






## Article

# Ontogenetic Change in the Venom of Mexican Black-Tailed Rattlesnakes (*Crotalus molossus nigrescens*)

Miguel Borja <sup>1,2</sup>, Edgar Neri-Castro <sup>3,4</sup>, Rebeca Pérez-Morales <sup>2</sup>, Jason L. Strickland <sup>5</sup>, Roberto Ponce-López <sup>3</sup>, Christopher L. Parkinson <sup>5,6</sup>, Jorge Espinosa-Fematt <sup>7</sup>, Jorge Sáenz-Mata <sup>1</sup>, Esau Flores-Martínez <sup>1</sup>, Alejandro Alagón <sup>3</sup> and Gamaliel Castañeda-Gaytán <sup>1,\*</sup>

<sup>1</sup> Facultad de Ciencias Biológicas, Universidad Juárez del Estado de Durango, Av. Universidad s/n. Fracc. Filadelfia, C.P. 35010 Gómez Palacio, Dgo., Mexico; alessandro\_53@hotmail.com (M.B.); jsaenz\_mata@ujed.mx (J.S.-M.); pollomtz97@hotmail.com (E.F.-M.)

<sup>2</sup> Facultad de Ciencias Químicas, Universidad Juárez del Estado de Durango, Av. Artículo 123 s/n. Fracc. Filadelfia, Apartado Postal No. 51, C.P. 35010 Gómez Palacio, Dgo., Mexico; rebecapms@ujed.mx

<sup>3</sup> Instituto de Biotecnología, Universidad Nacional Autónoma de México, Avenida Universidad 2001, Chamilpa, C.P. 62210 Cuernavaca, Mor., Mexico; neri@ibt.unam.mx (E.N.-C.); joserobertoponcelopez21@gmail.com (R.P.-L.); alagon@ibt.unam.mx (A.A.)

<sup>4</sup> Programa de Doctorado en Ciencias Biomédicas UNAM, C.P. 04510 México D.F., Mexico

<sup>5</sup> Department of Biological Sciences, Clemson University, 190 Collings St., Clemson, SC 29634, USA; jlstrock@clemson.edu (J.L.S.); viper@clemson.edu (C.L.P.)

<sup>6</sup> Department of Forestry and Environmental Conservation, Clemson University, 190 Collings St., Clemson, SC 29634, USA

<sup>7</sup> Facultad de Ciencias de la Salud, Universidad Juárez del Estado de Durango, Calz. Palmas 1, Revolución, 35050 Gómez Palacio, Dgo., Mexico; dr.jorge.espinosa@gmail.com

\* Correspondence: gamaliel.cg@gmail.com; Tel.: +52-871-715-2077

Received: 31 October 2018; Accepted: 26 November 2018; Published: 1 December 2018



**Abstract:** Ontogenetic changes in venom composition have important ecological implications due the relevance of venom in prey acquisition and defense. Additionally, intraspecific venom variation has direct medical consequences for the treatment of snakebite. However, ontogenetic changes are not well documented in most species. The Mexican Black-tailed Rattlesnake (*Crotalus molossus nigrescens*) is large-bodied and broadly distributed in Mexico. To document venom variation and test for ontogenetic changes in venom composition, we obtained venom samples from twenty-seven *C. m. nigrescens* with different total body lengths (TBL) from eight states in Mexico. The primary components in the venom were detected by reverse-phase HPLC, western blot, and mass spectrometry. In addition, we evaluated the biochemical (proteolytic, coagulant and fibrinogenolytic activities) and biological (LD<sub>50</sub> and hemorrhagic activity) activities of the venoms. Finally, we tested for recognition and neutralization of Mexican antivenoms against venoms of juvenile and adult snakes. We detected clear ontogenetic venom variation in *C. m. nigrescens*. Venoms from younger snakes contained more crotamine-like myotoxins and snake venom serine proteinases than venoms from older snakes; however, an increase of snake venom metalloproteinases was detected in venoms of larger snakes. Venoms from juvenile snakes were, in general, more toxic and procoagulant than venoms from adults; however, adult venoms were more proteolytic. Most of the venoms analyzed were hemorrhagic. Importantly, Mexican antivenoms had difficulties recognizing low molecular mass proteins (<12 kDa) of venoms from both juvenile and adult snakes. The antivenoms did not neutralize the crotamine effect caused by the venom of juveniles. Thus, we suggest that Mexican antivenoms would have difficulty neutralizing some human envenomations and, therefore, it may be necessary improve the immunization mixture in Mexican antivenoms to account for low molecular mass proteins, like myotoxins.

**Keywords:** antivenom; crotamine-like myotoxins; life history; phenotypic variability; sexual maturity; snake venom metalloproteinases; snake venom serine proteinases

**Key Contribution:** Clear ontogenetic venom variation was detected in *C. m. nigrescens* where venoms from juvenile snakes were, in general, more toxic and procoagulant but less proteolytic. Additionally, Mexican antivenoms were not effective at recognizing and neutralizing low molecular mass proteins, such as mycotoxins, predominantly found in juvenile venoms.

---

## 1. Introduction

Phenotypic variation as a result of ontogenetic changes during the life of an organism result from differential selection pressures through time [1]. Because of the different pressures, the realized niche also differs through time based on size and maturity. Differences can include the utilization of different habitats [2], risk to predation [3], and diet breadth [2,4,5]. For example, many species of snakes are known to change the prey composition of their diet based on their size [6–9]. The change in diet is possibly due to the increase in gape size through time [10]. For venomous snakes, there is evidence that many species undergo ontogenetic changes in venom composition that likely accompany the changes in diet [11–14]. The switch in phenotype tends to be associated with size at sexual maturity when the animals become capable of reproduction. For example, Margres et al. [15] reported that ontogenetic shift in venom composition of *C. adamanteus* occurred at 102 cm, the size at which the species reaches sexual maturity. Phenotypic changes through time have relevant ecological implications for the species as well as medical implications. Understanding ontogenetic venom variation is important from medical and biotechnological perspective because these phenotypic differences have direct consequences for the treatment of snake bites.

In general, more toxic and procoagulant venoms have been detected in newborn or juvenile snakes compared to adults [16–22]. These ontogenetic differences in biological activities of venoms could be reflected in different clinical manifestations in people bitten by newborn, juvenile or adult snakes. Differences in venom composition during different life-stages may cause antivenoms that are effective against adult venoms to be ineffective against the venom of juveniles [17,23]. Disentangling phenotypic differences due to ontogeny is challenging because many venom phenotypes, such as rattlesnakes, also have a high level of intraspecific and interspecific variability. Some rattlesnake species display different phenotype of venom (e.g., neurotoxic and hemorrhagic) though their distribution, where more severe envenomations may be expected in areas where rattlesnakes with neurotoxic venoms are distributed [24,25]. But, even when the differences are not as diverse as neurotoxic and hemorrhagic, there is still high levels of variability across the geographic distribution of many species [14,21,26].

Rattlesnake (Viperidae: *Crotalus* and *Sistrurus*) venoms are complex cocktails of proteins containing from 30 to over 100 toxins [27] with key ecological functions for prey acquisition and defense [28]. These proteins are grouped into families according to their sequence similarity, activity and structure [29]. The number and proportion of these protein families in snake venoms can vary at inter and intra-specific levels. Generally, the most abundant venom components in rattlesnakes are myotoxins (Myo), phospholipase A<sub>2</sub>s (PLA<sub>2</sub>s), snake venom metalloproteinases (SVMPs), and snake venom serine proteinases (SVSPs) [30]. Intraspecifically, venom variation has been observed in individuals from different geographic regions [31–35], age [13,14,19,21,36,37] and less commonly sex [38,39]. Snake venom variation may be mediated by genetic polymorphisms [40,41], presence/absence of genes that code for specific toxins (e.g., PLA<sub>2</sub>s and SVMPs) [42,43], and/or post-translational modifications [44,45]. Additionally, age-related venom variation has been associated more frequently to differences in the expression of some specific toxin families [15].

There are approximately 50 species of rattlesnakes which are only found in the New World. Mexico contains the highest number of rattlesnake species with 43 (42 *Crotalus* and 1 *Sistrurus*; Data taken from [www.reptiledatabase.org](http://www.reptiledatabase.org)). Recent studies have demonstrated important intraspecific venom variation in several rattlesnake species distributed in Mexico including: *Crotalus morulus* [46], *C. culminatus*, *C. simus*, *C. tzabcan* [47], *C. s. scutulatus* [33,48] and *C. polystictus* [21]. Additionally, in several species of rattlesnakes, ontogenetic changes have been documented that correspond with different toxin families. In species such as *C. culminatus* and *C. tzabcan*, a reduction in the amount of myotoxins in venoms as snakes increase their size has been detected [49], but in other species, the opposite occurs [50]. Additionally, SVMPs have been shown to increase as species such as *C. oreganus helleri*, *C. o. oreganus*, *Crotalus polystictus* and *C. simus* get larger [19,21,23,49]. However, even in relatively abundant and widely distributed rattlesnake species, there is little information on the composition of their venoms and the implications of possible ontogenetic venom variation in public health or in the efficacy of antivenoms.

The Black-tailed Rattlesnake (*Crotalus molossus*) is a large bodied species that reaches adult size at approximately 57.6 and 65.3 cm snout-vent length (SVLs), for males and females, respectively [51]. Due to the large size differences from neonate (total body length (TBL): ~30 cm) to adult (up to 152.4 cm TBL) [52,53], this species would likely undergo changes in diet composition through time which may be accompanied by venom differences. Balderas-Valdivia et al. [54] reported that *C. m. nigrescens* adults from La Reserva Ecológica del Pedregal in Mexico City feed predominately on rodents, while younger snakes consume predominately lizards. A similar pattern was reported by Werler and Dixon [55] for the closely related Eastern Black-tailed Rattlesnake, *C. ornatus*, from Texas, which shifts from lizards to rodents as they transition from juveniles to adults.

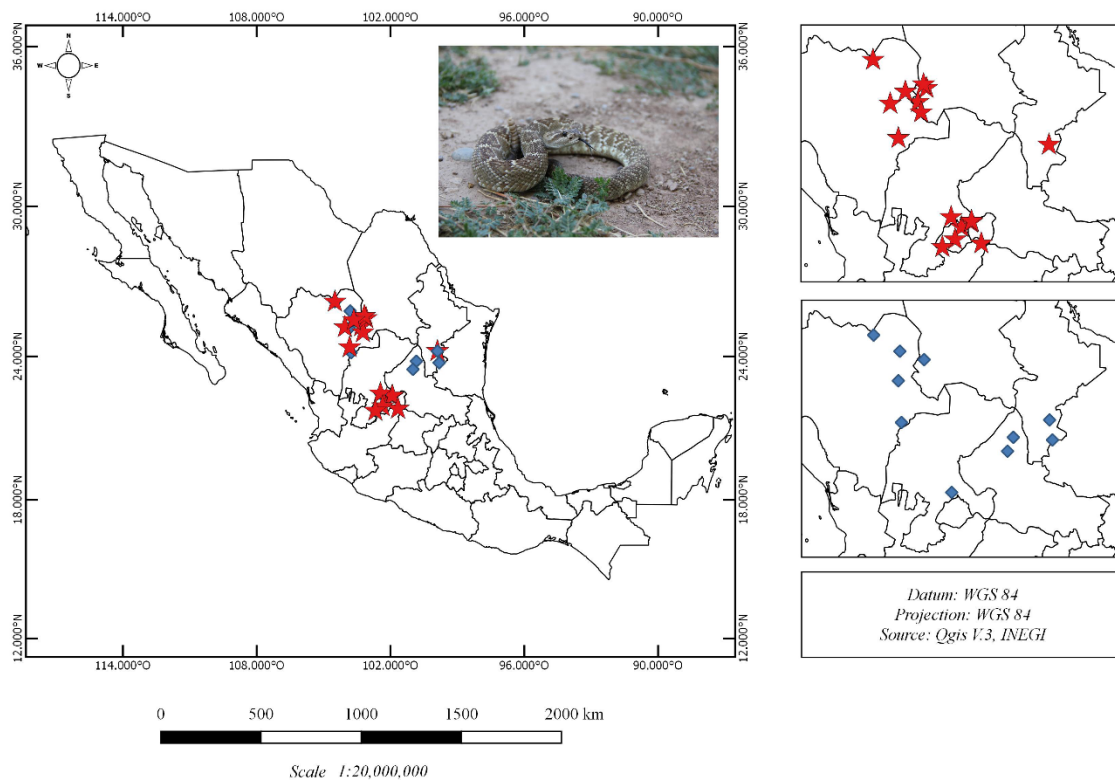
The Mexican Black-tailed Rattlesnake (*Crotalus molossus nigrescens*) is one of four subspecies within *C. molossus* (the other three are *C. m. estebanensis*, *C. m. molossus* and *C. m. oaxacus*) and has the largest distribution in Mexico. Its distribution extends from Northern Durango, Southern Chihuahua and Northern Nuevo León south to Western Veracruz and Northern Oaxaca [53]. Despite the fact that this subspecies is broadly distributed in Mexico, little is known about the biochemical and biological activities of its venom. Previous research reported that *C. m. nigrescens* venom is rich in hydrolytic enzymes such as thrombin-like proteases (21.4 kDa) and proteinase E (75 kDa) [56] and it can have myotoxins  $\alpha$  (crotamine-like) [57]. Additionally, ontogenetic venom variation in fibrinolytic and complement inactivation activities was detected in the Northern Black-tailed Rattlesnake (*C. m. molossus*) [58] so it is possible this change also occurs in *C. m. nigrescens*. Although there is no information available about human envenomations by *C. m. nigrescens*, it has been reported that *C. m. molossus* bites induce edema, ecchymosis, coagulopathy, thrombocytopenia, swelling, and hemorrhagic blebs [59,60]. Additionally, compartment syndrome was reported for one patient bitten by this subspecies [61].

Here, we use range-wide sampling of *C. m. nigrescens* to assess venom variability to test for ontogenetic changes in the biochemical and biologic activities of the venom. We were able to collect a range of sizes for this species and place the variability in a geographic context. This study represents the most complete characterization of the biochemical and biological properties of this subspecies and will be of help for future analyses on related subspecies and species.

## 2. Results

### 2.1. *Crotalus Molossus Nigrescens* Samples

Venom samples were obtained from twenty-seven *C. m. nigrescens* from eight states in Mexico (Figure 1; Table 1). Sixteen snakes were male, nine were females, and two were unknown (CM10 and CM28). The total body length (TBL) of the snakes ranged from 37 to 105 cm (Table 1), but could not be recorded for three individuals (CM21, CM26 and CM28). These three individuals were medium to large adults.



**Figure 1:** Geographic distribution of the twenty-seven *C. m. nigrescens* samples analyzed in this study. Snakes with a total body length (TBL) less than and greater than 70 cm are represented with blue diamonds and red stars, respectively.



**Table 1.** Biologic and biochemical properties of *C. m. nigrescens* venoms from Mexico sorted by total body length (TBL) from smallest to largest.

