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Venom variability and envenoming severity outcomes of the *Crotalus scutulatus scutulatus* (Mojave rattlesnake) from Southern Arizona

Daniel J. Massey[a], *Juan J. Calvete*[b,c,*], *Elda E. Sánchez*[d,**], *Libia Sanz*[c], *Kelvin Richards*[a], *Ryan Curtis*[a], *Keith Boesen*[a]

[a]*Arizona Poison and Drug Information Center, 1295 N Martin Room B308, Tucson, AZ 85721, United States*
[b]*Departamento de Biotecnología, Universidad Politécnica de Valencia, Valencia, Spain*
[c]*Laboratorio de Proteinomica Estructural, Instituto de Biomedicina de Valencia, CSIC, Jaime Roig 11, Valencia, Spain 46010*
[d]*National Natural Toxins Research Center & Department of Chemistry, Texas A&M University-Kingsville, MSC 158, Kingsville, TX 78363, United States*

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ABSTRACT

Twenty-one Mojave rattlesnakes, *Crotalus scutulatus scutulatus* (*C. s. scutulatus*), were collected from Arizona and New Mexico U.S.A. Venom proteome of each specimen was analyzed using reverse-phase HPLC and SDS-PAGE. The toxicity of venoms was analyzed using lethal dose 50 (LD_{50}). Health severity outcomes between two Arizona counties U.S.A., Pima and Cochise, were determined by retrospective chart review of the Arizona Poison and Drug Information Center (APDIC) database between the years of 2002 and 2009. Six phenotypes (A–F) were identified based on three venom protein families; Mojave toxin, snake venom metalloproteinases PI and PIII (SVMP), and myotoxin-A. Venom changed geographically from SVMP-rich to Mojave toxin-rich phenotypes as you move from south central to southeastern Arizona. Phenotypes containing myotoxin-A were only found in the transitional zone between the SVMP and Mojave toxin phenotypes. Venom samples containing the largest amounts of SVMP or Mojave toxin had the highest and lowest LD_{50s}, respectively. There was a significant difference when comparing the presence of neurotoxic effects between Pima and Cochise counties ($p=0.001$). No significant difference was found when comparing severity ($p=0.32$), number of antivenom vials administered ($p=0.17$), days spent in a health care facility ($p=0.23$) or envenomation per 100,000 population ($p=0.06$). Although not part of the original data to be collected, death and intubations, were also noted. There is a 10× increased risk of death and a 50× increased risk of intubations if envenomated in Cochise County.

* *Correspondence to:* J.J. Calvete, Laboratorio de Proteinomica Estructural, Instituto de Biomedicina de Valencia, CSIC, Jaime Roig 11, Valencia, Spain 46010. Tel.: +34 963391775; fax: +34 963690800.
** *Correspondence to:* E.E. Sánchez, National Natural Toxins Research Center & Department of Chemistry, Texas A&M University-Kingsville, MSC 158, Kingsville, TX 78363, United States.
E-mail addresses: daniel.massey@uahealth.com (D.J. Massey), jcalvete@ibv.csic.es (J.J. Calvete), elda.sanchez@tamuk.edu (E.E. Sánchez).

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1. Introduction

Venom represents an adaptive trophic trait that has played in the radiation of advanced snakes [1]. Snake venoms are well documented as having different venom compositions and toxicity based on taxonomic or geographical locations [2–5]. Differences can also be found within populations of the same species. Besides ecological and taxonomical implications, knowledge of the natural history and toxin composition of venoms is of fundamental importance for the treatment of bite victims and in the selection of specimens for the preparation of venom pools for antivenom production [6,7].

The *C. s. scutulatus* rattlesnake is found in parts of the southwestern United States and north central Mexico with noted geographical differences in venom composition and toxicity [8,9]. The most recognizable, and the most toxic, component of the *C. s. scutulatus* rattlesnake venom is the Mojave toxin. Nicknamed after the rattlesnake itself, this is a potent neurotoxin that binds presynaptically and is responsible for its "legendary" notoriety. Mojave toxin is built by two non-covalently associated subunits, a non-toxic acidic subunit (A), which lacks PLA_2 activity, and a weakly toxic basic subunit (B), which exhibits PLA_2 activity. The acidic subunit undergoes proteolytic processing to form three polypeptides held together by disulphide linkages [10,11]. Although the B-subunit exhibits neurotoxic activity, the native complex is at least one order of magnitude more potent than the B-subunit alone. The increase of toxicity in mice, rats and rabbits, appears to be due to the A-subunit acting as a chaperone blocking the binding to non-specific sites and guiding the B-subunit to its specific target site [9]. Similarly, the expression of the Mojave toxin-like presynaptic β-neurotoxic heterodimeric PLA_2 molecules, crotoxin (in Central and South American rattlesnake venoms) [12,13] and sistruxin (in *Sistrurus catenatus catenaus* and *S.c. tergeminus* venoms) [14,15] appear to be directly responsible for the high toxicity and the characteristic systemic neuro- and myotoxic effects observed in envenomations by these species. Most populations of the *C. s. scutulatus* rattlesnake throughout its geographic range contain Mojave toxin, although, there are isolated populations in central Arizona U.S.A. that lack this toxin [16]. Three venom phenotypes have been previously described in the literature [9]. Type A venom has large amounts of Mojave toxin and shows distinct neurotoxic effects. This phenotype is the most common throughout the *C. s. scutulatus* rattlesnakes range. Type B venom has large amounts of SVMP and shows hemorrhagic effects, and type A+B venom is a combination of the two and produces both neurotoxic and hemorrhagic effects. Type B and A+B phenotypes have only been documented in central Arizona U.S.A. in the counties of Pima and Pinal [9].

The purpose of this study was to determine if the geographical differences in venom of the *C. s. scutulatus* rattlesnake correlate with increased health severity outcomes due to rattlesnake envenomations. We chose two counties in Arizona based on the venom phenotypes found within each county. Pima County is documented to have venom phenotypes either lacking Mojave toxin (type B) or causing hemorrhagic

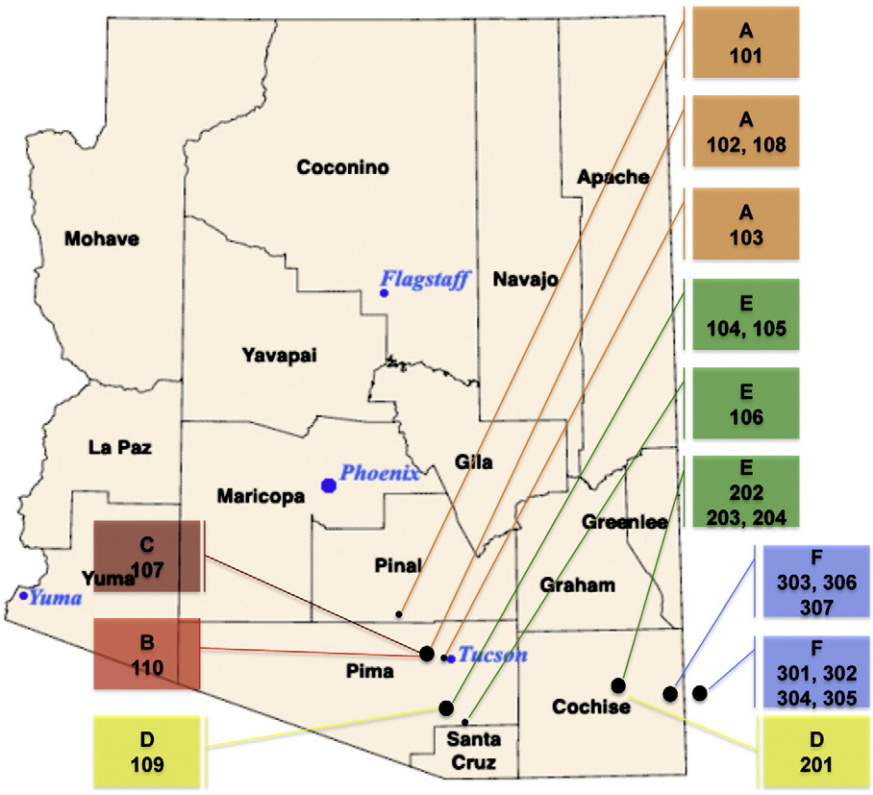

Fig. 1 – Geographical locations in Arizona U.S.A. of collected *C. s. scutulatus*. Colors and identification numbers correlate with phenotypes (A–F) and venom composition as listed in Figs. 4A, 4B and 5. Numbers 301, 302, 304 and 305 represent Hildalgo County New Mexico U.S.A.

effects when the Mojave toxin is present (type A+B). Cochise County is documented to have only venom phenotypes containing Mojave toxin which lacks hemorrhagic effects (type A) [6]. Based on this we hypothesized that Cochise County would have more severe envenomations when compared to Pima County.

