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Venom phenotypes of the Rock Rattlesnake (*Crotalus lepidus*) and the Ridge-nosed Rattlesnake (*Crotalus willardi*) from México and the United States

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ABSTRACT

Although the Mexican Highlands has the highest diversity of small-bodied rattlesnakes in the world, studies on the species found throughout this region have been relatively scarce. This has led to challenges with examining venom phenotypic characteristics, as well as species misidentifications and misclassifications. In the current study we investigated venom variation among four subspecies of *Crotalus lepidus* (*C. l. klaluberi, C. l. lepidus*, *C. l. maculosus*, *C. l. morulus*) and four subspecies of *C. willardi* (*C. w. amabilis*, *C. w. obscurus*, *C. w. silus*, and *C. w. willardi*) that inhabit regions of southwestern United States and central México. SDS-PAGE patterns show the presence of many of the major compounds found in other rattlesnake venoms, although minor variations in protein banding patterns and intensity are recognizable. Most notably, PI-metalloproteinase (SVMP) bands appear to be very faint to absent in northern *C. l. lepidus* and *C. l. klauberi* subspecies, but are fairly prominent in all other *C. lepidus* and *C. willardi* subspecies. Enzyme activity assays revealed that *C. lepidus* subspecies exhibit higher SVMP and thrombin-like activities when compared to *C. willardi* subspecies. Significant differences between subspecies were also observed for kallikrein-like serine protease, L-amino acid oxidase, and phosphodiesterase activities, although these differences appear to be random and fail to follow a geographical or phylogenetic trend. The same relationship was also observed for fibrinogenolytic and coagulation assays. Toxicity assays conducted on lab mice (*Mus musculus*), house geckos (*Hemidactylus frenatus*), and house crickets (*Acheta domestica*) revealed varying toxicities between subspecies, with *C. l klauberi* being the most toxic towards mice (LD_{50} = 1.36 μg/g) and house geckos (LD_{50} = 0.17 μg/g), and *C. w. silus* being most toxic to house crickets (LD_{50} = 1.94 μg/g). These results provide additional evidence that geographical isolation, natural selection, and adaptive evolution in response to diets may be driving forces contributing to population-level variation in venom composition.

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1. Introduction

An array of environmental factors can influence evolutionary diversification, and recognizing how a species adapts, and the mechanisms by which species arise, are fundamental areas of biological research. Large-scale landscape reconfiguration, such as the uplifting of mountains, can cause geographical isolation of previously continuous populations, interfere with gene flow, and drive patterns of phenotypic change (Scott and Reynolds, 1984; McCormack et al., 2008; Fjeldså et al., 2012; Noutsos et al., 2014). Spanning México, the Mexican highlands extend from the southern Rocky Mountains of the United States to the northern edge of Central America, comprising four major mountain ranges consisting of hundreds of kilometers of mountains and isolated peaks (Ferrusquía-Villafranca, 1990, 1993). These mountain ranges consist of the north-south trending Sierra Madre Occidental and Sierra Madre Oriental in north-central México, and the west-east trending Sierra Madre del Sur and the Trans-Mexican Volcanic Belt in south-central México (Ferrusquía-Villafranca, 1990, 1993). Vast

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intervening lowlands formed by the Chihuahuan Desert and Central Mexican Plateau have established complex but isolated assemblages of montane biotas ("sky islands"), creating a significant biodiversity hotspot for temperate, often endemic taxa (Ramamoorthy et al., 1993; Campbell, 1999; Mittermeier et al., 2005; Mastretta-Yanes et al., 2015). The Mexican highlands have therefore become a major landscape for exploring species adaptation and diversification in habitats with limited opportunities for biological dispersal (e.g., Bryson et al., 2011a).

Reptiles, and specifically rattlesnakes, represent ideal model organisms for investigating phenotypic adaptations driven by the geographical barriers of the Mexican highlands. In fact, this region is known to have the highest number of small rattlesnake species in the world (Alvarado-Díaz and Campbell, 2004), and most authors suggest that rattlesnakes originated in México and diversified in these mountainous areas (Gloyd, 1940; Klauber, 1956; Place and Abramson, 2004; Blair and Sanchez-Ramirez, 2016). Of these, the Rock Rattlesnake (*Crotalus lepidus*) is a diminutive species often found at high elevations (up to 3000 m) in central México and the southwestern United States (Campbell and Lamar, 1989; Lemos-Espinal et al., 2016, 2017). There are four subspecies of *C. lepidus* (*C. l. lepidus*, *C. l. klauberi*, *C. l. maculosus* and *C. l. morulus*; Campbell and Lamar, 1989) found throughout varying habitats that are often separated by large regions of unsuitable matrix. For instance, *C. l. lepidus* are found from southeastern New México and western Texas, south into the eastern Central Mexican Plateau in generally rocky habitat from 300 m to over 2000 m in elevation (Campbell and Lamar, 1989, 2004). *Crotalus l. klauberi* also occurs throughout the southwestern United States into northern and central México, primarily in rocky outcrops of desert grasslands and woodlands at elevations ranging from 1200 to 2500 m (Campbell and Lamar, 1989; Lowe et al., 1986). *Crotalus l. morulus*, recently elevated to a full species (Bryson et al., 2014) but retained here as a subspecies for simplicity, inhabits humid pine-oak forest in the Mexican states of Coahuila, Nuevo Leon, and Tamaulipas at elevations from 1200 to 2748 m (Campbell and Lamar, 2004), and *C. l. maculosus* is found in humid pine-oak forest in southwestern México in the states of Durango, Sinaloa, and Nayarit (Campbell and Lamar, 1989, 2004).

The Ridge-nosed Rattlesnake (*Crotalus willardi*) is a similar small-bodied rattlesnake, with adult males reaching an average length of 500 mm (Barker, 1992). *Crotalus willardi* is distributed from the sky islands of southwestern United States south into northwestern México, occurring mainly in the pine-oak woodlands of the Sierra Madre Occidental. *Crotalus willardi* is distinguishable from other species of rattlesnakes by the (usual) presence of a distinct facial pattern of pale stripes that converge dorsally to the canthus rostralis, and the tip of the snout that is distinctively raised, giving the species the common name of the Ridge-nosed Rattlesnake. Although all members of the *C. willardi* complex have this distinctive pattern of stripes, there is considerable variation in the color, breadth, and length of the stripe between populations (Campbell and Lamar, 2004), resulting in five recognized subspecies (or species, as recently proposed by Barker (2016)). *Crotalus w. willardi* is found in the Huachuca, Patagonia, Santa Rita, and Whetstone Mountains of Arizona and adjacent sky islands in northern Sonora, México (Campbell and Lamar, 1989, 2004). *Crotalus w. obscurus* has the lightest colored facial stripe (nearly absent in some individuals), is the largest of the five subspecies, and occurs in the Animas and Peloncillo Mountains of New México and in the Sierra de San Luis in northern Sonora and Chihuahua (Campbell and Lamar, 1989, 2004). *Crotalus w. silus* has the largest distribution of the five subspecies and can be found throughout the northern portion of the Sierra Madre Occidental in Sonora and Chihuahua. *Crotalus w. amabilis* appears to have the smallest known range and has only been documented from the canyons of the Sierra del Nido in north-central Chihuahua. *Crotalus w. meridionalis* is the southernmost subspecies and is found in the Mexican states of Durango and Zacatecas.

