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Venom composition of adult Western Diamondback Rattlesnakes (*Crotalus atrox*) maintained under controlled diet and environmental conditions shows only minor changes

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ABSTRACT

Many species of snakes produce venom as a chemical means of procuring potentially fractious prey. Studies have increasingly focused on venom compositional variation between and within individual snakes of the same species/subspecies, with significant differences often being observed. This variation in composition has been attributed to differences in age, season, diet, and environment, suggesting that these factors could help explain the inter- and intra-specific variation found in some snake venoms, perhaps via some type of feedback mechanism(s). To address several of these possible sources of variation, this study utilized wild-caught Western Diamondback Rattlesnakes (*Crotalus atrox*) from Cochise Co., AZ. Sixteen adult *C. atrox* were maintained in the lab on a diet of NSA mice for eight months to determine whether venom composition changed in captivity under a static diet in a stable environment. Reducing 1-D SDS-PAGE, fibrinogen degradation assays, reversed-phase HPLC, and MALDI-TOF mass spectrometry revealed only minor differences over time within individuals. Venom L-amino acid oxidase (LAAO) and phosphodiesterase activities significantly increased over the course of captivity, with no changes occurring in azocasein metalloproteinase, kallikrein-like serine proteinase (KLSP), or thrombin-like serine proteinase (TLSP) activities. Snake total length was positively correlated with TLSP activity and negatively correlated with LAAO and KLSP activity. There was typically a much higher degree of variation between individuals than within individuals for all analyses performed and measurements collected. Because the overall "fingerprint" of each snake's venom remained more/less constant, it is concluded that biologically significant changes in venom composition did not occur within individual *C. atrox* as a function of captivity/diet. However, this study does indicate that differences in activity levels do occur in minor venom enzyme components, but the differences observed are likely to be of minimal significance to the production of antivenom or to subsequent treatment of human envenomations.

1. Introduction

Snake venom is a complex mixture of various compounds, primarily proteins and peptides, which have evolved as a chemical means of immobilizing, killing, and digesting prey (e.g., Calvete et al., 2009b; Daltry et al., 1996b; Mackessy, 1988, 2008; 2010b; Tu et al., 1969). Although virtually all elapids, viperids and atractaspidids are venomous (Mackessy, 2010b), venoms and venom-delivery systems are also seen among many colubrid snakes (Fry et al., 2008; Junqueira-de-Azevedo et al., 2016; Mackessy, 2002; Mackessy and Saviola, 2016; Saviola et al., 2014). Recent work has indicated a high degree of intraspecific variation in venom composition, with some populations showing vastly different venom protein profiles (e.g., Kalita et al., 2018; Strickland et al., 2018). Identifying the causes of this variation could help provide a better understanding of what factors influence changes in snake venom composition, which in turn could help address the evolutionary advantages associated with utilizing a chemical means (venom) of disabling prey and how this varies with the biochemical composition of

Abbreviations: AZO MPr, Azocasein metalloproteinase; MPr, Metalloproteinase; LAAO, L-amino acid oxidase; PDE, Phosphodiesterase; KLSP, Kallikrein-like serine proteinase; TLSP, Thrombin-like serine proteinase; SP, Serine proteinase; PLA2, phospholipase A_2; CRiSP, cysteine-rich secretory protein; 1-D SDS-PAGE, One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis; RP-HPLC, Reversed-phase high performance/pressure liquid chromatography; MALDI-TOF MS, Matrix-assisted laser-desorption ionization time-of-flight mass spectrometry; kDa, kilo Daltons; LD_{50}, median lethal dose; DTT, Dithiothreitol

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venom. In addition, understanding the extent of this variation has very practical applications, as efficacy of antivenoms could be compromised if high levels of antigenic novelty exist in different populations of the same species.