2. Materials and methods

2.1. Snake and venom collection

Twenty-one adult *C. s. scutulatus* rattlesnake specimens were collected and identified as defined by Campbell and Lamar [17]. Photographs of all identifiable characteristics were taken and documented for each specimen; dorsal aspect of head (supraoculor and frontal scales), lateral aspect of head, dorsal patterns, and tail bands. Venom samples were obtained from all individuals. Each rattlesnake was given an identification number based on global positioning system (101–110, 201–204, 301–307) and recordings of elevation, length, sex, and dates were documented. Specimens were collected in the Arizona U.S.A. counties of Pinal, Pima, and Cochise and the New Mexico U.S.A. county of Hildalgo (Fig. 1). Manual venom extraction was performed by allowing each snake to bite into Parafilm covering a three-inch funnel that collected into a one-milliliter eppendorf tube. Samples were immediately stored at 0 °C until lyophilized (three weeks).

2.2. Isolation and characterization of venom proteins

Venom proteins were separated by reverse-phase HPLC using a Teknokroma Europa C_{18} (0.4 cm × 25 cm, 5 mm particle size, 300 Å pore size) column as described [18]. Protein detection was at 215 nm and peaks were collected manually and dried in a Speed-Vac (Savant). The relative abundances (% of the total venom proteins) of the different protein families in the venoms were estimated from the relation of the sum of the areas of the reverse-phase chromatographic peaks containing proteins from the same family to the total area of venom protein peaks. In a strict sense, and according to the Lambert–Beer law, the calculated relative amounts correspond to the "% of total peptide bonds in the sample", which is a good estimate of the % by weight (g/100 g) of a particular venom component. The relative contributions of different proteins eluting in the same chromatographic fraction were estimated by densitometry after SDS-PAGE separation.

Reverse-phase HPLC fractions were analyzed by SDS-PAGE (using 15% polyacrylamide gels) and N-terminal sequencing (using a Procise instrument, Applied Biosystems, Foster City, CA, USA). Amino acid sequence similarity searches were performed against the available databanks using the BLAST program implemented in the WU-BLAST2 search engine at http://www.bork.embl-heidelberg.de [19]. Molecular mass determination was performed by MALDI-TOF mass spectrometry (using an Applied Biosystems Voyager-DE Pro™ instrument) and electrospray ionization (ESI) mass spectrometry (using an Applied Biosystems QTrap™ 2000 mass spectrometer). Protein bands of interest were excised from Coomassie Brilliant Blue-stained SDS-PAGE gels and subjected to automated reduction, alkylation, and in-gel digestion with sequencing grade porcine pancreatic trypsin (Promega) using a ProGest™ digestor (Genomic Solutions). Doubly- or triply-charged ions of peptides selected from the MALDI-TOF mass fingerprint spectra of the tryptic digests were sequenced by CID-MS/MS using an Applied Biosystem's QTrap 2000. CID spectra were interpreted manually or using a licensed version of the MASCOT program (http://www.matrixscience.com) against a private database containing viperid protein sequences deposited in the SwissProt/TrEMBL database plus the previously assigned peptide ion sequences from snake

Table 1 – Scoring system was designed based on a clinically validated Snakebite Severity Score [20]. Our abbreviated version is based on the most common areas reported to the APDIC database and does not represent all variables originally presented.

Abbreviated Snakebite Severity Score (ASSS)	
Areas of interest	Points
Neurotoxic manifestations	
No symptoms/signs	Absent
Dizziness, chills, paresthesia, fasciculations in area of bite site, confusion, lethargy, seizures, psychosis	Present
Local wound effects	
No symptoms/signs	0
Pain, swelling, ecchymosis within 5–7 cm of bite site	1
Pain, swelling, ecchymosis involving less than half the extemity (7.5–50 cm from bite site)	2
Pain, swelling, ecchymosis involving half to all of extemity (50–100 cm from bite site)	3
Pain, swelling, ecchymosis extending beyond affected extremity (>100 cm from bite site)	4
Hematologic symptoms	
No symptoms/signs	0
Coagulation parameters slightly abnormal: PT <20 s; PTT <50 s; platelets 100 k–150 k/ml; or fibrinogen 100–150 mcg/ml	1
Coagulation parameters abnormal: PT <20–50 s; PTT <50–75 s; platelets 50 k–100 k/ml; or fibrinogen 50–100 mcg/ml	2
Coagulation parameters abnormal: PT <50–100 s; PTT <75–100 s; platelets 20 k–50 k/ml; or fibrinogen <50 mcg/ml	3
Coagulation parameters markedly abnormal: unmeasurable PT or PTT; platelets <20 k, or undetectable fibrinogen	4

PT = prothrombin time; PTT = partial thromboplastin time; k = thousand; mcg = microgram; ml = milliliter; s = seconds.
Based on data reported to the Arizona Poison and Drug Information Center.

venomics projects carried out in our laboratory. MS/MS mass tolerance was set to ±0.6 Da. Carbamidomethyl cysteine and oxidation of methionine were fixed and variable modifications, respectively.

2.3. *Lethal dose (LD_{50})*

The LD_{50} of venom samples was performed by a method of Sanchez et al. [20]. Using physiological saline, lyophilized *C. s. scutulatus* rattlesnake venom samples were re-suspended and serially diluted. Each solution was stored at 0 °C, then warmed to 37 °C prior to injection of mice. Five groups of eight BALB/C mice were used for each venom sample. The lethal toxicity was determined by injecting various concentrations of 0.2 ml of venom into the tail vein of each mouse (18–20 g female BALB/C mice). Negative control mice were injected with saline. Lethality endpoints were determined after 48 h.