The high endemism of the Mexican highlands suggests a strong role for these mountains in driving divergence and phenotypic adaptations among venomous snakes. Although several studies have addressed the phylogenetic relationship of rattlesnakes (Bryson et al., 2011a,b, 2014) and other pitvipers (Castoe et al., 2005, 2009) in this region, little is known about venom compositional patterns of these rattlesnakes. Recently, Martínez-Romero et al. (2013) explored the biochemical and toxicological properties of *C. l. lepidus, C. l. klauberi*, and *C. l. morulus* venoms, providing a profile of venom composition among these three subspecies (see also Forstner et al., 1997). However, an examination of the venom composition of *C. l. maculosus*, as well as the *C. willardi* subspecies complex, has not been completed. Examining the venom profiles of closely related but geographically isolated rattlesnake subspecies can provide insight into the mechanisms driving venom evolution, and provide a deeper understanding of the natural history and evolutionary relationships among venomous snakes. Therefore, the current study explored the protein compositional patterns and biological activities of the venoms from all four C. *lepidus* subspecies as well as four subspecies of the *C. willardi* complex (*C. w. amabilis, C. w. obscurus, C. w. silus,* and *C. w. willardi*).

2. Materials and methods

2.1. Venoms and reagents

All venoms (*C. l. klauberi* n = 4, *C. l. lepidus* n = 5, *C. l. maculosus* n = 5, *C. l. morulus* n = 5, *C. w. amabilis* n = 6, *C. w. obscurus* n = 7, *C. w. silus* n = 4, and *C. w. willardi* n = 6; see Appendix) were extracted from adult captive snakes using standard techniques (Mackessy, 1988), briefly centrifuged, lyophilized, and stored at −20 °C until used. Lyophilized samples were reconstituted in 18.3 MΩ Millipore-filtered water and prepared at a concentration of 4.0 mg/mL for experimental use. Protein gels, mass standards, and electrophoretic reagents were obtained from Invitrogen-Life Technologies (Grand Island, NY, USA), and all additional buffers, substrates, and reagents (analytical grade or better) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). *Crotalus lepidus klauberi* (United States) was sampled under permits from the Arizona Game and Fish Department (SPM: MCKSY000221). Collecting and research in México were conducted under permits issued by SEMARNAT (SEMARNAP D00.02.-2546, D00.02.-6390; SEMARNAT OFICIO NÚM/SGPA/DGVS/3394, 4267, 5431, 1643, 2847); the USFWS approved importation of the *C. w. obscurus* (MA053885-0).

2.2. Gel electrophoresis

Individual *C. lepidus* and *C. willardi* crude venom samples were assessed for the relative number and molecular masses of venom components by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under dithiothreitol (DTT) reducing conditions. Briefly, 20 μg of venom or 5 μL of Mark 12 standards were loaded onto precast NuPage bis-tris 12% acrylamide gels and run in MES SDS running buffer for 50 min at 175 V. Gels were stained in 0.1% Coomassie brilliant blue R-250, destained with 30% methanol/7% acetic acid, and photographed using a Bio-Rad gel imaging system (Munekiyo and Mackessy, 1998).

2.3. Enzyme assays

Venoms from each of the four *C. lepidus* subspecies and four *C. willardi* subspecies were assayed for activity of enzymes commonly found in rattlesnake venoms (Mackessy, 2008, 2010a). Metalloproteinase activity was assessed using azocasein as a substrate, and thrombin-like (TLE) serine protease and kallikrein-like (KLE) serine protease activities were measured using BzPheValArg p-nitroaniline and BzProPheArg p-nitroaniline substrates, respectively (Smith and Mackessy, 2016). These proteinase substrates are hydrolyzed by several different snake venom proteinases, but in our experience with rattlesnake venoms, only SVMPs hydrolyze azocasein, and thrombin-like and kallikrein-like activities in these venoms show much higher activity toward their respective substrates relative to other serine proteinases in the venoms (Mackessy, 1993a,b). L-kynurenine was used to measure L-amino acid oxidase (LAAO) activity, and phosphodiesterase (PDE) activity was measured using the substrate bis-p-nitrophenyl phosphate, as previously described (Smith and Mackessy, 2016); phospholipase A_2 (PLA_2) activity was determined using 4-nitro-3-(octanoyloxy)benzoic acid as substrate (Holzer and Mackessy, 1996). Metalloproteinase, thrombin-like serine protease, and kallikrein-like serine protease assays were run in triplicate. All other assays were performed in duplicate due to limited samples. Results were reported as product formed/minute/mg of venom protein.

2.4. Toxicity assays

The lethal activities of pooled venoms for each subspecies were determined using non-Swiss albino (NSA) mice (*Mus musculus*), House Geckos (*Hemidactylus frenatus*), and House Crickets (*Acheta domestica*). NSA mice were bred at the University of Northern Colorado (UNC) Animal Resource Facility; geckos were obtained from Bushmaster Reptiles (Longmont, CO, USA), and crickets were obtained from a local pet supply store. All doses were adjusted to individual test animal weights. Venom doses for NSA mice (0.5, 1.0, 3.0, and 5.0 μg/g mouse body weight) and geckos (1.0, 3.0, 5.0, 7.5, and 10.0 μg/g gecko body weight) were delivered intraperitoneally in a bolus of 100 μL and 50 μL, respectively (in 0.9% saline). Doses for house crickets were delivered into the body cavity in a bolus of 10 μL (venoms diluted in Insect Ringers solution; Munekiyo and Mackessy, 1998) at concentrations of 0.5, 1.0, 2.0, 2.5, 3.0, 5.0, and 10.0 μg/g cricket body weight. However, for *C. w. amabilis* venom, an additional group receiving a dose of 12.5 μg/g was included. Three animals were used at each dosage and were monitored for 24 h post injection. Toxicity for all three models was expressed as median lethal dose (LD_{50}) within 24 h (Reed and Muench, 1938), calculated in R (3.4.0).

2.5. Fibrinogenolytic activity

Fibrinogenolytic activity was visualized by incubating 10 μg of crude venom with 2 mg of human fibrinogen in 100 mM tris-HCl buffer (pH 8.0) at 37 °C (Modahl et al., 2016). At various time intervals (0, 1, 5, 10, 30, and 60 min), 15 μL of the incubation mixture was withdrawn and added to 15 μL of termination solution (4% SDS, 10% β mercaptoethanol, 20% glycerol). All samples were boiled for 5 min before being loaded onto a NuPage bis-tris 12% acrylamide gel and run in MES SDS running buffer for 50 min at 200 V. Gels were stained, destained and photographed as mentioned above.