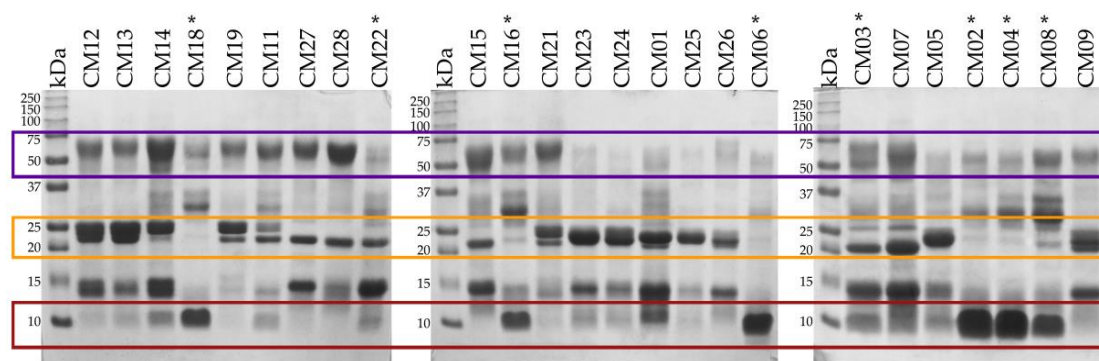
ID	Sex	TBL (cm)	Locality	% of Crotamine-Like Myotoxins in Venom	% of SVMPs in Venom	% of SVSPs in Venom	HPA Hydrolysis (U/mg)	Azocasein Hydrolysis (U/mg)	MCD-P (µg of Venom)	Fibrinogenolytic Activity
CM16	F	37	La Ascensión, N. L.	9.7	41.1	25.1	2.2 ± 0.04	0.31 ± 0.02	13.5	α
CM08	M	40	Ignacio López Rayón, Dgo.	15.3	31.8	38.0	14.0 ± 0.3	1.35 ± 0.01	10.3	α
CM18	F	47.5	Genaro Codina, Zac.	30.0	33.6	22.9	7.0 ± 0.3	0.32 ± 0.01	14.7	α
CM10	ND	49.5	Real de Catorce, S. L. P.	31.8	26.0	24.6	11.1 ± 0.1	ND	24.5	ND
CM04	F	60	Mapimí, Dgo.	48.6	14.0	18.7	7.0 ± 0.7	0.94 ± 0.08	416	α
CM02	M	64	Pedriceña, Dgo.	52.7	18.1	12.5	3.6 ± 0.08	1.4 ± 0.06	36	α
CM03	F	67	Matamoros, Coah.	8.8	44.6	15.8	12.8 ± 0.6	3.8 ± 0.13	390	α
CM17	M	67	Guadalupe del Carnicero, S. L. P.	6.0	41.5	25.4	50.5 ± 2.9	3.2 ± 0.01	301	α and β
CM22	F	68	Miquihuana, Tam.	2.2	35.6	29.5	9.6 ± 0.08	1.9 ± 0.06	44	ND
CM06	F	69	Agua Puerca, Dgo.	41.2	21.9	14.0	19.4 ± 0.7	1.9 ± 0.03	151	α
CM24	M	70	Matamoros, Coah.	1.8	57.32	6.22	178.3 ± 4.0	8.4 ± 0.27	>400 *	α and β
CM11	M	72	Tepezalá, Ags.	6.5	47.1	20.4	136.5 ± 4.2	4.2 ± 0.18	43.3	α and β
CM14	M	72	Genaro García, Zac.	4.5	61.3	14.5	152.7 ± 2.5	3.6 ± 0.19	271 *	α and β
CM05	M	72.5	Nazas, Dgo.	0	59.5	12.3	174.7 ± 2.6	7.5 ± 0.27	75	α and β
CM19	F	75	Genaro Codina, Zac.	3.2	56.5	11.5	163.8 ± 11.0	6.3 ± 0.16	>400	α and β
CM25	F	78.5	Agua Puerca, Dgo.	4.4	51.6	13.2	142.3 ± 4.3	7.7 ± 0.25	182	α and β
CM07	M	80	Barreal de Guadalupe, Coah.	4.3	49.3	13.2	24.9 ± 1.8	3.8 ± 0.15	350	α
CM15	M	80.5	La Ascensión, N. L.	0	50.1	21.6	68.0 ± 1.33	3.9 ± 0.01	470	α and β
CM12	M	82	Genaro García, Zac.	0	67.5	12.4	183.3 ± 6.5	6.0 ± 0.05	0 <sup>†</sup>	α and β
CM13	M	93	Genaro García, Zac.	0	67.7	5.1	203.8 ± 3.2	7.2 ± 0.21	0 <sup>†</sup>	α and β
CM27	F	102	Milpillas de Arriba, Ags.	0	44.8	14.8	99.8 ± 7.4	3.4 ± 0.20	33.5	α
CM01	M	105	Jimulco, Coah.	0	56.1	16.4	112.2 ± 4.1	5.8 ± 0.14	269*	α and β
CM09	M	105	Emiliano Zapata, Dgo.	0	45.2	14.9	184.9 ± 6.3	7.0 ± 0.12	81.8	α and β
CM23	M	105	Matamoros, Coah.	0	60.6	11.1	156.7 ± 12.0	7.0 ± 0.21	0 <sup>†</sup>	α and β
CM21	M	ND	Ojuelos, Jal.	0	61.8	14.5	180.3 ± 15.5	4.4 ± 0.10	177	ND
CM26	M	ND	La Loma, Dgo.	ND	ND	ND	134.6 ± 5.6	6.6 ± 0.40	267	α and β
CM28	ND	ND	Sierra del Laurel, Ags.	0	54.3	16.7	50.6 ± 4.4	3.8 ± 0.06	45.3	α and β

ND: not determined due to lack of venom; TBL: total body length; Sex: F (female); M (male); MCD-P: Minimum coagulant dose plasma; HPA: hide powder azure; SVMP: snake venom metalloproteinases; Ags. Aguascalientes; Coah: Coahuila; Dgo: Durango; Jal: Jalisco; N. L.: Nuevo León; S.L.P.: San Luis Potosí; Tam: Tamaulipas; Zac: Zacatecas; <sup>†</sup> No coagulant activity was observed even after adding up to 400 µg of venom; \* Only partial clot was detected.

## 2.2. SDS-PAGE

### 2.2. SDS-PAGE

In general, *C. m. nigrescens* venoms had diverse electrophoretic pattern in terms of number and intensity of bands (Figure 2). Venoms from six individuals (CM02, CM04, CM06, CM08, CM16, and CM18) displayed a prominent band near 10 kDa that was absent or less intense in the remaining venoms. These six venoms belong to individuals with a TBL of less than 70 cm. These six venoms also lacked a series of bands located between 20 and 25 kDa (PI-SVMs) that were detected in the remaining venoms. Other prominent differences were observed in the area located between 50 and 75 kDa (PII-SVMs), where 13 of the 27 individuals (CM03, CM07, CM11, CM12, CM13, CM14, CM15, CM16, CM18, CM21, CM27, and CM28) had a conspicuous band that was less intense in the remaining venoms (Figure 2).



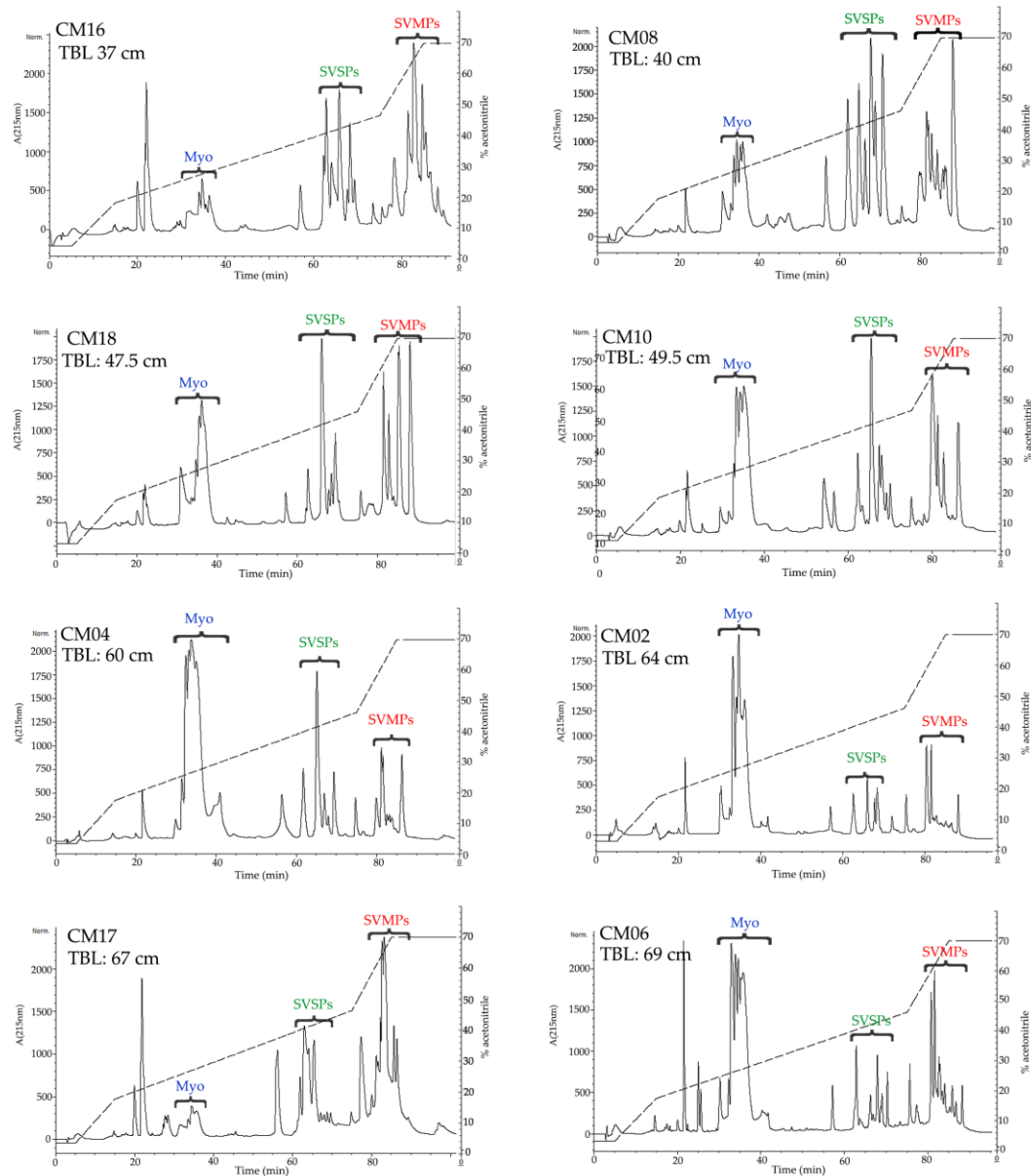
**Figure 2.** 15% SDS-PAGE of *C. m. nigrescens* venoms from Mexico under reducing conditions. Venoms of individuals with a TBL of less than 70 cm are marked with an asterisk (\*). Variation in the bands with molecular masses 10 kDa, from 20 kDa to 25 kDa, and from 50 kDa to 75 kDa are marked with red, yellow and purple boxes, respectively. Based on western blot and ESI-MS analyses, these bands correspond to crotoamine-like myotoxins, PI-SVMs and PII-SVMs, respectively. 15 µg of venom was loaded per lane and gels were stained with Coomassie Brilliant Blue G250.

### 2.3.2. Determination of the Primary Components in *C. m. nigrescens* Venoms

All but one venom sample was separated by RP-HPLC with the goal of visualizing differences in the protein profiles of the venoms (Figures 3 and 4). Figure 3 shows differences detected in the RP-HPLC profiles of *C. m. nigrescens* venoms related to the TBL of snakes. In general, chromatograms tended to be complex, in terms of total number of fractions and number of large or largeish peaks (>70 min TBL) (Figure 4). Six venoms had peaks between 30 and 40 min that were absent in the others (Figures 3 and 4). The seven largest measured individuals and the two unmeasured adults lacked these peaks and the thirteen smallest snakes had them. Of the snakes between 72.5 and 80 cm, only one lacked the peaks. Because peaks eluting from 30 to 40 min were highly abundant in the venom of CM04, we used this venom for further analysis to determine the composition of the peaks observed eluting from 30 to 40 min (Figure 5A) and correspond to proteins with an apparent molecular mass close to 10 kDa from SDS-PAGE (Figure 5B). From this venom (CM04), we collected fractions that eluted from 30 to 38 min (Fractions 6, 7, 8, 9, and 10; Figure 5A) and using ESI-MS mass spectrometry obtained seven proteins with molecular masses: 5129.17 Da (from fraction 6), 5071.90 Da (from fraction 6), 5143.14 Da (from fraction 7), 5180.00 Da (from fraction 7), 4958.53 Da (from fraction 8), 5143.60 Da (from fraction 9) and 5082.50 Da (from fraction 10) (Figure S2). In addition, fractions 6–10 all induced hind limb spastic paralysis in mice, a characteristic effect generated by crotoamine-like myotoxins. The *in vitro* ED<sub>50</sub> calculated for fraction 8 was 2.23 mg/kg. Additionally, through N-terminal sequencing of fraction 8, we determined that the partial protein sequence was comprised of 34 amino acids (KRGLKKGGHGHKFTVLSLPSSIDFGKMDGRWIKW) and showed 100% match to the partial sequence of crotoamine-3 from *C. regalis* and *C. butleri* (UniProt accession: A16601.1). fractions eluting between 30 and 40 min correspond to crotoamine-like myotoxins.

The final elution region with clear variation was the region corresponding to fractions that eluted after 78 min (Figures 3,4,S1). Using specific polyclonal antibodies against SVMPs, we determined that these fractions correspond to SVMPs with molecular masses in the range of 20 kDa to 75 kDa (Figure 5D).

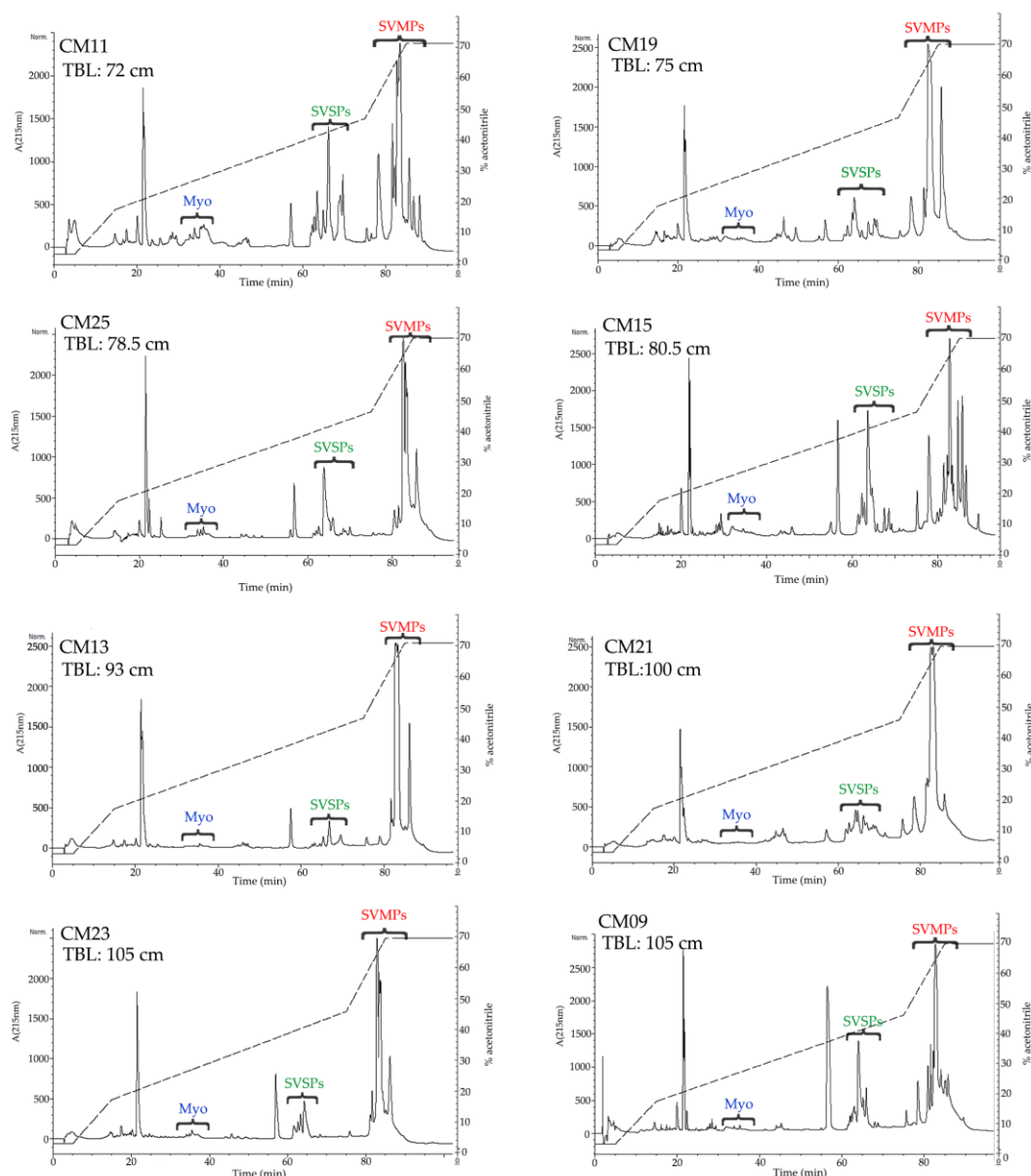
The percentage of crotamine-like proteins in venoms, calculated from chromatograms, ranged from 1.8% to 52.7% (Table 1). Interestingly, a significant relationship ( $r^2 = 0.27$ ;  $p = 0.009$ ) between the relative abundance of crotamine-like myotoxins in venoms and the TBL of snakes was identified. Thus, molecular mass, N-terminal sequence, and biological activity indicate that fractions eluting between 30 and 40 min correspond to crotamine-like myotoxins. The percentage of myotoxins in venom became less abundant in larger snakes and completely disappears in venoms of the largest snakes (Figure 6A). On the other hand, the calculated percentage of SVSPs in *C. m. nigrescens* venoms varied from 5.1% to 36% (Table 1) and again, there was a significant relationship ( $r^2 = 0.36$ ;  $p = 0.002$ ) with a reduction of SVSPs in venoms from larger snakes (Figure 6B). Finally, there was a significant relationship with TBL ( $r^2 = 0.30$ ;  $p = 0.005$ ) where SVMPs molecular mass between 25 kDa and 37 kDa (Figure 5C).