Ontogenetic and/or size-related changes in venom composition, in some cases paralleling a shift in the type/size of preferred prey, occurs in many species and is often most pronounced in viperids (Alape-Giron et al., 2008; Calvete et al., 2010; Daltry et al., 1996a; Gibbs et al., 2011; Mackessy, 1988, 1993; 2008; Mackessy et al., 2003, 2006; 2018; Zelanis et al., 2010). There has been speculation that these changes in gene expression levels have evolved in response to utilizing different prey types and sizes that snakes will encounter as they grow larger. Understanding whether venom composition is strictly genetically controlled or can be shaped by diet, environment or season will aid in comprehending how/why venom can sometimes be quite variable within a species and would also provide insight into producing more effective antivenoms. For example, if diet or other aspects of captivity affect venom composition, this information could influence the way venom labs commonly maintain snakes for antivenom production (typically maintained on a diet of mice, with periodic venom extractions), prompting changes in the feeding regime to include specific types of prey in order to bring about relevant changes in venom composition.

The Western Diamondback Rattlesnake (*Crotalus atrox*) is one of the leading causes of snake-related human morbidity and mortality in the United States (Gold et al., 2004), making *C. atrox* among the most medically-relevant American snakes for venom composition studies. It occurs commonly from southeastern California through Texas and much of northern Mexico (Campbell and Lamar, 2004; Stebbins, 1985), grows to large size (> 1.5 m) and can produce large amounts of venom (> 2.0 mL/extraction; pers. obs.). Principal venom components from *C. atrox* include metalloproteinases (MPr; P-I and P-III subtypes), serine proteinases (SP), L-amino acid oxidases (LAAO), phospholipase A_2s (PLA_2), cysteine-rich secretory proteins (CRiSP), C-type lectins, disintegrins and vasoactive peptides (Calvete et al., 2009a). Various aspects of venom from *C. atrox* have been analyzed for compositional variation between snake species and families (Aird, 2008; Bertke et al., 1966; Biardi and Coss, 2011; Bonilla and Horner, 1969; Calvete et al., 2009b; Glenn and Straight, 1978; Johnson, 1968; Mackessy, 2008, 2010b; Pahari et al., 2007; Straight et al., 1976), between populations (Calvete et al., 2009a; Minton, 1975; Minton and Weinstein, 1986), within populations (Gregory-Dwyer et al., 1986; Jimenez-Porras, 1961; Johnson, 1968; Minton, 1957), through ontogeny (Johnson, 1968; Minton and Weinstein, 1986) and/or age classes (Gregory-Dwyer et al., 1986; Minton, 1957, 1975; Reid and Theakston, 1978), and with regard to season (Gregory-Dwyer et al., 1986; Minton, 1957). For the current study, we evaluated venom composition of snakes maintained under a controlled diet and environment and predicted that a captive diet of only lab mice (*Mus musculus*) and residence in a climate-controlled room would affect venom composition of adult *C. atrox*.

2. Materials and methods

2.1. Snake collection/maintenance

Sixteen adult *C. atrox* (labeled A-P) were collected in Cochise Co., Arizona and maintained in 38–57 L (10–15 gallon) glass terraria in a room held at 25–28 °C, 14:10 light/dark cycle, and 40–50% humidity for an eight month period following capture in the UNC Animal Resource Facility, in accordance with UNC-IACUC protocol No. 9204.1; one individual, snake E, was retained for a longer period. Cages were supplied with a newspaper substrate, shelter and a water dish containing distilled water. Snakes were also provided with a diet of prekilled adult NSA mice, bred and maintained on-site, at two week intervals. The gender of each individual snake was determined using tail length data and was compared with Boyer (1957), with females having tail lengths of < 7.0% total body length and males having tail lengths of > 7.5%.

2.2. Snake venom extractions

Venoms were extracted manually using standard methods (Mackessy, 1988) immediately upon arrival to the lab and once every two-three months afterward, for a total of four extractions per snake over an eight month period; snake E, retained in the lab, was also extracted at 21 and 27 months. Venom samples were centrifuged, lyophilized and maintained frozen at −20 °C. All procedures utilized venom (~2–3 mg) solubilized in 18.2 MΩ H_2O at an apparent concentration of 4.0 μg/μl. Venom samples were kept at −20 °C when not used, and were thawed, vortexed at high speed, centrifuged at 9200 × *g* for 5 min, and kept on ice during use. Protein concentration of each venom sample was determined in quadruplicate using the method of Bradford (1976) as modified by Bio-Rad, with bovine γ-globulin as the standard, and all activities reported are based on these values.