2.6. Coagulopathy assay

One microgram of crude venom (pooled samples; 100 μL) was incubated with human fibrinogen in 100 μL of 50 mM tris-HCl buffer (pH 7.4) at 37 °C for 1.0 min on a BHL fibrometer. Human thrombin (1U/mL; 100 μL) was then added and the fibrometer was immediately started, and time to coagulation was measured in seconds.

2.7. Statistical analyses

Enzymatic activities and coagulation times of the venoms were analyzed with a Nested Analysis of Variance (ANOVA), allowing for comparisons between species and among subspecies. A Tukey HSD test was performed post–hoc to compare all possible pairs of means, and p-values < 0.05 were considered statistically significant.

3. Results

3.1. Electrophoresis

The electrophoretic profiles of 12% polyacrylamide gels showed 16–20 proteins in venoms of all subspecies of both *C. lepidus* and *C. willardi* (Fig. 1). Dominant proteins of most bands were identified based on published reports and electrophoretic patterns for several purified enzymes (Mackessy, 2008, 2010a). Enzymes such as phosphodiesterases (PDE) and L-amino acid oxidase (LAAO) were present in all *C. lepidus* and *C. willardi* venoms at varying concentrations; *C. l. morulus* venoms appeared to have the lowest levels. PIII-snake venom metalloproteinase (SVMP) bands were also found in all four subspecies of *C. willardi* and *C. lepidus* subspecies; however, a comparison of *C. lepidus* venoms indicated higher concentrations of these enzymes in the venoms of *C. l. maculosus* and *C. l. morulus*. PI-SVMPs were present in all four subspecies of *C. willardi* and in the venoms of *C. l. maculosus and C. l. morulus*, but these enzymes appeared to be completely absent from the venoms of *C. l. klauberi*, and only very faint bands were detected in two of the five *C. l. lepidus* venom samples. Serine proteases and phospholipase A_2 (PLA_2) enzymes also showed prominent bands in all *C. willardi* and *C. lepidus* venoms, with varying levels of intensity between the different groups, and PLA_2 bands were less intense in the venoms of *C. l. morulus* when compared to other *C. lepidus*. Small molecular mass proteins (~6 kDa), likely disintegrins, were noticeably absent in the venoms of *C. l. klauberi*, while the other three *C. lepidus* and all *C. willardi* subspecies showed minor bands.

3.2. Enzyme assays

All subspecies assayed showed activity for the six enzymes commonly found in *Crotalus* venoms (Fig. 2). The Nested ANOVA results for SVMP activity (Fig. 2A) showed a significant difference between *C. lepidus* and *C. willardi* species ($F_{1,33} = 143.28$, $p < 0.001$) as well as between the subspecies ($F_{6,33} = 3.62$, $p < 0.01$). Similarly, TLE activity (Fig. 2B) showed significant differences between species ($F_{1,33} = 60.23$, $p < 0.001$) and subspecies ($F_{6,33} = 8.93$, $p < 0.001$), as did KLE (Fig. 2C): species ($F_{1,33} = 4.306$, $p < 0.05$), subspecies ($F_{6,33} = 14.9$, $p < 0.001$). PLA_2 activity (Fig. 2D), on the other hand, was not significantly different between *C. lepidus* and *C. willardi* species ($F_{1,43} = 0.153$, $p > 0.05$); however, there was a significant difference between subspecies ($F_{6,43} = 3.414$, $p < 0.01$). Tukey's post-hoc comparison showed that only the comparison between *C. l. maculosus* and *C. w. silus* was statistically significant ($p < 0.05$). LAAO activity (Fig. 2E) was significantly different between species ($F_{1,33} = 57.69$, $p < 0.001$) and subspecies ($F_{6,33} = 8.288$, $p < 0.001$) as was PDE activity (Fig. 2F): species ($F_{1,32} = 9.389$, $p < 0.01$), subspecies ($F_{6,32} = 2.756$, $p < 0.05$). For the