2.4. *Data collection – rattlesnake envenomations*

A retrospective chart review of rattlesnake envenomation data was collected using the APDIC, U.S.A. database. Envenomations that occurred during an eight-year period between the dates of 01/01/2002 to 12/31/2009 were analyzed. Inclusion criteria: envenomation must have occurred in Pima or Cochise Counties U.S.A. Exclusion criteria: unable to confirm

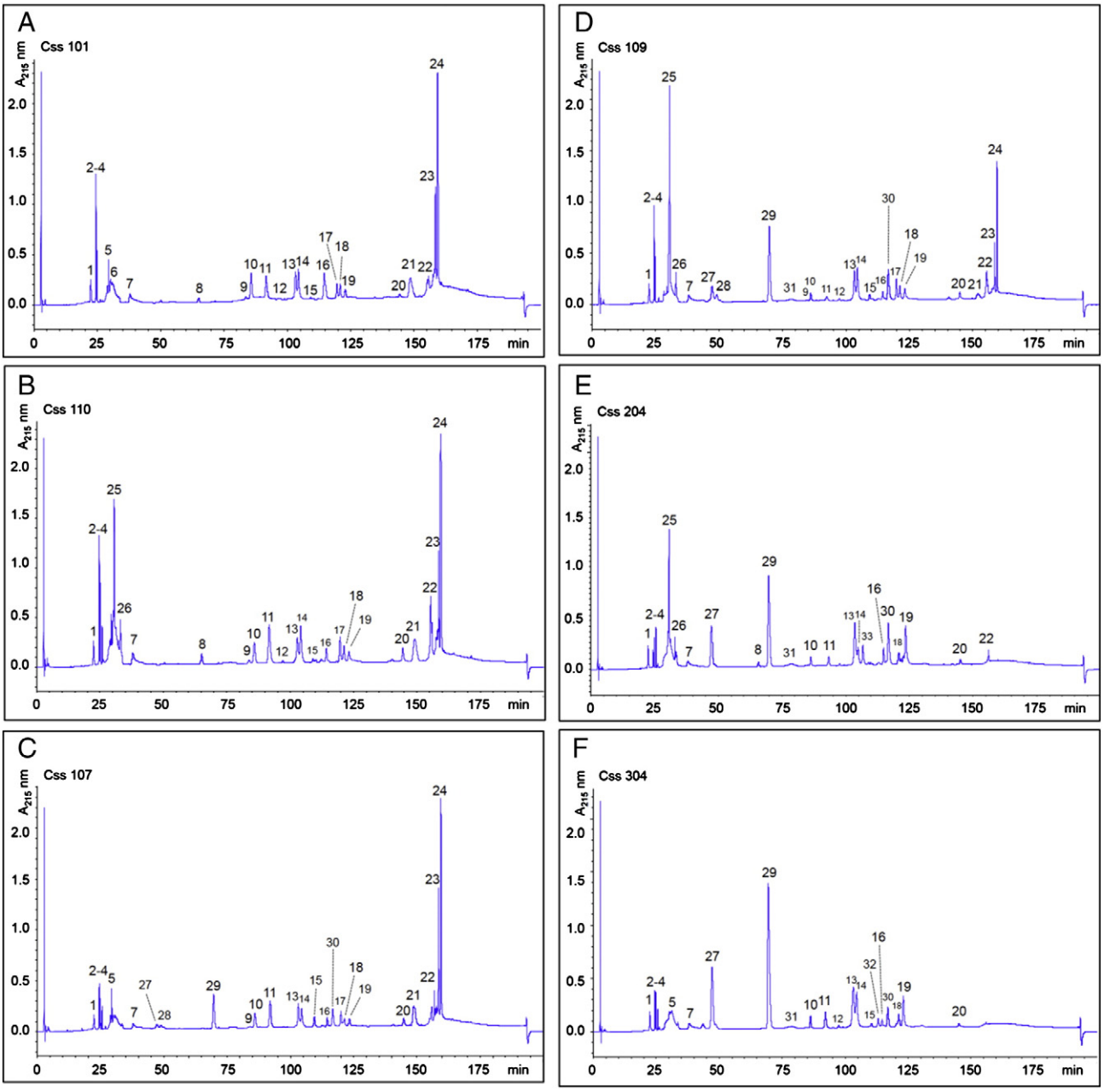

Fig. 2 – Representative reverse-phase HPLC profiles of the six different *C. s. scutulatus* venom phenotypes (A–F) characterized in *C. s. scutulatus* venoms collected from Arizona and New Mexico U.S.A. Proteomic-guided assignment of the chromatographic fractions to toxin families is shown in Table 2.

geographical bite location, venomous snake was not native to Pima or Cochise Counties U.S.A., intoxication (ethanol or illegal substances), acceptance into study for treatment which included a non FDA approved antivenom, or no signs or symptoms of envenomation present. Five areas of envenomation were analyzed; presence of neurotoxic effects vs. no neurotoxic effects, severity, number of antivenom vials administered, days spent in a health care facility, and risk of envenomation per 100,000 population.

Severity of envenomations is based on an abbreviated snakebite severity score (ASSS). The original snakebite severity score is an objective scale clinically validated based on six different aspects; pulmonary, cardiovascular, gastrointestinal, hematologic and nervous systems, and local wound effects [20]. Each area is given a point scale with a greater number indicating a more severe envenomation. We used an ASSS to differentiate the severity of envenomations based on the most common data reported to the APDIC; neurotoxic manifestations (present or absent), local wound effects (0–4), and hematologic abnormalities (0–4), range 0–8 (Table 1). A larger number indicated a more severe envenomation. The 2000 U.S. Census Bureau data was used to assess the difference in populations between Pima and Cochise Counties. Pima County has approximately 48% more square miles and 7.5 times the population than Cochise County. Based on this, envenomations per 100,000 population were analyzed.

2.5. *Statistics*

Chi-square test was used to evaluate the presents of neurotoxic effects and envenomations based on population. *T*-test was used to evaluate severity, number of antivenom vials administered, and days spent in a health care facility.

3. Results

3.1. *Venom proteomes of C. s. scutulatus rattlesnake*

The composition of the venoms of the *C. s. scutulatus* rattlesnake from the different localities investigated in this work (Fig. 1) was fractionated by reverse-phase HPLC (Fig. 2) and the chromatographic peaks analyzed by SDS-PAGE (Fig. 3) followed by N-terminal sequencing and venomic analysis (Table 2). Toxins belonging to 10 different classes were identified, although their relative occurrence in the different venoms varied (Table 3). Of these 10 protein families, 3 (SVMP, Mojave toxin, myotoxin-A) were used to group 6 distinct phenotypes labeled A–F. (Figs. 4A and 4B): A) SVMP-rich, Mojave toxin and myotoxin-A negative; B) SVMP+myotoxin-A, Mojave toxin negative; C) SVMP+Mojave toxin, myotoxin-A negative; D) SVMP+Mojave toxin+myotoxin-A; E) Mojave toxin+myotoxin-A, SVMP essentially negative; and F) Mojave toxin, myotoxin-A and SVMP negative. Average percent of SVMP, Mojave toxin and myotoxin-A, respectively within each phenotype are: A) 56.6, 0, 0; B) 48.2, 0, 5.5; C) 52.7, 7.2, 0; D) 13.2, 27.2, 22.2; E) <0.1, 27.6, 23.9; F) <0.1, 45.8, 0 (Table 4).

Figs. 4A, 4B, and 5 show trends of collected phenotypes. PI and PIII SVMP were the main components of *C. s. scutulatus* rattlesnake venom samples from south-central Arizona; 56.6%, 48.2% and 52.7% for phenotypes A, B and C, respectively. PI and PIII SVMP decreased as you move southeast and were absent in phenotypes E and F. Mojave toxins were found in the largest quantities from south-eastern Arizona venom samples; 45.8%, 27.6%, and 27.7% for phenotypes F, E, and D, respectively. Mojave toxin decreased as you move north-west and was absent in phenotypes A and B. Myotoxin-A was found in the "transitional zone" between south-central Arizona venom samples rich in PI and PIII SVMP and south-eastern Arizona venom samples rich in Mojave toxin; 5.5%, 22.2% and 23.9% for phenotypes B, D and E, respectively. Myotoxin-A was absent in phenotypes A, C, and F. (Table 4).

