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Rattling the border wall: Pathophysiological implications of functional and proteomic venom variation between Mexican and US subspecies of the desert rattlesnake *Crotalus scutulatus*

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Abstract

While some US populations of the Mohave rattlesnake (*Crotalus scutulatus scutulatus*) are infamous for being potently neurotoxic, the Mexican subspecies *C. s. salvini* (Huamantlan rattlesnake) has been largely unstudied beyond crude lethality testing upon mice. In this study we show that at least some populations of this snake are as potently neurotoxic as its northern cousin. Testing of the Mexican antivenom Antivipmyn showed a complete lack of neutralisation for the neurotoxic effects of *C. s. salvini* venom, while the neurotoxic effects of the US subspecies *C. s. scutulatus* were time-delayed but ultimately not eliminated. These results document unrecognised

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potent neurological effects of a Mexican snake and highlight the medical importance of this subspecies, a finding augmented by the ineffectiveness of the Antivipmyn antivenom. These results also influence our understanding of the venom evolution of *Crotalus scutulatus*, suggesting that neurotoxicity is the ancestral feature of this species, with the US populations which lack neurotoxicity being derived states.

1. Introduction

The Mohave rattlesnake, *Crotalus scutulatus scutulatus* Kennicott, 1861 (Kennicott, 1861), is a medically important snake that inhabits the southwestern United States in the arid regions of the Mohave, Sonoran, and Chihuahuan Deserts. Its distribution across the southwestern United States includes southern California, southern Nevada, and extreme southwestern Utah down into western and southern Arizona, extreme southwestern New Mexico, and trans-Pecos Texas (Campbell et al., 2004). In Mexico these snakes are documented from northern Sonora, eastward throughout most of Chihuahua, into Coahuila and western Nuevo León, south through a large portion of Durango and Zacatecas, extreme southwestern Tamaulipas, western San Luis Potosi, Aguascalientes and northeastern Jalisco south into northern Guanajuato (Campbell et al., 2004). It is a medium-sized rattlesnake, Klauber (1997) listing his largest measured male at 1231 mm (Klauber, 1997) while Mrinalini et al. 2015 documents a marginally larger size of 1236 mm. Cardwell (2016 and references within) refers to *C. s. scutulatus* as a dietary generalist that takes numerous small mammals, lizards, and other small vertebrates, with one California population eating a particularly high percentage (75%) of heteromyid rodents (Cardwell, 2016).

Venom from southwestern United States populations of *C. s. scutulatus* has long drawn the interest of researchers (Nair et al., 1976; Glenn and Straight, 1978; Nair et al., 1980; Ho and Lee, 1981; Glenn et al., 1983; Schwartz et al., 1984; Schwartz and Bieber, 1985; Henderson and Bieber, 1986; Glenn and Straight, 1989; Wilkinson et al., 1991; Rael et al., 1993; Wooldridge et al., 2001; Sánchez et al., 2005) and laymen alike. Amongst the latter, largely due to popular media coverage of the snakes' toxicity, *C. s. scutulatus* origins, resurrection capabilities, and reported venom toxicity have reached mythological proportions (Cochran, pers. obs). Geographic venom variation is documented in *C. s. scutulatus* and, historically, two distinct forms that showed an inverse relationship between more toxic neurotoxic and less potent haemorrhagic/proteolytic activity were recognised (Glenn and Straight, 1978; Glenn et al., 1983), with populations producing neurotoxic effects and lower intraperitoneal (i.p.) LD_{50} values designated as Type A and those with haemorrhagic/proteolytic activity and higher i.p. LD_{50} values designated as Type B (Glenn and Straight, 1978). The neurotoxicity observed in Type A venoms is largely attributed to the expression of a presynaptic neurotoxin called Mojave toxin (MT) (Gopalakrishnakone et al., 1980; Borja et al., 2014) while the haemorrhagic/proteolytic activity of Type B venoms is induced by PI and PIII SVMPs (Massey et al., 2012). A third venom phenotype (A + B), comprising both neurotoxic (MT) and proteolytic/haemorrhagic activities, was eventually discovered in individuals occupying the western and southern regions of those expressing Type B venoms (Glenn and Straight, 1989; Wilkinson et al., 1991). Recently, individuals with a venom composition dominated by myotoxin-A have been discovered in the transition zone between

the A and B phenotypes, and Massey et al. (2012) proposed the creation of an additional three venom phenotypes (Type A + M, Type B + M, Type A + B + M) to account for the varying expression of this protein family (Massey et al., 2012).

The Huamantlan rattlesnake, *Crotalus scutulatus salvini* Günther, 1895 (Günther, 1885–1902), ranges from extreme eastern Guanajuato through Querétaro, Hidalgo, possibly northern México, through Tlaxcala, and northern Puebla into western Veracruz where it is restricted to elevations above 1800 m (Campbell et al., 2004). *C. s. salvini* inhabits the open, high interior plains within the Temperate Pine-Oak and Mesquite-Grassland vegetation areas defined by Leopold (1950), with lava beds known to provide prime habitat (Leopold, 1950; Armstrong and Murphy, 1979). Two specimens were found to contain the remains of mammals upon examination (Klauber, 1997).