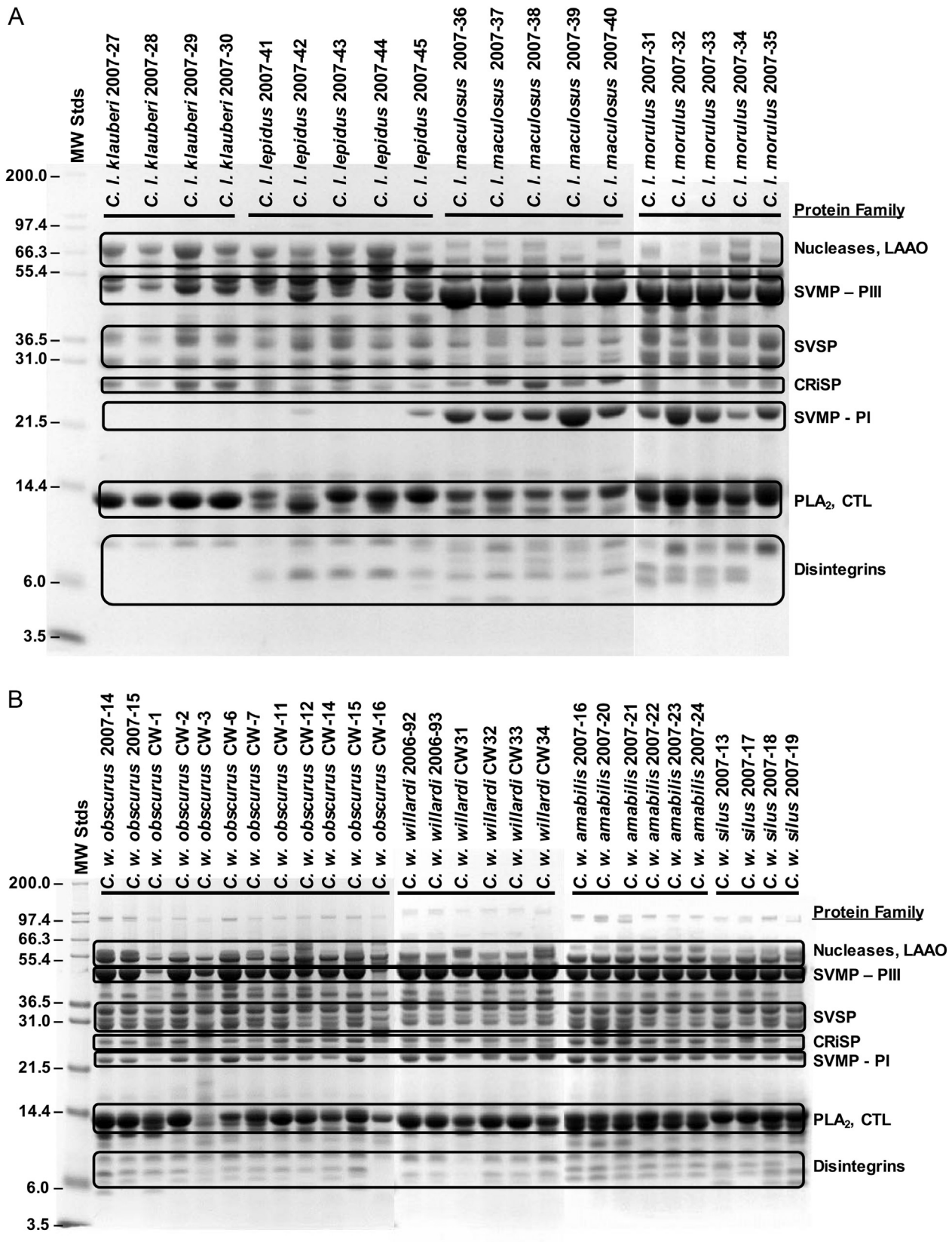

Fig. 1. Comparison of SDS-PAGE patterns of four subspecies of *Crotalus lepidus* (A) and *Crotalus willardi* (B) on NuPage gels (20 μg/lane). Typical protein families of bands of specific masses are indicated on the right; mass standards (MW Stds) are given in kilodaltons. Abbreviations: CTL – C-type lectin; LAAO – L-amino acid oxidase; PDE – phosphodiesterase; PLA_2 – phospholipase A_2; SVMP – snake venom metalloproteinase (PI and PIII classes); SVSP – snake venom serine proteinase. Note that PI-SVMPs are extremely faint to completely absent in *C. l. klauberi* and *C. l. lepidus* samples.

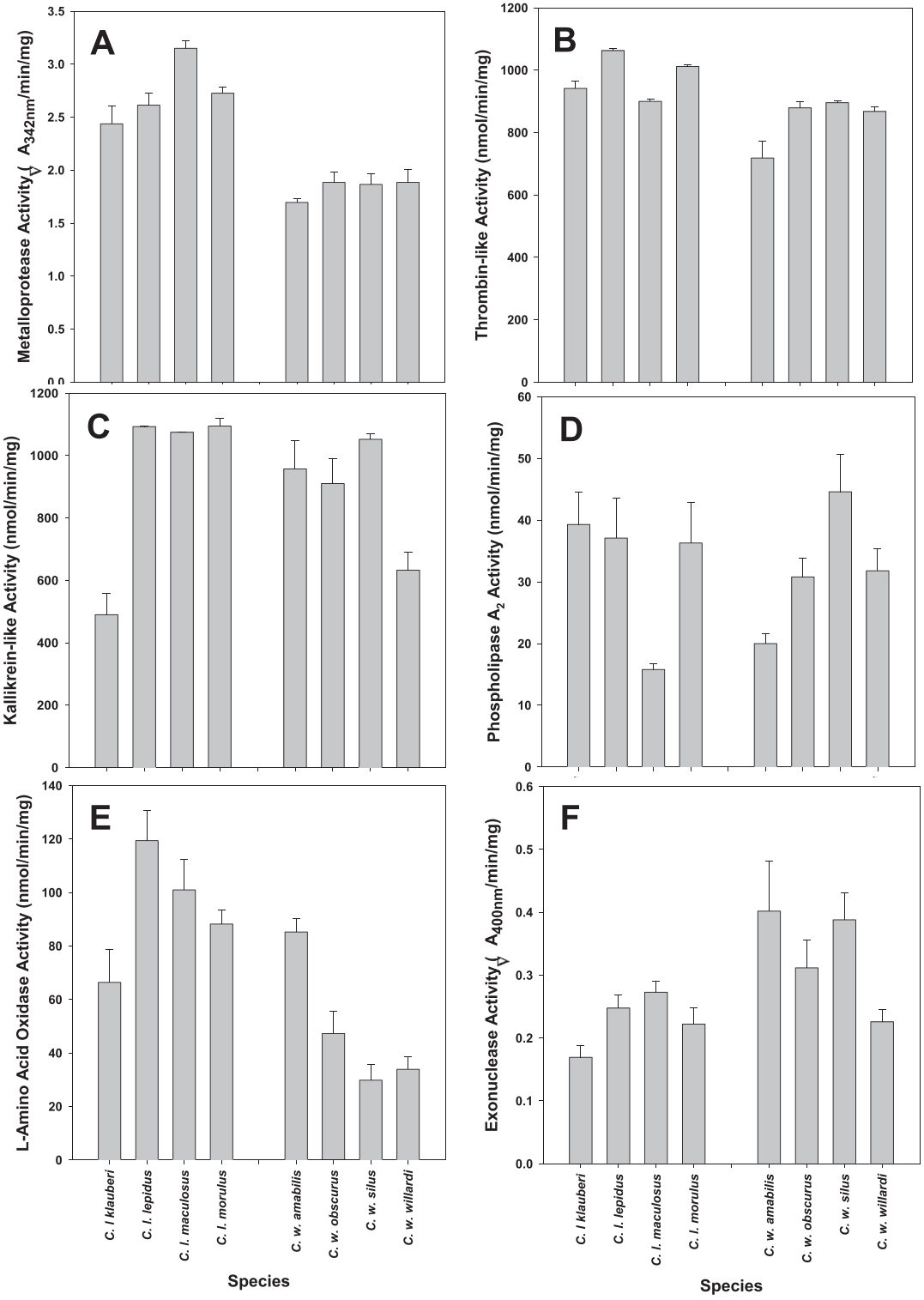

Fig. 2. Enzyme activity assays of venoms from four subspecies of *Crotalus lepidus* and *C. willardi*: metalloproteinase (SVMP) (A), thrombin-like (B) and kallikrein-like (C) serine proteases, phospholipase A_2 (D), L-amino acid oxidase (E), and phosphodiesterase (F). Activities are expressed as units/mg venom protein.

latter assay, the only comparison that was significantly different was between *C. w. amabilis* and *C. l. klauberi* ($p < 0.05$), whereas all other subspecies comparisons were not significant. Results of the Tukey's post-hoc comparisons for SVMP, TLE, KLE, and LAAO assays are shown in Table 1.

3.3. Toxicity assays

Venoms from *C. l. klauberi* and *C. l. lepidus* were more toxic toward mice than the other two subspecies, with LD_{50} values of 1.36 μg/g and 1.59 μg/g, respectively (Fig. 3). *Crotalus l. klauberi*

Table 1
Results of Tukey's post-hoc comparisons of SVMP, TLE, KLE, and LAAO enzymatic activity assays of crude venoms from four subspecies of *C. lepidus* and *C. willardi*.