**Figure 3.** Reverse-phase HPLC chromatograms of representative *C. m. nigrescens* venoms of snakes less than 70 cm ordered by total body length (TBL). An analytic C18 reverse-phase column (Vydac®, Deerfield, IL, USA, 218 TP 4.6 mm × 250 mm) was used. Retention time is along the x axis for each panel and labeled every twenty min. Proteins were detected at 215 nm and absorbance is indicated on the left axis. The acetonitrile gradient is shown in the HPLC graph and the percentage value corresponds to the right axis for each panel. Crotamine-like myotoxins (Myo), snake venom serine proteases (SVSPs), and snake venom metalloproteinases (SVMPs) are illustrated in blue, green and red, respectively.

Deerfield, IL, USA, 218 TP 4.6 mm × 250 mm) was used. Retention time is along the x axis for each panel and labeled every twenty min. Proteins were detected at 215 nm and absorbance is indicated on the left axis. The acetonitrile gradient is shown in the HPLC graph and the percentage value corresponds to the right axis for each panel. Crotamine-like myotoxins (Myo), snake venom serine proteases (SVSPs), and snake venom metalloproteinases (SVMPs) are illustrated in blue, green and red, respectively.

8 of 27



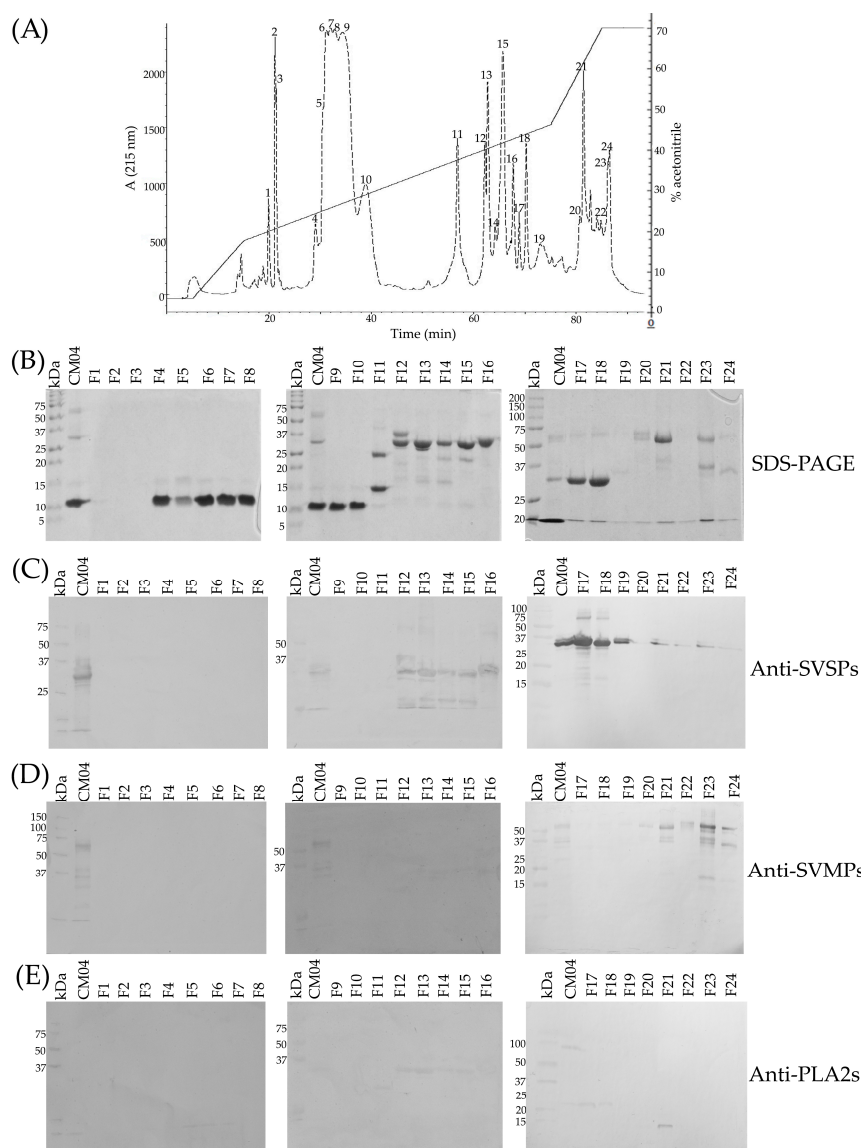
**Figure 4.** Reverse-phase HPLC chromatograms of representative *C. m. nigrescens* venoms of snakes greater than 70 cm ordered by total body length (TBL). An analytic C18 reverse-phase column (Vydac®, Deerfield, IL, USA, 218 TP 4.6 mm × 250 mm) was used. Retention time is along the x axis for each panel and labeled every twenty min. Proteins were detected at 215 nm and absorbance is indicated on the left axis. The acetonitrile gradient is shown in the HPLC graph and the percentage value corresponds to the right axis for each panel. The regions where crotamine-like myotoxins (Myo), snake venom serine proteases (SVSPs), and snake venom metalloproteinases (SVMPs) elute are illustrated in blue, green and red, respectively.

The final elution region with clear variation was the region corresponding to fractions that eluted after 78 min (Figures 3 and 4, Figure S1). Using specific polyclonal antibodies against SVMPs, we determined that these fractions correspond to SVMPs with molecular masses in the range of 20 kDa to 75 kDa (Figure 5D).

The percentage of crotamine-like proteins in venoms, calculated from chromatograms, ranged from 1.8% to 52.7% (Table 1). Interestingly, a significant relationship ( $r^2 = 0.27$ ;  $p = 0.009$ ) between the relative abundance of crotamine-like myotoxins in venoms and the TBL of snakes was identified. The percentage of myotoxins in venom became less abundant in larger snakes and completely

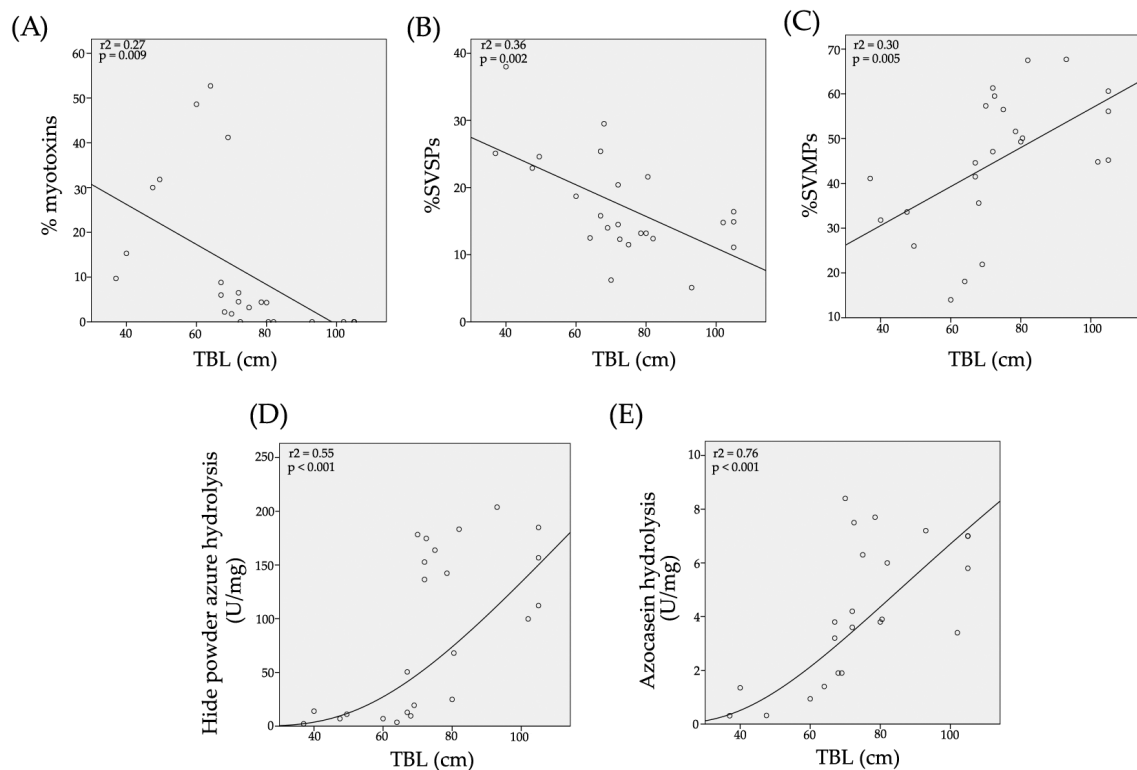


disappears in venoms of the largest snakes (Figure 6A). On the other hand, the calculated percentage of SVSPs in *C. m. nigrescens* venoms varied from 5.1% to 38% (Table 1) and again, there was a significant relationship ( $r^2 = 0.36$ ;  $p = 0.002$ ) with a reduction of SVSPs in venoms from larger snakes (Figure 6B). Finally, there was a significant relationship with TBL ( $r^2 = 0.30$ ;  $p = 0.005$ ) where SVMPs increased with greater TBL (Figure 6C, Table 1).



**Figure 5.** Elution profile of one representative *C. m. nigrescens* venom (CM04) by RP-HPLC, as well as SDS-PAGE and western blot analysis of its fractions. 5.5 mg of venom was fractionated on a C18 column. Twenty four fractions were collected (A) and separated by 15% SDS-PAGE under reducing conditions (B). All fractions were analyzed by western blot using polyclonal antibodies against SVSPs (C), SVMPs (D), and PLA2s (E). Antibodies were obtained from rabbit immunized with *C. simus* venom. RP-HPLC fractions 6, 7, 8, 9, and 10 were further analyzed by ESI-MS mass spectrometry to determine molecular mass of the proteins within those fractions. The acetonitrile gradient is shown in the HPLC graph and the percentage value corresponds to the right axis for each panel.





**Figure 6.** Approximate relative percentage of proteinase-like myotoxins (A), SVSPs (B), SVMPs (C), and proteolytic activity over hide powder azure (D) and azocasein (E) of crude venoms of *C. m. nigrescens* as a function of snake total length. Solid lines are linear curves (A–C) and sigmoidal curves (D,E).

#### 2.4. Hide Powder Azure (HPA) Hydrolysis

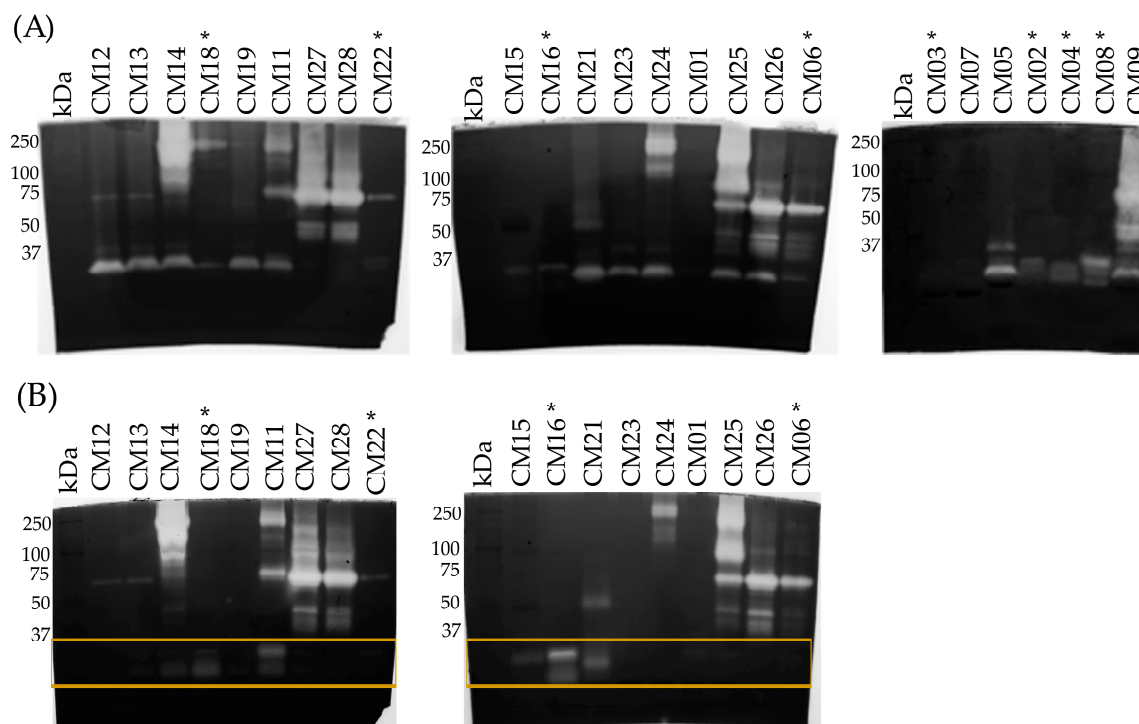
2.4.1. *C. m. nigrescens* HPA Hydrolysis  
All 27 *C. m. nigrescens* venoms analyzed had proteolytic activity on HPA. Venoms hydrolyzed the HPA in ranges from 2.2 U/mg (CM16) to 203.8 U/mg (CM13) (Table 1). A significant positive relationship ( $r^2 = 0.55$ ;  $p < 0.001$ ) between snake TBL and activity on HPA was detected. As the length of snakes increased, the proteolytic activity also increased (Figure 6D). Proteolytic activity of all venoms was completely abolished with ethylenediaminetetraacetic acid (EDTA) indicating this activity is mediated by SVMPs.

#### 2.5. Azocasein Hydrolysis

2.5.1. *C. m. nigrescens* Azocasein Hydrolysis  
Similar to what was observed with the HPA substrate, the 26 venoms tested were able to hydrolyze azocasein with a significant positive relationship ( $r^2 = 0.76$ ;  $p < 0.001$ ) between snake TBL and azocasein hydrolysis (Figure 6E). Venoms showed azocaseinolytic activity in the range from 0.31 U/mg (CM16) to 8.4 U/mg (CM24) (Table 1). Azocaseinolytic activity was completely inhibited with EDTA indicating that this activity is completely mediated by SVMPs.

2.6. Gelatin Hydrolysis  
To evaluate the gelatinolytic activity of venoms, zymograms with gelatin as the substrate were carried out on 25 of the 27 venoms. Venoms degraded gelatin in different regions of the gel and with different intensity (Figure 7A). Most of the venoms degraded gelatin below 37 kDa, however, gelatinolytic activity was more heterogeneous in the region above 37 kDa. For example, venoms such as CM09 and CM25 had proteolytic activity throughout the gel but mainly had low activity including gelatinolytic activity was more heterogeneous in the region above 37 kDa. For example, venoms such as CM09 and CM25 had proteolytic activity throughout the gel but many had low activity including CM01 (TBL: 105 cm), CM03 (TBL: 67 cm), CM07 (TBL: 80 cm), CM13 (TBL: 80.5 cm), CM15 (TBL: 80.5 cm), and CM22 (TBL: 68 cm). In general, venoms from snakes of smaller size (<70 cm) were less proteolytic than venoms of larger snakes (>70 cm); however, some venoms of individuals greater than 70 cm did not generate hydrolysis (CM01) or did in a reduced manner (CM07, CM15, and CM23).