The venom of the Huamantlan rattlesnake, *C. s. salvini*, has received far less attention in scientific literature to date (Nair et al., 1976; Glenn and Straight, 1978; Nair et al., 1979; Nair et al., 1980; Glenn et al., 1983; Zepeda et al., 1985; Henderson and Bieber, 1986). Glenn and Straight (1978) tested the venom of a single specimen from unlisted locality and found it to have a comparatively low intraperitoneal LD_{50} value (0.18 mg/kg) in laboratory mice, with values just above the average of two Utah, USA locality Type A (neurotoxic) *C. s. scutulatus* specimens (0.11 mg/kg, range 0.09–0.12) and below that of 28 Type A California-Arizona specimens (0.24 mg/kg, range 0.13–0.54) (Glenn and Straight, 1978). Glenn et al. (1983) continued to investigate i.p. LD_{50} values of *C. s. salvini* and *C. s. scutulatus*, though this time with an increased sample size of *C. s. salvini* (three individuals from Vera Cruz, Mexico). While the i.p. LD_{50} values of *C. s. salvini* venom (0.30 mg/kg, range 0.22–0.40) were higher compared to that of their previous findings, they again found the mean to be close to that of the Type A *C. s. scutulatus* venom tested (0.28 mg/kg, range 0.22–0.46) (obtained from six specimens: five from extreme southeastern Arizona and one from the northern city limits of Tucson, Arizona) (Glenn et al., 1983). In contrast, eleven venom samples of *C. s. scutulatus* collected in five localities at the South of Coahuila and Northeast of Durango—populations with Type B venom—presented high intravenous (i.v.) LD_{50} value (1.6 mg/kg, range 0.71–2.5) (Borja et al., 2014).

Geographic venom variation is well documented amongst members of the Viperidae (Jayanthi and Gowda, 1988, Daltry et al., 1996a, 1996b, Saravia et al., 2002, Núñez et al., 2009, Calvete et al., 2011), including members of the genus *Crotalus* (Glenn et al., 1983; Minton and Weinstein, 1986; Straight et al., 1991; Wilkinson et al., 1991; Forstner et al., 1997; Saravia et al., 2002; Sunagar et al., 2014), and is likely the norm rather than the exception. While venom variation between snake populations is becoming increasingly well-characterised from a functional and molecular perspective, the impact of such variation from a clinical perspective receives comparatively less research attention.

In our study we examined three populations of *C. s. scutulatus* and the subspecies *C. s. salvini* for their functional and proteomic variations in venom composition, and the relative impact this has upon the neutralising capacity of the antivenom for these medically important snakes.

2. Materials and methods

2.1. Venoms

Venoms from three adult male specimens for each venom were pooled to minimise individual variation. Collections localities for *C. scutulatus scutulatus* were Cochise Co. AZ, Culberson Co., TX, and Pima, Co. AZ. *C. scutulatus salvini* specimens were collected from unrelated captive animals of unknown locality.

2.2. Neurotoxicity studies

Male chicks (4–10 days) were killed by CO_2 and exsanguination. Both chick biventer cervicis nerve muscle preparations were isolated and mounted on wire tissue holders under 1 g resting tension in 5 mL organ baths containing Krebs solution (NaCl, 118.4 mM; KCl, 4.7 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; $CaCl_2$, 2.5 mM; $NaHCO_3$, 25 mM and glucose, 11.1 mM), maintained at 34 °C and bubbled with 95% O_2/ 5% CO_2. Indirect twitches were evoked by electrical stimulation of the motor nerve (supramaximal voltage, 0.2 ms, 0.1 Hz) using a Grass S_{88} stimulator (Grass Instruments, Quincy, MA). D-Tubocurarine (10 μM) was added, and subsequent abolition of twitches confirmed selective stimulation of the motor nerve, after which thorough washing with Krebs solution was applied to re-establish twitches. In the absence of electrical stimulation, contractile responses to acetylcholine (ACh; 1 mM for 30 s), carbachol (CCh; 20 μM for 60 s) and potassium (KCl; 40 mM for 30 s) were obtained prior to the addition of venom and at the conclusion of the experiment. The preparation was equilibrated for 30 min or until a stable twitch tension was observed prior to the addition of venom. Venoms were left in contact with the preparation for a maximum of 3 h to test for slow developing effects. Efficacy of Antivipmyn (Instituto Bioclon, Mexico; 10 μL/mL) was assessed via a 10 minute pre-incubation with the chick biventer 5 mL organ bath preparation in the organ bath prior to the administration of venom.

Twitch tension was measured from the baseline in two minute intervals. Responses were expressed as a percentage of twitch tension prior to the addition of the venom. Contractile responses to agonists obtained at the conclusion of the experiment were measured and expressed as a percentage of the response obtained prior to the addition of venom. The time taken to inhibit 90% of twitch contractions (t_{90}) was measured as a quantitative means of measuring neurotoxicity. Values for t_{90} were measured by the time elapsed to reach 10% twitch tension amplitude following addition of venom. Where indicated, a two-way analysis of variance (ANOVA) followed by a Bonferroni-corrected post-hoc test was used to determine statistical significance of responses. Statistical analysis was performed using the Prism 5 (GraphPad Software, San Diego, CA, USA) software package. Unless otherwise indicated, data are expressed as mean ± S.E.M.

These experiments were approved by the SOBS-B Monash University Animal Ethics Committee.

2.3. Fibrinogen degradation studies

1 mm 12% SDS-PAGE gels were prepared using the following recipe for resolving gel layer: 3.3 mL deionised H_2O, 2.5 mL 1.5 M Tris-HCl buffer pH 8.8 (Tris - Sigma-Aldrich, St.