	CLK	CLL	CLMa	CLMo	CWA	CWO	CWS	CWW
SVMP Activity								
CLK	–	NS	*	NS	**	NS	NS	NS
CLL	NS	–	NS	NS	***	**	**	**
CLMa	*	NS	–	NS	***	***	***	***
CLMo	NS	NS	NS	–	***	***	***	***
CWA	**	***	***	***	–	NS	NS	NS
CWO	NS	**	***	***	NS	–	NS	NS
CWS	NS	**	***	***	NS	NS	–	NS
TLE Activity								
CLK	–	NS	NS	NS	***	NS	NS	NS
CLL	NS	–	*	NS	***	**	NS	***
CLMa	NS	*	–	NS	**	NS	NS	NS
CLMo	NS	NS	NS	–	***	NS	NS	*
CWA	***	***	**	***	–	**	**	*
CWO	NS	**	NS	NS	**	–	NS	NS
CWS	NS	NS	NS	NS	**	N	–	NS
KLE Activity								
CLK	–	***	***	***	***	**	***	NS
CLL	***	–	NS	NS	NS	NS	NS	***
CLMa	***	NS	–	NS	NS	NS	NS	***
CLMo	***	N	NS	–	NS	NS	NS	***
CWA	***	S	NS	NS	–	NS	NS	*
CWO	**	NS	NS	NS	NS	–	NS	NS
CWS	***	NS	NS	NS	NS	NS	–	**
LAAO Activity								
CLK	–	*	NS	NS	NS	NS	NS	NS
CLL	*	–	NS	NS	NS	***	***	***
CLMa	NS	NS	–	NS	NS	**	***	***
CLMo	NS	NS	NS	–	NS	NS	**	**
CWA	NS	NS	NS	NS	–	NS	**	**
CWO	NS	***	**	NS	NS	–	NS	NS
CWS	NS	***	***	**	**	NS	–	NS

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS – not significant, $p > 0.05$. CLK – *C. l. klauberi*; CLL – *C. l. lepidus*; CLMa – *C. l. maculosus*; CLMo – *C. l. morulus*; CWA – *C. w. amabilis*; CWO – *C. w. obscurus*; CWS – *C. w. silus*; CWW – *C. w. willardi*.

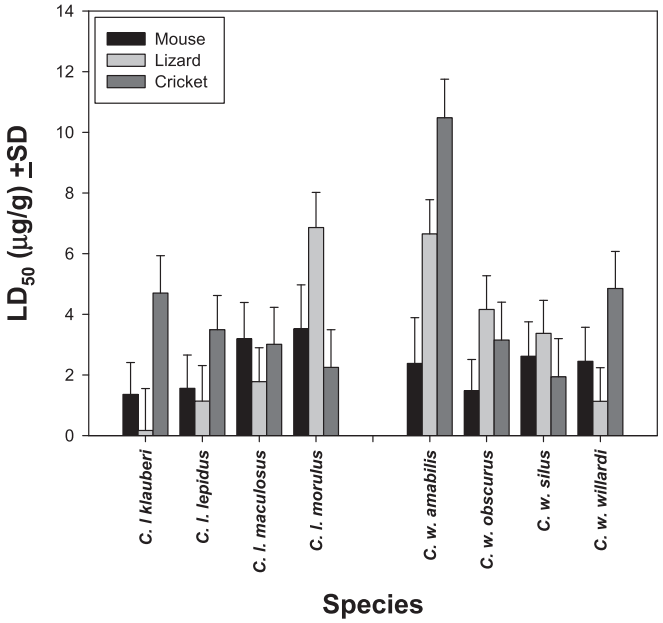

Fig. 3. Lethal toxicity (LD_{50}) of venoms toward mouse, lizard and cricket species. For *C. lepidus*, *C. l. klauberi* venom was most toxic toward both mice and lizards, while *C. l. morulus* venom was most toxic toward crickets; for *C. willardi*, *C. w. obscurus* venom was most toxic toward mice. For both species, the northern-most subspecies showed the most toxic venoms toward vertebrates.

venom was also the most toxic toward lizards (0.17 μg/g) and the least toxic toward crickets (4.70 μg/g). Venoms from *C. l. morulus* showed the opposite pattern, being the least toxic toward lizards (6.68 μg/g) and the most toxic towards crickets (2.25 μg/g). Among the four *C. willardi* subspecies, venoms from *C. w. obscurus* were more toxic toward mice, with an LD_{50} value of 1.58 μg/g. Of the four subspecies, *C. w. willardi* venom was the most toxic toward lizards (1.13 μg/g) while the venom from *C. w. silus* was the most toxic toward crickets (1.94 μg/g).

Venoms from *C. w. amabilis* showed lower toxicity toward crickets (10.48 μg/g) and lizards (6.65 μg/g). The numerical results of toxicity assays are summarized in Table 2.

3.4. Fibrinogenolytic activity

Venoms from *C. l morulus* and *C. l. maculosus* showed potent fibrinogenolytic activity and completely degraded the alpha and beta fibrinogen subunits within 1 min of incubation (Fig. 4A). Venoms from *C. l. klauberi* and *C. l. lepidus* also degraded the alpha and beta subunits of fibrinogen, after 5 min incubation (Fig. 4A). Similarly, venoms from *C. w. willardi* completely digested the alpha and beta subunits within the first 5 min of incubation, whereas venoms from *C. w. silus* digested the alpha subunit within the 5 min of incubation, but the beta subunit, although appearing to show a noticeably lower concentration after 30 min incubation, was not completely degraded even after 60 min (Fig. 4B). The degradation pattern of *C. w. amabilis* was similar to that observed for *C. w. silus*, with the alpha subunit being completely degraded after 1 min of incubation, while the beta subunit was not completely degraded until 30 min. The fibrinogenolytic pattern produced by venom of *C. w. obscurus* was clearly different than those of the other three subspecies, and the alpha subunit of fibrinogen was not completely degraded until after 10 min of incubation. The beta subunit was stable for 30 min and began to show signs of degradation only after 60 min of incubation. None of the venoms of either subspecies degraded the gamma chain.

3.5. Coagulation assay

The serine protease thrombin plays an important role in the blood coagulation cascade by converting native fibrinogen into fibrin (Tanaka et al., 2009), a critical step in the polymerization reaction for clot formation. Results of the clot prolongation assay indicate that there was no difference between *C. lepidus* and *C. willardi* species ($F_{1,20} = 0.147$, $p > 0.05$); however, there was a significant difference at the subspecies level ($F_{8,20} = 34.373$, $p < 0.01$). For *C. lepidus* subspecies, *C. l. maculosus* had the highest average prolonged clotting time (46 s), followed by *C. l. morulus* (34 s) and *C. l. klauberi* (30 s), while *C. l. lepidus* had the lowest

Table 2
Lethal toxicity of crude venoms from four subspecies of *Crotalus lepidus* and *C. willardi* toward three model organisms. Mean LD_{50} values are expressed with 95% confidence intervals (C.I.) in parentheses.