3.2. *Venom lethal dose 50*

Table 5 shows the LD_{50} of venom samples from twenty-one adult *C. s. scutulatus* rattlesnakes. Venom potency increased in correlation with increased Mojave toxin and decreased in correlation with increased PI and PIII SVMP. Meaning, LD_{50} decreased as you move from south central to southeastern

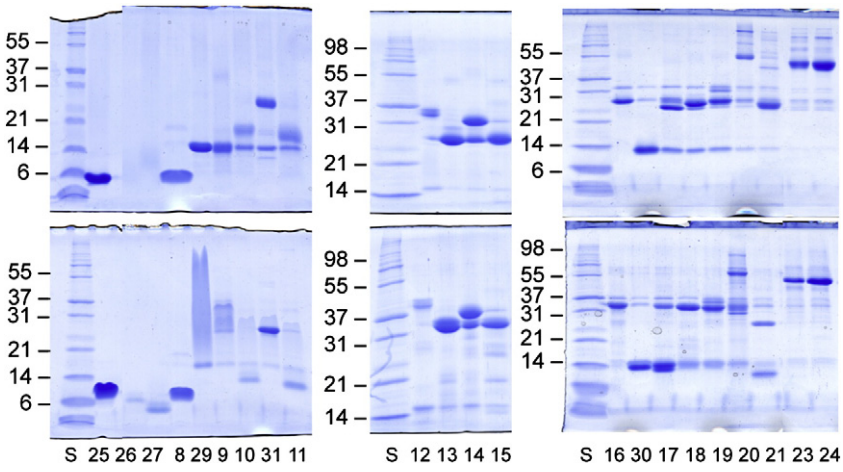

Fig. 3 – SDS-PAGE of the reverse-phase HPLC separated *C. s. scutulatus* venom proteins (Fig. 2) run under non-reduced (top) and reduced (down) conditions. Molecular mass markers (lane S) are indicated (in kDa) at the side of each gel.

Table 2 – Assignment of the reverse-phase-HPLC-separated proteins of *C. s. scutulatus* venoms (Fig. 2A–F) to protein families by N-terminal Edman sequencing, mass spectrometry, and collision-induced fragmentation by nESI-MS/MS of selected peptide ions from in-gel digested protein bands separated by SDS-PAGE (Fig. 3). In MS/MS-derived sequences, J=Ile or Leu; Z, pyrrolidone carboxylic acid; Cp, propionamide cysteine. Unless otherwise stated, for MS/MS analyses, cysteine residues were carbamidomethylated; molecular masses of native proteins were determined by electrospray-ionization (±0.02%) mass spectrometry. Apparent molecular masses were determined by SDS-PAGE of non-reduced (■) and reduced (▼) samples. np, non-peptidic material found; N.A., not analyzed.

HPLC fraction	N-terminal sequence	Molecular mass	Peptide		MS/MS-derived ion sequence	Protein/peptide class
			m/z	z		
1	TPPAGPDVGPR	1063.8	532.4	2	TPPAGPDVGPR	Bradykinin-inhibitory peptide [~P85025]
2–4	Mixture of peptides		621.3	2	(423.3)GGGEGGTTAJ	Unknown
			632.3	2	ZQTESJRNJPP	Bradykinin-potentiating peptide
5,6	n.p.					
7	Blocked		608.4	2	ZJWPRPQJPP	Bradykinin-potentiating peptide [P0C7S6]
8	Blocked	4456.1	501.7	2	JGCEPJDAK	Unknown
			741.8	2	(198.2)ECpEQENFCR	
9	SLVQFETLIMKIAGR	16754.8	838.4	2	JTGCpDPTTDVYTYR	D49-PLA_2 ~ [P08878]
			655.4	2	SLVQFETLIMK	
10	NLLQFNKMIKMMTKK	14200.4				D49-PLA_2 [AF403138]
11	NLVQFELLIMKVAKR	13623.9	649.7	2	YGYMFYPDSR	D49-PLA_2 ~ [AAM80565]
			753.6	2	CCFVHDCCYGK	
12	N.A.	38 kDa▼	763.8	2	IIGGDECNINEHR	Serine proteinase
13	VIGGHPCNINEHRSL	31 kDa▼				Serine proteinase
14	IIGGDECNINEHR(S/F)L	37 kDa▼				Serine proteinase
15	IIGGDECNINEHRFL	33 kDa▼	763.8	2	IIGGDECNINEHR	Serine proteinase
			604.7	2	JMGWGTJTTTK	
			698.6	2	AAYPEYGJPATSR	
	VIGGDECNINEHRFL	31 kDa▼	595.3	2	WDKDJMJJR	Serine proteinase
			756.8	2	VIGGDECNINEHR	
16	VIGGDECNINEHRFL	33 kDa▼				Serine proteinase
17	V(I/V)GGDECNINEHRSL	33 kDa▼	604.7	2	JMGWGTJTTTK	Serine proteinase
			595.3	2	WDKDJMJJR	
			756.8	2	VIGGDECNINEHR	
			588.3	2	AAYPEJPATSR	
		14 kDa▼	753.6	2	CCFVHDCCYGK	D49-PLA_2
			552.3	2	TVJYTYSEK	
18	VVGGDECNINEHRSL	33 kDa▼				Serine proteinase
		14 kDa▼	519.3	2	JDJFWGMR	C-type lectin-like
			567.3	2	AKPECJVCR	
19	VIGGDECNINEHRFL	33 kDa▼				Serine proteinase
20	AHDRNPLEECFRETDY	56 kDa▼	532.8	2	NPLEECFR	L-amino acid oxidase [O93364]
			618.8	2	SAAQJYVESJR	
			485.8	2	VQVHFNAR	
			462.8	2	FEPPJPPK	
			743.9	2	ETDYEEFJEJAK	
			611.8	2	DWYANJGPMR	
			881.4	2	EDJQTFCHPSMJQR	
			782.8	3	IYFAGEYTAQFHGWJDSTJK	
			630.8	2	FWEDDGJHGGK	
			677.9	3	JNEFSQENENAWYFJK	
			689.1	3	DCADJVJNDJSJIHEJPK	
21	NPEHQRYVELFIVVD	23 kDa■/ 12 kDa▼	605.3	3	YVELFIVVDHGMYTK	PI-SVMP ~ [P34182]
	NLNPEHQRYVELFIV		518.9	3	STGVVQDHSEINJR	
			555.2	2	TJNSFGEWR	
			534.3	2	YNGDSDKJR	
22	DCPSGWSSYEGHCYR	14 kDa▼				C-type lectin-like
	Blocked	69 kDa▼	657.2	2	YVEJVJVADHR	PIII-SVMP
			502.6	2	GNDYGYCR	
23,24	Blocked	48 kDa▼	526.8	2	GNYYGCR	PIII-SVMP [Q90282, Q2QA02]
			604.9	2	FVEJFJVVDK	
			822.4	2	MYEJVNTVNEJYR	
			797.9	2	HDNAQLLTAIDLDR	
			684.7	3	JTVKPEAGYTJNAFGEWR	
25	YKQCHKKGGHCFP	4822.2	593.8	2	ICIPPSSDLGK	Myotoxin-A [P01476]

(continued on next page)

Table 2 (*continued*)

HPLC fraction	N-terminal sequence	Molecular mass	Peptide m/z	Peptide z	MS/MS-derived ion sequence	Protein/peptide class
26	N.A.	5 kDa▼	593.8	2	ICIPPSSDLGK	~[Myotoxin-A [P01476]
27	SSYGCYCGAGGQG	9742.8				Mojave toxin acid chain (38–80; 84–119; 127–138)
	SPENCQGESQPC					[P18998]
28	YSSYGCYCGAGGQG	9905.4				Mojave toxin acid chain (37–80; 84–119; 127–138)
	SPENCQGESQPC					[P18998]
29	HLLQFNKMIKFETRK	14186.3	871.8	2	GTWCEEQICECDR	Mojave toxin basic chain [AAC59674]
			753.3	2	CCFVHDCCYGK	
			719.9	3	NAIPFYAFYGCYCGWGGR	
30	SLVQFEALIMKIAGR	13646.3	753.3	2	CCFVHDCCYGK	D49-PLA_2~[AF403134, P00624]
31	N.A.	23 kDa▼	769.3	2	MEWYPEAAANAER	CRISP
			563.8	2	JVDJHNFJR	
			569.7	2	SVDFDSESPR	
			617.2	2	SJVQQAGCQDK	
			629.3	3	QMQSDCPAJCFCQNK	
32	VIGGDECNINEHRSL	33 kDa▼	604.7	2	JMGWGTJTTTK	Serine proteinase
			595.3	2	WDKDJMJJR	
			756.8	2	VIGGDECNINEHR	
33	IIGGDECNINEHR(S/F)L	32 kDa▼	698.6	2	AAYPEYGJPATSR	Serine proteinase

Arizona. Potency (least to greatest) based on average LD_{50} of each phenotypes are; A, B, C, E, D and F, respectively.

