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Evolutionary trends in venom composition in the Western Rattlesnakes (*Crotalus viridis* sensu lato): Toxicity vs. tenderizers

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ABSTRACT

The Western Rattlesnake (*Crotalus viridis* sensu lato, now including *Crotalus oreganus*) is broadly distributed across the western half of the United States, northwestern Mexico and southwestern Canada, and eight subspecies are currently recognized. Although some venom characteristics have been noted for most subspecies, a systematic study of venoms from all subspecies has not been reported. Venom was extracted from snakes collected from approximate geographic range centers for all subspecies and analyzed using SDS-PAGE, MALDI-TOF mass spectrometry, enzyme and toxicity assays. Electrophoretic and mass spectrometric analyses demonstrated that small myotoxins, disintegrins and PLA_2 were abundant in most venoms. PIII and PI metalloproteinases (~54 kDa and 23 kDa, respectively) were common to all venoms except *C. o. concolor*, *C. o. caliginis* and *C.o. helleri*. Metalloproteinase activity was highest in *C. o. cerberus* and lowest in *C. o. concolor* venoms (~100-fold difference). Conversely, *C. o. concolor* venom was the most toxic and *C. o. cerberus* venom was least toxic (15-fold difference). In general, venoms with high metalloproteinase activity were less toxic (type I venoms), while venoms which were highly toxic showed low protease activity (type II venoms). Within the *C. viridis*/*oreganus* complex, these two extremes of venom compositional phenotypes are observed, and it appears that high metalloproteinase activity and high toxicity are incompatible qualities of these venoms. The functional significance of these biochemical characteristics likely relates to characteristics of prey consumed, and venoms with low metalloproteinase activity may constrain snake prey selection or foraging activity patterns.

1. Introduction

The Western Rattlesnake (*Crotalus viridis* sensu lato) complex occurs in the United States west of the Mississippi and extends from southern Canada to northern Mexico (Klauber, 1956). Many of the nine previously described subspecies occur in the southwestern United States, and local variation in morphology and coloration is often observed (e.g., Ashton, 2001). In addition, disjunct populations occur in much of the mountainous west, resulting in potential disruption of gene flow and local variation. The complex has been subject of several molecular studies to reevaluate the taxonomic status of these subspecies, and all have used mtDNA sequences (Pook et al., 2000; Ashton and de Queiroz, 2001; Douglas et al., 2002). These three studies reached somewhat different conclusions concerning species status of complex members, but the consensus opinion (see Crothers et al., 2003) largely follows Ashton and de Queiroz (2001), which recognized two species, *C. viridis* (Prairie Rattlesnake: 2 subspecies) and *Crotalus oreganus* (Western Rattlesnake: 6 subspecies). In the current paper, the classification of Ashton and de Queiroz (2001) will be followed, with the following notations: Western Rattlesnake (*C. oreganus*/*viridis*) will refer to the entire

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complex of both species, and the Coronado Island Rattlesnake, no longer recognized as a valid taxon (subsumed into *C. o. helleri*), will be referred to as *C. o. caliginis* (the previously recognized subspecies).

The evolution of venoms has allowed many species of advanced snakes to utilize chemical rather than mechanical means of dispatching prey (Kardong et al., 1997; Greene, 1997). As trophic adaptations, venoms have been shaped by many life history factors, and in most rattlesnake species, at least 20–40 protein components in the venom of a single species can be visualized by 1D electrophoresis (Munekiyo and Mackessy, 1998; Mackessy et al., 2003; Mackessy, 2008, 2009). Two-dimensional electrophoresis and mass spectrometry have indicated that there may be well over one hundred protein and peptide components (including isoforms) in most viperid venoms (Fox et al., 2002; Sanz et al., 2006). This diversity of components begs the question of why venoms should show such complex composition and why numerous apparently similar activities (such as multiple metalloproteinases: Bjarnason and Tu, 1978; Tu, 1982; Bjarnason and Fox, 1995; Fox and Bjarnason, 1995; Munekiyo and Mackessy, 1998; Fox and Serrano, 2005; and serine proteases: Pahari et al., 2007) should exist in a single venom. Venoms in general target homeostatic mechanisms of prey (e.g., Fox and Serrano, 2005), and because of a continuing "arms race" between snakes and their prey (Heatwole et al., 1999; Mackessy et al., 2003; Mackessy, 2008), multiple component venoms appear to have been subject to selection pressures to offset the possibility of development of prey immunity. In addition, at least some snake venoms show taxon-specific differences in toxicity (Mackessy et al., 2006; Pawlak et al., 2006, 2009; Gibbs and Mackessy, 2009), and these differential effects may result from selection for toxins with effects specific to a given prey type. Because the Western Rattlesnake occurs across a broad geographical area and shows age-related changes in prey, it represents an ideal species group to investigate questions of what differences in venom composition occur, why these differences evolve and how composition affects the biological role(s) of venom.

Venoms are known to vary in composition as a function of phylogenetic affinities (Tu, 1977, 1982, 1991; Mackessy, 2009), snake age (Mackessy, 1985, 1988, 1993a,b; Mackessy et al., 2003; Alape-Girón et al., 2008; Calvete et al., 2010), geography (Glenn et al., 1983; Alape-Girón et al., 2008; Núñez et al., 2009) and diet (Mackessy, 1988; Daltry et al., 1996; Mackessy et al., 2003; Sanz et al., 2006; Gibbs and Mackessy, 2009; Barlow et al., 2009), and it is likely that the venoms of the wide-ranging Western Rattlesnake are also affected by all of these factors. Although various compositional parameters have been described for many of the subspecies (e.g., Young et al., 1980; Glenn and Straight, 1982; Aird, 1984; Aird and Kaiser, 1985; Ownby and Colberg, 1987; Mackessy, 1988, 1996; Mackessy et al., 2003), there has not been a comprehensive study of numerous venom parameters by a single investigator. The present study evaluates venom composition of the nominate subspecies of the Western Rattlesnakes collected from near the center of their presumed distributions; therefore, these samples should be representatives of the "typical" composition of adult venom for each (former) subspecies. It is recognized that the above-mentioned sources of individual variation will limit the capacity of results of this study to be generalized to all populations of the Western Rattlesnake complex (but see Mackessy, 1988, 2008, 2009; Mackessy et al., 2003), but it represents an important step toward characterizing the variation present in these venoms.

