venoms should be evaluated further, as the PLA_2 toxin homologs unique to the southern populations may have biological and functional significance.

Conflicts of interest

The authors have no conflicts of interest with this work.

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 to this article can be found online at https://doi.org/10.1016/j.toxicon.2019.09.011.

References

Peterson, A.T., Navarro-Sigüenza, A.G., 1999. Alternate species concepts as bases for determining priority conservation areas. Conserv. Biol. 13, 427–431.
Arbuckle, K., Rodríguez de la Vega, R.C., Casewell, N.R., 2017. Coevolution takes the sting out of it: evolutionary biology and mechanisms of toxin resistance in animals. Toxicon 140, 118–130.

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Armstrong, B.L., Murphy, J., 1979. The Natural History of Mexican Rattlesnakes. University of Kansas Press, Lawrence, KS.

Arriaga, L., Moreno, E., Aguilar, C., 2005. An overview of the floristic richness and conservation of the arid regions of Northern Mexico. In: Gottfried, G.J., Gebow, B.R., Eskew, L.G., Edminster (compilers, C.B. (Eds.), Connecting Mountain Islands and Desert Seas: Biodiversity and Management of the Madrean Archipelago II. USDA Forest Service Proceedings RMRS-P-36, pp. 171–175.

Barlow, A., Pook, C.E., Harrison, R.A., Wüster, W., 2009. Coevolution of diet and prey-specific venom activity supports the role of selection in snake venom evolution. Proc. Biol. Sci. 276, 2443–2449.

Beever, E.A., Brussard, P.F., Berger, J., 2003. Patterns of apparent extirpation among isolated populations of Pikas (*Ochotona princeps*) in the Great Basin. J. Mammal. 84, 37–54.

Biardi, J.E., Chien, D.C., Coss, R.G., 2006. California ground squirrel (*Spermophilus beecheyi*) defenses against rattlesnake venom digestive and hemostatic toxins. J. Chem. Ecol. 32, 137–154.

Blair, C., Sánchez-Ramírez, S., 2016. Diversity-dependent cladogenesis throughout western Mexico: evolutionary biogeography of rattlesnakes (Viperidae: Crotalinae: *Crotalus* and *Sistrurus*). Mol. Phylogenetics Evol. 97, 145–154.

Brodie III, E.D., Brodie Jr., E.D., 1990. Tetrodotoxin resistance in garter snakes: an evolutionary response of predators to dangerous prey. Evolution 44, 651–659.

Brodie Jr., E.D., Ridenhour, B.J., Brodie III., E.D., 2002. The evolutionary response of predators to dangerous prey: hotspots and coldspots in the geographic mosaic of coevolution between garter snakes and newts. Evolution 56, 2067–2082.

Bryson, R.W., Murphy, R.W., Graham, M.R., Lathrop, A., Lazcano, D., 2011a. Ephemeral Pleistocene woodlands connect the dots for highland rattlesnakes of the *Crotalus intermedius* group. J. Biogeogr. 38, 2299–2310.

Bryson, R.W., Murphy, R.W., Lathrop, A., Lazcano-Villareal, D., 2011b. Evolutionary drivers of phylogeographical diversity in the highlands of Mexico: a case study of the *Crotalus triseriatus* species group of montane rattlesnakes. J. Biogeogr. 38, 697–710.

Campbell, J.A., Lamar, W.W., 2004. The Venomous Reptiles of the Western Hemisphere, vol. II Cornell University Press, Ithaca, NY.

Castoe, T.A., Parkinson, C.L., 2006. Bayesian mixed models and the phylogeny of pitvipers (Viperidae: Serpentes). Mol. Phylogenetics Evol. 39, 91–110.

Coblentz, D., Riitters, K., 2005. A quantitative topographic analysis of the Sky Islands: a closer examination of the topography-biodiversity relationship in the Madrean Archipelago. In: Gottfried, G.J., Gebow, B.R., Eskew, L.G., Edminster, C.B. (Eds.), Connecting Mountain Islands and Desert Seas: Biodiversity and Management of the Madrean Archipelago II. USDA Forest Service Proceedings RMRS-P-36, pp. 171–175.