70 cm did not generate hydrolysis (CM01) or did in a reduced manner (CM07, CM15, and CM23) (Figure 7A). Gelatinolytic activity was only slightly inhibited with EDTA, indicating that proteases other than SVMs (possibly SVSPs) generated most of the activity (Figure 7B).



**Figure 7.** (A) Gelatinolytic activity of crude venoms of *C. m. nigrescens*. 1 mg/mL of gelatin was added to the 12% SDS PAGE; 10 µg of venom was loaded per lane. Venoms of individuals with a TBL of less than 70 cm are marked with an asterisk (\*). Bands containing proteolytic activity are seen as a clear band on the dark background. (B) Gelatinolytic activity of crude venoms of *C. m. nigrescens* preincubated with 20 mM EDTA for 30 min at 37 °C. Panel B shows the disappearance of activity at 37 kDa (yellow boxes), particularly for venoms CM12, CM13, CM14 (partially), CM18, CM19, CM21, CM23, CM24, CM25, CM26 and CM06. Additionally, an inhibition of the activity in the high molecular weight region (>100 kDa) was observed for the venoms CM18, CM19 and CM21. These data indicate that there are metalloproteases of type PII and PIII that have been inhibited under the aforementioned conditions.

2.7. Minimum Coagulant Dose (MCD-P)

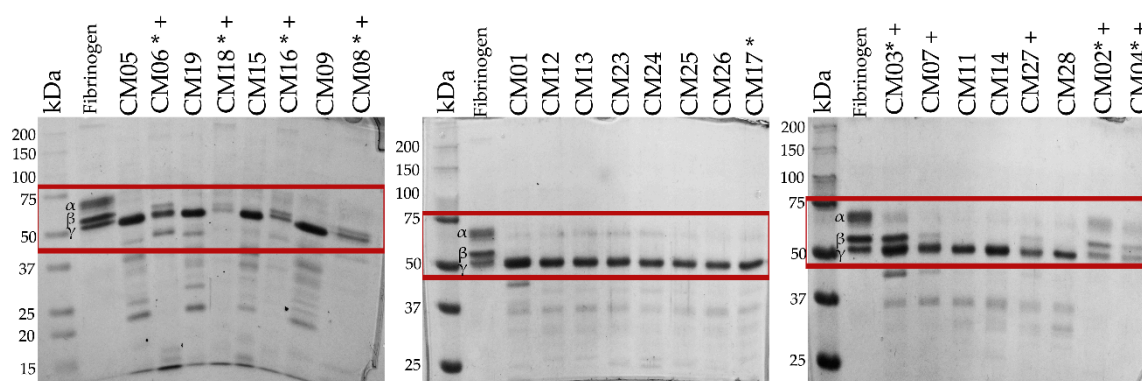
Twenty-four of the twenty-seven venoms produced a fibrin clot when they were incubated with human plasma, although at different rates. Of these, three venoms (CM01, CM14, and CM24) only formed partial clots while the remaining twenty-one venoms generated complete clots (Table 1). Three venoms (CM12, CM13, and CM23) of individuals greater than 70 cm TBL lacked coagulant activity despite adding 400 µg of venom (Table 1). Although the venoms from CM19 and CM24 had procoagulant activity, it was not possible to determine their MCD-P because adding up to 400 µg of venom only produced a blood clot after 10 min of reaction. The MCD-P of venoms ranged from 10.3 µg to up to >400 µg (Table 1). Noticeably, venoms of individuals with a TBL of less than 50 cm (CM08, CM10, CM16, and CM18) showed the highest procoagulant activity, with values in the range of 10.3–24.6 µg. Although, in general, venoms of smaller snakes exhibited more thrombin-like action on plasma, some venoms of snakes longer than 100 cm had coagulant activity (CM23 and CM09, MCD-P: 24.6 µg and 81.8 µg, respectively). Incubation of venoms with phenylmethylsulfonyl fluoride (PMSF) completely abolished the coagulant activity of all venoms while incubation with EDTA did not inhibit P: 33.5 µg and 81.8 µg, respectively). Incubation of venoms with phenylmethylsulfonyl fluoride (PMSF) completely abolished the coagulant activity of all venoms while incubation with EDTA did not inhibit the activity. This indicates that coagulant activity is mediated by SVSPs and not by metal-dependent proteolytic enzymes like SVMs.

the activity. This indicates that coagulant activity is mediated by SVSPs and not by metal-dependent proteolytic enzymes like SVMPs.

12 of 27

## 2.8. Fibrinogenolytic Activity

All 24 *C. m. nigrescens* venoms analyzed hydrolyzed fibrinogen, however, only five venoms hydrolyzed both the  $\alpha$  and  $\beta$  chains of fibrinogen and nine venoms hydrolyzed only the  $\alpha$  chain of fibrinogen (Table 2) (Figure 8). Five of the five venoms that cleaved both fibrinogen chains belonged to individuals with a TBL greater than 70 cm, while seven of the nine venoms that hydrolyzed only the  $\alpha$  chain of fibrinogen corresponded to snakes with a TBL less than 70 cm. Interestingly, venoms with specificity for specific fibrinogen chains were not observed with venoms from snakes with a TBL greater than 70 cm, which indicates that SVSPs are responsible for the fibrinogenolytic activity in these venoms.



**Figure 8.** Fibrinogenolytic activity of crude venoms of *C. m. nigrescens*. 150  $\mu$ g of fibrinogen were incubated with 5  $\mu$ g of each venom for 15 min at 37 °C. Venoms of individuals with a TBL of less than 70 cm are marked with an asterisk (\*). Venoms marked with a cross (+) generated a fibrinogen clot. Fibrinogenolytic activity was visualized on 12% SDS-PAGE and it is marked with a red box. 100  $\mu$ g of fibrinogen/venom mix were loaded per lane. Gels were stained with Coomassie Brilliant Blue.

## 2.9. Median Lethal Dose (LD<sub>50</sub>)

To reduce the number of mice sacrificed, only nine representative venoms were analyzed for LD<sub>50</sub>. *C. m. nigrescens* venoms had toxicity in the range of 1.13 mg/kg to 4.3 mg/kg (Table 2). Five of the six venoms of snakes with a TBL of less than 70 cm were significantly more toxic (with a median LD<sub>50</sub> of 1.5 mg/kg) than venoms of snakes with a TBL greater than 70 cm (with a median LD<sub>50</sub> of 4.03 mg/kg;  $t = 0.0015$ ;  $p = 0.03$ ). The one venom (CM04) corresponding to an individual (with a size of 60 cm) had an LD<sub>50</sub> of 4.1 mg/kg. This toxicity value was similar to those displayed by venoms of snakes with a TBL greater than 70 cm (Table 2). In addition, venoms from snakes less than 70 cm (CM03, CM04, CM06, CM08, CM16 and CM18) provoked hind limb spastic paralysis of all mice injected. This effect was not observed in the mice injected with venoms from snakes less than 70 cm (CM05, CM07, CM09, CM15 and CM19). This effect was not observed in the mice injected with venoms from snakes greater than 70 cm (CM09, CM15 and CM19).



**Table 2.** Toxicity and minimum hemorrhagic dose (MHD) of representative *C. m. nigrescens* venoms from Mexico.

ID	TBL (cm)	LD <sub>50</sub> (mg/kg)	MHD (μg)	Neutralization (ED <sub>50</sub> ) Antivipmyn® (μLAV/3LD <sub>50</sub> )	Neutralization (ED <sub>50</sub> ) Faboterapico Polivalente Antiviperino® (μLAV/3LD <sub>50</sub> )
CM16	37	1.26 *	6.10	ND	ND
CM08	40	1.71 *	19.30	ND	ND
CM18	47.5	1.13 *	4.0	222.0 (204.4–241.1) **	240.1 (184.1–313.1) **
CM04	60	4.10 *	>25	ND	ND
CM03	67	1.71 *	ND	ND	ND
CM06	69	1.72 *	21.90	246.2 (214.1–283.0) **	158.0 (121.7–205.1) **
CM19	75	4.20	10.26	>450	>450
CM15	80.5	3.60	8.30	ND	ND
CM09	105	4.30	10.95	322.1 (309.3–335.3)	411.1 (401.7–420.7)

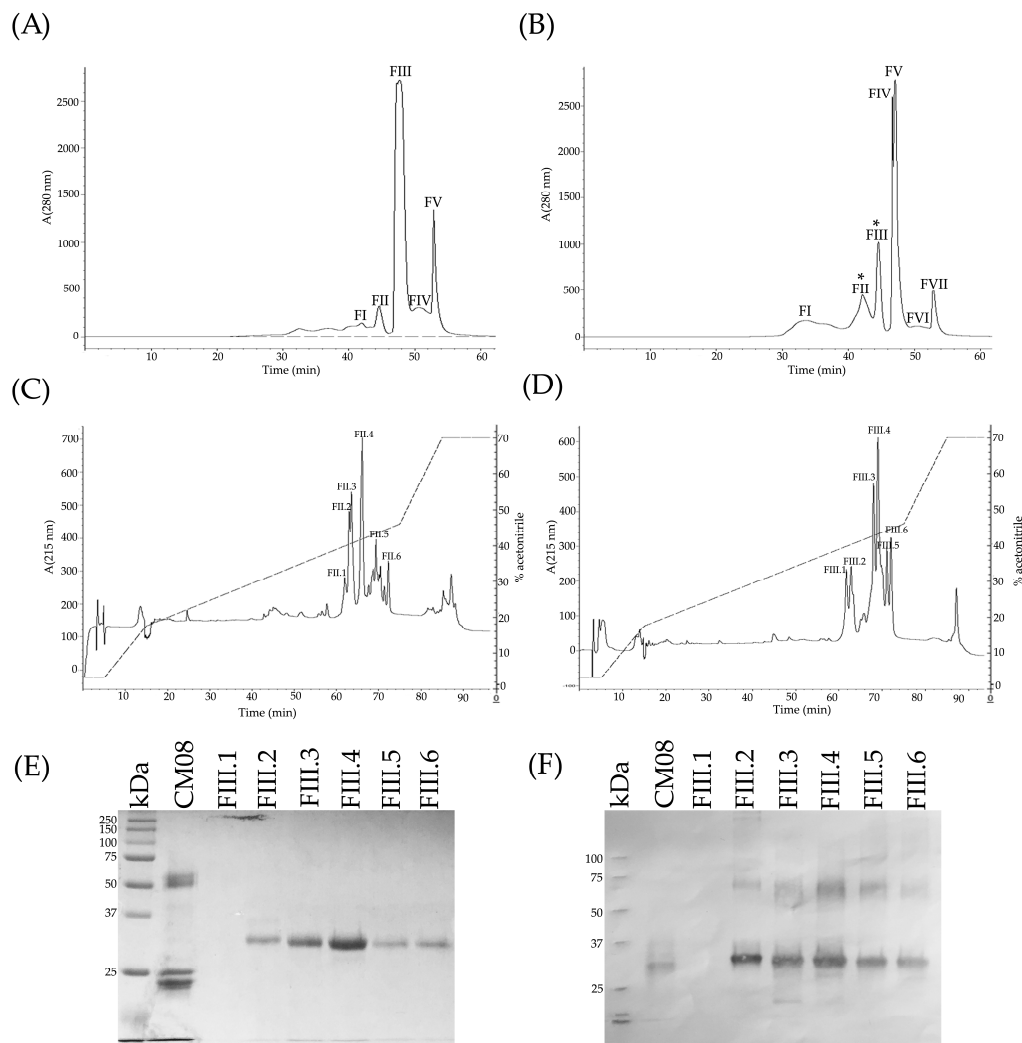
\* Provoked hind limb spastic paralysis of all mice injected during LD<sub>50</sub> tests. \*\* Mice presented hind limb spastic paralysis during neutralization studies. ED<sub>50</sub>: effective dose 50%; neutralizing potency expressed as microliters of antivenom that neutralized 3 times the median lethal dose (LD<sub>50</sub>). >450: Mice did not survive with volumes less than or equal to 450 μL. ND: not determined.

## 2.10. Minimum Hemorrhagic Dose (MHD)

To reduce the number of mice sacrificed, only eight representative *C. m. nigrescens* venoms were analyzed for MHD. With the exception of the venom from CM04 which lacked hemorrhagic activity despite injecting up to 25 μg, the remaining venoms displayed hemorrhagic activity (Table 2). The most hemorrhagic venoms were CM18, CM16, and CM15 with MHD values of less than 4.0 μg, 6.1 μg and 8.3 μg, respectively. Of the remaining venoms with hemorrhagic activity, venom CM06 was the least active with a MHD of 21.9 μg (Table 2). In addition, no apparent relationship between hemorrhagic activity and TBL of snakes was observed.

## 2.11. Toxic Components in Juvenile *C. m. nigrescens* Venoms

With the goal of determining the toxic components in venoms from juvenile snakes, CM04 and CM08 venom were separated by size-exclusion chromatography. Five and seven fractions were obtained from CM04 and CM08 venoms, respectively (Figure 9A,B, respectively). Only fraction three (FIII) from CM04 venom and fractions two, three and four (FII, FIII and FIV) from CM08 venom were able to kill mice after 60 μg were injected i.v. (Table 3). However, only FIII from CM08 venom is toxic to mice after injecting only 20 μg (Table 3). Interestingly, while FIII from CM04 and FIV from CM08 caused hind limb spastic paralysis when injected in mice (indicating that they contained crotoamine-like myotoxins), FII and FIII from CM08 did not induced this effect but instead induced ophisthosis and bleeding. Thus, FII and FIII from CM08 venom were further separated by RP-HPLC (Figure 9C,D, respectively). Both molecular exclusion fractions eluted proteins in the range from 60 to 72 min by RP-HPLC, however all RP-HPLC fractions lost their toxic activity in mice (possibly due to protein denaturalization by acetonitrile used in RP-HPLC) since they were unable to kill mice despite adding 25 μg. SDS-PAGE revealed that RP-HPLC fractions obtained from CM08 FIII contain a unique band between 25 kDa and 37 kDa (Figure 9E) and it was demonstrated by western blot using polyclonal antibodies against SVSPs that this band correspond to SVSPs (Figure 9F). Unexpectedly, an additional band between 50 kDa and 75 kDa was observed in the western blot, indicating that at least two SVSPs generated most of the toxicity in juvenile snakes (Figure 9F).



**Figure 9.** Size-exclusion chromatography of CM04 and CM08 venoms, RP-HPLC of size-exclusion fractions, and SDS-PAGE and western blot of RP-HPLC fractions. Nine and five milligrams of venoms CM04 (A) and CM08 (B), respectively, were loaded in a Cosmosil column (Diol 300, 7.5 × 600 mm) and eluted by size-exclusion chromatography. Two hundred micrograms of FII (C) and FII (D) fractions (the main fractions to mice) from CM04 and CM08 venoms were further separated by RP-HPLC. RP-HPLC fractions from FII were analyzed by SDS-PAGE (E) and Western blot (F) using anti-cyclophosphamide antibodies against SVSPs. Faint SVSPs with molecular masses in the range from 25 kDa to 75 kDa were detected by Western blot, indicating that SVSPs are the main components for generating toxicity of the toxic snakes.