2. Materials and methods

2.1. Reagents

NuPage bis-tris and casein Zymogram tris-glycine electrophoretic gels, buffers and Mark 12 molecular weight standards were obtained from Invitrogen, Inc. (San Diego, CA, USA). Protein concentration reagents were purchased from BioRad, Inc. (San Diego, CA, USA). All other reagents used (analytical grade or better) were obtained from Sigma Chemical Corp. (St. Louis, MO, USA).

2.2. Snakes and venoms

Representatives of all nine subspecies of the Western Rattlesnake were collected in the approximate center of their known distribution by Mr. Thom Moisi (California Polytechnic University, Pomona; Fig. 1); all nine snakes were adults, and only these venoms were used for toxicity assays and for venom yields. Additional samples (at least 2) were obtained for of all subspecies except *C. v. caliginis* (one sample only). Venoms were extracted manually as previously described (Mackessy, 1988) and were lyophilized and stored frozen until used. All venoms were reconstituted in ultrapure Millipore-filtered water at apparent concentrations of 4.0 mg/mL and were stored frozen at −20 °C between assays. All venoms were assayed for protein concentration (Bradford, 1976 as modified by BioRad) in triplicate (bovine γ-globulin as standard), and all activities reported are based on these values.

2.3. Enzyme assays

Metalloproteinase (azocasein), serine protease (thrombin-like: benzoyl PheValArg paranitroaniline; kallikrein-like: BzProPheArg-pNA; and plasmin-like: BzVal-LeuLys-pNA), L-amino acid oxidase, phospholipase A_2 and phosphodiesterase activities were assayed as described previously (Munekiyo and Mackessy, 1998). All assays were run in triplicate and results were reported as product formed/minute/mg venom protein (±1 SD).

2.4. Electrophoresis

NuPage bis-tris 12% acrylamide gels were loaded with 24 μg venom protein or 5 μL Mark 12 standards per lane and run in MES (N-morpholino-ethane sulfonic acid) running buffer for 50 min at 200 V. Gels were stained in 0.1% Coomassie brilliant blue R-250, destained and photographed using an Alpha Imager system. Zymogram gels (copolymerized casein) were loaded with 0.5–1.0 μg venom protein per lane and run in standard tris-glycine-SDS buffer at 4 °C

Fig. 1. Map of North America (centered on the United States) showing approximate locations of specimens sampled. Abbreviations: Ab, *Crotalus oreganus abyssus*; Ca, *C. o. caliginis*; Ce, *C. o. cerberus*; Co, *C. o. concolor*; He, *C. o. helleri*; Lu, *C. o. lutosus*; Or, *C. o. oreganus*; Nu, *C. viridis nuntius*; Vi, *C. v. viridis.*

for approximately 2 h at 100 V; gels were then developed as described previously (Heussen and Dowdle, 1980; Munekiyo and Mackessy, 1998).

2.5. Mass spectrometry

Venoms from each subspecies were subjected to analysis using an ABI Voyager DE Pro matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometer operating in linear mode. Venoms (approximately 1.0 μg) were spotted onto sinapinic acid matrix (10 mg/mL 50% acetonitrile in water) and spectra were acquired in the mass range of 0.5–15 kDa.

2.6. Toxicity assays

Venoms were evaluated for intravenous lethal toxicity using female NSA mice weighing 25–30 g (3 mice per dose). All doses were adjusted to individual weights and delivered via the caudal vein in a bolus of 100 μL (0.9% saline), and toxicity was expressed as median lethal dose (LD_{50}). Procedures were conducted as approved by the UNC-IACUC (protocol 9401).

3. Results

3.1. Venoms

Venom yields, snake masses and lengths, and general localities are shown in Table 1. Volume of venom yields increases exponentially with length as has been observed for other species (e.g., Mackessy, 1988; Mackessy et al., 2003), and the largest yields were obtained from the largest snakes.

3.2. Enzyme assays

Crude venoms were assayed for seven enzyme activities commonly found in rattlesnake venoms (Fig. 2). Thrombin-like and kallikrein-like serine protease activities were generally high in all venoms but were quite low in *C. o. cerberus* venom. Plasmin-like serine protease activity was

Table 1
Venom yields and snake sizes: subspecies of *Crotalus oreganus* and *C. viridis*.

Subspecies of *Crotalus oreganus*	Lyophilized venom mass (mg)	Snake length (SV/Tail; mm)	General locality
oreganus	159.2	830/54	San Luis Obispo Co., CA
abyssus	44.8	660/44	Colorado River, cent. AZ
caliginis	58.7	875/75	Isla Coronado, MX
cerberus	169.8	920/82	Graham Co., AZ
concolor	27.3	605/40	S. Utah
helleri	218.8	930/85	Los Angeles Co., CA
lutosus	25.3[a]	610/39	Las Vegas, NV
Subspecies of *Crotalus viridis*			
viridis	58.0	730/60	Weld Co., CO
nuntius	16.8	500/33	N. Arizona

[a] Partial yield only.