Davis, M.A., Douglas, M.R., Webb, C.T., Collyer, M.L., Holycross, A.T., Painter, C.W., Kamees, L.K., Douglas, M.E., 2015. Nowhere to go but up: impacts of climate change on demographics of a short-range endemic (*Crotalus willardi obscurus*) in the sky-islands of southwestern North America. PLoS One 10 (6), e0131067. https://doi.org/10.1371/journal.pone.0131067.

Favé, M.J., Johnson, R.A., Cover, S., Handschuh, S., Metscher, B.D., Müller, G.B., Gopalan, S., Abouheif, E., 2015. Past climate change on Sky Islands drives novelty in a core developmental gene network and its phenotype. BMC Evol. Biol. 15, 183.

Gibbs, H.L., Mackessy, S.P., 2009. Functional basis of a molecular adaptation: prey-specific toxic effects of venom from *Sistrurus* rattlesnakes. Toxicon 53, 672–679.

Gottfried, G.J., Hodges, D., 2005. Preface. In: Gottfried, G.J., Gebow, B.R., Eskew, L.G., Edminster (compilers, C.B. (Eds.), Connecting Mountain Islands and Desert Seas: Biodiversity and Management of the Madrean Archipelago II. USDA Forest Service Proceedings RMRS-P-36, pp. iii–iiv.

Heyborne, W.H., Mackessy, S.P., 2013. Identification and characterization of a taxon-specific three-finger toxin from the venom of the Green Vinesnake (*Oxybelis fulgidus*; family Colubridae). Biochimie 95, 1923–1932.

Holycross, A.T., Mackessy, S.P., 2002. Variation in the diet of *Sistrurus catenatus edwardsii* (Desert Massasauga). J. Herpetol. 36, 454–464.

Jones, L.L.C., Lovich, R.E., 2009. Lizards of the American Southwest: A Photographic Field Guide. Rio Nuevo Publishers, Tucson, AZ.

Jones, B.K., Saviola, A.J., Reilly, S.B., Stubbs, A.L., Arida, E., Iskandar, D.T., McGuire, J.A., Yates III, J.R., Mackessy, S.P., 2019. Venom composition and diversity in a phenotypically variable pitviper (*Trimeresurus insularis*) across the Lesser Sunda Archipelago. J. Proteome Res. 18, 2206–2220.

Kishimoto, M., Takahashi, T., 2001. A spectrophotometric microplate assay for l- amino acid oxidase. Anal. Biochem. 298, 136–139.

Lomolino, M.V., Brown, J.H., Davis, R., 1989. Island biogeography of montane forest mammals in the American southwest. Ecology 70, 180–194.

Mackessy, S.P., 1988. Venom ontogeny in the Pacific rattlesnakes *Crotalus viridis helleri* and *Crotalus viridis oreganus*. Copeia (1), 92–101.

Mackessy, S.P., 2008. Venom composition in rattlesnakes: trends and biological significance. In: Hayes, W.K., Beaman, K.R., Cardwell, M.D., Bush, S.P. (Eds.), The Biology of Rattlesnakes. Loma Linda University Press, Loma Linda, CA, pp. 495–510.

Mackessy, S.P., 2010a. The field of reptile toxinology: snakes, lizards, and their venoms. In: Mackessy, S.P. (Ed.), Handbook of Venoms and Toxins of Reptiles. CRC Press, Boca Raton, FL, pp. 1–21.

Mackessy, S.P., 2010b. The evolution of venom composition in the Western Rattlesnakes (*Crotalus viridis* sensu lato): toxicity versus tenderizers. Toxicon 55, 1463–1474.

Mackessy, S.P., Sixberry, N.M., Heyborne, W.H., Fritts, T., 2006. Venom of the BrownTreesnake (*Boiga irregularis*): ontogenetic shifts and taxa-specific toxicity. Toxicon 47, 537–548.

Mastretta-Yanes, A., Moreno-Letelier, A., Pinero, D., Jorgensen, T.H., Emerson, B.C., 2015. Biodiversity in the Mexican highlands and the interaction of geology, geography and climate within the Trans-Mexican Volcanic Belt. J. Biogeogr. 42, 1586–1600.

McDonald, J.A., 1993. In: Ramamoorthy, T.P., Bye, R., Lot, A., Fa, J. (Eds.), Phytogeography and History of the Alpine–Subalpine Flora of Northeastern Mexico. Biological Diversity in Mexico: Origins and Distribution. Oxford University Press, New York, pp. 681–703.

