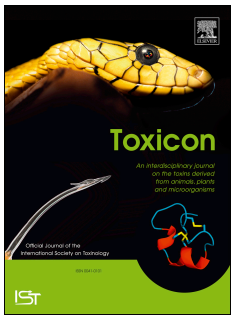

Ontogenetic change in the venom composition of one Mexican black-tailed rattlesnake (*Crotalus molossus nigrescens*) from Durango, Mexico

Miguel Borja, Edgar Neri-Castro, Arelí Gutiérrez-Martínez, Richard Bledsoe, Vanessa Zarzosa, Bruno Rodriguez-López, Jason L. Strickland, Jorge Becerra-López, Sara Valenzuela-Ceballos, Christopher L. Parkinson, Alejandro Alagón, Gamaliel Castañeda-Gaytán

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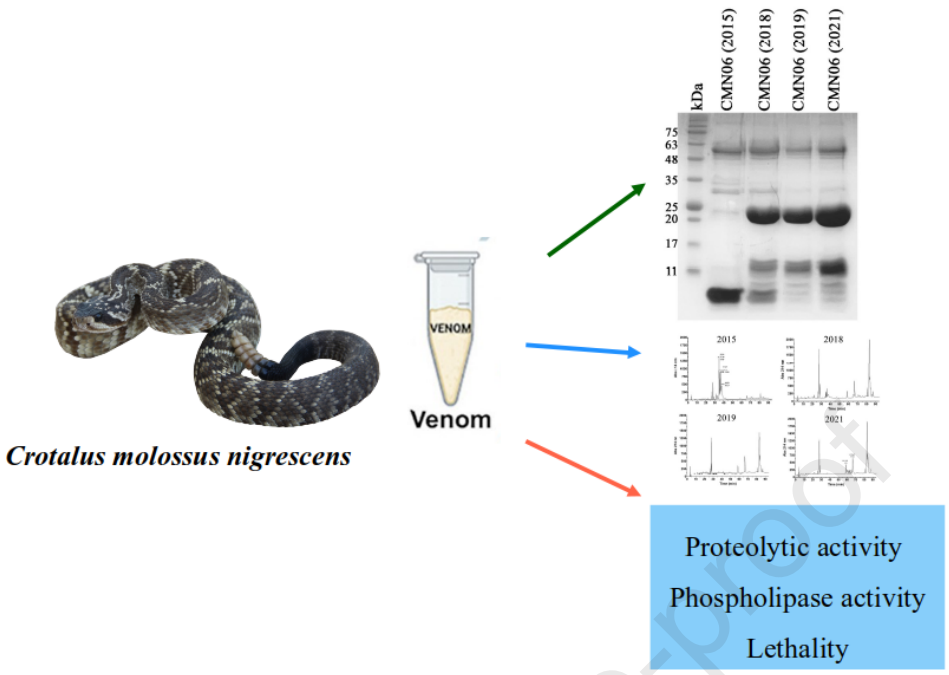
CRediT authorship contribution statement

Miguel Borja: Conceptualization, Methodology, Investigation, Formal analysis, Writing - Original draft preparation, Funding acquisition, Validation and Writing - Reviewing and Editing. **Edgar Neri-Castro**: Conceptualization, Methodology, Investigation, Formal analysis, Writing - Original draft preparation, Funding acquisition, Validation and Writing - Reviewing and Editing **Areli Gutiérrez-Martínez:** Methodology, Investigation, Formal analysis, and Writing **Richard Bledsoe**: Methodology, Reviewing and Editing **Vanessa Zarzosa:** Methodology, Investigation, Formal analysis. **Bruno Rodriguez-López**: Reviewing and Editing. **Jason L. Strickland**: Reviewing and Editing. **Jorge Becerra-López**: Reviewing and Editing. **Sara Valenzuela-Ceballos**: Reviewing and Editing. **Christopher L. Parkinson:** Reviewing and Editing. **Alejandro Alagón**: Reviewing and Editing, and Funding acquisition. **Gamaliel Castañeda-Gaytán**: Conceptualization, Validation and Writing - Reviewing and Editing.