3.3. *Envenomation data*

Pima County had a total of 644 envenomations reported to the APDIC, 203 excluded, 441 included. Cochise County had a total of 118 envenomations reported, 43 excluded, 75 included. Neurotoxic effects (Table 6) were noted in 8 of 441 (1.8% or approximately 1 in 50) and 7 of 75 (9.5% or approximately 1 in 10) for Pima and Cochise Counties, respectively, $p=0.001$. An eight-year average was analyzed for severity based on our ASSS, number of antivenom vials administered, and days spent in a health care facility. For Pima and Cochise Counties, respectively: severity 2.7 and 2.5 ($p=0.32$), number of antivenom vials administered 12.5 and 10.8 ($p=0.17$), days spent in a health care facility 3.3 and 3.1 ($p=0.23$). When comparing the eight-year average of envenomations per 100,000

Table 3 – Relative occurrence of proteins/peptides (in percentage of the total reverse-phase HPLC-separated components) of different classes in the different venom phenotypes of *C. s. scutulatus*.

Phenotype/ID number	VAPep	D49-PLA_2	SerProt	CRISP	LAO	CTL	PI-SVMP	PIII-SVMP	Mojave	Myo	Unkn
A-101	5.1	11.1	24.6	ND	1.1	0.1	8.1	49.8	ND	ND	0.5
A-102	4.6	13.8	23.9	ND	1.1	0.1	7.7	46.9	ND	ND	0.9
A-103	5.3	12.1	21.9	ND	1.3	0.2	9	50.2	ND	ND	0.1
A-108	4.8	15.2	23.8	ND	1.2	0.2	9.6	45	ND	ND	0.2
B-110	5.7	14.3	22.2	ND	2.1	0.2	7.1	41.1	ND	5.5	1.8
C-107	4.7	13.6	18.9	<0.1	1.5	0.1	7.8	44.9	7.6	ND	0.8
D-109	4.1	16.5	9.6	ND	1.2	ND	3.5	15.9	21.2	26.2	1.6
D-201	3.8	17.6	19.3	ND	1.5	ND	ND	3.4	34.2	18.1	2.1
E-104	3.4	12.8	28.9	ND	1.3	ND	ND	<0.1	17.3	34.1	2.3
E-105	2.9	8.9	37.6	ND	<0.1	ND	ND	<0.1	17.7	29.6	3.3
E-106	5.1	10.3	28.1	0.3	1.2	ND	ND	<0.1	27.7	24.4	2.9
E-202	1.9	10.8	30.9	ND	1.4	ND	ND	<0.1	39.7	12.5	2.8
E-203	1.7	7.6	32.1	ND	0.8	ND	ND	<0.1	35.7	19.2	2.9
E-204	2.2	10.8	30.1	0.3	0.7	ND	ND	1.1	27.6	23.9	3.3
F-301	4.4	8.9	29.8	<0.1	1.1	ND	ND	0.3	53.3	ND	2.2
F-302	4.6	15.6	40.2	0.3	0.7	ND	ND	<0.1	35.9	ND	2.6
F-303	3.9	10.5	36.2	ND	<0.1	ND	ND	<0.1	47	ND	2.4
F-304	3.8	8.7	33.5	0.2	<0.1	ND	ND	<0.1	51.4	ND	2.2
F-305	3.5	7.4	41.1	0.1	<0.1	ND	ND	<0.1	44.1	ND	2.8
F-306	3.9	7.6	41.5	0.1	<0.1	ND	ND	<0.1	43.9	ND	2.8
F-307	3.2	6.9	41.6	0.1	<0.1	ND	ND	<0.1	45.3	ND	2.9

VAPep=vasoactive peptides (BIP and BPP, bradykinin-inhibitory and bradykinin-potentiating peptides respectively), D49-PLA_2=phospholipase A_2, SerProt=serine proteinase, CRISP=cysteine-rich secretory protein, LAO=L-amino acid oxidase, CTL=C-type lectin-like protein, PI and PIII-SVMP=snake venom metalloproteinases of class I and III respectively, Mojave=mojave toxin, Myo=myotoxin-A, Unk=unknown, ND=not detected.

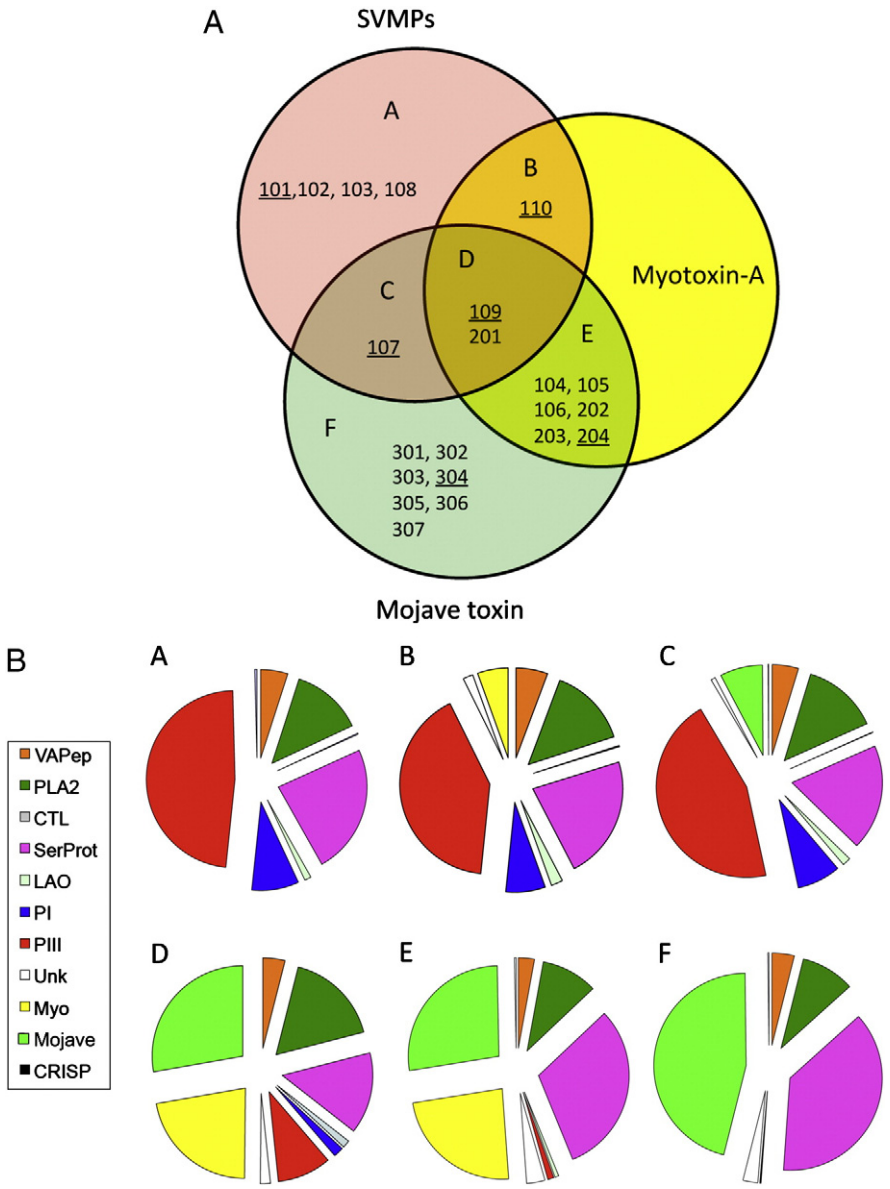

Fig. 4 – Panel A, Venn diagram showing the relations between the six phenotypes (A–F) identified based on three protein families: Mojave toxin, snake venom metalloproteinases (SVMP), and myotoxin-A. Panel B, relative occurrence of proteins from different toxin families in the different *C. s. scutulatus* venom phenotypes. The percentages of the different toxin families in the each of the 21 venoms sampled are listed in Table 3.