Louis, MO, USA; HCl - Univar, Wilnecote, UK), 100 μL 10% SDS (Sigma-Aldrich, St. Louis, MO, USA), 4 mL 30% acrylamide mix (Bio-Rad, Hercules, CA, USA), 100 μL 10% APS (Bio-Rad, Hercules, CA, USA), 4 μL TEMED (Bio-Rad, Hercules, CA, USA); and stacking gel layer: 1.4 mL deionised H_2O, 250 μL 0.5 M Tris-HCl buffer pH 6.8, 20 μL 10% SDS (Sigma-Aldrich, St. Louis, MO, USA), 330 mL 30% acrylamide mix (Bio-Rad, Hercules, CA, USA), 20 μL 10% APS (Bio-Rad, Hercules, CA, USA), 2 μL TEMED (Bio-Rad, Hercules, CA, USA). 10× gel running buffer was prepared using the following recipe: 250 mM Tris (Sigma-Aldrich, St. Louis, MO, USA), 1.92 M glycine (MP Biomedicals), 1% SDS (Sigma-Aldrich, St. Louis, MO, USA), pH 8.3.

Lyophilised human fibrinogen was reconstituted to a concentration of 2 mg/mL in isotonic saline solution, flash frozen in liquid nitrogen, and stored at −80 °C until use. Freeze-dried venom was reconstituted in deionised H_2O and concentrations were measured using a Thermo Scientific™ NanoDrop 2000. Assay concentrations were a 1:10 ratio of venom:fibrinogen, in comparison to 1:5 ratios used in other snake venom testing (Weldon and Mackessy, 2010). The following was conducted in triplicate for each venom: Five "secondary" aliquots containing 10 μL buffer (5 μL of 4× Laemmli sample buffer (Bio-Rad, Hercules, CA, USA), 5 μL deionised H_2O, 100 mM DTT (Sigma-Aldrich, St. Louis, MO, USA)) were prepared. A "primary" aliquot of fibrinogen (volume/concentration as per the above) was warmed to 37 °C in an incubator. 10 μL was removed from the primary aliquot ("0 minute incubation" fibrinogen control) and added to a secondary aliquot, pipette mixed, and boiled at 100 °C for 4 min. 4 μg (dry weight) of venom was then added to the primary aliquot of fibrinogen (amounting to 0.1 mg/mL of venom and 1 mg/mL of fibrinogen in 40 μL total volume), pipette mixed, and immediately returned to the incubator. At each incubation time period (1 min, 5 min, 20 min, and 60 min), 10 μL was taken from the primary aliquot, added to a secondary aliquot, pipette mixed, and boiled at 100 °C for 4 min. The secondary aliquots were then loaded into the gels and were run in 1× gel running buffer at room temperature for 20 min at 90 V (Mini Protean3 power-pack from Bio-Rad, Hercules, CA, USA) and then 120 V until the dye front neared the bottom of the gel. Gels were stained with colloidal coomassie brilliant blue G250 (34% methanol (VWR Chemicals, Tingalpa, QLD, Australia), 3% orthophosphoric acid (Merck, Darmstadt, Germany), 170 g/L ammonium sulfate (Bio-Rad, Hercules, CA, USA), 1 g/L coomassie blue G250 (Bio-Rad, Hercules, CA, USA)), and destained in deionised H_2O.

2.4. Enzymatic substrate cleavage studies

A working stock solution of freeze dried venom was reconstituted in a buffer containing 50% deionised H_2O/50% glycerol (> 99.9%, Sigma) at a 1:1 ratio to preserve enzymatic activity and reduce enzyme degradation with the final venom concentration of 1 mg/mL, and then stored at −20 °C. For assessing the PLA_2 activity a fluorescence substrate assay was used (EnzChek® Phospholipase A_2 Assay Kit, ThermoFisher Scientific). Venom solution (0.1 μg in dry venom weight) was brought up to 12.5 μL in 1× PLA_2 reaction buffer (250 mM Tris-HCL, 500 mM NaCl, 5 mM $CaCl_2$, pH 8.9) and plated out in triplicates on a 384 well plate. Triplicates were measured by adding 12.5 μL quenched 1 mM EnzChek® Phospholipase A_2 substrate per well (total volume 25 μL/well) over 100 cycles at an excitation of 485 nm and emission of 520 nm, using a Fluoroskan Ascent™ Microplate

Fluorometer (ThermoFisher Scientific). The negative control consisted of PLA_2 reaction buffer and substrate only. For testing on RDES substrates, venom solutions (0.1 μg in dry venom weight) were plated in triplicates on a 384 well plate and measured by adding 90 μL quenched fluorescent substrate per well (total volume 100 μL/well; 10 μL/5 mL enzyme buffer - 150 mM NaCl, 50 mM Tri-HCl, 5 mM $CaCl_2$, pH 7.4, Fluorogenic Peptide Substrate, R & D systems Cat#ES0011, Minneapolis, Minnesota). Fluorescence was monitored (excitation at 390 nm and emission at 460 nm for RDES011; 320/405 for all other substrates) over 400 min or until activity ceased.