Crotalus molossus nigrescens

Ontogenetic change in the venom composition of one Mexican Black-tailed Rattlesnake (*Crotalus molossus nigrescens*) from Durango, Mexico

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Running title: Ontogenetic venom variation in one *C. m. nigrescens* individual

Keywords: Ontogenetic venom variation; median lethal dose; myotoxins; proteolytic activity; SVMPs; PLA_2s

Abstract

To corroborate the ontogenetic shift in the venom composition of the Mexican Black-tailed Rattlesnake (*Crotalus molossus nigrescens*) previously reported through the census approach, we evaluated the shift in the protein profile, lethality, and proteolytic and phospholipase activities of four venom samples obtained in 2015, 2018, 2019, and 2021 from one *C. m. nigrescens* individual (CMN06) collected in Durango, Mexico. We demonstrated that the venom of *C. m. nigrescens* changed from a myotoxin-rich venom to a phospholipase A_2 and

snake venom metalloproteinase-rich venom. Additionally, the proteolytic and phospholipase activities increased with age, but the lethality decreased approximately three times.

Mexico is the country with the most rattlesnake species (*Crotalus* and *Sistrurus*) in the world with 47 (Uetz et al., 2023), although few studies about their venom have been carried out (Neri-Castro et al., 2020). The ontogenetic venom shift has been reported in some rattlesnake species from Mexico using the census approach (Arnaud et al., 2021; Borja et al., 2018; Colis-Torres et al., 2022; Mackessy et al., 2018). The "census approach" characterizes venoms of individuals from different localities and snake sizes (Schonour et al., 2020). However, these studies have limitations and biases as the venom composition of juveniles and adults may vary slightly among populations (Margres et al., 2015), reducing the chance to describe, in detail, the pattern of venom change during one individual´s lifetime.

Crotalus molossus nigrescens are medium-sized pitvipers distributed broadly in Mexico with a maximum total body length (TBL) of 105 cm (Borja et al., 2018), while newborn snakes have an average TBL of 27.8 cm (Fernández-Badillo and Torres-Angeles, 2018). Previously, it has been demonstrated that the venom of *C. m. nigrescens* displays differences in composition and biological activities in individuals with different TBL (Borja et al., 2018). For instance, smaller individuals (e.g., TBL<60 cm) contained more crotamine-like myotoxins (MYO) than larger individuals (e.g., TBL>80 cm); however, the opposite occurred with the snake venom metalloproteinases (SVMPs). In addition, the venoms of the largest individuals, which contained more SVMPs, were more proteolytic but less lethal than those of the smallest individuals. Similar results were obtained by Colis-Torres et al. (2022), where the venom of adult individuals of *C. basiliscus*, a species closely related to *C. m. nigrescens*, was significantly more proteolytic than the venom of juveniles. To corroborate the ontogenetic shift in the venom composition of *C. m. nigrescens* previously reported through the census approach, we evaluated the shift in the protein profile, lethality, and proteolytic and phospholipase activity of four samples obtained in different years of the venom from one *C. m. nigrescens* individual collected in Durango, Mexico.

To evaluate ontogenetic shifts in the venom composition of *C. m. nigrescens*, we used venom samples from one female *C. m. nigrescens* individual (CMN06) collected in 2015 from Agua Puerca, Durango, Mexico (26.228341° and -104.492504°) under the collection