population, Pima County has a risk of 45 and Cochise County a risk of 57 ($p=0.06$). Also noted during data collection; 1 death and 4 intubations in Cochise County, zero in Pima. The percent difference between Pima and Cochise Counties, respectively: deaths 0.01, 0.001, intubations 0.05, 0.001 (Table 7).

4. Discussion

4.1. *Distribution and venom of the C. s. scutulatus rattlesnake*

The *C. s. scutulatus* rattlesnake ranges from Southern California to Western Texas U.S.A. into Northern Mexico and was first described by Kennicott in 1861. The venom has been well documented as being more potent when compared to other rattlesnakes with noted variation in venom composition based on geographical location [8,9,21]. Bieber and Tu were the first to isolated a homogeneous and monomeric form of toxin from the *C. s. scutulatus* rattlesnake venom and designated it as "Mojave toxin" [22]. This is the toxin responsible for the increased potency and neurotoxic effects. Glenn and Straight showed different venom phenotypes and LD_{50s} base on geographical locations within Arizona U.S.A. [8,9]. They designated these phenotypes; type A, type B and type A+B. Type A venom contains Mojave toxin and produces neurotoxic effects but lacks hemorrhagic and proteolytic activities. Type B contains SVMP and produces hemorrhagic and proteolytic effects but lacks neurotoxic activities. Type A+B contains both the neurotoxic Mojave toxin and the hemorrhagic proteolytic activities of SVMP. LD_{50} was lowest

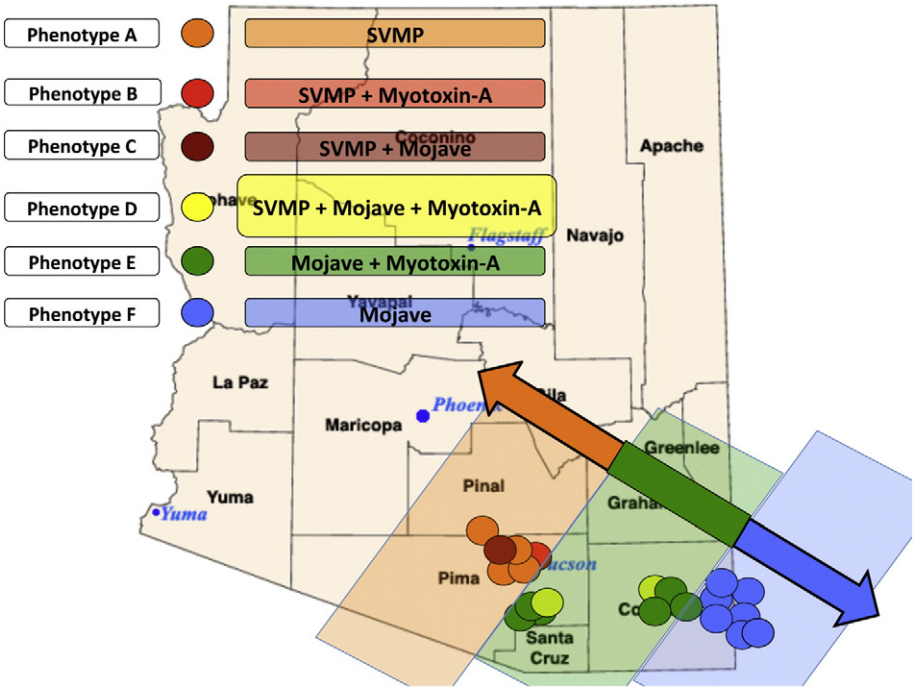

Fig. 5 – Phenotype composition of venoms based on geographical location of collected *C. s. scutulatus* from Arizona and New Mexico. Identification numbers for each specimen are listed in Fig. 1.

in type A venoms and increased with type A+B and type B, respectively.

4.2. *Phenotypes and LD_{50} of collected specimens*

The following phenotype and LD_{50} descriptions will be based on the familiarity of Glenn and Straight documentation of venom "types" in past literature; type A, type B, and type A+B. [8,9]. Table 4 can be used to compare these descriptions to phenotypes isolated from our research.

Table 4 – Phenotypes based on three main protein families found in venom samples of *C. s. scutulatus*.

Phenotype	Protein family	Average percent
A	SVMP	56.6
	Mojave toxin	0
	Myotoxin-A	0
B	SVMP	48.2
	Mojave toxin	0
	Myotoxin-A	5.5
C	SVMP	52.7
	Mojave toxin	7.2
	Myotoxin-A	0
D	SVMP	13.2
	Mojave toxin	27.2
	Myotoxin-A	22.2
E	SVMP	<0.1
	Mojave toxin	27.6
	Myotoxin-A	23.9
F	SVMP	<0.1
	Mojave toxin	45.8
	Myotoxin-A	0

SVMP = snake venom metalloproteinase.

Table 5 – Toxicity of venom samples from *C. s. scutulatus* based on phenotypes.

Phenotype	ID number	County	State	LD_{50} mg/kg
A	101	Pinal	AZ	3.2
A	102	Pima	AZ	2.3
A	103	Pima	AZ	4
A	108	Pima	AZ	3.02
				Mean; 3.13
B	110	Pima	AZ	2.9
C	107	Pima	AZ	2.6
D	109	Pima	AZ	1.4
D	201	Cochise	AZ	0.72
				Mean; 1.06
E	104	Pima	AZ	1.7
E	105	Pima	AZ	1.33
E	106	Pima	AZ	1
E	202	Cochise	AZ	0.56
E	203	Cochise	AZ	0.878
E	204	Cochise	AZ	1
				Mean; 1.07
F	301	Hildago	NM	0.34
F	302	Hildago	NM	0.926
F	303	Cochise	AZ	0.63
F	304	Hildago	NM	0.737
F	305	Hildago	NM	0.55
F	306	Cochise	AZ	0.854
F	307	Cochise	AZ	0.538
				Mean; 0.65

AZ = Arizona U.S.A., NM = New Mexico U.S.A.

Table 6 – Neurotoxic signs or symptoms noted in 15 of the 516 envenomations analyzed. Neurotoxic manifestations occurred in 8 of 441 from Pima County and 7 of 75 from Cochise County. Patients may have presented with one or more of the listed neurotoxic effects.

Neurotoxic manifestations as reported to the Arizona Poison and Drug Information Center		
Neurotoxic symptoms	Pima County	Cochise County
Seizures/combative	0	3
Fasiculations [a]	1	1
Edema of airway	0	2
Intubation [b]	0	4
Numbness [a]	3	2
Paresthesia [a]	7	2
Paresthesia + edema [c]	5	0
Vision changes	0	1
Dizzy	0	1
Emesis	1	3
Diarrhea	0	1
Death [b]	0	1

[a] Symptom noted in general area of bite site.
[b] Not part of original data to be analyzed.
[c] Parethesia presenting with significant edema at bite site.