2.5. LD_{50} studies

Five groups of eight mice (18–20 g, Male and Female BALB/c) for each venom were used. The endpoint of lethality of the mice was determined after 48 h. The venom was dissolved in 0.85% saline at the highest test dose per mouse. Serial dilutions of 2-fold using saline were made to obtain four additional concentrations. The venom lethality was found by injecting 0.2 mL of venom into the tail veins. The injections were administered using a 1-mL syringe fitted with a 30-gauge, 0.5-inch needle. Saline controls were used. The LD_{50} was calculated by the Spearman-Karber method. This protocol was approved by the Texas A&M University-Kingsville Institutional Animal Care and Use Committee (IACUC protocol #: 2015-12-09-A5).

2.6. Proteomic studies

In order to establish the proteomic variations, 1D gradient gels were run under both reducing and non-reducing conditions using the manufacturer (Bio-Rad) protocol. Gels were prepared as follows: 0.05 mL deionised H_2O, 2.5 mL 30% acrylamide mix, 1.5 mL 1.0 M Tris-HCl, pH 8.45, 0.480 mL glycerol, 20 μL 10% APS, 2 μL TEMED (spreading gel); 0.760 mL deionised H_2O, 0.760 mL 30% acrylamide mix, 0.760 mL 1.0 M Tris-HCl, pH 8.45, 15 μL 10% APS, 2 μL TEMED (spacer gel); 1.560 mL deionised H_2O, 0.340 mL 30% acrylamide mix, 0.630 mL 1.0 M Tris-HCl, pH 8.45, 15 μL 10% APS, 2 μL TEMED (stacking gel). Spreading gel was cast first. After it was set the spacer gel was slowly layered atop of it, and after spacer gel was set the stacking gel was layered atop of it. Running buffers were: 0.2 M Tris-HCl, pH 8.9 (anode buffer); 0.1 M Tris-tricine-HCl pH 8.45. The gels were run at 100 V for 3 h at room temperature. 30 μg of venom was reconstituted in Tricine loading buffer (Bio-Rad) with 10 mM DTT added to provide reduced conditions. Gels were stained overnight with colloidal Coomassie brilliant blue G250 (34% methanol, 3% phosphoric acid, 170 g/L ammonium sulfate, 1 g/L Coomassie blue G250). After the staining was complete, water was used to remove excess dye.

In order to identify the toxin types present, digested gel spot samples were processed using an Agilent Zorbax stable bond C18 column (2.1 mm by 100 mm, 1.8 μm particle size, 300 Å pore size) at a flow rate of 400 μL per minute and a gradient of 1–40% solvent B (90% acetonitrile, 0.1% formic acid) in 0.1% formic acid over 15 min or 4 min for shotgun samples and 2D–gel spots respectively on a Shimadzu Nexera UHPLC coupled with an AB SCIEX 5600 Triple TOF mass spectrometer. MS2 spectra are acquired at a rate of 20 scans per second with a cycle time of 2.3 s and optimised for high resolution. Precursor ions were selected between 80 and 1800 m/z with a charge state of 2–5 and of an intensity of at least

120 counts per second with a precursor selection window of 1.5 Da. The isotopes within 2 Da were excluded for MS2. MS2 spectra were searched against known translated transcriptome libraries or UniProt database with Proteinpilot v4.0 (ABSciex) using a thorough identification search, specifying iodoacetamide as an alkylation method, trypsin digestion, and allowing for biological and chemical modifications (ethanolyl C or deamidated N in particular) and amino acid substitutions, including artifacts induced by the preparation or analysis processes. This was done to maximize the identification of protein sequences.

3. Results and discussion

Venoms of *C. s. scutulatus* (Cochise Co., AZ and Culberson Co., TX) and *C. s. salvini* caused rapid blockade of nerve-mediated twitches in the chick biventer cervicis nerve-muscle preparation at the 3 μg/mL concentration (Fig. 1). The two *C. s. salvini* samples were congruent in this respect. In contrast, *C. s. scutulatus* (Pima Co., AZ) had no appreciable effect even at 10 μg/mL. All three neurotoxic venoms did not significantly affect the contractile responses to exogenous agonists acetylcholine (ACh; 1 mM), carbachol (CCh; 20 mM) and potassium chloride (KCl; 40 mM) (Fig. 1, $P > 0.05$, $n = 3$), indicating that these neurotoxins act on the presynaptic site.

Antivipmyn antivenom (16.6 μL:1 μg venom) did not eliminate the action of the two neurotoxic *C. s. scutulatus* venoms (from Cochise Co., AZ, and Culberson Co., TX), though delays in the induction of the venoms' neurotoxic effects were generated by the addition of the anti-venom (Fig. 1). No effect upon the neurotoxicity of the *C. s. salvini* venom was evident (Fig. 1). *C. s. scutulatus* (Cochise Co., AZ) had a t90 of 25.7 ± 1.5 which antivenom shifted to 47 ± 1.4, *C. s. scutulatus* (Culberson Cu., TX) had a t90 of 22.3 which antivenom shifted to 39.6 ± 7.6, and *C. s. salvini* had a t90 of 30.6 ± 4.16, which in the presence of antivenom was unchanged (30.3 ± 1.5). This ratio (16.6 μL:1 μg venom) is considerably higher than the stated potency of the antivenom in neutralisation tests measured against lethality produced by the "challenge dose" of *C. simus* venom (10 μL antivenom neutralising 5.7–6.5 μg venom) (Benard-Valle et al., 2015).