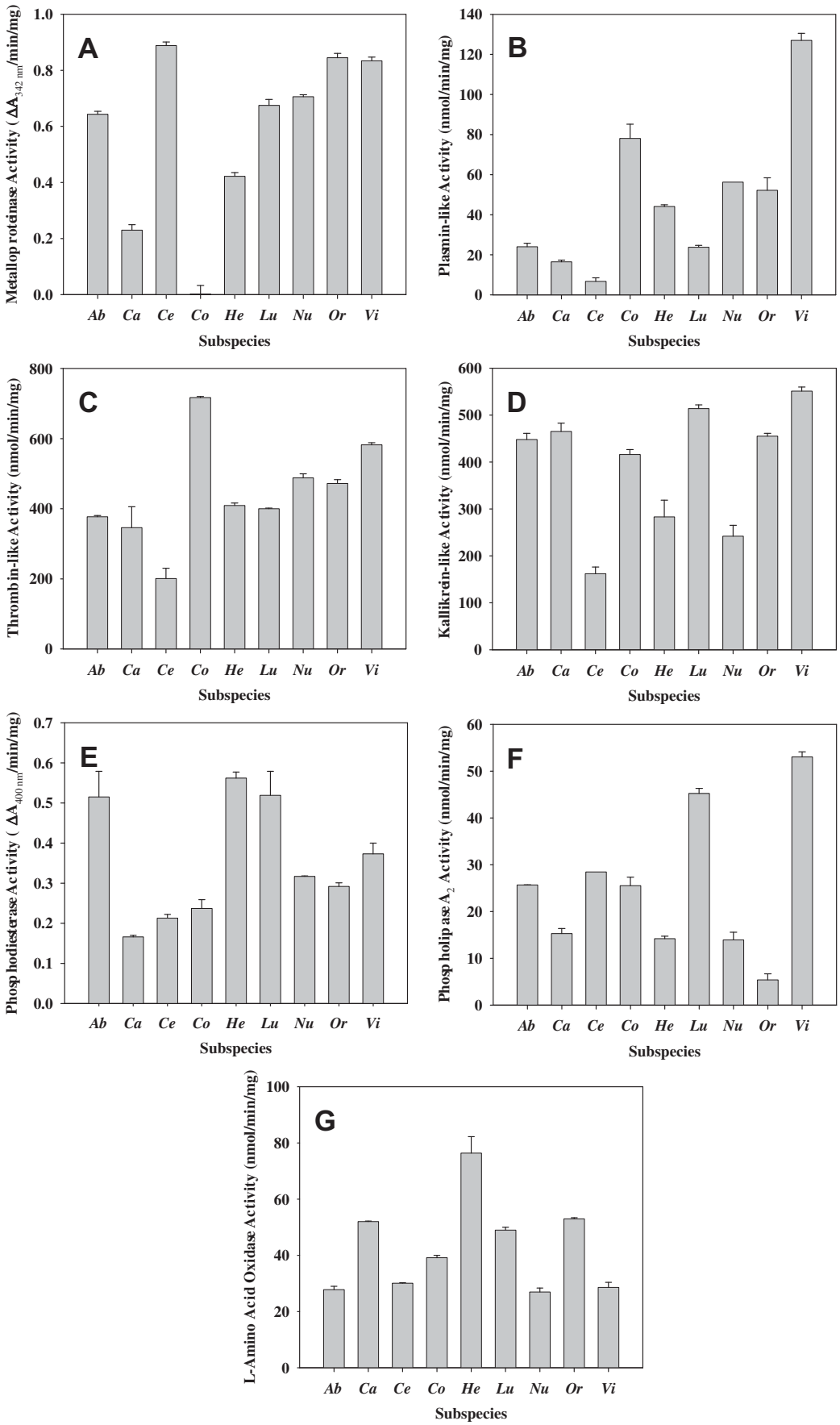

Fig. 2. Enzyme activities of venoms from the nine nominate subspecies of the *Crotalus viridis/oreganus* complex. Note that metalloproteinase activity is very low in *C. o. concolor* venom and very high in *C. o. cerberus* venom. All activities are expressed as units/mg venom protein; error bars are 1 SD. *Abbreviations: Ab, Crotalus oreganus abyssus; Ca, C. o. caliginis; Ce, C. o. cerberus; Co, C. o. concolor; He, C. o. helleri; Lu, C. o. lutosus; Or, C. o. oreganus; Nu, C. viridis nuntius; Vi, C. v. viridis.*

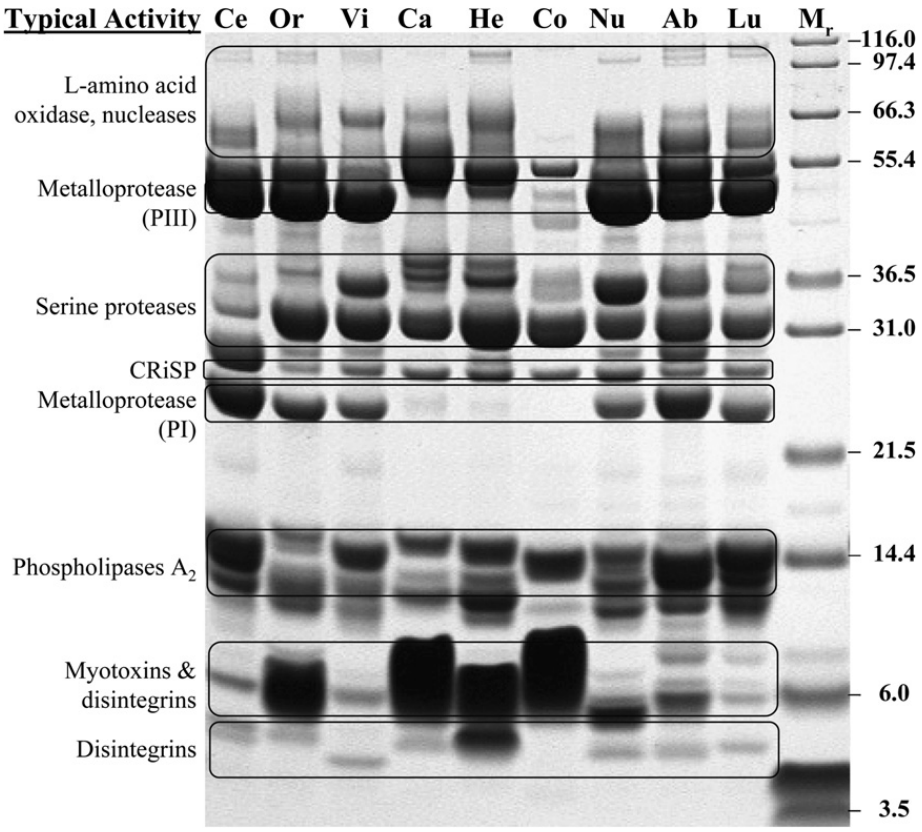

Fig. 3. Comparison of electrophoretic patterns of the nine nominate subspecies of the *Crotalus viridis*/*oreganus* complex on a bis-tris NuPage gel (24 μg venom/lane). Overall patterns are similar, but note the lack of PI and PIII metalloproteinases in *C. o. concolor, C. o. caliginis* and *C. o. helleri* venoms; differences in myotoxin amounts (band size and density) are also apparent among subspecies. Activities typical of bands of given masses are indicated. Abbreviations as in Fig. 1; M_r, Novex Mark 12 molecular weight standards (in kilodaltons).

generally low in all venoms except *C. o. concolor* and *C. v. viridis* venoms; activity was again lowest in *C. o. cerberus* venom. Metalloproteinase activity was generally high, but it was barely detectable in *C. o. concolor* venom and was very low in *C. o. caliginis* venom; activity was highest in *C. o. cerberus* venom.