Our study of twenty-one venom samples of the *C. s. scutulatus* rattlesnake collected from four counties in Arizona and New Mexico U.S.A. supports and extends the findings of Glenn et al. [8,9]. We describe six different phenotypes that consist of Mojave toxin (A), hemorrhagic and proteolytic toxins PI and PIII SVMP (B), and a third previously undocumented component myotoxin-A (M). The phenotypes isolated consisted of type A, type B, and type A+B as previously described, but also included three undocumented phenotypes: type A+M, type B+M, and type A+B+M. Again, consistent with past literature, these phenotypes differed based on geographical location. Seventeen of the twenty-one venom samples (81%) fell into three of the six different phenotypes describe. Type B, type A+M and type A, which changed as you move from South Central Arizona to South Eastern Arizona, respectively. The average composition and number of specimens within each of these three phenotypes are: four in type B consisting of 56.6% SVMP, 0% Mojave toxin, 0% myotoxin-A; six specimens in type A+M consisting of <0.1% SVMP, 27.6% Mojave toxin and 23.9% myotoxin-A, and seven specimens in type A consisting of 0% SMVP, 45.8% Mojave toxin and 0% myotoxin-A.

Table 7 – Arizona Poison and Drug Information Center retrospective chart review of rattlesnake envenomations between 01/01/2002 and 12/31/2009.

Original data to be collected during chart review	Pima County	Cochise County
Total envenomations reviewed	644	118
Excluded	203	43
Included	441	75
Neurotoxic effects noted	8	7
Severity (based on ASSS) [a]	2.7	2.5
Antivenom vials administered [a]	12.5	10.8
Days in health care facility [a]	3.3	3.1
Envenomations/100,000 population [a]	45	57
Other significant data		
Death [b]	0	1
Intubation [b]	0	4

[a] Eight year average.
[b] Noted during retrospective chart review.

The LD_{50} of the six different phenotypes in our study followed the basic trend as described by Glenn and Straight [23]. Type A had the lowest LD_{50} and type B the highest. The difference was when looking at phenotypes containing myotoxin-A. Type B+M (48.2% SVMP, 5.5% myotoxin-A) and type A+B+M (13.2% SVMP, 27.7% Mojave toxin, 22.2% myotoxin-A) had lower LD_{50} than type B (56.6% SVMP) and type A+B (52.7% SVMP, 7.6% Mojave toxin). This shows a decreased LD_{50} when myotoxin-A is present. When a phenotype is lacking SVMP, myotoxin-A does not seem to decrease potency. Type A (45.8% Mojave toxin) had a lower LD_{50} than type A+M (27.6% Mojave toxin, 23.9% myotoxin-A).

4.3. *Reasoning and difficulties of envenomation reviews*

Based on past literature, there has been a presumption that more severe envenomations may occur in South Eastern Arizona U.S.A. when compared to other areas of Arizona. This is primarily due to the documented decreased LD_{50} of Mojave rattlesnake in South Eastern Arizona [8,9,21,23]. When attempting to identify envenomations caused by a specific species such as the *C. s. scutulatus* rattlesnake, there are multiple variables to take into consideration. Most individuals envenomated by a rattlesnake do not know the specific species, do not have a clear photograph of the snake, or do not know how to properly identify similar species. Inevitably, if the rattlesnake cannot be identified, the culprit is a "Mojave". There is also documentation of neurotoxic effects caused by other species of rattlesnakes found within the *C. s. scutulatus* range; *C. oreganus helleri* (Southern Pacific rattlesnake) and the *C. tigris* (Tiger rattlesnake) [24–27]. Mojave toxin has been isolated from the venom of other rattlesnakes as well, *C. atrox* (Western diamondback) and *C. viridis viridis* (Prairie rattlesnake) thought to be due to interbreeding [28,29]. Whether or not these interbred specimens have enough Mojave toxin to cause neurotoxic symptoms is unclear.

There is limited literature available describing specific envenomations caused by the *C. s. scutulatus* rattlesnake. Hardy et al. [27] discussed fifteen positively identified envenomations involving *C. s. scutulatus* in Arizona. Fourteen of these individuals were located in the "Type B" venom range and lacked any neurotoxic effects. One was located within the "Type A" venom range with eyelid ptosis noted 36 h post envenomation. Farstad et al. reported thirteen envenomations from Southern California having signs and symptoms of neurotoxic effects but only four were positively identified as *C. s. scutulatus* [24]. The other nine envenomations were within the range of the *C. oreganus helleri*, which is known to cause similar effects.

4.4. *Data collection and review*

As for neurotoxic manifestations, Cochise County *C. s. scutulatus* rattlesnakes are well documented as having neurotoxic

effects of type A venoms and presumably lack the hemorrhagic effects of type B venoms. This was confirmed when evaluating the 7 envenomations documented with neurotoxic manifestations in Cochise County. Based on our ASSS (Table 1) each of the 7 envenomations showed one or more neurotoxic effects and lacked any significant local wound or hematologic symptoms. It is more difficult to assess neurotoxic manifestations in Pima County *C. s. scutulatus* rattlesnakes due to well documented venoms of either type B or type A+B, each of which would presumably have some hematologic symptoms. This was also confirmed when evaluating the 8 envenomations documented with neurotoxic manifestations in Pima County. Again, based on our ASSS each of the 8 envenomations showed one or more neurotoxic effects but also showed significant local wound and hematologic symptoms. Of note, 5 of 7 cases involving paresthesias of the hand or foot also had significant swelling of that effected limb. It is unclear if this is the presents of type A+B venom or due to edema and local tissue damage.

Also noted during data collection was death and intubation. Cochise County had one death and four cases requiring intubation, Pima County had zero in both instances. When looking at a percent difference between the counties, Cochise County shows a 10× greater risk of death and a 50× greater risk of being intubated. These are conservative estimates since when calculating percents a zero cannot be placed in the numerator; therefore Pima County was given 0.5 for both death and intubation.

4.5. *Concluding remarks*

The nature of a retrospective chart review limits drawing generalized conclusions of future envenomations. Data available for analysis was limited to what is reported and documented at the APDIC. Due to APDIC being a consult service, specifics regarding the pulmonary, cardiovascular, and gastrointestinal systems were rarely noted and therefore not included in this review. Of the data included, neurological manifestations, local wound affects, and hematologic system, only hematologic findings is non subjective. Local wound affects such as swelling, pressure, and pain varies between individual subjects as well as individuals reporting these clinical aspects. Envenomations required an average of 3 days in a health care facility; therefore numerous different health care providers reported these effects (medical attending, medical resident, nurse, clinical pharmacist). As for the neurotoxic effects, paresthesia could be caused by restricted blood flow and dizziness or confusion may be the result of pharmacology such as pain management.

Venom sample size of twenty-one, although comparable to past literature of Glenn et al.[5,6] and Sanchez et al.[16], is an overall small sample of the actual population of *C. s. scutulatus* rattlesnakes within Arizona and New Mexico U.S.A. Our goal was to collect specimens through out its geographical distribution instead of pure number of specimens. Collection sites included South Central to South Eastern Arizona, extreme South Western New Mexico, and did not include the western half Arizona or southern California. In addition, although some specifics on species were noted in the APDIC database (*C. s. scutulatus*, *C. atrox*, *C. tigris*, *C. lepidus klauberi*, *C. cerastes*), very few specimens were identified by experts and therefore could not be confirmed or denied.