Consistent with the variance in neurotoxicity, *C. s. scutulatus* (Pima Co., AZ) displayed a dramatically lower level of lethality in comparison to the other three venoms, having an LD_{50} of 4.7 mg/kg (Cantu et al., 2017) compared to 0.998 mg/kg for *C. s. scutulatus* (Cochise Co., AZ), 0.493 mg/kg for *C. s. scutulatus* (Culberson Co., TX), and 0.648 mg/kg for *C. s. salvini*. Consistent with the *C. s. scutulatus* population (Culberson Co., TX) demonstrating the most potent neurotoxicity (Fig. 1), it was also the population with the highest lethality in the LD_{50} tests.

Differential fibrinogenolytic activity was evident in the fibrinogen cleavage tests. While all venoms showed some activity in degrading fibrinogen chains, only *C. s. scutulatus* (Pima Co., AZ), was potent in rapidly degrading both the Aα and Bβ chains (Figs. 2 and 3). Only *C. s. scutulatus* (Culberson Co., TX) was limited in its activity on the Bβ chain. The variation between all snakes in their degradation of the Aα-chain was significant ($P < 0.001$), as were the variations in degrading the Bβ-chain with the exception of *C. s.*

scutulatus (Pima Co., AZ) vs *C. s. salvini*. There was an inverse relationship between neurotoxicity and fibrinogen chain destruction for *C. s. scutulatus* (Culberson Co., TX) and *C. s. scutulatus* (Pima Co., AZ), suggesting that these venoms are dominated by toxins targeting the nerves as opposed to those targeting the haemostatic system. However, such a relationship was not evident for the venom of *C. s. scutulatus* (Cochise Co., TX) or *C. s. salvini*. Each venom possessed both strong neurotoxic and fibrinogenolytic activities, with the former having moderate fibrinogen Aα chain destruction activity (0.55 out of 1 relative activity) and strong Bβ chain activity (0.80) and the latter possessing strong relative activity on both chains (0.73 and 0.94, respectively), relative to the normalised (1) value for *C. s. scutulatus* (Pima Co., AZ) on both chains (Figs. 2 and 3), while having neurotoxicity similar in potency to that of *C. s. scutulatus* (Cochise Co., TX) in impeding nerve conductance (Fig. 1).

Other enzymatic tests also produced variation in activity between venoms. In the PLA_2 assay, *C. s. salvini* displayed extremely high levels of activity compared to each of the *C. s. scutulatus* venoms, with *C. s. scutulatus* (Pima Co., AZ) being notable for exhibiting negligible activity (Fig. 4). *C. s. scutulatus* (Pima Co., AZ) was the only venom active upon the metalloprotease substrate RDSE001 and also displayed higher activity upon the metalloprotease substrate RDSE005 than each of the other venoms (Fig. 4). *C. s. scutulatus* (Cochise Co., TX) was much less active upon the serine protease substrate RDSE002 than all other venoms and, along with *C. s. scutulatus* (Pima Co., AZ), was significantly less active upon the serine protease substrate RDES011 (Fig. 4).

In the proteomic examinations, venom from the Pima Co., AZ, population of *C. s. scutulatus* possessed higher concentrations of P-III SVMP (consistent with the RDES001 and RDSE005 enzyme substrate results in Fig. 5) and CRiSP proteins than other populations. There was also differential presence of PLA_2, with the most neurotoxic population (*C. s. scutulatus* (Culberson Co., TX)) possessing only one PLA_2 type, lacking the lower molecular weight form present in the others.

That we witnessed intersubspecific and intrasubspecific venom variation in neurotoxicity, fibrinogen degradation, PLA_2 enzymatic activity, affinity/activity on both metalloprotease and serine protease substrates, and concentrations of P-III SVMP and CRiSP proteins, is not particularly surprising as variation in snake venom components is well documented as occurring at all trophic levels (Glenn and Straight, 1978; Glenn et al., 1983; Minton and Weinstein, 1986; Glenn and Straight, 1989; Forstner et al., 1997; da Silva and Aird, 2001; Saravia et al., 2002; Fry et al., 2003; Sanz et al., 2006; Calvete et al., 2007; Angulo et al., 2008; Fry et al., 2008; Mackessy, 2008; Zelanis et al., 2008; Gibbs and Mackessy, 2009; Calvete et al., 2010; Calvete et al., 2011; Castro et al., 2013; Sunagar et al., 2014; Rogalski et al., 2017). Evidence for co-evolutionary arms races between predators and prey has been documented in the discovery of prey specific toxins and geographic variance in prey susceptibility (Poran et al., 1987; Heatwole and Poran, 1995; Daltry et al., 1996a, 1996b; da Silva and Aird, 2001; Li et al., 2005; Pawlak et al., 2006; Barlow et al., 2009; Gibbs and Mackessy, 2009; Jansa and Voss, 2011). Unfortunately, adequate studies documenting the feeding ecology of *C. s. scutulatus* and *C. s. salvini* in relation to geography are unavailable. The species appear to be dietary generalists (Cardwell, 2016) but detailed dietary studies,

particularly in the areas where *C. s. scutulatus* experiences shifts in venom profiles, may prove informative.

While variation between populations is not a novel finding, these results are the first investigation into the composition and action of the medically important subspecies, *C. s. salvini*, and the first documentation of its potent neurotoxic effect. Our results also show the inability of the regionally specific antivenom, Antivipmyn, to neutralise the neurotoxins in *C. s. salvini* venom, highlighting a crucial consideration for treatment of envenomation by this subspecies and the implications this may have on the pathology experienced by envenomed patients.