**Table 3.** Toxicity on mice of size-exclusion chromatography fractions from CM04 and CM08 venoms.

Table 3. Toxicity on mice of size-exclusion chromatography fractions from CM04 and CM08 venoms.											
Fraction/Venom		Mortality with Different Amounts of Venom (µg/mice)									
		CM04 (60)		CM08 (60)		CM04 (45)		CM08 (45)		CM04 (20)	
		Mortality with Different Amounts of Venom (µg/mice)									
Fraction/venom		CM04	CM08	CM04	CM08	CM04	CM08	CM04	CM08	CM04	CM08
		(60)	(60)	(45)	(45)	(20)	(20)	(20)	(20)	(20)	(20)
FI		0/3	0/3	NA	NA	NA	NA	NA	NA	NA	NA
FII		0/3	3/3	NA	NA	1/3	NA	NA	NA	NA	0/3
FIII		3/3	3/3	1/3	2/3	1/3	2/3	0/3	0/3	1/3	1/3
FIV	FI	0/3	0/3	3/3	0/3	NA	NA	1/3	NA	NA	0/3
FV	FII	0/3	0/3	0/3	3/3	NA	NA	NA	1/3	NA	0/3
FVI	FIII	NA	3/3	0/3	3/3	NA	1/3	NA	2/3	NA	1/3
FVII	FIV	NA	0/3	0/3	3/3	NA	NA	NA	1/3	NA	0/3
	FV	0/3	NA	Not applicable	NA	NA	NA	NA	NA	NA	NA



FVI	NA	0/3	NA	NA	NA	NA
FVII	NA	0/3	NA	NA	NA	NA

NA: Not applicable.

### 2.12. Detection of Crotoxin-Like Neurotoxins at the Protein Level

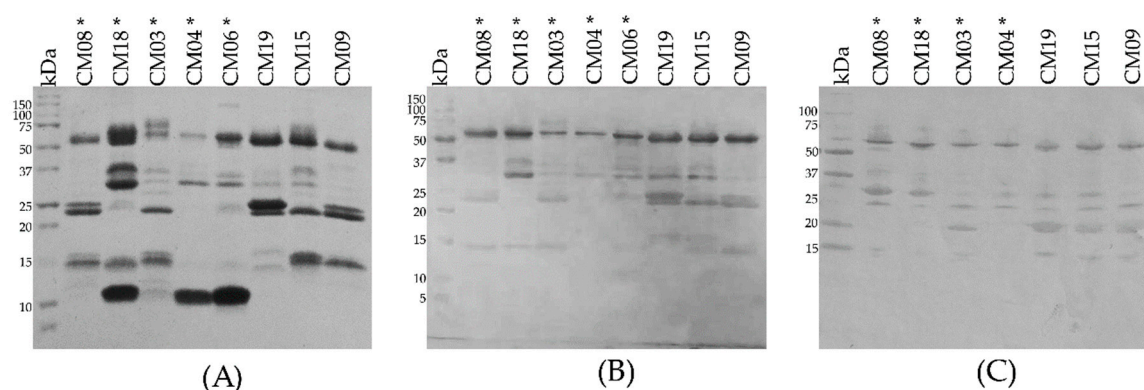
To determine if neurotoxins similar to Crotoxin/Mojave toxin were present at the protein level, an ELISA analysis was made using monoclonal antibodies against the basic subunit of crotoxin. As previously reported in literature, none of the *C. m. nigrescens* individuals were positive for neurotoxins in their venom [62,63]. As expected, the Type A *C. s. scutulatus* venom used as a control was positive for Mojave toxin.

### 2.13. Neutralization Studies

Neutralizing activity of Mexican antivenoms Antivipmyn® and Faboterapico polivalente antiviperino® were evaluated using venoms from four *C. m. nigrescens* individuals with different TBL (CM06, CM09, CM18 and CM19). In general, both antivenoms neutralized venoms of juvenile snakes (CM06 and CM18) better than venoms of the adults (CM09 and CM19). Antivipmyn® neutralized the title of the venoms tested, effective (ED<sub>50</sub>) (ED<sub>50</sub> values of 246/31 D<sub>50</sub>/332/31 D<sub>50</sub> and 222/31 D<sub>50</sub>/31 D<sub>50</sub> for CM06, CM09 and CM18 and CM19, respectively (Table 2). Similarly, Faboterapico polivalente® antivenom neutralized the same three venoms that Antivipmyn® did. The ED<sub>50</sub> values were 158/61 D<sub>50</sub>/413 D<sub>50</sub>/41 D<sub>50</sub> and 240 D<sub>50</sub>/43 D<sub>50</sub> for CM06, CM09 and CM18 and CM19, respectively. Neither of the antivenoms were able to neutralize CM19 despite adding 50 to 450 µL of antivenom and adding hind limb spastic paralysis induced by the toxins in CM06 and CM18 venoms was not neutralized by any antivenom.

### 2.14. Immune Recognition of Antivenom

Although most proteins were recognized by the Mexican antivenoms Antivipmyn® and Faboterapico polivalente antiviperino® (Figure 10B,C, respectively), proteins with molecular masses lower than 20 kDa were weakly or not recognized. Importantly, crotoxin-like myotoxins (~10 kDa by SDS-PAGE, Figure 10A) of juvenile venoms (CM04, CM06 and CM18) were not recognized by antivenoms, a result congruent with what was observed in the neutralization test where antivenoms did not neutralize the activity of these proteins.



**Figure 10.** SDS PAGE and western blot analysis of representative *C. m. nigrescens* venoms. (A) Venoms (10 µg) were separated by 15% SDS-PAGE. Venoms (5 µg) on nitrocellulose were detected with Antivipmyn® (B) and Faboterapico polivalente antiviperino® (C). Venoms of individuals with a TBL of less than 70 cm are marked with an asterisk (\*).

## 3. Discussion

### 3. Discussion

The number and relative abundance of the protein families in rattlesnake venom varies broadly at the intra-specific level. The number and relative abundance of the protein families in rattlesnake venom varies broadly at the intra-specific level. The factors commonly associated with intra-specific venom variation are the snake's age and their geographic distribution [13,32,44,63]. In our study, we detected clear variation, in terms of relative abundance and presence/absence of proteins specifically associated with the length of individual snakes. The protein profiles of *C. m. nigrescens* venoms as demonstrated by SDS-PAGE

and RP-HPLC analyses, showed marked differences in venoms from snakes of different sizes classes. The most abundant components in *C. m. nigrescens* venoms were SVMPs, SVSPs, and crotamine-like myotoxins. However, the proportion of each of these components was variable among venoms from smaller and larger snakes, where small snakes displayed more crotamine-like myotoxins and SVSPs compared to larger snakes, but the opposite occurred with SVMPs (Figure 6A–C; Table 1). In the current study, the threshold of venom change of *C. m. nigrescens* was at approximately 70 cm TBL. Snakes with a size below this threshold were, in general, more toxic and procoagulant, but less proteolytic. In addition, snakes with TBL less than 70 cm had venoms containing crotamine-like myotoxins that were reduced or absent in the snakes with a TBL greater than 70 cm. It is likely that the ontogenetic venom change observed in *C. m. nigrescens* corresponds with its sexual maturity, since this subspecies acquires sexual maturity at approximately 70 cm of TBL [51].

Crotamine is a small, non-enzymatic protein composed of about 42 amino acids (4.8 kDa) that was first isolated in the venom of *C. durissus terrificus* [66]. Among other effects, crotamine is capable of inducing spastic paralysis in the hind limbs of mice that are injected with the toxin and generates necrosis in muscle cells [67,68]. Previously, it had been reported that some rattlesnakes species, including *C. m. nigrescens*, contain crotamine-like myotoxins (e.g.,  $\alpha$ -myotoxins) in their venom [57]. In the current study, sixteen *C. m. nigrescens* venoms contained crotamine-like myotoxins in different proportion as demonstrated by RP-HPLC (Figure 3, Figure S1; Table 1). Interestingly, venoms of snakes larger than 80 cm completely lacked this small toxin (Figure 4, Figure S1; Table 1). In addition, only venoms containing crotamine-like myotoxins were able to cause hind limb spastic paralysis of all mice injected. Crotamine-like myotoxins were also visualized by SDS-PAGE as a prominent band near 10 kDa in venoms with the highest percentage of these proteins (e.g., CM02, CM04, CM06, CM08, CM16, and CM18). This apparent molecular mass obtained by SDS-PAGE is similar to those described in other analyses for this basic protein [23,47,69]. From venom CM04, we isolated at least seven crotamine-like myotoxin isoforms with molecular masses ranging from 4958.53 Da to 5180.00 Da and obtained the N-terminal sequence of one of them. Presence of multiple forms of crotamine-like myotoxins in the same venom has been reported in other *Crotalus* venoms and these isoforms can differ only in few amino acids [70], what could explain the variation in the molecular masses of the isoforms detected in *C. m. nigrescens* venom. Additionally, the i.v. LD<sub>50</sub> calculated for one of these isoforms was 2.23 mg/kg, a value within the range reported for the toxicity of crotamine (from 0.07 to 32.76 mg/kg) [67]. Presence of myotoxins in the venom of juvenile snakes and their consequent reduction in adult snakes has been reported in *C. adamanteus* and *C. tzabcan* (the juvenile has 34% while the adult has 18.5% of myotoxins) [15,49]. However, an inverse pattern has been detected in *C. oreganus concolor* and *C. viridis viridis*, where venoms of adult individuals expressed a greater amount of myotoxins compared to juvenile snakes [14,71].

Thrombin-like SVSPs (TL-SVSPs) are able to hydrolyze both  $\alpha$  and  $\beta$  chains of fibrinogen, releasing fibrinopeptides A and B, respectively, but most of them preferentially act upon only on one of the fibrinogen chains [72]. On the other hand, fibrinogenases can be classified as  $\alpha$ -fibrinogenases and  $\beta$ -fibrinogenases, where the first group hydrolyses preferentially (although not exclusively)  $\alpha$ -chain of fibrinogen, while the second group has more affinity for  $\beta$ -chain of fibrinogen. Unlike TL-SVSP, fibrinogenases do not release fibrinopeptides A or B, do not induce fibrin clot formation and most of them are SVMPs [73,74]. All venoms of *C. m. nigrescens* analyzed had fibrinogenolytic activity. Interestingly, while fifteen venoms (most of them corresponding to snakes with a TBL greater than 70 cm) hydrolyzed both the  $\alpha$ - and  $\beta$ -chains of fibrinogen, ten venoms (most of them belonging to snakes with a TBL of less than 70 cm) hydrolyzed only the  $\alpha$ -chain of this plasmatic protein and generated a fibrin clot. In addition, *C. m. nigrescens* venoms of snakes with a TBL less than 60 cm were the most procoagulant on human plasma with MCD-P values in the range of 10.3  $\mu$ g to 24.6  $\mu$ g. Pre-incubation of venoms with SVSPs inhibitor (PMSF) completely inhibited the coagulant activity of venoms, indicating that this activity is mediated solely by SVSPs. Similar results has been reported in species such as *C. durissus cumanensis* [18] and *C. d. terrificus* [75] where venoms from neonate

or juvenile snakes had MCD-P lower (more coagulant) than venoms of adult snakes. These results suggest that fibrinogenases are responsible for activity in *C. m. nigrescens* venoms breaking both  $\alpha$ - and  $\beta$ -chains of fibrinogen, while TL-SVSPs would be responsible of the activity in venoms with affinity only for the  $\alpha$ -chain of fibrinogen.

Within *C. m. nigrescens*, there was also an increase in the relative abundance of SVMPs in venom as the snakes increase in TBL. Similarly, a positive significant relationship was found between TBL of snakes and the proteolytic activity on HPA and azocasein of their venoms. Specifically, the longer the snake, the greater the proteolytic activity. Thus, taking into account that EDTA completely inhibited the proteolytic activity over both substrates, it is likely that the increase in proteolytic activity in venoms as the snakes get longer is related to the increase in the content of SVMPs in venom. Interestingly, gelatinolytic activity did not increase with the length of snakes and was not completely inhibited by EDTA which indicates that not only metal dependent proteolytic enzymes are associated with this activity. An increase of SVMPs abundance and proteolytic activity with increasing snake size has been reported in venom of other rattlesnakes such as *C. oreganus helleri*, *C. o. oreganus*, *C. polystictus*, and *C. simus* [19,21,23]. Though, species such as *C. o. concolor* and *C. viridis viridis* exhibit a reduction in the amount and activity of SVMPs as the snakes increase their longitude [14,71].

It has been proposed that juvenile snakes require toxic venoms to ensure rapid prey immobilization when prey abundance is limited and since eating frequently allows them to reach the adult stage (a less vulnerable stage against predators) more rapidly [15,76]. Comparatively, for adults that feed on larger prey, having venoms rich in proteases (e.g., metalloproteases) is more important to properly digest their prey than having toxic venoms [15,76]. In *C. m. nigrescens*, most of the venoms of snakes with a TBL of less than 70 cm were more toxic and were equally hemorrhagic compared to venoms of snakes with larger size. The main components related to toxicity in small *C. m. nigrescens* snakes were SVSPs (with molecular masses between 25 kDa and 75 kDa) and coincidentally, venom of juvenile snakes contained, in general, more SVSPs than venom of adult snakes. In addition, mice injected with these SVSPs showed opisthotonos and bleeding. A group of TL-SVSPs (e.g., gyroxin, crotoalase, ancrod, leucurobin, and LM-TL) with molecular masses in the range from 30 kDa to 60 kDa produce temporary episodes characterized by opisthotonos and rotations around the long axis of mice [77,78] and can have high toxicity (e.g., LD<sub>50</sub> of 0.07  $\mu$ g/g in mice) [77]. Our results suggest that a combination of myotoxic (crotoamine-like myotoxins), procoagulant (TL-SVSPs) and hemorrhagic (SVMPs) components could induce faster paralysis and death in prey of juveniles *C. m. nigrescens*, compared to venoms of adult snakes that lack myotoxins and have limited or no procoagulant activity.

Although venom variation related to age of snakes was demonstrated for *C. m. nigrescens*, venom variation related to geographic distribution was not as clear. In general, *C. m. nigrescens* individuals with similar TBL but from different localities from Mexico displayed similar venoms in terms of protein profile, toxicity, and proteolytic activity related to SVMPs. Gelatinolytic, fibrinogenolytic, and coagulant activities were more variable among venoms but even in these activities no patterns were associated with geographic distribution. Rattlesnake venoms can be classified in two groups according to their toxicity and SVMP mediated proteolytic activity, where Type I corresponds to venoms with low or moderate toxicity and high proteolytic activity, while Type II venoms are more toxic and less proteolytic [76]. Nevertheless, this classification can be made more complex if we take into account that the main components of these venom groups, such as neurotoxins, SVMPs, and myotoxins, can vary widely in terms of presence and relative abundance [25,34]. Further, some rattlesnake species, such as *C. helleri*, *C. horridus*, *C. scutulatus*, and *C. simus*, can have populations with Type I, Type II, and/or Type I + II venoms [31,33,34,47,79,80]. Taking into account their low toxicity (compared to neurotoxic venoms), high proteolytic activity and lack of the Mojave toxin (in agreement with other reports [62,63]), *C. m. nigrescens* venoms would be classified exclusively as Type I.

Both Mexican antivenoms (Antivipmyn<sup>®</sup> and Faboterapico polivalente antiviperino<sup>®</sup>) effectively neutralized lethal activity of two juvenile *C. m. nigrescens* venoms. However, the western blot analysis revealed that both Mexican antivenoms did not recognize proteins with molecular mass

close to 10 kDa (e.g., crotamine-like myotoxins) from juvenile *C. m. nigrescens* venoms, suggesting that both antivenoms lack specific antibodies against these small proteins. Another possibility is that epitopes in small proteins (~10 kDa) were disrupted due to the reduced conditions used in SDS-PAGE, so that antivenoms were not able to recognize them. However, neither antivenom neutralized the activity of crotamine-like myotoxins *in vivo*, suggesting that antivenoms did not recognize and were unable to neutralize denatured or native small proteins. In contrast, both antivenoms had difficulties neutralizing lethality of venoms of adult snakes; one of the adult venoms was not neutralized by either antivenom. It has been reported that venoms that have high LD<sub>50</sub>s need greater volume of antivenom to neutralize the venom which could explain the lack of neutralization for one adult. For example, *C. culminatus* venom with LD<sub>50</sub>s ranging from 66 to 162 µg/mouse is not neutralized with volumes less than equal to 460 µL of antivenom [81]. The opposite relationship has been reported for snake species such as *Bothrops asper* and *C. durissus durissus*, where antivenoms from Costa Rica were less effective neutralizing newborn snake venoms compared to adult ones [17,82]. Other studies have demonstrated that some experimental antivenoms from Costa Rica reacted weakly with proteins with molecular masses lower than 20 kDa of *B. asper* venom [83]. Nevertheless, CroFab® antivenom did recognize myotoxin in a juvenile and adult *Crotalus viridis viridis* venoms [71], indicating that a representative selection of immunization mixtures in antivenoms production is crucial to have antibodies able to neutralize intraspecific venom variation. Results suggest that Mexican antivenoms could have problems neutralizing human envenomation by some adult *C. m. nigrescens* and a higher than normal dose would be necessary to effectively neutralize the venom.