permit SGPA/DGVS/03562/15 from SEMARNAT. Venom extraction was performed as previously described by Román-Domínguez et al (2019). Due to samples from years 2016, 2017, and 2020 being used for other studies and therefore unavailable, we evaluated only four venom samples from the years 2015, 2018, 2019, and 2021 during which the snake measured 64 cm, 71 cm, 75 cm, and 79 cm snout-vent length (SVL) and a total body length (TBL) of 69 cm, 76 cm, 80 cm, and 84 cm, respectively. Two milligrams of each venom were weighed and dissolved in 1 mL of phosphate-buffered saline (PBS) pH 7.2 (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). The protein concentration for each sample was determined using the Bradford assay. The electrophoretic profile of the venom was determined using 20 µg of venom from each sample dissolved in sample buffer (50 mM Tris-HCl, pH 6.8, 25% SDS, 10% glycerol, and 0.002% bromophenol blue) and 5% β-mercaptoethanol. A 12.5% polyacrylamide gel was run under reducing conditions using a discontinuous system as in Borja et al. (2018). The four venom samples (1 mg in 900 µl of 0.1% TFA) were fractionated by reversed-phase high-performance liquid chromatography (RP-HPLC). RP-HPLC was carried out on a C-18 analytical column (Agilent, 250 x 4.6 mm) using an Agilent 1100 system as described by Colis-Torres et al. (2022). The protein fractions were detected at 214 nm. We estimated the relative percentage of the most variable fractions of the four samples using the area under the curve of chromatograms. To determine the molecular mass, we selected the RP-HPLC fractions that displayed notable dissimilarities (in terms of height and area) among the CMN06 venom samples obtained in 2015 and 2021. ESI-MS obtained the intact masses on an LCQ Fleet Ion Trap Mass Spectrometer (Borja et al., 2018). The proteolytic activity was assessed using azocasein (Sigma-Aldrich, St. Louis, MO, USA) as substrate, following Colis-Torres et al. (2022). One unit of proteolytic activity was defined as a change of 0.2 in absorbance per min (Gutiérrez et al., 2008). The synthetic substrate 4-nitro-3-(octanoyloxy)-benzoic acid (4-NOBA) was used to determine the PLA_2 activity of the complete venom and of the fractions with a molecular mass expected for PLA_2. We followed the procedure described by Holzer and Mackessy (1996) with some modifications. The lethal potency of the CMN06 venom from 2015 and 2021 was evaluated through LD_{50} following the method initially described by Lorke (1983) with modifications described by Neri-Castro et al. (2022). Animal experiments were approved by the Bioethics Committee of the Universidad Nacional Autonoma de Mexico (UNAM) Biotechnology Institute, project 374.

Differences in composition were detected in the electrophoretic and chromatographic profiles of the four venom samples of the CMN06 individual. The most notable changes in electrophoretic gels were observed in the bands smaller than 17 kDa and the bands between 20 kDa to 25 kDa (**Fig. 1A**). For example, the major band in the venom from the year 2015 was located below 11 kDa (likely containing mostly MYO). However, this band gradually decreased in intensity in the subsequent years as the snake aged and increased in size. This result was confirmed by RP-HPLC, where the venom sample from 2015 had four major fractions (representing approximately 69% of the venom (**Table 1**)) that eluted between 30 and 40 min and according to their molecular masses (4959-5187 Da) they correspond to MYO. These fractions were drastically reduced in height in the sample from 2018 and completely disappeared in the samples from 2019, and 2021 (**Fig. 1B**). In contrast, the bands with a molecular mass just above 11 kDa (likely containing phospholipases A_2s (PLA_2s) and C-type lectins (CTLs)) and between 20 kDa and 25 kDa (likely containing PI- and PII-SVMPs and cysteine-rich secretory proteins (CRISP)) were almost absent in the 2015 venom sample but increased in intensity in the remaining samples until becoming the dominant bands in the venom from the year 2021 (**Fig. 1A**). RP-HPLC fractions eluting after 50 min notably increased height and area in the samples from 2018, 2019, and 2021 which corroborated the SDS-PAGE. Particularly, the intact molecular masses of the fractions that eluted at 58.5 min (FV), 65.7 min (FVI), and 66.5 min (FVII) were 13,772 Da, 13,813 Da, and 13,797 Da, respectively (**Fig. 1B**), a molecular mass expected for PLA_2s. The N-terminus of fraction FVII (SLVQFEILIMKVAKRSGLFSYSAYGCYCGWGGH) confirmed that this fraction is an acidic PLA_2. These three fractions comprised approximately 24% of the venom in the sample from 2021 but only 7.5% of the venom in the sample from 2015 (**Table 1**). The highest peaks in the venoms from 2018, 2019, and 2021 were eluted after 80 min, with percentages of 44.6, 49.6, and 53.6, respectively. In contrast, fractions in this area constituted only 3.4% of the sample from 2015 (**Table 1**). According to a previous publication (Borja et al., 2018), these fractions contain mostly SVMPs.