Phosphodiesterase levels were lowest in *C. o. caliginis* venom and high in *C. o. abyssus*, *helleri* and *lutosus* venoms. All venoms showed moderately high L-amino acid oxidase activity, and levels were highest in *C. o. helleri* venom. Phospholipase A_2 activity was highest in *C. v. viridis* venom and lowest in *C. o. oreganus* venom.

3.3. Electrophoresis

Electrophoresis on 12% acrylamide gels demonstrated the presence of 16–30 protein bands in the venoms (Fig. 3). General identification of bands was based on comparable migration of several purified enzymes (Mackessy, 1996, 2008, and unpubl. data) and on masses reported in the literature. Metalloproteinase bands were prominent in all venoms except *C. o. concolor*, *C. o. caliginis* and *C. o. helleri* venoms, concordant with results of enzyme assays. Phosphodiesterases and other nucleases, L-amino acid oxidase, serine proteases and phospholipase A_2s were present in all venoms, though apparent concentrations varied. Low molecular weight myotoxins (molecular weight ~4 kDa) were present in all venoms; levels were very high in *C. o. caliginis*, *C. o. concolor*, *C. o. helleri* and *C. o. oreganus* venoms and in low concentrations in venoms of *C. o. cerberus*, *C. o. lutosus* and *C. v. viridis.*

All venoms showed multiple metalloproteinase activities on zymogram gels (Fig. 4), typically with several of apparent molecular masses of 19–37 kDa and 53 kDa. A high molecular mass protease (approximately 74 kDa) was observed only in the venoms of *C. o. cerberus* and *C. v.*

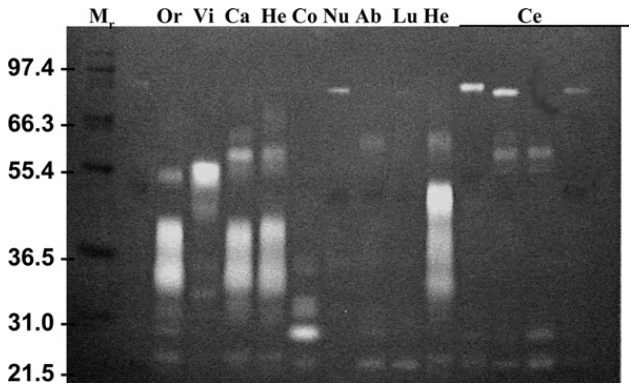

Fig. 4. Metalloproteinase activity of venoms following zymogram electrophoresis on casein copolymerized gels; 0.5 μg venom was loaded in each lane. Protease activity appears as a clear band or region. High molecular weight proteases (~75 kDa) were seen only in *C. o. cerberus* and *C. v. nuntius* venoms, and *C. o. concolor* venom apparently contains only lower mass (<35 kDa) proteases. Venoms from *C. o. oreganus*, *caliginis* and *helleri* showed similar activity profiles. Abbreviations as in Fig. 1.