Venom	LD_{50},μg/g (C.I.)		
	Mice	Lizards	Crickets
C. l klauberi	1.36 (1.04–1.65)	0.17 (0.109–0.240)	4.70 (2.6–6.87)
C. l. lepidus	1.59 (.845–2.33)	1.16 (0.919–1.41)	3.41 (2.58–4.23)
C. l. maculosus	3.19 (2.8–3.65)	1.78 (1.36–2.18)	3.01 (2.21–3.84)
C. l. morulus	3.52 (2.62–4.28)	6.86 (4.60–8.0)	2.25 (1.64–2.68)
C. w. amabilis	2.38 (1.4–3.41)	6.65 (5.69–7.4)	10.48 (8.7–11.77)
C. w. obscurus	1.58 (0.94–2.22)	4.22 (3.31–5.13)	3.15 (2.56–3.76)
C. w. silus	2.62 (1.71–3.53)	3.37 (2.64–4.06)	1.94 (0.24–3.58)
C. w. willardi	2.45 (1.93–2.99)	1.13 (0.57–1.69)	4.85 (3.79–5.91)

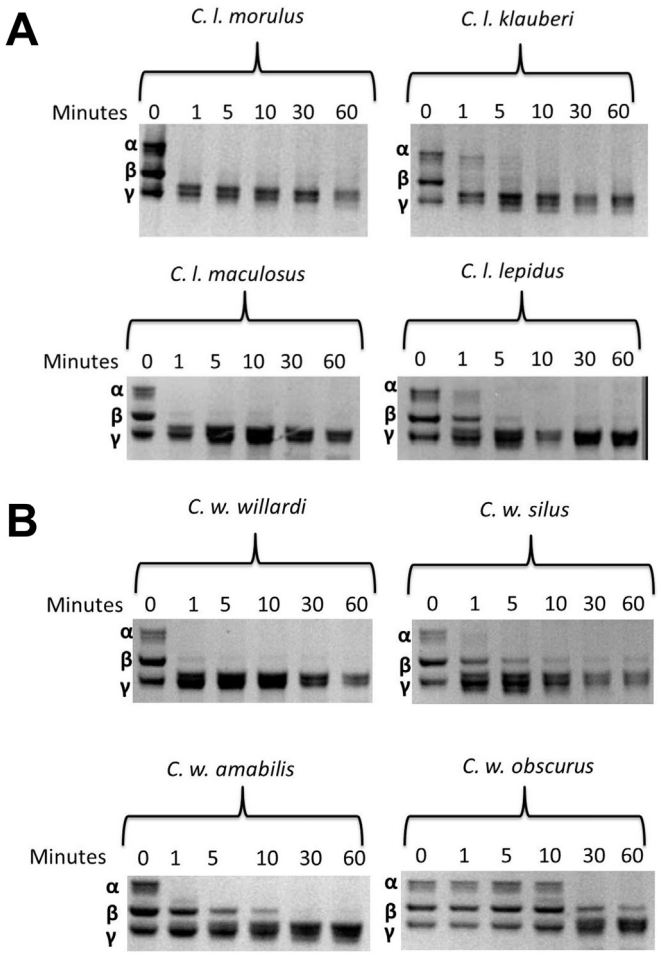

Fig. 4. Fibrinogen digest gel assays showing the degradation of subunits after incubation with venom from subspecies of *Crotalus lepidus* (A) and *Crotalus willardi* (B) during specific time intervals (1, 5, 10, 30, and 60 min). Note that all venoms rapidly catalyze the hydrolysis of the alpha subunit, with *C. w. obscurus* venom showing very weak activity toward either alpha or the beta subunits. Figures show one representative experiment for each subspecies; assays of all samples for each subspecies showed very similar results.

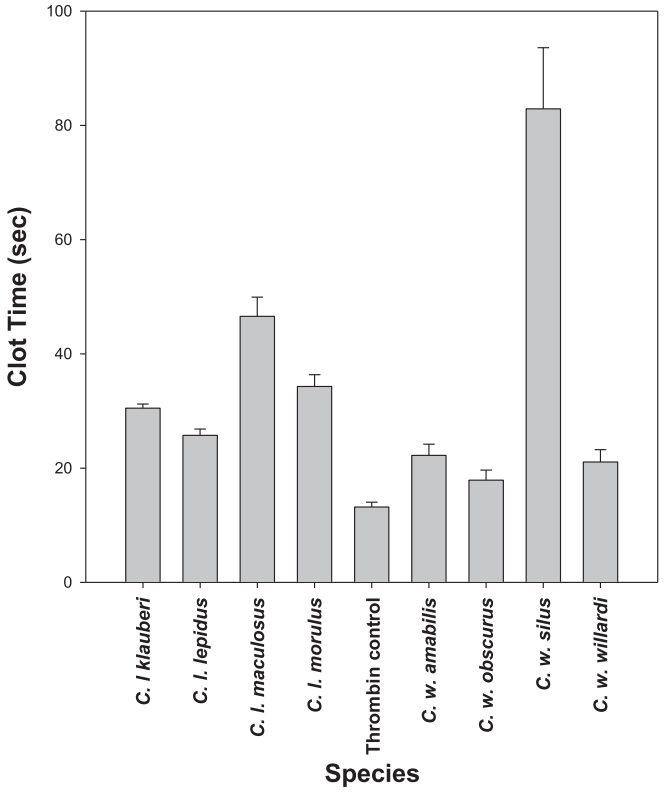

Fig. 5. Prolongation of thrombin-induced clotting time (in sec) observed from the venom of four subspecies of *Crotalus lepidus* (A) and *Crotalus willardi* (B). All clotting times for venom-treated samples were statistically different from the thrombin controls (Tukey HSD test).

clotting time (25 s) (Fig. 5). None of these comparisons were significantly different following Tukey's post-hoc analysis (all p's > 0.05). Both *C. l maculosus* and *C. l. morulus* venoms were statistically significant when compared to the thrombin control ($p < 0.05$), and *C. l. maculosus* was significantly different compared to *C. w. amabilis* and *C. w. obscurus* venoms (both p's < 0.05). For *C. willardi* subspecies, *C. w. silus* exhibited the highest average clotting time (83 s) followed by *C. w. amabilis* (22 s), and then *C. w. willardi* (21 s), while *C. w. obscurus* had the lowest clotting time (18 s) of all four subspecies venoms. Tukey's post-hoc analysis indicated that, in addition to the thrombin control, *C. w. silus* venom was significantly different when compared to all other *C. willardi* and *C. lepidus* subspecies (all p's < 0.05). No other comparisons were statistically significant (all p's > 0.05).

4. Discussion

The complex biogeographical, geological, and climatic history of the Mexican Highlands has turned this North American biodiversity hotspot into an area of considerable scientific research (Bryson et al., 2011a,b, 2014, 2017; Mastretta-Yanes et al., 2015; McCormack et al., 2008). Studies conducted in this region have greatly contributed to our understanding of the diversification of North American fauna and flora (Mastretta-Yanes et al., 2015). Historically, however, Mexican montane rattlesnakes have been difficult to define unambiguously, leading to misidentifications and misclassifications (Alvarado-Díaz and Campbell, 2004; Bryson et al., 2014), as well as presenting challenges for examining their venom phenotypes.