2.3. Reagents

Gel electrophoresis materials (NuPAGE Bis-Tris gels, MES buffer, LDS sample buffer and Mark 12 molecular weight standards) were acquired from Life Sciences/Invitrogen, Inc. (San Diego, CA, USA). Dye and protein standards for the protein concentration assay were obtained from BioRad, Inc. (San Diego, CA, USA). Kallikrein substrate (Bz-ProPheArg-pNA) was purchased from AnaSpec, Inc., Fremont, CA, USA. All other reagents were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

2.4. Gel electrophoresis

Reducing (using DTT) one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1-D SDS-PAGE) was performed on 12% acrylamide NuPage gels using 24 μg of each venom sample per lane (5 μl of Mark 12 molecular weight standards) and stained in 0.1% Coomassie Brilliant Blue R-250, as described previously (Mackessy, 2010a). Band identity designation (toxin family) was based on many published accounts, comparison with purified components, previous venomic studies on *Crotalus* (including *C. atrox*; Calvete et al., 2009a) and many years experience with electrophoretic analyses of snake venoms.

2.5. Fibrinogen degradation assay

A fibrinogen degradation assay was performed on a select number of venom samples, chosen based on their enzyme activity changes (or lack thereof) across the four extractions, using a procedure modified from Ouyang and Huang (1979). Twelve μg (3 μl) of venom was incubated at 37 °C with 240 μg (120 μl) of human fibrinogen (fraction 1 from human plasma; Sigma) in buffer (100 mM tris-HCl, pH 8.0). A 15 μl aliquot was removed after 0, 1, 5, 10, 30, and 60 min and added to an equivalent amount of termination solution (4% SDS, 10% β-mercaptoethanol, 20% glycerol). After placing these samples in a boiling water bath for 5 min, six μl were removed and added to an equal amount of 2x LDS buffer. Ten μl of each sample was then run on 1-D SDS-PAGE as above.

2.6. Reversed-phase high performance liquid chromatography

Reversed-phase high pressure liquid chromatography (RP-HPLC) was performed on all extractions from a subset of snakes, chosen based on their enzyme activity changes (or lack thereof) across the extractions. Two different protocols, utilizing the same solvents A (0.1% TFA in 18.2 MΩ H_2O) and B (80% acetonitrile (ACN) in H_2O, 0.1% TFA), were run on a Waters HPLC system at a flow rate of 1 ml/min using Empower Pro 5 software. Eight hundred μg of venom from snake A were injected onto a protein C4 Vydac RP-HPLC column (300 Å,

150 × 4.6 mm, 5 μm; Grace Davison Discovery Sciences); total run time was 124 min (5% solvent B for 6 min, 5–15% solvent B over 3 min, 15–80% solvent B over 98 min, 80–100% solvent B over 2 min, 100% solvent B for 5 min, 100-5% solvent B for 2 min, and then 5% solvent B for 8 min). To conserve time and sample, two hundred μg of venom from snakes E and K were run on a Jupiter 5u C4 RP-HPLC column (300 Å, 150 × 4.6 mm, 5 μm; Phenomenex) for 46 min (5–15% solvent B over 5 min, 15–70% solvent B over 23 min, 70–100% B over 1 min, 100% solvent B for 5 min, 100-5% solvent B over 1 min, and then 5% solvent B for 11 min). Fractions were collected and identities of peaks were determined using 1-D SDS-PAGE and known composition of *C. atrox* venom (Calvete et al., 2009a). Absorbance was recorded at 220 nm and 280 nm, with 280 nm chromatograms presented here.