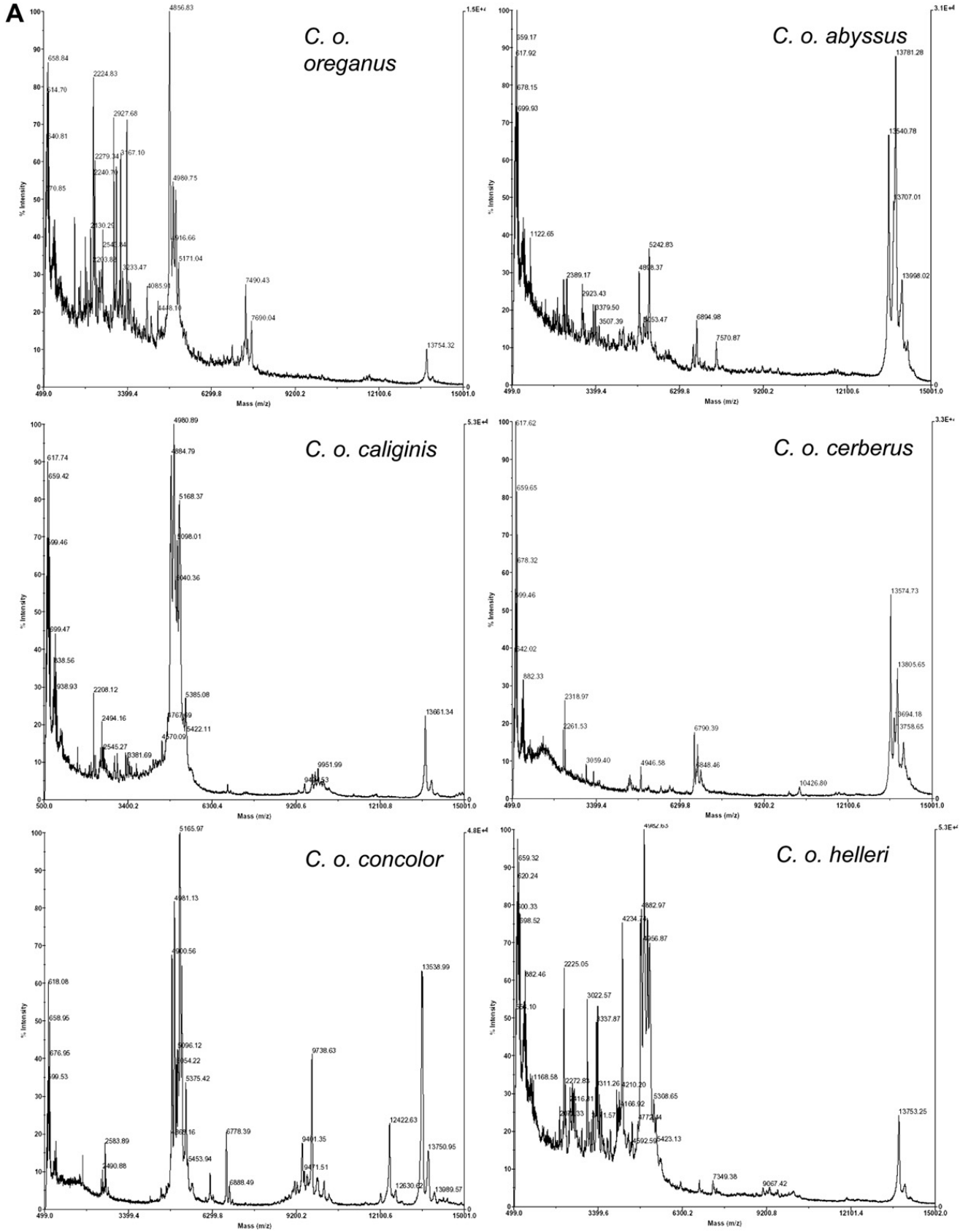

Fig. 5. MALDI-TOF mass spectra of venoms from the nine nominate subspecies of the Western Rattlesnake complex. Spectra were obtained on a Voyager DE MALDI-TOF mass spectrometer operating in linear mode with a window of 0.5–15 kDa (*m*/*z*).

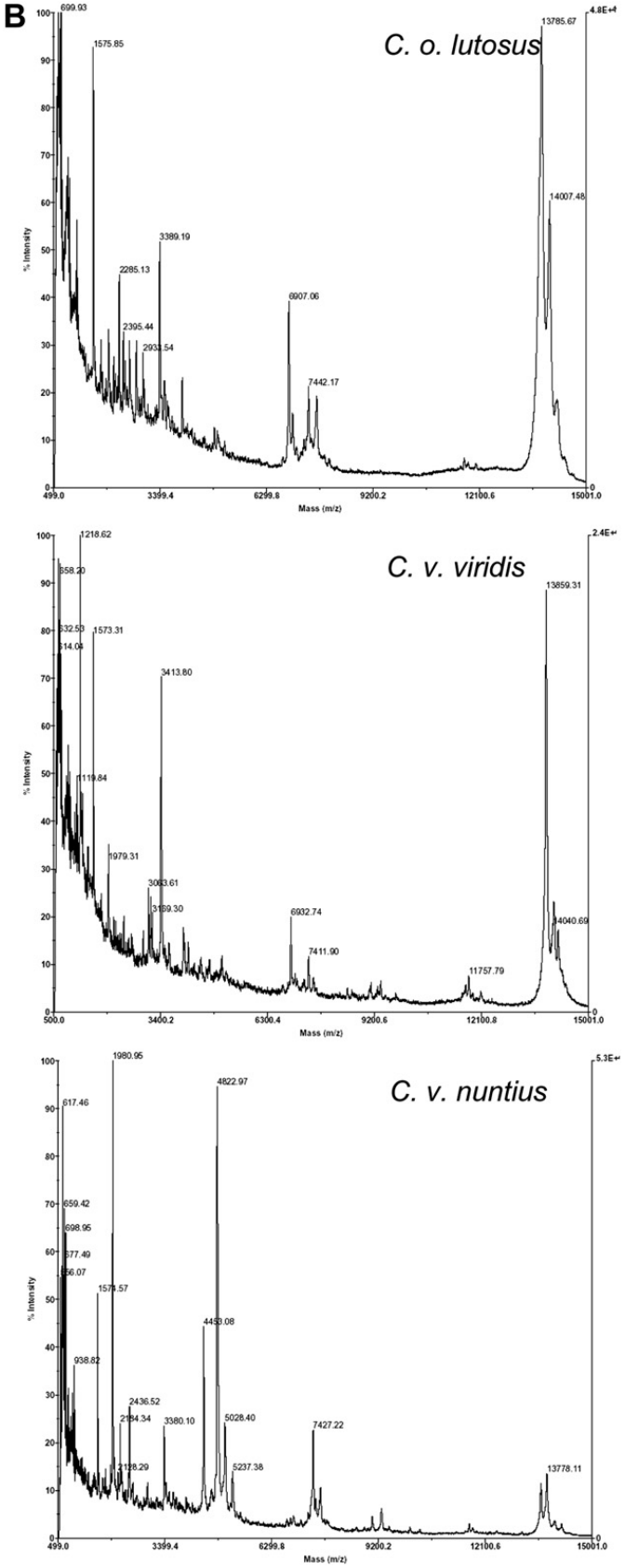

Fig. 5. *(continued).*

Table 2
Molecular masses (2–5.5 kDa) observed following MALDI-TOF mass spectrometry of crude venoms from *Crotalus oreganus* and *C. viridis* subspecies. Bold indicates peaks above 30% relative intensity.