Based solely on laboratory findings of venom samples, one would expect to see more neurological symptoms with envenomations that occurred in Cochise County when compared to Pima. Other areas that affect not only the severity of envenomation, but also what clinical manifestations may appear, need to be considered as well. Factors such as amount of venom injected, species of rattlesnake and time to health care facility are of note. Amount of venom injected is dependent on multiple factors; size, age, differences in anatomical features between species, time of year, whether or not the snakes had recently eaten, etc. Also, time to health care facility and time to first administration of antivenom may cause significant differences in presentations due to the envenomation as time progresses.

Venom composition and LD_{50} of the *C. s. scutulatus* rattlesnake changed based on geographical location, which is consistent with past literature. Six phenotypes were identified based on three toxin families, Mojave toxin (A), PI and PIII SVMP (B), and myotoxin-A (M). Three described in past literature; type A, type B, type A+B, and three previously undocumented; type A+M, type B+M, type A+B+M. Myotoxins decreased the LD_{50} in type B+M and type A+B+M when compared to type B and type A+B alone. Type A and type B had the lowest and highest LD_{50}, respectively. Myotoxin-A was only identified in the transitional zone between venoms rich in SVMP and venoms rich in Mojave toxins. When comparing envenomations between Pima and Cochise Counties Arizona, there was a significant difference between the presents of neurotoxic effects, $p=0.001$. Envenomations in Cochise County had an occurrence of 1 in 10 having neurotoxic manifestations as compared to Pima County having an occurrence of 1 in 50. No significant difference was found when comparing the eight year average of: severity $p=0.32$, number of antivenom vials administered $p=0.17$, days spent in health care facility $p=0.23$, or envenomations per 100,000 population $p=0.06$. Not part of the original data to be collected, death and intubation were also noted. There is a 10× increased risk of death and a 50× increased risk of intubations if envenomated in Cochise County. Although neurotoxic effects do correlate with the venom phenotypes found within Pima and Cochise Counties, no significant difference was found in envenomation outcomes based on our original data.

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REFERENCES

[1] Greene HW. Snakes: The Evolution of Mystery in Nature. Berkeley: University of California Press; 1997.
[2] Boldrini-Franca J, Carréa-Netto C, Silva MMS, Rodrigues RS, De La Torre P, Pérez A, et al. Snake venomics and antivenomics of *Crotalus durissus* subspecies form Brazil: assessment of geographic variation and its implication on snakebite management. J Proteomics 2010;73:1758–76.
[3] Chippaux JP, Williams V, White J. Snake venom variability: methods of study, results, and interpretation. Toxicon 1991;29:1279–303.
[4] Glenn JL, Straight R. The miget faded rattlesnake (*Crotalus viridis concolor*) venom: lethal toxicity and individual variability. Toxicon 1977;15:129–32.
[5] Githens TS, George ID. Comparative studies on the venoms of certain rattlesnakes. Bull Antivenin Inst Am 1931;5:31.
[6] Williams DJ, Gutiérrez JM, Calvete JJ, Wüster W, Ratanabanangkoon K, Paiva O, et al. Ending the drought: new strategies for improving the flow of affordable, effective antivenoms in Asia and Africa. J Proteomics 2011;74:1735–67.
[7] Calvete JJ. Proteomic tools against the neglected pathology of snake bite envenoming. Expert Rev Proteomics 2011;8:739–58.
[8] Glenn JL, Sraight RC, Wolfe MC, Hardy DL. Geographical variation in *Crotalus scutulatus scutulatus* (Mojave rattlesnake) venom properties. Toxicon 1983;21:119–30.
[9] Glenn JL, Sraight RC. Intergradation of two different venom populations of the Mojave rattlesnake (*Crotalus scutulatus scutulatus*) in Arizona. Toxicon 1989;27:411–8.
[10] John TR, Smith LA, Kaiser II. Genomic sequences encoding the acidic and basic subunits of Mojave toxin: unusually high sequence identity of non-coding regions. Gene 1994;139:229–34.
[11] Faure G, Xu H, Saul FA. Crystal structure of crotoxin reveals key residues involved in the stability and toxicity of this potent heterodimeric β-neurotoxin. J Mol Biol 2011;412:176–91.
[12] Bon C. Multicomponent neurotoxic phospholipases A_2. In: Kini RM, editor. Venom Phospholipase A_2 Enzymes: Structure, Function and Mechanism. Chichester: Wiley; 1997. p. 269–85.
[13] Sampaio SC, Hyslop S, Fontes MR, Prado-Franceschi J, Zambelli VO, Magro AJ, et al. Crotoxin: novel activities for a classic beta-neurotoxin. Toxicon 2010;55:1045–60.
[14] Chen YH, Wang YM, Hseu MJ, Tsai IH. Molecular evolution and structure-function relationships of crotoxin-like and asparagine-6-containing phospholipases A_2 in pit viper venoms. Biochem J 2004;381:25–34.
[15] Sanz L, Gibbs HL, Mackessy SP, Calvete JJ. Venom proteomes of closely related Sistrurus rattlesnakes with divergent diets. J Proteome Res 2006;5:2098–112.
[16] Wooldridge BJ, Pineda G, Banuelas-Ornelas JJ, Dagda RK, Gasanov SE, Rael ED, et al. Mojave rattlesnakes (*Crotalus scutulatus scutulatus*) lacking the acidic subunit DNA sequence lack Mojave toxin in their venom. Comp Biochem Physiol B Biochem Mol Biol 2001;130:169–79.
[17] Campbell JA, Lamar WW. The Venomous Reptiles of the Western Hemisphere, vol. 2. Ithaca, NY: Comstock Publishing Associates; 2004.
[18] Calvete JJ, Juárez P, Sanz L. Snake venomics. Strategy and applications. J Mass Spectrom 2007;42:1405–14.
[19] Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25:3389–402.
[20] Dart RC, Hurlbut KM, Garcia R, Boren J. Development of a severity score for the assessment of crotalid snakebite. Ann Emerg Med 1996;27:321–6.
[21] Sanchez EE, Galan JA, Powell RL, Reyes SR, Sots JG, Russell WK, et al. Disintegrin, hemorrhagic, and proteolytic activities of Mojave rattlesnake, *Crotalus scutulatus scutulatus* venoms lacking Mojave toxin. Comp Biochem Phys C 2005;141:124–32.
[22] Bieber AL, Tu T, Tu AT. Studies of an acidic cardiotoxin isolated from the venom of the Mojave rattlesnake (*Crotalus scutulatus*). Biochim Biophys Acta 1975;400:178–88.
[23] Glenn JL, Straight R. Mojave rattlesnake *Crotalus scutulatus scutulatus* venom: variation in toxicity with geographical origin. Toxicon 1978;16:81–4.
[24] Farstad D, Thomas T, Chow T, Bush S, Stiegler P. Mojave rattlesnake envenomations in southern California: a review of suspected cases. Wilderness Environ Med 1997;8:89–93.
[25] Weinstein SA, Smith LA. Preliminary fractionation of tiger rattlesnake (*Crotalus tigris*) venom. Toxicon 1990;28:1447–55.
[26] Minton SA, Weinstein SA. Protease activity and lethal toxicity of venoms from some little known rattlesnakes. Toxicon 1984;22:828–30.
[27] Hardy DL. Envenomations by the Mojave rattlesnake (*Crotalus scutulatus scutulatus*) in southern Arizona, U.S.A. Toxicon 1983;21:111–8.
[28] Pook CE, Wuster W, Thorpe RS. Historical biogeography of the Western Rattlesnake (Serpentes: Viperidae: *Crotalus viridis*), inferred from mitochondrial DNA sequence information. Mol Phylogenet Evol 2000;15:269–82.
[29] Mackessy SP. Venom ontogeny in the Pacific Rattlesnakes *Crotalus viridis helleri* and *C. v. oreganus*. Copeia 1988;1:92–101.