Metcalfe, S.E., O'Hara, S.L., Caballero, M., Davies, S.J., 2000. Records of Late Pleistocene-Holocene climatic change in Mexico—a review. Quat. Sci. Rev. 19, 699–721.

Minton, S.A., Weinstein, S.A., 1984. Protease activity and lethal toxicity of venoms from some little known rattlesnakes. Toxicon 5, 828–830.

Modahl, C.M., Mrinalini, Frietze, S.E., Mackessy, S.P., 2018. Adaptive evolution of prey-specific three-finger toxins in the Amazon Puffing Snake, *Spilotes sulphureus*. Proc. R. Soc. Lond. B Biol. Sci. 285, 20181003.

Pawlak, J., Mackessy, S.P., Fry, B.G., Bhatia, M., Mourier, G., Fruchart-Gaillard, C., Servent, D., Menez, R., Stura, E., Menez, A., Kini, R.M., 2006. Denmotoxin, a three-finger toxin from the Colubrid snake *Boiga dendrophila* (Mangrove Catsnake) with bird-specific activity. J. Biol. Chem. 281, 29030–29041.

Pawlak, J., Mackessy, S.P., Sixberry, N.M., Stura, M.H.L.D., Menez, R., Foo, C.S., Menez, A., Nirthanan, S., Kini, M., 2009. Irditoxin, a novel covalently linked heterodimeric three-finger toxin with high taxon-specific neurotoxicity. FASEB J. 23, 534–545. https://doi.org/10.1096/fj.08-113555.

Poran, N.S., Coss, R.G., Benjamini, E., 1987. Resistance of California ground squirrels (*Spermophilus beecheyi*) to the venom of the northern Pacific rattlesnake (*Crotalus viridis oreganus*): a study of adaptive variation. Toxicon 25, 767–777.

Prival, D.B., 2016. Twin-spotted rattlesnake (*Crotalus pricei*). In: In: Schuett, G.W., Feldner, M.J., Smith, C.F., Reiserer, R.S. (Eds.), Rattlesnakes of Arizona, vol. 1. Eco Publishing, Rodeo, NM, pp. 508–530.

Prival, D.B., Schroff, M.J., 2012. A 13- year study of a northern population of Twin-spotted Rattlesnakes (*Crotalus pricei*): growth, reproduction, survival, and conservation. Herpetol. Monogr. 26, 1–18.

Rex, C.J., Mackessy, S.P., 2019. Venom composition of adult Western Diamondback Rattlesnakes (*Crotalus atrox*) maintained under controlled diet and environmental conditions shows only minor changes. Toxicon 164, 51–60.

Saviola, A.J., Pla, D., Sanz, L., Castoe, T.A., Calvete, J.J., Mackessy, S.P., 2015. Comparative venomics of the Prairie Rattlesnake (*Crotalus viridis viridis*) from Colorado: identification of a novel pattern of ontogenetic changes in venom composition and assessment of the immunoreactivity of the commercial antivenom CroFab®. J. Proteom. 121, 28–43.

Saviola, A.J., Gandara, A.J., Bryson Jr., R.W., Mackessy, S.P., 2017. Venom phenotypes of the rock rattlesnake (*Crotalus lepidus*) and the ridge-nosed rattlesnake (*Crotalus willardi*) from México and the United States. Toxicon 138, 119–129.

Smiley-Walters, S., Farrell, T.M., Gibbs, H.L., 2018. The importance of species: Pygmy rattlesnake venom toxicity differs between native prey and related non-native species. Toxicon 144, 42–47.

Smith, C.F., Mackessy, S.P., 2016. The effects of hybridization on divergent venom phenotypes: characterization of venom from *Crotalus scutulatus scutulatus x Crotalus oreganus helleri* hybrids. Toxicon 120, 110–123.

Thompson, R.S., Anderson, K.H., 2000. Biomes of western North America at 18,000, 6000 and 0 ^{14}C yr BP reconstructed from pollen and packrat midden data. J. Biogeogr. 27, 555–584.

van Valen, L., 1973. A new evolutionary law. Evol. Theory 1, 1–30.