#### 4. Conclusions

It has been demonstrated that rattlesnakes species such as *C. o. helleri*, *C. o. oreganus*, *C. o. concolor*, and *C. v. viridis* undergo ontogenetic changes in their diet, in which they go from feeding mainly on ectothermic animals (e.g., lizards) in juvenile stage to consume endothermic animals (e.g., mammals) in the adult stage [14,19,71]. These same species undergo a marked change in the composition of their venom in relation to age, indicating that diet plays an important role in the regulation of expression of protein families in venom. Based on what is known in the *C. molossus* complex about diet [54,55], it is likely that venom compositional changes are due to diet differences through time.

Therefore, it is possible to hypothesize that the ontogenetic venom variation observed in the current study could be associated, at least in part, with differences in prey consumed by this subspecies during its life cycle. However, it is necessary to corroborate this hypothesis with a diet analysis.

Based on this study and previous studies on rattlesnake venom variation, rattlesnakes appear to evolve unique strategies given different environmental pressures exerted on them. In the case of *C. m. nigrescens*, an ontogenetic venom change seems to be more advantageous than changes in the venoms of adult individuals across their distribution. It is likely that further analysis of venom in the remaining subspecies (*C. m. estebanensis*, *C. m. molossus*, and *C. m. oaxacus*) and species (*C. ornatus*, *C. basiliscus*, and *C. totonacus*) in the *Crotalus molossus* species complex will find the same pattern that we did in *C. m. nigrescens*.

#### 5. Materials and Methods

##### 5.1. Ethics Statement

We followed the guidelines described by the live animal use committees of the Facultad de Ciencias Biológicas at UJED, the Universidad Nacional Autónoma de México, and the Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT) of Mexico as well as developing our protocols in accordance with the American Society of Ichthyologists and Herpetologists guidelines for use of live amphibians and reptiles. SEMARNAT issued collecting permits (SGPA/DGVS/002288/18, 01090/17 and 03562/15; 13 March 2018; 10 February 2017; 7 April 2015, respectively, for samples collected in Mexico.



### 5.2. *Crotalus Molossus Nigrescens* Sampling

Snakes were captured in the field, venom was sampled, and then the animal was immediately released after venom extraction and measurements were taken. Samples were obtained from twenty-seven *C. m. nigrescens* from eight states (Aguascalientes, Coahuila, Durango, Jalisco, Nuevo León, San Luis Potosí, Tamaulipas and Zacatecas) in Mexico (Table Table 1). When possible, total body length (TBL) was measured and sex determined for each snake. Venom was extracted manually by allowing each snake to bite sterile 100 mL plastic containers covered with parafilm. Venoms were centrifuged at 14,000 rpm for 1 min to remove debris and the supernatant was lyophilized and stored at  $-20^{\circ}\text{C}$  until use. Tests using mice were carried out only with representative venoms to reduce the number of animals sacrificed.

### 5.3. Protein Concentration Determination

The protein concentration of each venom was obtained using the Pierce<sup>®</sup> Bicinchoninic Acid (BCA) Protein Assay (Thermo Scientific, Rockford, IL, USA) following the manufacturer's instructions and using bovine serum albumin (BSA) as a standard.

### 5.4. SDS-PAGE

15% polyacrylamide gels were made on a Miniprotean III system (Bio-Rad, Hercules, CA, USA) using the discontinuous system. Fifteen micrograms of each venom were dissolved in sample buffer (50 mM Tris-HCl, pH 6.8, 25% SDS, 10% glycerol, and 0.002% bromo-phenol blue) and then we added 5%  $\beta$ -mercaptoethanol. Each of the 27 samples were boiled for 5 min and run at 120 V. Gels were stained with 0.2% Coomassie brilliant blue R-250, 10% acetic acid, and 25% methanol for 8 h and rinsed in 10% acetic acid and 10% methanol [33]. Standard molecular mass markers (Bio-Rad, Carlsbad, CA, USA) were used as references.

### 5.5. Reverse-Phased High Performance Liquid Chromatography HPLC

Separation of 26 venom samples by RP-HPLC was carried out on an analytic C18 reverse-phase column (Vydac<sup>®</sup>, Deerfield, IL, USA, 218 TP 4.6 mm  $\times$  250 mm) using an Agilent 1100 chromatograph. One milligram of each venom was dissolved in 1.7 mL of water containing 0.1% trifluoroacetic acid (TFA). Elution was performed as described by Castro et al. [47] at 1 mL/min by applying a gradient toward solution B (acetonitrile, containing 0.1% TFA), as follows: 0% B for 5 min, 0 to 15% B over 10 min, 15 to 45% B over 60 min, 45 to 70% B over 10 min, and 70% B for 9 min. Proteins were detected at 215 nm.

### 5.6. Identification of Protein Families in Representative *C. m. nigrescens* Venom by Western Blot

In order to determine the identity of the main fractions obtained from the RP-HPLC, fractions and whole CM04 venom were analyzed by western blot using polyclonal antibodies against SVMPs, SVSPs and PLA<sub>2</sub>s. Antibodies against SVMPs, SVSPs, or PLA<sub>2</sub>s were obtained from serum of rabbits hyperimmunized with *C. simus* venom. An immunopurification step was carried out to get specific antibodies against every protein family. Depending of the antibodies, a final step of negative immunopurification was used to remove antibodies with cross-reactivity or residual cross-contamination to SVMPs, SVSPs, or PLA<sub>2</sub>s.

Western blot was carried out as follow. Two micrograms of each fraction and five micrograms of whole venom were separated by 15% SDS-PAGE. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Trans-blot 0.45  $\mu\text{m}$ , Bio-Rad, Merck, Cork, Ireland) using a model HEP-1<sup>®</sup> semi-dry immunotransference chamber (Thermo Scientific, Carlsbad, CA, USA). After transference at 420 mA for 1 h, the membrane was blocked with 5% non-fat dry milk diluted in TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20, pH 8.4) for 2 h. The membrane was then rinsed three times with TBST and incubated by shaking gently for 1 h at room temperature with



antibodies against SVMs, SVSPs, or PLA<sub>2</sub>s diluted 1 µg/mL in TBST. After three washes with TBST, the membrane was incubated at room temperature for 1 h with goat antibodies against rabbit IgG conjugated to alkaline phosphatase (Invitrogen, Eugene, OR, USA) diluted 1:7000. The membrane was again rinsed with TBST and then developed by adding the BCIP/NPT substrate for alkaline phosphatase (Invitrogen, Camarillo, CA, USA) [33].

#### 5.7. Molecular Mass and N-Terminal Sequence Determination of Crotonamine-Like Myotoxins

We determined the intact masses of crotonamine-like myotoxins of venom from one individual (CM04) using ESI-MS on an LCQ Fleet Ion Trap Mass Spectrometer. Amino-terminal sequencing of one crotonamine-like myotoxin (RP-HPLC fraction 8) was determined by automated Edman degradation on a PPSQ-31A Protein Sequencer (Shimadzu, Tokyo, Japan).

#### 5.8. Hide Powder Azure (HPA) Hydrolysis

We used the Hide Powder Azure (HPA) hydrolysis assay to determine proteolytic activity for each of the 27 samples. Hydrolysis of HPA was determined by adding 25 µg of venom to 1 mL of a solution containing 5 mg of HPA in 0.1 M Tris-HCl, pH 8.0. After 2 h of incubation, the reaction was stopped by centrifugation at 14,000 rpm for 5 min. The supernatant was obtained and the absorbance at 595 nm was measured. The absorbances of samples corresponding to HPA solutions incubated with 0.1 M Tris-HCl, pH 8.0, were subtracted from the absorbances of venom-containing samples. Analyses were conducted in triplicate. A standard curve of HPA hydrolysis was created by adding 50 µL of trypsin (2 mg/mL) to three concentrations of HPA (2, 4 and 6 mg/mL) and venom enzymatic activity was calculated using the standard curve. A unit of enzymatic activity (U) was defined as the amount of venom necessary to digest 1 mg of HPA in a 2 h period at room temperature and reported as specific activity (U/mg) [33].

#### 5.9. Azocasein Hydrolysis

Proteolytic activity of 26 venoms was evaluated using azocasein (Sigma-Aldrich, St. Louis, MO, USA) as the substrate, as described by Castro et al. [47]. Ten micrograms of venom, dissolved in 20 µL 0.15 M NaCl, were added to 100 µL of 10 mg/mL azocasein, dissolved in 50 mM Tris 0.15 M NaCl, 5 mM CaCl<sub>2</sub>, pH 8.0. After 60 min of incubation at 37 °C, the reaction was stopped by adding 200 µL of 5% trichloroacetic acid. The mixture was centrifuged and 150 µL of the supernatant was added to 150 µL of 0.5 M NaOH. Absorbances were recorded at 450 nm. The absorbances of samples corresponding to azocasein solutions incubated with 0.15 M NaCl were subtracted from the absorbances of venom-containing samples. One unit of proteolytic activity was defined as a change of 0.2 in absorbance per min.

#### 5.10. Gelatinolytic Activity

Gelatin zymography was performed in 12% SDS-PAGE gels containing 1 mg/mL gelatin (Bio-Rad, Carlsbad, CA, USA) for all 25 samples. The venoms (10 µg) were solubilized (5:1 venom/buffer) in sample buffer (50 mM Tris-HCl, pH 6.8, 25% SDS, 10% glycerol, and 0.002% bromophenol blue) in the absence of β-mercaptoethanol and without boiling. After electrophoresis at 120 V, the gels were placed sequentially in renaturing buffer (5% Triton X-100 in 0.1 M Tris-HCl, pH 8) for 2 h and developing buffer (0.1 M Tris-HCl, pH 8) for 10 min. Gels were then incubated in developing buffer at 37 °C for 12 h with gentle agitation. Gels were stained with staining solution (0.2% Coomassie brilliant blue R-250, 10% acetic acid, and 25% methanol) and destained with 10% acetic acid plus 10% methanol. Standard molecular mass markers (Bio-Rad, Carlsbad, CA, USA) were used as references [46,48].

#### 5.11. Minimum Coagulant Dose Plasma (MCD-P)

For all samples, various amounts of venom (from 10 µg to 400 µg), dissolved in 100 µL 0.15 M NaCl, was added to 200 µL of citrated human plasma, previously incubated at 37 °C. Clotting times were recorded and coagulant activity was expressed as the Minimum Coagulant Dose in plasma (MCD-P) defined as the dose of venom inducing clotting in 60 s [84]. As a negative control, 100 µL of 0.15 M NaCl without venom was added to citrated human plasma.

#### 5.12. Fibrinogenolytic Activity

Fibrinogenolytic activity of the venoms was tested using the procedure described by Borja et al. [46]. Human fibrinogen (150 µg; Sigma, St. Louis, MO, USA) was incubated with 24 individual venoms (5 µg) in 0.05 M Tris-HCl buffer, pH 8.0, at 37 °C for 15 min. The reaction was stopped by adding reducing SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 25% SDS, 10% glycerol, 0.002% bromophenol blue, and 5% β-mercaptoethanol) and heating to 100 °C for 5 min. Fibrinogenolytic activity was demonstrated by 12% SDS-PAGE. As a negative control, human fibrinogen without venom was added to gels. Standard molecular mass markers (Bio-Rad, Carlsbad, CA, USA) were used as references.

#### 5.13. Enzymatic Inhibition Analysis

To evaluate the influence of SVMPs in proteolytic activities over HPA, azocasein, gelatin, and human plasma, venoms were preincubated with 20 mM EDTA for 30 min at 37 °C before carrying out the analyses [40]. Similarly, coagulant activity over human plasma was carried out after preincubating venoms with 5 mM PMSF (inhibitor of serine proteases) for 15 min at 37 °C [85]. A concentrated stock of PMSF (167 mM) was prepared in 100% isopropanol. Then 3 µL of inhibitor (167 mM) were added to 97 µL of PBS containing venom samples and incubated for 10 min before being added to human plasma. A control of 3 µL of 100% isopropanol without inhibitor was used to demonstrate that isopropanol itself does not inhibit enzyme activity.

#### 5.14. Median Lethal Dose (LD<sub>50</sub>)

Nine representative venoms were dissolved in phosphate buffer solution (PBS), pH 7.2. The lethal dose was determined by injecting different quantities of venom (from 10 µg to 150 µg) diluted in a total volume of 0.5 mL into the caudal vein of male and female ICR-CD1 mice (18 to 20 g) in groups of three [86]. The percentage of dead mice 24 h after inoculation was plotted against the logarithm of the quantity of venom injected and analyzed with nonparametric methods using the program GraphPad Prism V4 (GraphPad Software, La Jolla, CA, USA, 2005) to determine the LD<sub>50</sub> value for each venom.

#### 5.15. Minimum Hemorrhagic Dose (MHD)

Different quantities of eight representative venoms (from 5 µg to 25 µg) were injected intradermally, in a volume of 50 µL PBS, to groups of three (male and female) ICR-CD1 mice (25–28 g); 3 h later, they were sacrificed by CO<sub>2</sub>, their skin removed, and the area of the hemorrhagic spot was measured. The minimum hemorrhagic dose was defined as the dose of venom which induced a lesion of 10 mm diameter [87].

#### 5.16. Detection of Crotoxin-Like Neurotoxins at the Protein Level

Detection of crotoxin-like neurotoxins was carried out using the procedure previously described to detect neurotoxins in *C. s. scutulatus* venoms [33]. Venom from two Mojave Rattlesnakes with Type A and Type B venom from Arizona were used as control.

### 5.17. Neutralization Studies

Neutralization studies were carried out using a modification of the procedure described by Gutierrez et al. [88]. Venoms of four *C. m. nigrescens* individuals with different TBL (CM06, CM09, CM18 and CM19) were challenged against two Mexican antivenoms: Antivipmyn<sup>®</sup> manufactured by Bioclon (Lot. B-9F-22) and Faboterapico polivalente antiviperino<sup>®</sup> manufactured by Birmex (Lot. FV030A). Amounts equivalent to 3 times the LD<sub>50</sub> of each venom were incubated with different volumes of each antivenom (from 50 µL to 450 µL of antivenom) dissolved in PBS in a constant final volume of 500 µL for 30 min at 37 °C. Next, 500 µL of solution containing venom/antivenom were i.v. injected in groups of three (male and female) ICR-CD1 mice (18 to 20 g) and the survival percentage 24 h after inoculation was recorded. As controls, groups of three mice were injected with venom alone (positive) or antivenom alone (negative). Neutralization was expressed as Effective Dose 50% (ED<sub>50</sub>), the volume of antivenom that prevents the death of half of the injected population [81].

### 5.18. Immune Recognition of Antivenom

To observe the immune recognition of both Mexican antivenoms (Antivipmyn<sup>®</sup> and Faboterapico polivalente antiviperino<sup>®</sup>) towards the components of eight representative *C. m. nigrescens* venoms (CM03, CM04, CM06, CM08, CM09, CM15, CM18, CM19), 10 µg of whole venom was separated by 15% SDS-PAGE. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Trans-blot 0.45 µm, Bio-Rad, Merck, Cork, Ireland) using a model HEP-1<sup>®</sup> semi-dry immunotransference chamber (Thermo Scientific). After transference at 420 mA for 1 h, the membrane was blocked with 5% non-fat dry milk diluted in TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20, pH 8.4) for 2 h. The membrane was then rinsed three times with TBST and incubated by shaking gently for 1 h at room temperature with Antivipmyn<sup>®</sup> or Favoterapico polivalente antiviperino<sup>®</sup> diluted 1:800 in TBST. After three washes with TBST, the membrane was incubated at room temperature for 1 h with goat antibodies against house IgG conjugated to alkaline phosphatase (Jackson Immuno Research) diluted 1:7000. The membrane was again rinsed with TBST and then developed by adding the BCIP/NPT substrate for alkaline phosphatase (Sigma<sup>®</sup>).