The proteolytic activity of the CMN06 venom was 1.05±0.03, 6.94±0.41, 7.02±0.32, and 7.05±0.35 U/mg for the years 2015, 2018, 2019, and 2021, respectively (**Table 1)**. The phospholipase activity for venoms from 2015, 2018, 2019, and 2021 was 1.4±0.4, 3.1±0.2, 3.2±0.2, and 3.7±0.2 ΔA 450 nm, respectively (**Table 1**). The only RP-HPLC fraction with

phospholipase activity (2.5±0.4) was the one eluting at 66.4 min (FVII). The CMN06 venom from 2015 (LD_{50}: 1.86 µg/g (C.I.=1.79-1.93)) was approximately three times more lethal to mice than CMN06 venom from 2021 (LD_{50}: 5.64 µg/g (C.I.=5.33-5.96)) (Table 1). In addition to the shift in lethality, the venom from 2015 generated hind limb spastic paralysis in mice, an effect that was not observed in mice injected with venom from 2021.

In general, two basic venom compositional "strategies" have been observed in rattlesnakes: venoms with high levels of SVMPs but lower lethality (type I), and venoms with reduced amounts of SVMPs but higher lethality (type II) (Mackessy, 2010). Our results suggest that *C. m. nigrescens* venom shifts from a type II venom to a type I venom as they grow up and mature; and support that lethal toxicity and venom metalloproteinase activity are negatively associated (Mackessy, 2010). Crotamine-like myotoxins (MYOs) are basic peptides comprised of between 42 and 45 amino acids (~4.8 kDa) (Porta et al., 2021), which induce myonecrosis and spastic paralysis in the hind limbs of mice, rats, rabbits, and dogs (Gonçalves, 1956). Because MYOs rapidly paralyze mammalian prey, it is likely that the high amounts of this toxin in juvenile *C. m. nigrescens* ensure the rapid immobilization of prey and prevent escape. Additionally, the high abundance of MYO in juvenile venoms suggests that this toxin could be responsible for their increased toxicity; however, previously, it has been shown that the LD_{50} of MYO is relatively high (1.5-3.0 mg/kg for intravenous (iv)) (Marinovic et al. 2017); therefore, it is likely that several toxins acting in synergy, including MYO, may induce the higher toxicity in juvenile venoms.

PLA_2s and SVMPs are two of the most abundant components in rattlesnake venoms (Tasoulis and Isbister, 2017). PLA_2s have a molecular mass ranging from 13-16 kDa and can generate diverse biological effects including, neurotoxicity, myotoxicity, cardiotoxicity, among others (Lomonte and Križaj, 2021). In addition to enzymatic PLA_2s, viperid venoms may also contain phospholipase A_2-like proteins, which lack catalytic activity but induce myotoxic effects (Lomonte, 2023). SVMPs are classified into three groups (PI, PII, and PIII-SVMPs) based on the number of domains that they contain. The range of molecular masses for PI, PII, and PIII-SVMP is 20-30 kDa, 30-60 kDa, and 60-100 kDa, respectively (Olaoba et al., 2020). Our results suggest that, at least in this individual in particular, the amount of PLA_2s, PI and PII SVMPs in venom tends to increase with the age and size, while the expression of MYO tends to decrease. It has been suggested that, in addition to their

contribution to the toxicity of venom, PLA_2s and PI-SVMPs play a role in digestion (Bernardoni et al., 2014; Thomas and Pough, 1979), particularly in environmental temperatures lower than the optimal for the snake. Although little is known about the diet of *C. m. nigrescens* (Balderas-Valdivia et al., 2009; Carbajal-Márquez et al., 2023), it is likely that adults consume large mammal prey, which would be difficult to digest, and the enzymes in venom may help in the digestive process.

Previously, it was reported that the probable threshold size for the shift in the venom composition of *C. m. nigrescens* is when the snakes reach a TBL close to 70 cm (Borja et al., 2018). In accordance with the above, the most drastic change in the electrophoretic profile of the venom of the CMN06 individual was between 69 cm (2015 sample) and 76 cm (2018 sample) of TBL. Nevertheless, the electrophoretic and chromatographic profiles appear to show a gradual interchange of certain toxin families instead of an abrupt change where some toxin families completely disappear, and others appear. Unfortunately, we did not evaluate all the years in this range (e.g., we missed the years 2016, 2017, 2020). This assumption needs to be tested by evaluating the ontogenetic shift in more individuals from birth to adulthood.

In conclusion, our results corroborate the differences previously reported in the venom composition of different *C. m. nigrescens* individuals with different SVL. However, it is important to note that monitoring the venom composition of more individuals from different geographic regions from birth to adulthood is needed to completely describe the ontogenetic shift in this species and all the toxin families implied.