Potential ID	*Crotalus oreganus* subspecies							*C. viridis* subspecies	
	abyssus	*caliginis*	*cerberus*	*concolor*	*helleri*	*lutosus*	*oreganus*	*nuntius*	*viridis*
C type BPP?					2072.3				
–							**2130.3**	2128.3	
–		2208.1					2203.9	2184.3	
–					**2225.1**		**2224.8**		
–							**2240.7**		
–			2261.5		2272.8	**2285.1**	**2279.3**		
–			2319.0						
–	2389.2				2416.8	2395.4		2436.5	
–		2494.2		2490.9					
–		2545.3		**2583.9**			**2543.8**		
–	2923.4					2933.5	**2927.7**		
–			3059.4		**3022.6**				3063.6
–					3171.8		**3167.1**		3169.3
–					3311.3		3233.5		
–	3379.5	3381.7			**3337.8**	**3389.2**		3380.1	**3413.8**
–	3507.4								
CTx A homolog?					4166.9		4085.9		
–					4210.2				
CTx A homolog?					**4234.7**				
–							4448.1	**4453.1**	
–		4570.1			4592.6				
–		4767.7			4772.4				
Myotoxin							**4856.8**	**4823.0**	
Myotoxin	**4898.4**	**4884.8**		4868.2	**4883.0**		**4916.7**		
Myotoxin			4946.6	**4900.6**	**4956.9**				
Myotoxin		**4980.9**		**4981.1**	**4982.6**		**4980.8**		
Myotoxin	5053.5	**5040.4**		**5054.2**				5028.4	
Myotoxin		**5098.0**		**5096.1**					
Myotoxin	**5242.8**	**5168.4**		**5166.0**			5171.0	5237.4	
Myotoxin		5385.1		**5375.4**	5308.7				
Myotoxin		5422.1		5453.9	5423.1				

Abbreviations: C type BPP, C type bradykinin-potentiating peptide; CTx A, crotoxin acidic subunit A.

nuntius, and only low molecular mass protease bands were seen in venom from *C. o. concolor*. It should be noted that mass estimates based on zymogram gels are somewhat inaccurate, so these values are most useful as relative masses. Metalloproteinase banding patterns of *C. o. caliginis*, *C. o. helleri* and *C. o. oreganus* were quite similar and were distinct from other subspecies.

3.4. Mass spectrometry

MALDI-TOF mass spectrometry of venoms revealed approximately 31 peptides with masses between 2 and 5.5 kDa in venoms of the nine nominate subspecies (Fig. 5; Table 2). In addition, approximately 26 peptides with masses of 6–15 kDa were observed in the same venoms (Fig. 5; Table 3). Peptide peaks with intensities of greater than 30% appear bolded in the tables, but it should be noted that this mass spectrometry method is not quantitative; some compounds (such as PLA_2s and CRISPs) ionize readily and efficiently, whereas others do not, so peak intensity is dependent on ionization efficiency rather than relative concentration. Peptides from different taxa were considered homologous (same line in Tables) if the masses were within 0.3% (cf. Sanz et al., 2006). Tentative protein family identifications based on mass are also given. Myotoxins and phospholipases A_2 were found in all venoms, often with several apparent isoforms, but disintegrins (7–7.8 kDa) appeared to be absent from the venoms of *C. o. abyssus*, *C. o. caliginis*, *C. o. concolor*, *C. o. helleri* and *C. v. viridis*. However, disintegrins have been isolated from some of these venoms, such as *C. v. viridis* (viridin; Scarborough et al., 1993). Further, the venoms of *C. o. abyssus*, *C. o. concolor* and *C. o. helleri* also showed ions in the range of 6.8–6.9 kDa, which may be disintegrins (Table 3, indicated by ?). Individual variation in composition, ion suppression and failure to ionize could also give rise to negative results for some venoms.

3.5. Toxicity assays

A 15-fold range in lethal toxicity toward NSA mice was observed for the nine subspecies (Table 4). Venom from *C. o. concolor* was exceptionally toxic (0.36 μg/g), and *C. o. cerberus* venom was the least toxic (5.4 μg/g). Toxicity as a function of metalloproteinase activity showed a weak correlation ($r^2 = 0.3$), and more toxic venoms tended to show lower metalloproteinase activity (Fig. 6); notably, *C. o. cerberus* and *C. o. concolor* venoms were at extreme opposites of this trend. An overlay of toxicity data on the hypothesis of phylogenetic relations among the Western Rattlesnakes (Ashton and de Queiroz, 2001) suggested that type I venom (less toxic, higher metalloproteinase activity) is a more basal trait in the *C. oreganus* clade (Fig. 7), but note that the outgroup (*Crotalus scutulatus*) utilized by Ashton

Table 3
Molecular masses (6–15 kDa) observed following MALDI-TOF mass spectrometry of crude venoms from *Crotalus oreganus* and *C. viridis* subspecies. Bold indicates peaks above 30% relative intensity.

Potential ID	*Crotalus oreganus* subspecies							*C. viridis* subspecies	
	abyssus	*caliginis*	*cerberus*	*concolor*	*helleri*	*lutosus*	*oreganus*	*nuntius*	*viridis*
–	6769.7			**6773.5**			6790.4		
Disintegrin?		6832.3	6845.0				6848.5	6817.5	
Disintegrin?	6890.2		6890.1	6888.5		**6897.4**		6893.9	6931.5
Disintegrin			**7013.6**						
Disintegrin			7038.8						
Disintegrin			7129.4						
Disintegrin							7300.2		
Disintegrin						7375.7	7355.6	**7380.3**	
Disintegrin						**7433.0**	7439.7	**7430.7**	
Disintegrin							**7493.3**		
Disintegrin	7570.9						7561.1		
Disintegrin						**7649.5**	**7693.1**	**7631.1**	
CTx A homolog?				9401.4					
CTx A homolog?		9488.1		9471.5					
CTx homolog?				**9729.3**			9699.0		
PLA_2				**12,422.6**					
PLA_2				12,630.6					
PLA_2	**13,487.2**								
PLA_2	**13,533.1**		13,531.4	**13,539.0**					
PLA_2			**13,565.2**						
PLA_2	**13,702.1**	**13,656.4**	**13,686.5**			**13,710.0**		**13,621.6**	
PLA_2	**13,775.3**		**13,796.4**	13,751.0	**13,748.6**	**13,777.4**	**13,747.5**	**13,780.6**	
PLA_2	**13,994.5**	13,868.7	13,895.9	13,989.6	13,958.1	**13,991.9**			**13,855.3**
PLA_2			**14,013.7**					13,992.7	
PLA_2			**14,083.1**						14,067.3
PLA_2	14,183.8		14,222.9			14,166.2		14,178.0	

Abbreviations: PLA_2, phospholipase A_2; CTx A, crotoxin acidic chain homolog.

and de Queiroz (2001) has one of the most toxic venoms among rattlesnakes.