### 5.19. Statistical Analysis

The SigmaPlot<sup>®</sup>11 (Systat Software Inc. (SSI), San Jose, CA, USA, 2008) statistical program was used to determine the regression that best fit our data. We regressed the relative abundance of myotoxins, SVSP, and SVMPS in the venom to the total body length of the snake and evaluated the fit with linear curves. The relationship of proteolytic activity of venoms to snake total body length was assessed by sigmoidal curves. Differences in LD<sub>50</sub> between snakes with a TBL less than and greater than 70 cm were compared by using the Mann-Whitney *U* test.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2072-6651/10/12/501/s1>, Figure S1. Reverse-phase HPLC chromatograms of remaining *C. m. nigrescens* venoms of snakes with different total body length (TBL). An analytic C18 reverse-phase column (Vydac<sup>®</sup>, Deerfield, IL, USA, 218 TP 4.6 mm × 250 mm) was used. Retention time is along the x axis for each panel and labeled every twenty min. Proteins were detected at 215 nm and absorbance is indicated on the left axis. The acetonitrile gradient is shown in the HPLC graph and the percentage value corresponds to the right axis for each panel. The regions where crotoamine-like myotoxins (Myo), snake venom serine proteases (SVSPs), and snake venom metalloproteinases (SVMPS) elute are illustrated in blue, green and red, respectively. Figure S2. Mass spectrometry of crotoamine-like myotoxins. RP-HPLC fractions 6 (A), 7 (B), 8 (C), 9 (D) and 10 (E) obtained from CM04 venom were analyzed using ESI-MS on an LCQ Fleet Ion Trap Mass Spectrometer. Molecular masses calculated were 5129 Da (A), 5071 Da (A), 5143 Da (B), 5180 Da (B), 4958 Da (C), 5143 Da (D), and 5082 Da (E).

**Author Contributions:** M.B., E.N.-C. and A.A. conceived and designed the experiments; M.B., G.C.-G., J.L.S., E.F.-M. and C.L.P. carried out field work and sampling; M.B., E.N.-C., R.P.-L. and E.F.-M. performed the experiments; M.B., E.N.-C., G.C.-G., J.L.S., J.E.-F., A.A., R.P.-M. and J.E.-F. analyzed the data. R.P.-M., A.A., G.C.-G., C.L.P., J.E.-F. and J.S.-M. contributed reagents, materials, analysis tools and/or funding; M.B., E.N.-C., G.C.-G., J.L.S. and A.A. wrote the paper; all authors edited and approved the final submission.

**Funding:** This research was funded by Consejo de Ciencia y Tecnología del Estado de Durango (COCYTED) grant number COCYTED 2018 01: 15088 and Dirección General de Asuntos del Personal Académico (DGAPA) grant number DGAPA-PAPIIT: IN207218.

**Acknowledgments:** We would like to thank Juan Castañeda, Melisa Bernard, Alejandro Olvera, Flavio C. García, Cristóbal Moreno, Alan Salas, Fernando Hernandez, Manuel Rodríguez, Sara Valenzuela, Jorge Becerra, Leonardo Badillo, Jesus Sigala, Ricardo Ramirez, Raul Solis, Ivan Villalobos, Gustavo Quintero, Gustavo Zavala, Ivan Ahumada, Erick Rivas, Arely Gutierrez, Jason Jones, Chris Grünwald, Hector Franz, Eduardo Fanti, and Andrew Mason for their help in the field and laboratory. We thank Bruno Rodriguez for assistance making the maps for this manuscript. We thank Fernando Zamudio for his help with determining the masses of and doing the N-terminal sequencing for crotoamine-like myotoxins. We thank Rhett Rautsaw and Claudia Avitia for making suggestions that improved this manuscript. In addition, we would like to thank the Consejo Nacional de Ciencia y Tecnología (CONACYT) for supporting the scholarship grant to the postgraduate student M.B. (No. 247437), the National Science Foundation to C.L.P. (DEB 1638879 and DEB 1822417), Clemson University through faculty startup to C.L.P., Prairie Biotic Research Inc. (J.L.S.), Sigma Xi Grants-in-aid-of-research (J.L.S.), SnakeDays Research Grant (J.L.S.), the Theodore Roosevelt Memorial Fund through the American Museum of Natural History (J.L.S.) and the Howard McCarley Student Research Award of the Southwestern Association of Naturalist (M.B. and J.L.S.). We thank the Universidad Juárez del Estado de Durango for provided facilities and vehicles.

**Conflicts of Interest:** The authors declare no conflict of interest. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

## References

- Booth, C.L. Evolutionary significance of ontogenetic colour change in animals. *Biol. J. Linn. Soc.* **1990**, *40*, 125–163. [\[CrossRef\]](#)
- Lind, A.J.; Welsh, H.H.J. Ontogenetic changes in foraging behaviour and habitat use by the Oregon garter snake, *Thamnophis atratus hydrophilus*. *Anim. Behav.* **1994**, *48*, 1261–1273. [\[CrossRef\]](#)
- Brodin, T.; Johansson, F. Conflicting selection pressures on the growth/predation risk trade-off in a damselfly. *Ecology* **2004**, *85*, 2927–2932. [\[CrossRef\]](#)
- Natusch, D.J.D.; Lyons, J.A. Relationships between ontogenetic changes in prey selection, head shape, sexual maturity, and colour in an Australasian python (*Morelia viridis*). *Biol. J. Linn. Soc.* **2012**, *107*, 269–276. [\[CrossRef\]](#)
- Herrel, A.; Joachim, R.; Vanhooydonck, B.; Irschick, D.J. Ecological consequences of ontogenetic changes in head shape and bite performance in the Jamaican lizard *Anolis lineatopus*. *Biol. J. Linn. Soc.* **2006**, *89*, 443–454. [\[CrossRef\]](#)
- Webber, M.M.; Jezkova, T.; Rodríguez-Robles, J.A. Feeding Ecology of Sidewinder Rattlesnakes, *Crotalus cerastes* (Viperidae). *Herpetologica* **2016**, *72*, 324–330. [\[CrossRef\]](#)
- Holycross, A.T.; Mackessy, S.P. Variation in the diet of *Sistrurus catenatus* (Massasauga), with emphasis on *Sistrurus catenatus edwardsii* (Desert Massasauga). *J. Herpetol.* **2002**, *36*, 454–464. [\[CrossRef\]](#)
- Rulon, W.C. Diet of the Timber Rattlesnake, *Crotalus horridus*. *J. Herpetol.* **2002**, *36*, 494–499.
- Macartney, J.M. The Ecology of the Northern Pacific Rattlesnake, *Crotalus Viridis* Oreganus. Master's Thesis, University of Victoria, Victoria, BC, Canada, 1985.
- King, R.B. Predicted and observed maximum prey size—Snake size allometry. *Funct. Ecol.* **2002**, *16*, 766–772. [\[CrossRef\]](#)
- Daltry, J.C.; Wüster, W.; Thorpe, R.S. Diet and snake venom evolution. *Nature* **1996**, *379*, 537–540. [\[CrossRef\]](#) [\[PubMed\]](#)
- Cipriani, V.; Debono, J.; Goldenberg, J.; Jackson, T.N.W.; Arbuckle, K.; Dobson, J.; Koludarov, I.; Li, B.; Hay, C.; Dunstan, N.; et al. Correlation between ontogenetic dietary shifts and venom variation in Australian brown snakes (*Pseudonaja*). *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **2017**, *197*, 53–60. [\[CrossRef\]](#) [\[PubMed\]](#)
- Zelanis, A.; Tashima, A.K.; Rocha, M.M.T.; Furtado, M.F.; Camargo, A.C.M.; Ho, P.L.; Serrano, S.M.T. Analysis of the Ontogenetic Variation in the Venom Proteome/Peptidome of *Bothrops jararaca* Reveals Different Strategies to Deal with Prey. *J. Proteome Res.* **2010**, *9*, 2278–2291. [\[CrossRef\]](#) [\[PubMed\]](#)
- Mackessy, S.P.; Williams, K.; Ashton, K.G. Ontogenetic Variation in Venom Composition and Diet of *Crotalus oreganus concolor*: A Case of Venom Paedomorphosis? *Copeia* **2003**, *4*, 769–782. [\[CrossRef\]](#)

15. Margres, M.J.; Wray, K.P.; Seavy, M.; McGivern, J.J.; Sanader, D.; Rokyta, D.R. Phenotypic integration in the feeding system of the eastern diamondback rattlesnake (*Crotalus adamanteus*). *Mol. Ecol.* **2015**, *24*, 3405–3420. [[CrossRef](#)] [[PubMed](#)]
16. Reid, H.A.; Theakston, R.D.G. Changes in coagulation effects by venoms of *Crotalus atrox* as snakes age. *Am. J. Trop. Med. Hyg.* **1978**, *27*, 1053–1057. [[CrossRef](#)] [[PubMed](#)]
17. Saravia, P.; Rojas, E.; Arce, V.; Guevara, C.; López, J.C.; Chaves, E.; Velásquez, R.; Rojas, G.; Gutiérrez, J.M. Geographic and ontogenic variability in the venom of the neotropical rattlesnake *Crotalus durissus*: Pathophysiological and therapeutic implications. *Rev. Biol. Trop.* **2002**, *50*, 337–346. [[PubMed](#)]
18. Céspedes, N.; Castro, F.; Jiménez, E.; Montealegre, L.; Castellanos, A.; Cañas, C.; Arévalo-Herrera, M.; Herrera, S. Biochemical comparison of venoms from young Colombian *Crotalus durissus cumanensis* and their parents. *J. Venom. Anim. Toxins Incl. Trop. Dis.* **2010**, *16*, 268–284. [[CrossRef](#)]
19. Mackessy, S.P. Venom ontogeny in the pacific rattlesnake *Crotalus viridis helleri* and *C. v. oreganus*. *Copeia* **1988**, *1*, 92–100. [[CrossRef](#)]
20. Rokyta, D.R.; Margres, M.J.; Ward, M.J.; Sanchez, E.E. The genetics of venom ontogeny in the eastern diamondback rattlesnake (*Crotalus adamanteus*). *PeerJ* **2017**, *5*, e3249. [[CrossRef](#)] [[PubMed](#)]
21. Mackessy, S.; Leroy, J.; Mociño-Deloya, E.; Setser, K.; Bryson, R.; Saviola, A. Venom Ontogeny in the Mexican Lance-Headed Rattlesnake (*Crotalus polystictus*). *Toxins* **2018**, *10*, 271. [[CrossRef](#)] [[PubMed](#)]
22. Neri-Castro, E.; Ponce-Lopez, R. Variación ontogénica en el veneno de *Crotalus simus* en México. *Aridociencia* **2018**, *3*, 42–47.
23. Calvete, J.J.; Sanz, L.; Cid, P.; de la Torre, P.; Flores-Díaz, M.; Dos Santos, M.C.; Borges, A.; Bremo, A.; Angulo, Y.; Lomonte, B.; et al. Snake Venomics of the Central American Rattlesnake *Crotalus simus* and the South American *Crotalus durissus* Complex Points to Neurotoxicity as an Adaptive Paedomorphic Trend along *Crotalus* Dispersal in South America. *J. Proteome Res.* **2010**, *9*, 528–544. [[CrossRef](#)] [[PubMed](#)]
24. Farstad, D.; Thomas, T.; Chow, T.; Bush, S.; Stiegler, P. Mojave rattlesnake envenomation in southern California: a review of suspected cases. *Wilderness Environ. Med.* **1997**, *8*, 89–93. [[CrossRef](#)]
25. Massey, D.J.; Calvete, J.J.; Sánchez, E.E.; Sanz, L.; Richards, K.; Curtis, R.; Boesen, K. Venom variability and envenoming severity outcomes of the *Crotalus scutulatus scutulatus* (Mojave rattlesnake) from Southern Arizona. *J. Proteom.* **2012**, *75*, 2576–2587. [[CrossRef](#)] [[PubMed](#)]
26. Minton, S.A.; Weinstein, S.A. Geographic and ontogenic variation in venom of the western diamondback rattlesnake (*Crotalus atrox*). *Toxicon* **1986**, *24*, 71–80. [[CrossRef](#)]
27. Kang, T.S.; Georgieva, D.; Genov, N.; Murakami, M.T.; Sinha, M.; Kumar, R.P.; Kaur, P.; Kumar, S.; Dey, S.; Sharma, S.; et al. Enzymatic toxins from snake venom: Structural characterization and mechanism of catalysis. *FEBS J.* **2011**, *278*, 4544–4576. [[CrossRef](#)] [[PubMed](#)]
28. Hayes, W.K.; Herbert, S.S.; Reiling, G.C.; Gennaro, J.F. Factors that influence venom expenditure in viperids and other snake species during predatory and defensive contexts. In *Biology of the Vipers*; Schuett, G.W., Hoggren, M., Douglas, M.E., Greene, H.W., Eds.; Eagle Mountain Pub Lc: Eagle Mountain, UT, USA, 2002; pp. 207–233. [[CrossRef](#)]
29. Wu, C.H.; Huang, H.; Yeh, L.S.L.; Barker, W.C. Protein family classification and functional annotation. *Comput. Biol. Chem.* **2003**, *27*, 37–47. [[CrossRef](#)]
30. Tasoulis, T.; Isbister, G. A Review and Database of Snake Venom Proteomes. *Toxins* **2017**, *9*, 290. [[CrossRef](#)] [[PubMed](#)]
31. Sunagar, K.; Undheim, E.A.B.; Scheib, H.; Gren, E.C.K.; Cochran, C.; Person, C.E.; Koludarov, I.; Kelln, W.; Hayes, W.K.; King, G.F.; et al. Intraspecific venom variation in the medically significant Southern Pacific Rattlesnake (*Crotalus oreganus helleri*): Biodiscovery, clinical and evolutionary implications. *J. Proteom.* **2014**, *99*, 68–83. [[CrossRef](#)] [[PubMed](#)]
32. Alape-Girón, A.; Sanz, L.; Escolano, J.; Flores-Díaz, M.; Madrigal, M.; Sasa, M.; Calvete, J.J. Snake venomics of the lancehead pitviper *Bothrops asper*. Geographic, individual, and ontogenetic variations. *J. Proteome Res.* **2008**, *7*, 3556–3571. [[CrossRef](#)] [[PubMed](#)]
33. Borja, M.; Neri-Castro, E.; Castañeda-Gaytán, G.; Strickland, J.L.; Parkinson, C.L.; Castañeda-Gaytán, J.; Ponce-López, R.; Lomonte, B.; Olvera-Rodríguez, A.; Alagón, A.; Pérez-Morales, R. Biological and proteolytic variation in the venom of *Crotalus scutulatus scutulatus* from Mexico. *Toxins* **2018**, *10*, 35. [[CrossRef](#)] [[PubMed](#)]
34. Strickland, J.L.; Mason, A.J.; Rokyta, D.R.; Parkinson, C.L. Phenotypic variation in Mojave Rattlesnake (*Crotalus scutulatus*) venom is driven by four toxin families. *Toxins* **2018**, *10*, 135. [[CrossRef](#)] [[PubMed](#)]