Despite the wide variety of habitats and extensive home ranges occupied by *C. lepidus* and *C. willardi* subspecies, examination of the SDS-PAGE banding patterns revealed a high degree of conservation between all *C. willardi* and all *C. lepidus* venoms. Specifically, all venoms consist predominately of enzymatic LAAOs, SVMPs, serine proteases, and PLA_2, and the non-enzymatic C-type lectins, cysteine-rich secretory proteins (CRISP), and disintegrins. A noticeable difference is seen, however, with the absence of PI-SVMP bands in the two northern *C. lepidus* subspecies, *C. l. lepidus* and *C. l. klauberi*, whereas the two southern subspecies, *C. l. maculosus* and *C. l. morulus*, exhibited prominent PI-SVMP bands, and PIII-SVMP bands are also of much greater intensity in these latter two subspecies. However, when examining SVMP activity toward azocasein, only the comparison between *C. l. maculosus* and *C. l. klauberi* venoms exhibited significantly different activity. The high SVMP activity observed in *C. l. maculosus* and *C. l. morulus* venom (though *C. l. morulus* was not statistically significant from the other *C. lepidus* subspecies) is likely due to the fact that PI-SVMPs generally have higher proteolytic activity than other venom components (Herrera et al., 2015; Gonçalves-Machado et al., 2016). Martínez-Romero et al. (2013) also reported a similar pattern of SVMP activity towards azocasein, with *C. l. morulus* exhibiting the highest activity, followed by *C. l. lepidus* and *C. l. klauberi*; *C. l. maculosus* was not

evaluated in their study. Interestingly, although all *C. willardi* subspecies exhibited similar patterns of both PI and PIII-SVMP protein bands, and similar SVMP activities, their activities were overall significantly lower when compared to *C. lepidus*.

In addition to SVMP activity, the venoms of all subspecies demonstrated activities towards thrombin-like serine protease (Fig. 2B), kallikrein-like serine protease (Fig. 2C), PLA_2 (Fig. 2D), LAAO (Fig. 2E) and PDE (Fig. 2F) substrates, at varying levels, and in general the observed differences appear to be random and do not follow any apparent geographical or phylogenetic trend. However, there are quantitative differences regarding toxicity towards mammalian, reptilian, and invertebrate prey models that may be indicative of natural prey preference for *C. lepidus* and *C. willardi* subspecies. For example, Holycross et al. (2002a) reported that the diet of *C. l. klauberi* consisted of 55.4% lizards, 28.3% centipedes, and 13.8% mammals, and our results show that venoms from *C. l. klauberi* were most toxic toward our lizard model. This phenomenon was also seen in the venom of *Sistrurus catenatus edwardsii*, which showed a correlation between higher venom toxicity toward lizards and a greater reliance on lizard prey (Holycross and Mackessy, 2002; Gibbs and Mackessy, 2009). Comprehensive studies regarding the diet of *C. lepidus* are scarce, although the literature is rich with natural history notes reporting a variety of prey items. A generalist diet in the *Crotalus lepidus* clade may suggest that venom components evolved to target various prey items to allow for optimal foraging success. However, the venom from *C. l. morulus*, which was least toxic toward lizards (LD_{50} of 6.86 μg/g) and the most toxic towards insects (LD_{50} of 2.25 μg/g), may suggest a diet where invertebrates are a major component, but once again a more comprehensive analysis of snake diet is needed. Our results with mammals are consistent with those of Martínez-Romero et al. (2013), which showed that *C. l. klauberi* venom was the most toxic. Increased toxicity toward mammal prey may be due to a Mojave-toxin like compound that has been previously identified in several *C. l. klauberi* populations (Glenn and Straight, 1987; Rael et al., 1992; Martínez-Romero et al., 2013). However, geographical differences in the presence or absence of this compound have been reported (Glenn and Straight, 1987), and based on HPLC profiles (data not shown), the *C. l. klauberi* venoms assayed here lack Mojave toxin homologs.

Although there is limited knowledge on the diet of *C. willardi* range-wide, it has been suggested that this species consumes primarily lizard prey (Klauber, 1956). However, Holycross et al. (2002b) reported that the diet of *C. w. obscurus* consist of 26.4% lizards and 62.3% mammals. *Crotalus w. obscurus* venom also exhibited the highest toxicity towards mice, suggesting that a diet consisting predominantly of rodents has led to the evolution of a more toxic venom towards mammalian prey, as observed in *Sistrurus* (Gibbs and Mackessy, 2009). When compared to mouse LD_{50} values, *C. w. willardi* had higher toxicity toward lizards, and *C. w. silus* had the highest overall toxicity toward crickets among all *C. willardi* and *C. lepidus* subspecies. Although diet information for *C. w. silus* is also lacking, the high toxicity of this subspecies' venom towards crickets may suggest a diet high in invertebrate prey. This correlation has been seen in both *Echis carinatus* and *E. pyramidum*, which have diets consisting predominately of arthropods, and both species had significantly more toxic venoms towards scorpions when compared to vertebrate-feeding *Echis* species (Barlow et al., 2009). Clearly, comprehensive diet studies from the other subspecies of *C. lepidus* and *C. willardi* are needed to determine if the venom toxicity observed toward our model prey species correlates with toxicity toward natural prey.

Under normal physiological conditions, the serine protease thrombin specifically cleaves fibrinogen, causing the release of fibrinopeptides A and B, and polymerization into insoluble fibrin clots and blood coagulation (Tanaka et al., 2009). Venom thrombin-like serine proteases, and some SVMPs, cleave fibrin(ogen) at different sites, thus depleting major clotting factors and preventing clot formation (Mackessy, 1993a,b, 2010b; Swenson and Markland, 2005; Markland and Swenson, 2013) that leads to the severe and uncontrolled bleeding often observed in snakebite victims (Gutiérrez and Rucavado, 2000). From a trophic standpoint, this action inhibits blood coagulation and likely facilitates the distribution of additional venom components throughout the prey's circulation. Experimentally, it can be hypothesized that venoms that fail to digest fibrinogen during a 1-minute incubation period (fibrinogen digest test) will have a shorter thrombin-induced clotting time (coagulopathy assay) since fibrinogen remains available for specific hydrolysis by thrombin (the experimental control). Conversely, venoms that digest fibrinogen in less than 1 min of incubation will have less substrate available for thrombin, resulting in a longer thrombin-induced coagulation time. Venoms from *C. l. lepidus* failed to digest fibrinogen completely after 1 min and also had the shortest coagulation time (25 s), a surprising result given the clear presence of serine proteases and PIII SVMPs in this venom by SDS-PAGE, and because *C. l. lepidus* had the highest thrombin-like activity out of all of *C. lepidus* and *C. willardi* subspecies venoms examined here. Further, venom from *C. l. maculosus* completely digested fibrinogen during the 1 min incubation period and, not surprisingly, had the longest clotting time (45 s); interestingly, venoms from *C. l. maculosus* had the lowest thrombin-like serine protease activity, but obvious presence of PI-SVMPs (by SDS-PAGE). This suggests that PI-SVMPs, which are essentially absent in *C. l. lepidus* but present in *C. l. maculosus* venoms, may be a major venom component contributing to the fibrinogenolytic activity in *C. lepidus* venoms. Although the venoms of all four subspecies of *C. willardi* showed distinct patterns of fibrinogenolytic activity, the results from the coagulation assays show relatively similar clotting times for all subspecies except *C. w. silus*, which had the highest coagulation time (83 s) of all *C. willardi* and *C. lepidus* subspecies. *Crotalus w. silus* venom also exhibited the highest thrombin-like and kallikrein-like activities; however, the slight differences in these activities when compared to other *C. willardi* subspecies does not appear to be sufficient enough to explain the significantly longer coagulation. A detailed venomics analysis of venom composition may identify quantitative differences in thrombin-like, kallikrein-like, or SVMP isoforms, which when coupled with enzymatic activities may help elucidate mechanisms driving the differences observed here. It is important to note that other venom compounds, including PLA_2s, LAAOs, and C-type lectins, exhibit anticoagulant activities (see Kini, 2006 for review); however, their mechanism of action, unlike many SVMPs and TLEs, is not direct acting towards fibrinogen.