2.7. MALDI-TOF mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on selected samples, chosen based on the criteria stated for RP-HPLC. Approximately one μg of crude venom from snake A and E's first and last extractions was spotted onto one μl of sinapinic acid matrix and then subjected to MALDI-TOF MS using a Bruker Ultraflex MALDI-TOF/TOF mass spectrometer operated in linear positive mode (CSU Metabolomics and Proteomics Facility, Fort Collins, CO). Samples were analyzed using a window of 0–84 kDa (m/z) to identify the masses of specific peptides in this range.

2.8. Enzyme assays

Enzyme assays were performed in triplicate as described previously (Munekiyo and Mackessy, 1998; Smith and Mackessy, 2016) to determine azocasein metalloproteinase (AZO MPr; azocasein), L-amino acid oxidase (LAAO; L-kynurenine), phosphodiesterase (PDE; bis-p-nitrophenylphosphate, Na^+ salt), and serine proteinase (kallikrein-like, KLSP: benzoyl-Pro-Phe-Arg-paranitroaniline; thrombin-like, TLSP: N-Bz-Phe-Val-Arg-pNA hydrochloride) activities, expressed as amount product formed (or change in absorbance)/minute/mg venom protein.

2.9. Analysis of data

Gel images and chromatograms were analyzed qualitatively, and graphical (regression analysis) and statistical (Mann-Whitney test, one-way ANOVA, and Tukey's HSD) analyses were performed on enzyme assay data sets using Minitab 16. p-values for significance were set to 0.05. Data were analyzed for normality assumptions using boxplots (plotting the mean, median, quartile, and outlier values) and probability plots (plotting the original data against the mean and standard deviation of the original data). Although normality was commonly violated, non-parametric tests were only used when comparing uneven groups (e.g., between sexes) since ANOVA is robust to such normality violations when the sample size between the groups is the same (each was n = 16).

3. Results

3.1. Snake demographics and venom yields

Snakes ranged from 720 to 1275 mm in total body length, with a mean of 1015 ± 172 mm, and all were considered sexually mature adults. Male (1028 ± 172 mm, n = 9) and female (997 ± 164 mm, n = 7) total body lengths were similar (p = 0.916), and males possessed significantly longer relative tail lengths (8.23 ± 0.68%) than females (5.96 ± 0.64%; p = 0.001), similar to previous reports (Boyer, 1957).

Among all 16 *C. atrox* venom extractions over the four sampling periods, venom yield had a strong positive correlation with total length

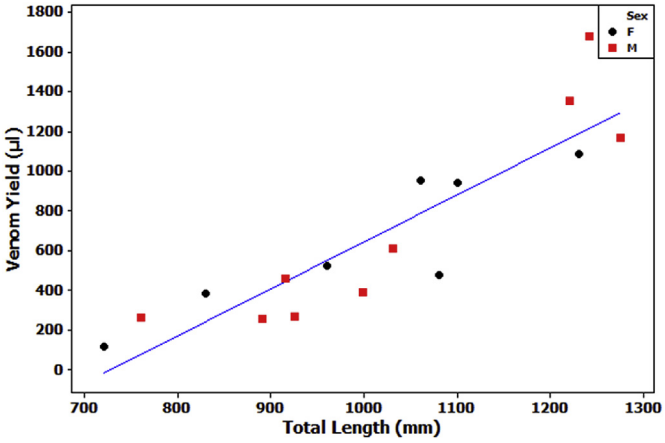

Fig. 1. Scatterplot showing average venom yield as a function of total body length for all 16 *C. atrox*, separated by sex. There is a strong positive correlation between venom yield and total length (r^2 = 0.70); the equation for this relation is venom yield (in μl) = 2.376 (total length in mm) – 1720. Male (n = 9) and female (n = 7) venom yields did not differ relative to total body length.

(r^2 = 0.70, p < 0.001; Fig. 1); there was also no significant difference in venom yield between extractions (n = 14; p = 0.780), and male and female snake yields did not differ (p > 0.655). No differences in venom yields related to the time of the year (season) that the venom samples were collected was noted.