4. Discussion

Venoms of snakes serve several different biological roles, but biochemical features which enhance trophic functions are likely most important. Over most of its range, *C. oreganus*/*viridis* feed on small prey (lizards, neonate rodents) as juveniles and then take larger prey (small mammals and occasionally birds) as adults (Klauber, 1956). Because *C. oreganus*/*viridis* is found over much of western North America, and because diet is generally similar for most subspecies, broad patterns of variation in venom composition were predicted to depend on geographic factors. However, there are overlying constraints on composition which result in the expression of only one of two broad venom compositional phenotypes (see also Mackessy, 2008).

Table 4
Lethal toxicity toward inbred mice of venom from the nine subspecies of *Crotalus oreganus* and *Crotalus viridis*.

Crotalus oreganus subspecies	Mouse IV LD_{50} (μg/g)
abyssus	2.05
caliginis	2.30
cerberus	5.40
concolor	0.36
helleri	3.75
lutosus	2.88
oreganus	4.75
Crotalus viridis subspecies	
nuntius	1.33
viridis	1.55

Moderate to high levels of seven common viperid venom enzymes are observed for all subspecies, with several notable deviations from species-wide trends. The Arizona Black Rattlesnake (*C. o. cerberus*) has venom with extremely low levels of three serine proteases, which are involved in the disruption of hemostasis of prey, but metalloproteinase activity, which is implicated in predigestion of prey (e.g., Thomas and Pough, 1979; Mackessy, 1988), is exceptionally high. Conversely, the Midget Faded Rattlesnake (*C. o. concolor*) produces a venom with high to very high levels of serine proteases, but metalloproteinase activity is barely detectable (see also Mackessy et al., 2003);

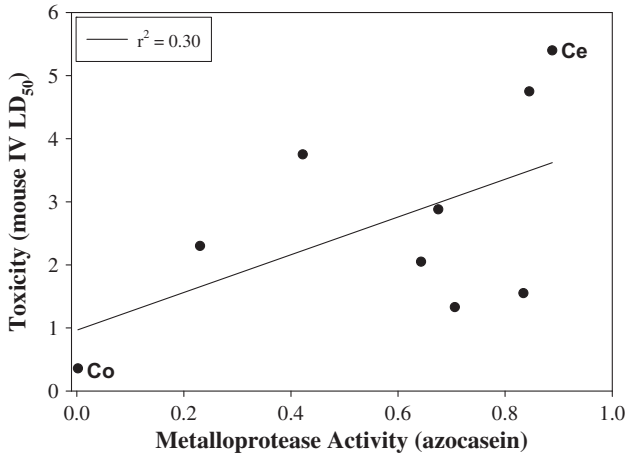

Fig. 6. Relationship of venom toxicity and metalloproteinase activity; a weak correlation is observed, but note that the most toxic venom (*C. o. concolor*, Co) shows the lowest metalloproteinase activity, and the least toxic venom (*C. o. cerberus*, Ce) shows the highest activity.

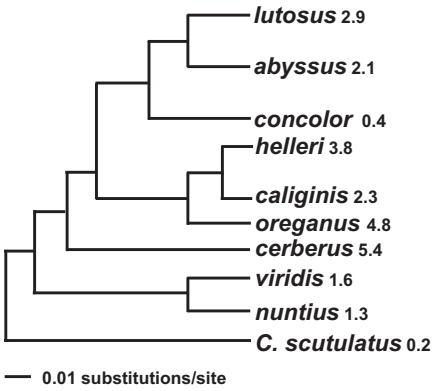

Fig. 7. Phylogenetic relationship of the Western Rattlesnakes (based on strict consensus tree of Ashton and de Queiroz, 2001) with venom 24 h LD_{50} values (NSA mouse, IV). Note the basal position of *C. o. cerberus* (least toxic, highest metalloproteinase activity) and the more derived position of *C. o. concolor* (most toxic, lowest metalloproteinase activity), suggesting that within the *Crotalus oreganus* clade, type I venom may be the ancestral trait.

PIII and PI metalloproteinases also appear to be lacking from this venom. Two other venoms (*C.o. caliginis* and *C. o. helleri*) showed the next lowest metalloproteinase activity and also lacked prominent PIII and PI bands. The absence of significant levels of metalloproteinase activity is particularly noteworthy because this family of proteins is most often a dominant constituent of viperid venoms (Sanz et al., 2006; Calvete et al., 2007). Interestingly, *C. o. cerberus* is considered to be basal to all other members of the Western Rattlesnake clade (Fig. 6), and *C. o. concolor* is considered one of the more derived subspecies (Pook et al., 2000; Ashton and de Queiroz, 2001; Douglas et al., 2002). Phospholipase A_2 enzymatic activity, associated with myonecrosis, disruption of platelet and muscle function, and cell membrane damage, is very high in venom of the Prairie Rattlesnake (*C. v. viridis*) but is very low in venom of the Northern Pacific Rattlesnake (*C. o. oreganus*). Accelerated evolution of the phospholipase A_2 gene (e.g., Ohno et al., 2002) has often resulted in multiple isoforms of PLA_2 in a single venom (but see Pahari et al., 2007), one of which, a two subunit toxin (crotoxin and homologs) present in venoms of some viperids, consists of an acidic subunit and a basic phospholipase A_2. This toxin is a potent presynaptic neurotoxin which is characteristically present in venoms of *C. o. concolor* (Pool and Bieber, 1981; Aird and Kaiser, 1985; Bieber et al., 1990) and it is a major contributor to the lethal toxicity of this venom. Homologs of this toxin have also been found in venoms of snakes from a few localized populations of *C. o. helleri* (French et al., 2004) and several other rattlesnake species, but they appear to be generally absent from venoms of other *C. oreganus*/*viridis* subspecies.