35. Segura, Á.; Herrera, M.; Villalta, M.; Vargas, M.; Uscanga-Reynell, A.; de León-Rosales, S.P.; Jiménez-Corona, M.E.; Reta-Mares, J.F.; Gutiérrez, J.M.; León, G. Venom of *Bothrops asper* from Mexico and Costa Rica: Intraspecific variation and cross-neutralization by antivenoms. *Toxicon* **2012**, *59*, 158–162. [[CrossRef](#)] [[PubMed](#)]
36. Zelanis, A.; Travaglia-Cardoso, S.R.; De Fátima Domingues Furtado, M. Ontogenetic changes in the venom of *Bothrops insularis* (Serpentes: Viperidae) and its biological implication. *S. Am. J. Herpetol.* **2008**, *3*, 43–50. [[CrossRef](#)]
37. Saldarriaga, M.M.; Otero, R.; Núñez, V.; Toro, M.F.; Díaz, A.; Gutiérrez, J.M. Ontogenetic variability of *Bothrops atrox* and *Bothrops asper* snake venoms from Colombia. *Toxicon* **2003**, *42*, 405–411. [[CrossRef](#)]
38. Menezes, M.C.; Furtado, M.F.; Travaglia-Cardoso, S.R.; Camargo, A.C.M.; Serrano, S.M.T. Sex-based individual variation of snake venom proteome among eighteen *Bothrops jararaca* siblings. *Toxicon* **2006**, *47*, 304–312. [[CrossRef](#)] [[PubMed](#)]
39. Furtado, M.F.D.; Travaglia-Cardoso, S.R.; Rocha, M.M.T. Sexual dimorphism in venom of *Bothrops jararaca* (Serpentes: Viperidae). *Toxicon* **2006**, *48*, 401–410. [[CrossRef](#)] [[PubMed](#)]
40. Dagda, R.K.; Gasanov, S.; De La Oiii, Y.; Rael, E.D.; Lieb, C.S. Genetic basis for variation of metalloproteinase-associated biochemical activity in venom of the Mojave rattlesnake (*Crotalus scutulatus scutulatus*). *Biochem. Res. Int.* **2013**, *2013*, 1–11. [[CrossRef](#)] [[PubMed](#)]
41. Whittington, A.C.; Mason, A.J.; Rokyta, D.R. A Single Mutation Unlocks Cascading Exaptations in the Origin of a Potent Pitviper Neurotoxin. *Mol. Biol. Evol.* **2017**, *35*, 887–898. [[CrossRef](#)] [[PubMed](#)]
42. Dowell, N.L.; Giorgianni, M.W.; Kassner, V.A.; Selegue, J.E.; Sanchez, E.E.; Carroll, S.B. The Deep Origin and Recent Loss of Venom Toxin Genes in Rattlesnakes. *Curr. Biol.* **2016**, *26*, 2434–2445. [[CrossRef](#)] [[PubMed](#)]
43. Dowell, N.L.; Giorgianni, M.W.; Griffin, S.; Kassner, V.A.; Selegue, J.E.; Sanchez, E.E.; Carroll, S.B. Extremely Divergent Haplotypes in Two Toxin Gene Complexes Encode Alternative Venom Types within Rattlesnake Species. *Curr. Biol.* **2018**, *28*, 1–11. [[CrossRef](#)] [[PubMed](#)]
44. Casewell, N.R.; Wagstaff, S.C.; Wuster, W.; Cook, D.A.N.; Bolton, F.M.S.; King, S.I.; Pla, D.; Sanz, L.; Calvete, J.J.; Harrison, R.A. Medically important differences in snake venom composition are dictated by distinct postgenomic mechanisms. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 9205–9210. [[CrossRef](#)] [[PubMed](#)]
45. García, L.T.; Parreiras, E.; Silva, L.T.; Ramos, O.H.P.; Carmona, A.K.; Bersanetti, P.A.; Selistre-De-Araujo, H.S. The effect of post-translational modifications on the hemorrhagic activity of snake venom metalloproteinases. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **2004**, *138*, 23–32. [[CrossRef](#)] [[PubMed](#)]
46. Borja, M.; Lazcano, D.; Martínez-Romero, G.; Morlett, J.; Sánchez, E.; Cepeda-Nieto, A.C.; Garza-García, Y.; Zugasti-Cruz, A. Intra-specific variation in the protein composition and proteolytic activity of venom of *Crotalus lepidus morulus* from the Northeast of Mexico. *Copeia* **2013**, *4*, 707–716. [[CrossRef](#)]
47. Castro, E.N.; Lomonte, B.; del Carmen Gutiérrez, M.; Alagón, A.; Gutiérrez, J.M. Intraspecific variation in the venom of the rattlesnake *Crotalus simus* from Mexico: Different expression of crotoxin results in highly variable toxicity in the venoms of three subspecies. *J. Proteom.* **2013**, *87*, 103–121. [[CrossRef](#)] [[PubMed](#)]
48. Borja, M.; Castañeda, G.; Espinosa, J.; Neri, E.; Carbajal, A.; Clement, H.; García, O.; Alagon, A. Mojave Rattlesnake (*Crotalus scutulatus scutulatus*) with Type B Venom from Mexico. *Copeia* **2014**, *1*, 7–13. [[CrossRef](#)]
49. Durban, J.; Sanz, L.; Trevisan-Silva, D.; Neri-Castro, E.; Alagón, A.; Calvete, J.J. Integrated venomomics and venom gland transcriptome analysis of juvenile and adult Mexican rattlesnakes *Crotalus simus*, *C. tzabcan*, and *C. culminatus* revealed miRNA-modulated ontogenetic shifts. *J. Proteome Res.* **2017**, *16*, 3370–3390. [[CrossRef](#)] [[PubMed](#)]
50. Hofmann, E.P.; Rautsaw, R.M.; Strickland, J.L.; Holding, M.L.; Hogan, M.P.; Mason, A.J.; Rokyta, D.R.; Parkinson, C.L. Comparative venom-gland transcriptomics and venom proteomics of four Sidewinder Rattlesnake (*Crotalus cerastes*) lineages reveal little differential expression despite individual variation. *Sci. Rep.* **2018**, *8*, 15534. [[CrossRef](#)] [[PubMed](#)]
51. Goldberg, S.R. Reproduction in the Blacktail Rattlesnake, *Crotalus molossus* (Serpentes: Viperidae). *Tex. J. Sci.* **1999**, *5*, 323–328.
52. Platt, S.G.; Rainwater, T.R. A new maximum size record for *Crotalus molossus* (Baird and Girard, 1853). *J. Kansas Herpetol.* **2008**, *28*, 14–15.
53. Ernst, C.H.; Ernst, E.M. *Venomous Reptiles of the United States, Canada, and northern Mexico*; Johns Hopkins University Press: Baltimore, MD, USA, 2011; Volume 2, ISBN 0801898765.

54. Balderas-Valdivia, C.J.; Barreto-Oble, D.; Madrid-Sotelo, C.A. Contribución a la historia natural de *Crotalus molossus*. *Biodivers. del Pedregal San Ángel* **2009**, 363–369.
55. Werler, J.E.; Dixon, J.R. *Texas Snakes: Identification, Distribution, and Natural History*; University of Texas Press: Austin, TX, USA, 2000; ISBN 9780292791305.
56. Ramírez, G.A.; Fletcher, P.L.; Possani, L.D. Characterization of the venom from *Crotalus molossus nigrescens* Gloyd (Black tail rattlesnake): Isolation of two proteases. *Toxicon* **1990**, 28, 285–297.
57. Bober, M.A.; Glenn, J.L.; Straight, R.C.; Ownby, C.L. Detection of myotoxin a-like proteins in various snake venoms. *Toxicon* **1988**, 26, 665–673. [[CrossRef](#)]
58. Rael, E.D.; Rivas, J.Z.; Chen, T.; Maddux, N.; Huizar, E.; Lieb, C.S. Differences in fibrinolysis and complement inactivation by venom from different northern blacktailed rattlesnakes (*Crotalus molossus molossus*). *Toxicon* **1997**, 35, 505–513. [[CrossRef](#)]
59. Hardy, D.L.; Jeter, M.; Corrigan, J.J. Envenomation by the northern blacktail rattlesnake (*Crotalus molossus molossus*): report of two cases and the vitro effects of the venom on fibrinolysis and platelet aggregation. *Toxicon* **1982**, 20, 487–493. [[CrossRef](#)]
60. Yarema, M.C.; Curry, S.C. Envenomation by the Northern Blacktail rattlesnake (*Crotalus molossus molossus*): Case report. *Pediatr. Emerg. Care* **2005**, 21, 40–42. [[CrossRef](#)] [[PubMed](#)]
61. Hardy, D.L.; Zamudio, K.R. Compartment syndrome, fasciotomy, and neuropathy after a rattlesnake envenomation: aspects of monitoring and diagnosis. *Wilderness Environ. Med.* **2006**, 17, 36–40. [[CrossRef](#)]
62. Rael, E.D.; Salo, R.J.; Zepeda, H. Monoclonal antibodies to Mojave toxin and use for isolation of cross-reacting proteins in *Crotalus* venoms. *Toxicon* **1986**, 24, 661–668. [[CrossRef](#)]
63. Wooldridge, B.J.; Pineda, G.; Banuelas-Ornelas, J.J.; Dagda, R.K.; Gasanov, S.E.; Rael, E.D.; Lieb, C.S. 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, 169–179. [[CrossRef](#)]
64. Chippaux, J.P.; Williams, V.; White, J. Snake venom variability: Methods of study, results and interpretation. *Toxicon* **1991**, 29, 1279–1303. [[CrossRef](#)]
65. Wray, K.P.; Margres, M.J.; Seavy, M.; Rokyta, D.R. Early significant ontogenetic changes in snake venoms. *Toxicon* **2015**, 96, 74–81. [[CrossRef](#)] [[PubMed](#)]
66. Goncalves, J.M.; Polson, A. The electrophoretic analysis of snake venoms. *Arch. Biochem.* **1947**, 13, 253–259. [[PubMed](#)]
67. Oguiura, N.; Boni-Mitake, M.; Rádis-Baptista, G. New view on crotamine, a small basic polypeptide myotoxin from South American rattlesnake venom. *Toxicon* **2005**, 46, 363–370. [[CrossRef](#)] [[PubMed](#)]
68. Kerkis, I.; Hayashi, M.A.F.; Prieto Da Silva, A.R.B.; Pereira, A.; De Sá Júnior, P.L.; Zaharenko, A.J.; Rádis-Baptista, G.; Kerkis, A.; Yamane, T. State of the art in the studies on crotamine, a cell penetrating peptide from South American rattlesnake. *BioMed Res. Int.* **2014**, 2014. [[CrossRef](#)] [[PubMed](#)]
69. Lourenço, A.; Zorzella Creste, C.F.; Curtolo de Barros, L.; Delazari dos Santos, L.; Pimenta, D.C.; Barraviera, B.; Ferreira, R.S. Individual venom profiling of *Crotalus durissus terrificus* specimens from a geographically limited region: Crotamine assessment and captivity evaluation on the biological activities. *Toxicon* **2013**, 69, 75–81. [[CrossRef](#)] [[PubMed](#)]
70. Ownby, C.L. Structure, function and biochemical aspects of the myotoxin from snake venoms. *Toxicon* **1998**, 17, 213–238.
71. Saviola, A.J.; Pla, D.; Sanz, L.; Castoe, T.A.; Calvete, J.J.; Mackessy, S.P. Comparative venomomics of the Prairie Rattlesnake (*Crotalus viridis viridis*) from Colorado: Identification of a novel pattern of ontogenetic changes in venom composition and assessment of the immunoreactivity of the commercial antivenom CroFab®. *J. Proteom.* **2015**, 121, 28–43. [[CrossRef](#)] [[PubMed](#)]
72. Castro, H.C.; Zingali, R.B.; Albuquerque, M.G.; Pujol-Luz, M.; Rodrigues, C.R. Snake venom thrombin-like enzymes: From reptilase to now. *Cell Mol. Life Sci.* **2004**, 61, 843–856. [[CrossRef](#)] [[PubMed](#)]
73. Swenson, S.; Markland, F.S. Snake venom fibrin(ogen)olytic enzymes. *Toxicon* **2005**, 45, 1021–1039. [[CrossRef](#)] [[PubMed](#)]
74. Markland, F.S. Snake venom fibrinogenolytic and fibrinolytic enzymes: An updated inventory. On behalf of the Registry of Exogenous Hemostatic Factors of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. *Thromb. Haemost.* **1998**, 79, 668–674. [[CrossRef](#)] [[PubMed](#)]

75. Furtado, M.F.D.; Santos, M.C.; Kamiguti, A.S. Age-related biological activity of South American rattlesnake (*Crotalus durissus terrificus*) venom. *J. Venom. Anim. Toxins Incl. Trop. Dis.* **2003**, *9*, 186–201. [[CrossRef](#)]
76. Mackessy, S.P. Venom composition in rattlesnakes: Trends and biological significance. In *The Biology of the Rattlesnakes*; Hayes, W.K., Beaman, K.R., Cardwell, M.D., Bush, S.P., Eds.; Loma Linda University Press: Loma Linda, CA, USA, 2008; pp. 495–510, ISBN 978-159410-011-6.
77. da Silva, N.J.; Aird, S.D.; Seebart, C.; Kaiser, I.I. A gyroxin analog from the venom of the bushmaster (*Lachesis muta muta*). *Toxicon* **1989**, *27*, 763–771. [[CrossRef](#)]
78. Phillips, D.; Swenson, S.D.; Markland, F.S., Jr. Thrombin-like snake venom serine proteinases. In *Handbook of Venoms and Toxins of Reptiles*; Mackessy, S.P., Ed.; CRC Press: Boca Raton, FL, USA, 2009; pp. 139–154, ISBN 9780849391651.
79. French, W.J.; Hayes, W.K.; Bush, S.P.; Cardwell, M.D.; Bader, J.O.; Rael, E.D. Mojave toxin in venom of *Crotalus helleri* (Southern Pacific Rattlesnake): Molecular and geographic characterization. *Toxicon* **2004**, *44*, 781–791. [[CrossRef](#)] [[PubMed](#)]
80. Glenn, J.L.; Straight, R.C.; Wolt, T.B. Regional variation in the presence of canebrake toxin in *Crotalus horridus* venom. *Comp. Biochem. Physiol. Part C Pharmacol.* **1994**, *107*, 337–346. [[CrossRef](#)]
81. Bénard-Valle, M.; Neri-Castro, E.; Fry, B.; Boyer, L. Antivenom research and development. In *Venomous Reptiles and Their Toxins*; Fry, B.G., Ed.; Oxford University Press: New York, NY, USA, 2015; pp. 61–72, ISBN 978-0-19-930939-9.
82. Chaves, F.; Gutiérrez, J.; Brenes, F. Pathological and biochemical changes induced in mice after intramuscular injection of venom from newborn specimens of the snake *Bothrops asper* (Terciopelo). *Toxicon* **1992**, *30*, 1099–1109. [[CrossRef](#)]
83. Gutiérrez, J.M.; Sanz, L.; Flores-Díaz, M.; Figueroa, L.; Madrigal, M.; Herrera, M.; Villalta, M.; León, G.; Estrada, R.; Borges, A.; et al. Impact of regional variation in *Bothrops asper* snake venom on the design of antivenoms: Integrating antivenomics and neutralization approaches. *J. Proteome Res.* **2010**, *9*, 564–577. [[CrossRef](#)] [[PubMed](#)]
84. Theakston, R.D.; Reid, H.A. Development of simple standard assay procedures for the characterization of snake venom. *Bull. World Health Organ.* **1983**, *61*, 949–956. [[PubMed](#)]
85. Zaqueo, K.D.; Kayano, A.M.; Domingos, T.F.S.; Moura, L.A.; Fuly, A.L.; da Silva, S.L.; Acosta, G.; Oliveira, E.; Albericio, F.; Zanchi, F.B.; et al. BbrzSP-32, the first serine protease isolated from *Bothrops brazili* venom: Purification and characterization. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **2016**, *195*, 15–25. [[CrossRef](#)] [[PubMed](#)]
86. Lorke, D. A new approach to practical acute toxicity testing. *Arch. Toxicol.* **1983**, *54*, 275–287. [[CrossRef](#)] [[PubMed](#)]
87. Gutiérrez, J.M.; Gené, A.; Rojas, G.; Cerdas, L. Neutralization of proteolytic and hemorrhagic activities of Costa Rican snake venoms by a polyvalent antivenom. *Toxicon* **1985**, *23*, 887–893. [[CrossRef](#)]
88. Gutiérrez, J.; Rojas, G.; Lomonte, B.; Gené, J.; Chaves, F.; Alvarado, J.; Rojas, E. Standardization of assays for testing the neutralizing ability of antivenoms. *Toxicon* **1990**, *28*, 1127–1129. [[CrossRef](#)]