This study also reinforces neurotoxicity as a plesiotypic feature of *C. scutulatus ssp.*, as suggested elsewhere (such as Dowell et al., 2016) with populations lacking this function (such as Pima County, AZ) representing a derived state. This derived state may be considered a reversal condition back to the Type I (high levels of metalloprotease activity) from the Type II condition (neurotoxin rich) (Mackessy, 2010). This reinforces the inherent plasticity of snake venoms and the evolutionary as well as clinical implications of such variance.

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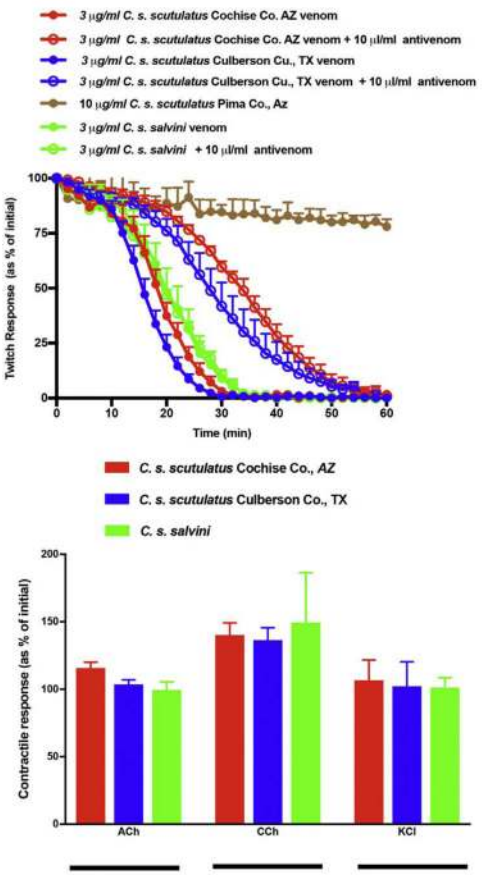

Fig. 1.

Effect of *C. s. scutulatus* and *C. s. salvini* venoms in the absence and presence of Antivipmyn antivenom (3.33 μL:1 μg venom) on (a) nerve-mediated twitches of the chick biventer nerve-muscle preparation and (b) responses to exogenous ACh (1 mM), CCh (20 μM) and KCl (40 mM), compared to initial response, $n = 3$.

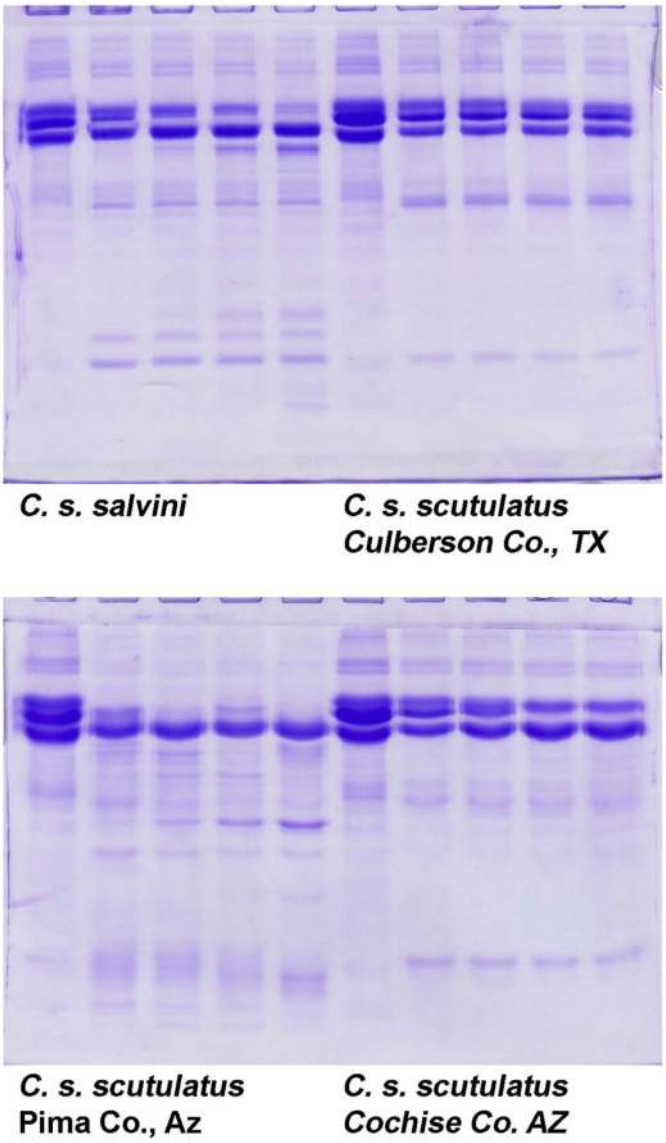

Fig. 2.
Differential ability to degrade the alpha, beta and gamma chains of fibrinogen.