Venoms from subspecies of *C. oreganus*/*viridis* show differences in toxicity (Glenn and Straight, 1982; this study, Table 2), but most subspecies produce venoms with mouse IV LD_{50}s of 1.5–3 μg/g. However, two notable exceptions exist: *C. o. concolor* venom is highly toxic, and *C. o. cerberus* venom is the least toxic of all subspecies. *C. o. cerberus* venom also apparently lacks an abundance of the small myotoxins characteristic of *C. o. concolor* venom (see Mackessy et al., 2003 and Figs. 3 and 5, this study), and SDS-PAGE and zymogram analyses show that the metalloproteinase profiles of the two venoms are quite distinct. Unlike crotoxin homologs, myotoxins are not highly toxic, but they are functionally quite potent venom components, and purified myotoxins rapidly immobilize/kill mice (S.D. Aird, pers. comm.; Bober et al., 1988). Venom lethal toxicity and venom metalloproteinase activity are negatively associated, and this relationship is most pronounced at the extremes. This observation has been generalized to rattlesnakes (*Crotalus*, *Sistrurus*) as an entire clade (Mackessy, 2008): type I venoms show high metalloproteinase activity and lower toxicity, while type II venoms are very toxic ($LD_{50} < 1.0$ μg/g) but have low to very low levels of metalloproteinase activity. High lethal toxicity and high metalloproteinase (tissue-degrading) activity of the venoms of rattlesnakes therefore appear to be incompatible.

Efficient predigestion of prey likely requires some delocalization of venom from the site of injection, and rapid prey death precludes this. Highly toxic but weakly predigestive venom, as seen in *C. v. concolor*, ensures prey retention but may place constraints on utilizable prey size, particularly at higher latitude. In a population of *C. v. concolor* from southern Wyoming (ele. ~2240 m), the largest prey taken by adults were deer mice (*Peromyscus maniculatus*, approx. 30 g) except for one woodrat (*Neotoma*) taken by a large adult during July, the month with the warmest and most stable temperatures (Mackessy et al., 2003). In general, snakes "opt" for either one or the other of these two basic venom compositional "strategies" (type I or II), and each permits and constrains aspects of diet (and perhaps foraging mode). However, rapid prey immobilization is necessary irrespective of level of lethal toxicity (LD_{50}), and the general occurrence of myotoxin a homologs in all venoms analyzed suggests that this component may facilitate immobilization in the absence of crotoxin homologs. Interestingly, *C. v. concolor* venom also showed one of the highest levels of myotoxins (see also Mackessy et al., 2003); unlike the toxicity vs. tenderizer dichotomy, myotoxin presence appears to be compatible with either type I or II venom compositional strategy.

Snake venom metalloproteinases are abundant components of viperid venoms and contribute significantly to the occurrence of hemorrhage and necrosis following envenomation (Gutiérrez et al., 2009). An important role of rattlesnake venom in prey digestion was first shown by Thomas and Pough (1979), and the metalloproteinases have been implicated as the major family of proteins involved in this biological role (e.g., Mackessy, 1988). On the other hand, a more recent study indicated that at 30 °C, digestive efficiency (including SDA, gut passage time and assimilation) was not enhanced by envenomation of prey (McCue, 2007), suggesting that the role of venom in prey digestion is minimal. At this higher temperature, it is not surprising that venom has little effect, and Thomas and Pough's (1979) study also showed temperature dependence of venom-enhanced digestion, with a highly pronounced effect at cooler temperatures and little at higher temperatures. Snake gastric processes are highly efficient at "normal operating temperatures", and even bones are digested (Secor, 2008), but venom metalloproteinases likely provide the additional tissue-

degrading enhancement which facilitates efficient digestion of the prey at suboptimal temperatures encountered in the field. Most biological systems have inherent redundancies, and it was suggested previously that highly proteolytic activity of venoms allowed rattlesnakes and other temperate vipers to expand their foraging repertoire to include ambient conditions less than optimal (highly variable daily temperatures), essentially compensating for low temperature-induced compromise of gastrointestinal digestion (Mackessy, 1988). However, a very recent study suggested that there was no effect of envenomation on digestion efficiency among two species of *Trimeresurus* (Chu et al., 2009), and we are currently re-testing the conclusions of Thomas and Pough (1979) using several species of temperate rattlesnakes and different thermal regimes.

Within *C. oreganus*, both extremes of venom compositional strategies (type I vs. type II) are observed, and either high metalloproteinase activity or high toxicity appears selected for in venoms. A general biochemical trend among venomous snakes is to produce either venoms rich in specific toxins (commonly neurotoxic PLA_2s or three-finger toxins, including neurotoxins, cardiotoxins, cytotoxins, etc.) but poor in metalloproteinases and other enzymes (characteristic of most elapids; Tan and Ponnudurai, 1990, 1992; Tu, 1991, 1998), or venoms with the opposite characteristics, exemplified by many viperids. The mechanism(s) by which venom variation is produced are incompletely known, but based on venom proteomic (Sanz et al., 2006) and venom gland transcriptomic (Pahari et al., 2007; Doley et al., 2008) studies of *Sistrurus catenatus edwardsii* (Desert Massasauga Rattlesnake), post-transcriptional regulation of mRNA transcripts can also significantly affect final venom composition. Paedomorphosis, the retention of certain juvenile characteristics in the adult forms of later evolutionary descendents, was invoked to explain the existence of highly toxic, low metalloproteinase activity venoms in *C. o. concolor* (Mackessy et al., 2003), and this condition appears to explain the occurrence of type I vs. type II venoms in *C. oreganus/viridis* (this study) and among rattlesnakes generally (Mackessy, 2008), perhaps via heterochrony. In several recent studies, type I vs. type II venom characteristics were also shown to occur in lineages of Central and South American rattlesnakes, also via paedomorphosis (Calvete et al., 2010; Núñez et al., 2009), and so this phenomenon is likely characteristic of rattlesnakes generally. However, the proximate factors, including possible environmental constraints, which lead to both type I and II venom phenotypes being expressed in different members of closely related clades/subspecies, remain elusive. We are continuing to investigate the interplay of venom composition and snake natural history, and broadly-occurring species such as *C. oreganus/viridis* represent ideal organisms for such studies.

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Conflict of interest statement

None declared.

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