PDE and LAAO activities were detected at varying levels in the venoms of all *C. lepidus* and *C. willardi* subspecies tested. Although the biological roles of PDEs and LAAOs are somewhat elusive, the wide distribution of these compounds in viperid and elapid venoms (Dhananjaya and D'souza, 2010; Izidoro et al., 2014) suggests that these enzymes play a pivotal role in prey envenomation. PDEs can generate purine nucleosides, which may contribute to prey immobilization via hypotension (Russell et al., 1963; Aird, 2002). LAAOs, on the other hand, may contribute to venom toxicity due to the production of hydrogen peroxide during the enzymatic reaction. Some LAAOs have demonstrated activity on platelet aggregation, both induction (Rodrigues et al., 2009) and inhibition (Samel et al., 2006), in addition to inducing hemorrhage (Souza et al., 1999) and apoptotic cell death *in vitro* (Samel et al., 2006; Lee et al., 2014; Mukherjee et al., 2015). However, the exact contribution of these two enzymes to effects of envenomation by *C. lepidus* and *C. willardi* remains to be explored.

Rattlesnake venoms have been classified into type I venoms that exhibit high SVMP activity and low toxicity (>1.0 μg/g mouse body weight) and type II venoms with high toxicity (<1.0 μg/g mouse body weight) and low SVMP activity (Mackessy, 2008, 2010a). Venoms from all *C. lepidus* and *C. willardi* subspecies most closely follow a type I venom pattern, with moderate SVMP activity and median mouse toxicity values. However, *C. l. klauberi* and *C. l. lepidus* venoms showed significantly higher toxicity towards mice, approaching values that would be considered as type II venoms, but both venoms also showed moderately high SVMP activity with no evidence of Mojave toxin-like presynaptic neurotoxins. This basic observation was also reported by Martínez-Romero et al. (2013), who reported a type I classification for *C. l. morulus* venom and type II venoms for *C. l. klauberi* and *C. l. lepidus*. *Crotalus willardi* subspecies had overall lower SVMP activity when compared to *C. lepidus* subspecies, but three subspecies (*C. w. amabilis*, *C. w. silus* and *C. w. willardi*) had mouse LD_{50} values > 2 μg/g and can clearly be classified as type I venoms. Studies in very different taxa using more natural prey models to investigate venom toxicities have demonstrated strong correlations between greater toxicity and preferred prey (Mackessy, 1988; Jorge da Silva and Aird, 2001; Barlow et al., 2009), indicating that natural selection and adaptive evolution in response to diet likely drives the evolution of some venom characteristics. To understand venoms as a trophic adaptation, it is therefore most appropriate to conduct LD_{50} studies against the natural prey species in order to assess relative toxicity more appropriately. Such data can provide a better understanding of the interaction between venom compositional evolution and diet, but this approach is often not possible, because specific prey species may not be known, native species may be difficult to obtain, and institutional regulations can prohibit such assays.

Our results indicate that regardless of the relative uniform patterns of venom protein composition by SDS-PAGE, there are observable differences in enzymatic (including fibrinogenolytic) activities, prey toxicities, and effects on coagulation time of venoms from these montane rattlesnakes, though these variations do not follow any apparent trend. Venom variability may therefore be in response to local diet, and as gape-limited predators, smaller snakes must consume smaller, often ectothermic, prey items. Ontogenetic shifts in feeding habit are routinely documented among rattlesnakes, with adults consuming larger, often endothermic prey, and neonates taking smaller, generally ectothermic prey (Mackessy, 1988). This shift in prey preference often correlates with ontogenetic changes in venom composition (Mackessy, 1988; Gutiérrez et al., 1991; Alape-Girón et al., 2008; Gibbs et al., 2011) where venoms generally shift towards lower toxicities and increased enzymatic (SVMP) activity in adult snakes when compared to neonates. However, a reversed trend has recently been reported in a population of Prairie Rattlesnakes (*C. v. viridis*) from Colorado (Saviola et al., 2015), and venom paedomorphism, where adults retain venom characteristics observed in neonates, has also been documented (Mackessy et al., 2003; Calvete et al., 2009). It is likely that adaptive responses to sympatric prey populations, and isolated predator-prey arms races, contribute to the metapopulation differences commonly observed in many species venom (Barlow et al., 2009).

5. Conclusions

This study provides the first comprehensive examination of the venom characteristics from four subspecies of *C. willardi* and examines additional venom enzymatic activities and toxicities of *C. lepidus* not previously reported by Martínez-Romero et al. (2013). Venoms of these diminutive rattlesnakes appear to follow evolutionary trends seen in larger, lower-elevation species, but it should be noted that the *C. lepidus* and *C. willardi* complexes span a wide range of largely non-contiguous habitat, and our study evaluated representatives of only some populations. Phylogeographic studies have shown that the evolution of montane rattlesnakes has been strongly influenced by the formation of sky islands and geographical barriers (Bryson et al., 2011a,b), and many populations have been geographically isolated for several million years. It is likely that regional diet also contributes to the variation observed in the reported enzymatic activities and toxicities towards select prey. However, the close similarities between *Crotalus lepidus* and *Crotalus willardi* toxin families as observed by SDS-PAGE may also be explained by the fact that these two species occur sympatrically over much of their range, and in fact a hybrid individual was reported in 1989 in a more northern population (Campbell and Lamar 1989). Our study provides a baseline for species-level variation in venoms from these montane rattlesnakes that can guide future studies of these and other isolated venomous snakes. The present data, combined with future detailed proteomic analyses of both *C. willardi* and *C. lepidus* subspecies venoms, can further help elucidate the evolutionary processes that contribute to venom evolution.

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

Localities of rattlesnakes used in this study. *C. l klauberi*. USA: Arizona: Cochise Co. México: Chihuahua: Mojarachic. *C. l. lepidus*. USA: western Texas. *C. l. maculosus*. México: Durango: Los Bancos. *C. l. morulus*. México: Nuevo León: Pablillo. México: Nuevo León: Sierra Peña Nevada. *C. w. amabilis*. México: Chihuahua: Sierra del Nido. *C. w. obscurus*. México: Chihuahua/Sonora border: Sierra de San Luis. *C. w. silus*. México: Chihuahua: Mojarachic. *C. w. willardi*. USA: captive individuals originating in Arizona.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.toxicon.2017.08.016.

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