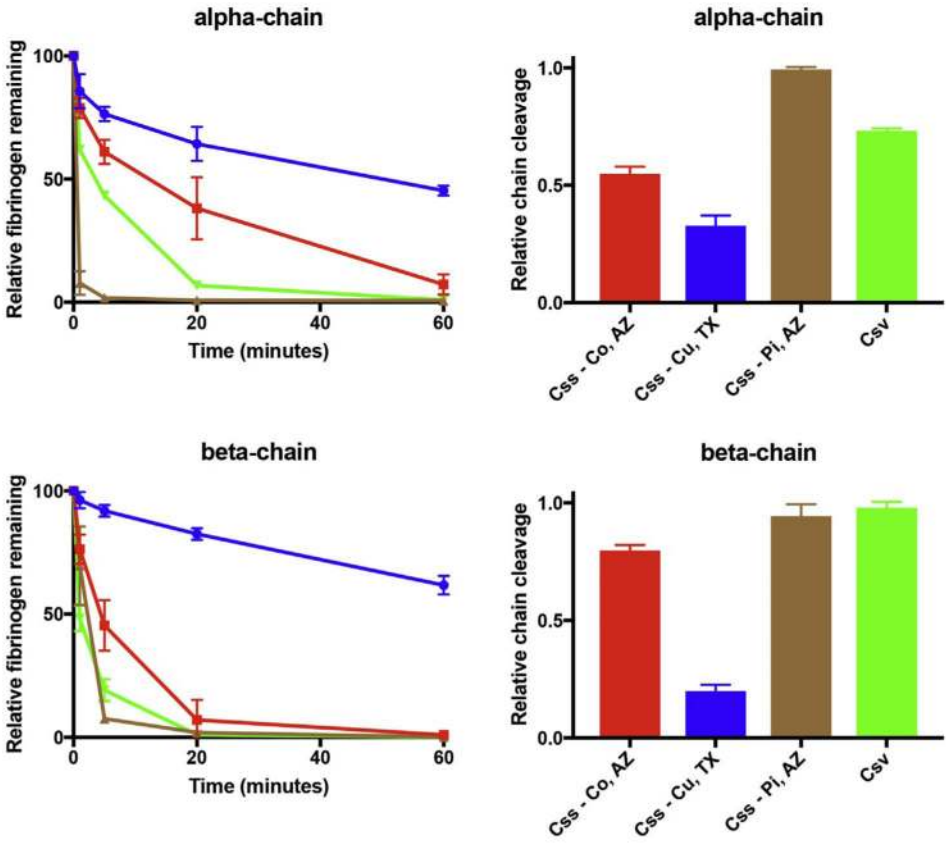

Fig. 3.

Differential ability to degrade the alpha, beta and gamma chains of fibrinogen. **Css–Co** = *C. s. scutulatus* (Cochise Co., AZ), **Css–Cu** = *C. s. scutulatus* (Culberson Co., TX), **Css–Pi** = *C. s. scutulatus* (Pima Co., AZ), and **Csv** = *C. scutulatus salvini* (locality unknown).