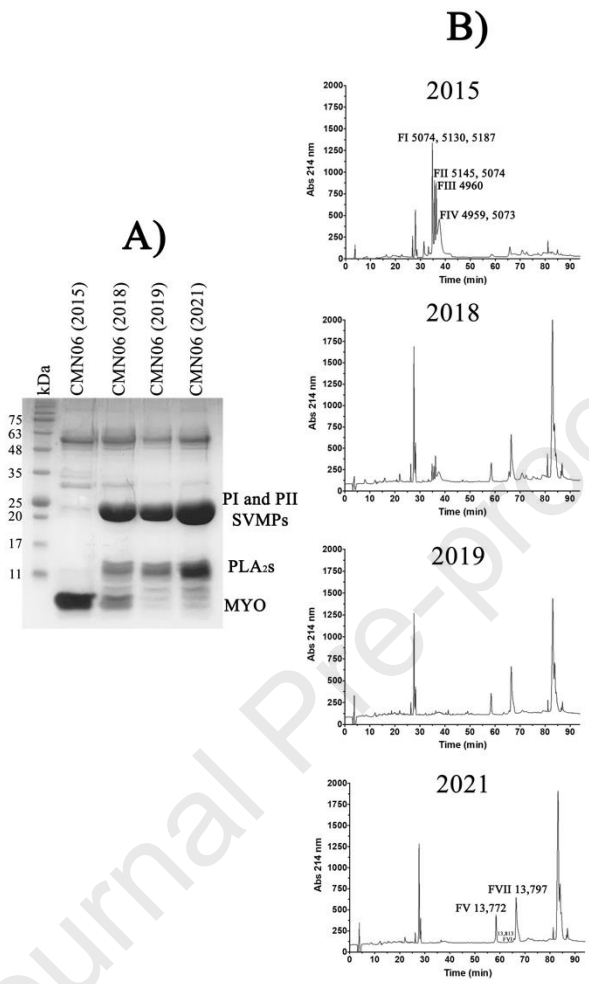

Fig. 1. SDS-PAGE and RP-HPLC of four venoms from one *C. m. nigrescens* individual (CMN06) milked in the years 2015 (SVL: 64 cm), 2018 (SVL: 71 cm), 2019 (SVL: 75 cm) and 2021 (SVL: 79 cm). **A)** Twenty micrograms of venom were loaded per line in a 15% reducing-SDS-PAGE. A protein ladder was used as a reference to estimate the molecular mass in kDa of venom bands. Note the transition from high to low MYO prevalence and low to high prevalence in SVMPs and PLA_2s. **B)** One milligram of venom was separated in each run. Proteins were detected at 214 nm and absorbance is indicated on the left axis. The molecular mass (Da) for selected fractions is shown in the chromatograms of venoms from 2015 and 2021. Note the reduction of earlier eluting proteins and the increase in later eluting proteins over time.

Table 1. Relative percentages of PLA_2s, MYOs, and SVMPs estimated from the area under the curve of chromatograms of CMN06 samples from 2015, 2018, 2019, and 2021 and toxic, proteolytic, and phospholipase activities for each venom sample.

Sample	%PLA_2	%MYO	%SVMPs	Proteolytic activity (U/mg)	Phospholipase activity (ΔA 450 nm)	LD_{50} (µg/g)
CMN06 2015	7.4	69.1	3.2	1.05±0.03	1.4 ±0.4	1.86
CMN06 2018	17.6	8.7	44.7	6.94±0.41	3.1±0.2	ND
CMN06 2019	27.6	0	49.6	7.02±0.32	3.2±0.2	ND
CMN06 2021	23.9	1.5	53.6	7.05±0.35	3.7±0.2	5.64

ND: Not determined

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Highlights

- The ontogenetic venom shift in *C. m. nigrescens* is reported using a single animal.
- Venom composition transitioned from myotoxin-rich to rich in PLA_2s and SVMPs.
- Ontogenetic venom shift predicts proteolytic, phospholipase, and toxic activities.

Ethical Statement

The protocols for the use of these animals were authorized by the Institutional Committee for Animal Care at the Biotechnology Institute of the National Autonomous University of Mexico.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

The protocols for the use of these animals were authorized by the Institutional Committee for Animal Care at the Biotechnology Institute of the National Autonomous University of Mexico.
We also declare that we have no competing financial interest influencing the work reported on our manuscript.