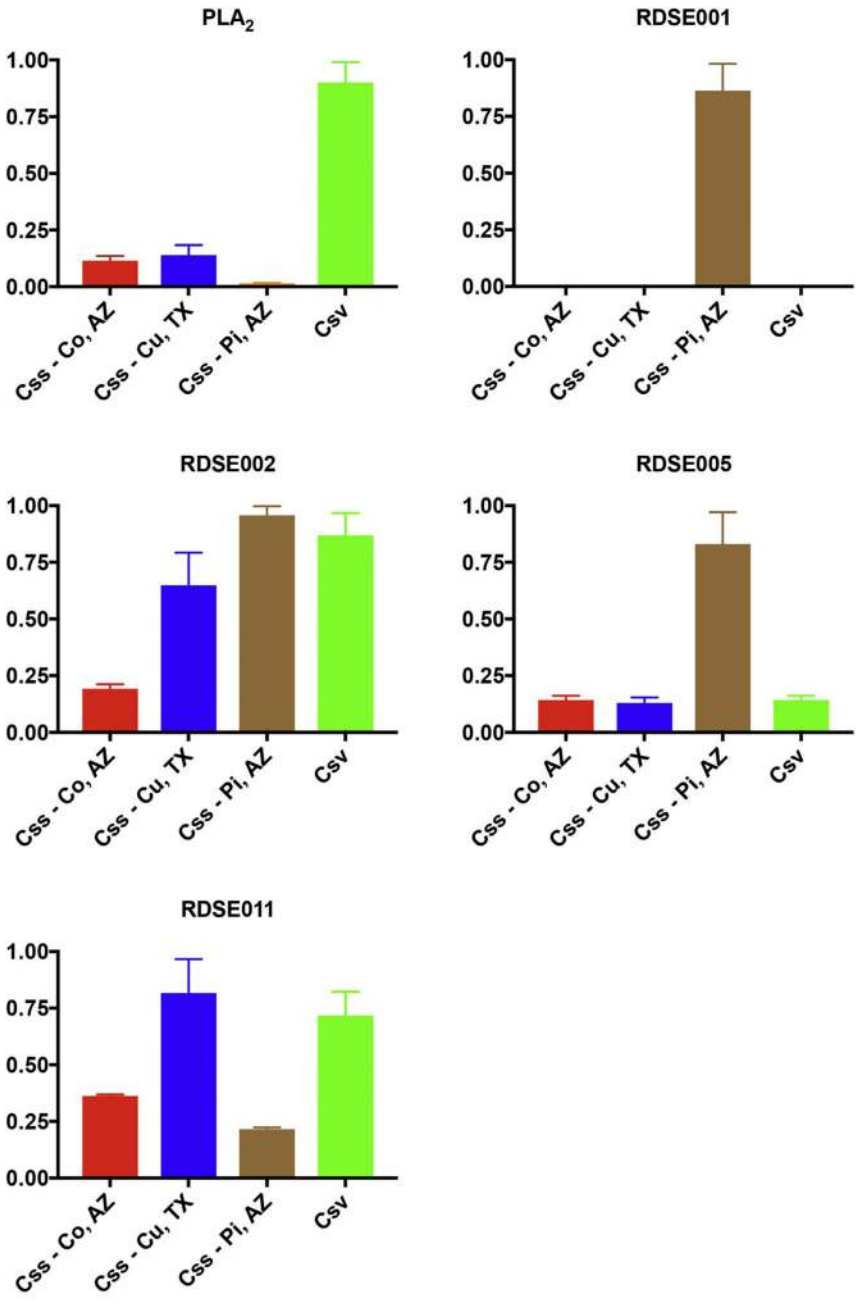

Fig. 4.

Differential activity upon PLA_2, metallo-protease (RDSE001 and RDSE005) and serine protease (RDSE002 and RDES011) substrates.

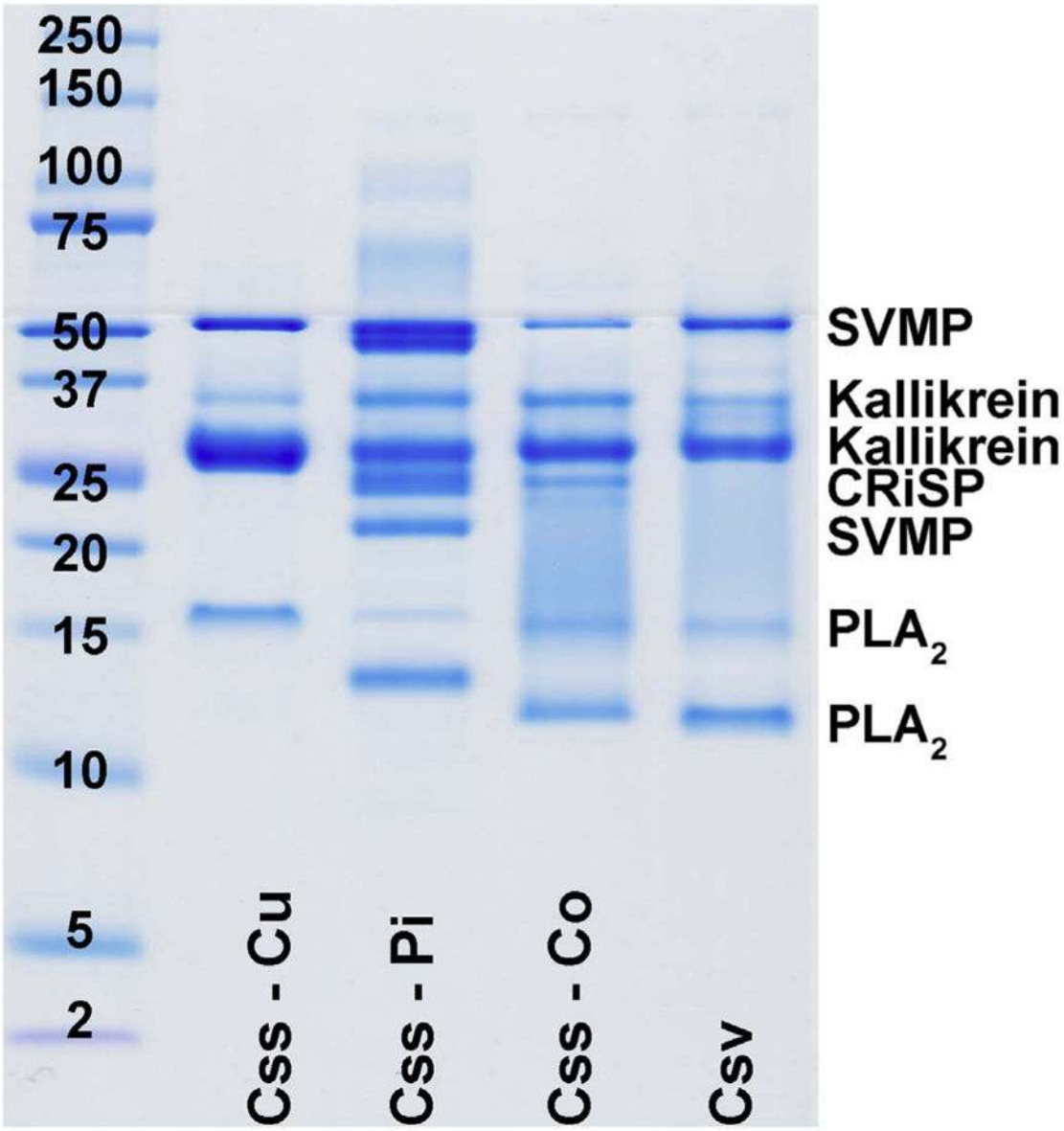

Fig. 5.

1D gel variation with toxin types identified by MS/MS. **Css–Cu** = *C. s. scutulatus* (Culberson Co., TX), **Css–Pi** = *C. s. scutulatus* (Pima Co., AZ), **Css–Co** = *C. s. scutulatus* (Cochise Co., AZ), and **Csv** = *C. scutulatus salvini* (locality unknown).