3.2. 1-D SDS-PAGE

Gel band regions were labeled with their predominant protein families, as previously described (Figs. 2 and 3); protein banding patterns in this system are very consistent for rattlesnakes, and the complete proteome of *C. atrox* has been determined (Calvete et al., 2009a; Mackessy, 2008). A moderate degree of variation in banding pattern/intensity between individuals is apparent in most mass ranges, reflecting expected population-level variation.

However, for each individual, none to only minor differences were observed over the four extractions (Fig. 3), and differences were primarily in the higher mass region (~60 kDa). One notable exception is sample K2, in which a band appears at ~80 kDa and a band at ~65 kDa increases greatly in intensity. Those two bands seem to return to the "initial state" of the first extraction by the fourth extraction (Fig. 3).

3.3. Fibrinogen degradation assay

Three individual snakes were selected for this assay based on the results of the enzyme assays (snake A showed "typical" results and snake K showed more "atypical" results) and the fact that snake E was kept in the lab longer than the eight month time period (extraction six was 27 months following capture). In all three snakes, the time required to digest the subunits and the mass of the most visible degradation products (on 1-D SDS-PAGE) do not appear to change significantly from the first to last extraction (Fig. 4). An exception is seen in K4, as the γ-subunit region appears to be more completely hydrolyzed at 30 and 60 min. Venoms of snakes A, E, and K all digest the α-subunit within 1 min and are incapable of completely hydrolyzing the γ-subunit within 60 min. Interestingly, venoms from snakes A and K take 30 min to digest the β-subunit completely, while it takes only 10 min for venom from snake E to do so.

3.4. RP-HPLC

Chromatogram peak contents were broadly categorized using migration patterns on 1-D SDS-PAGE (data not shown; Mackessy, 2008)

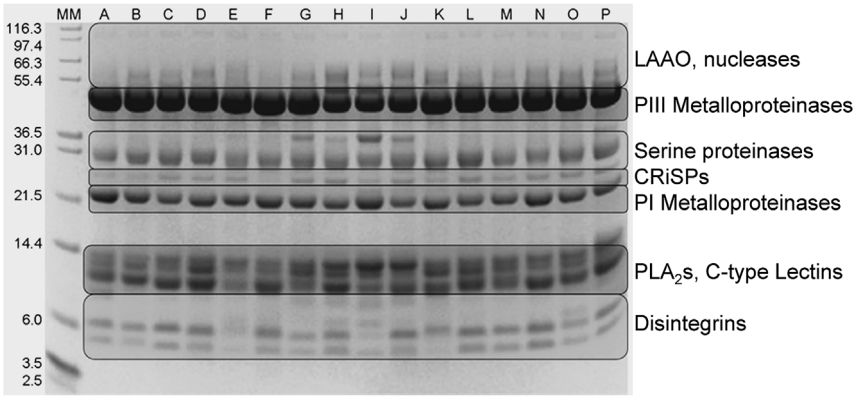

Fig. 2. 1-D SDS-PAGE of venoms from the first extractions of all 16 *C. atrox* (A-P). Lane 1 is the protein standard (MM), and bands are labeled in kDa. Each individual snake is labeled A-P in lanes 2–17. Typical protein families of bands are indicated on right side.

and results from Calvete et al. (2009a; Fig. 5).

In general, there is very little variation throughout the HPLC chromatograms for the first and last venom extractions of *C. atrox* A, E, or K (Fig. 6). Differences in peak height are seen for some samples, but these differences are minimal. Due to room temperature variations in the lab during separations, there are slight shifts in retention times for the chromatographic profiles of the first and last venom extractions of *C. atrox* E and K (Fig. 6). Note that in spite of a different protocol and column used for snake A, profiles within an individual snake are highly conserved.

3.5. MALDI-TOF MS

MALDI-TOF MS spectra in the mass range 0–84000 m/z were recorded and allowed evaluation of qualitative changes in the mass ranges of proteins/peptides present due to captivity/diet effects, but not quantitative changes in the relative amounts of proteins/peptides in the venom. Neither *C. atrox* A nor E displayed substantial variations in

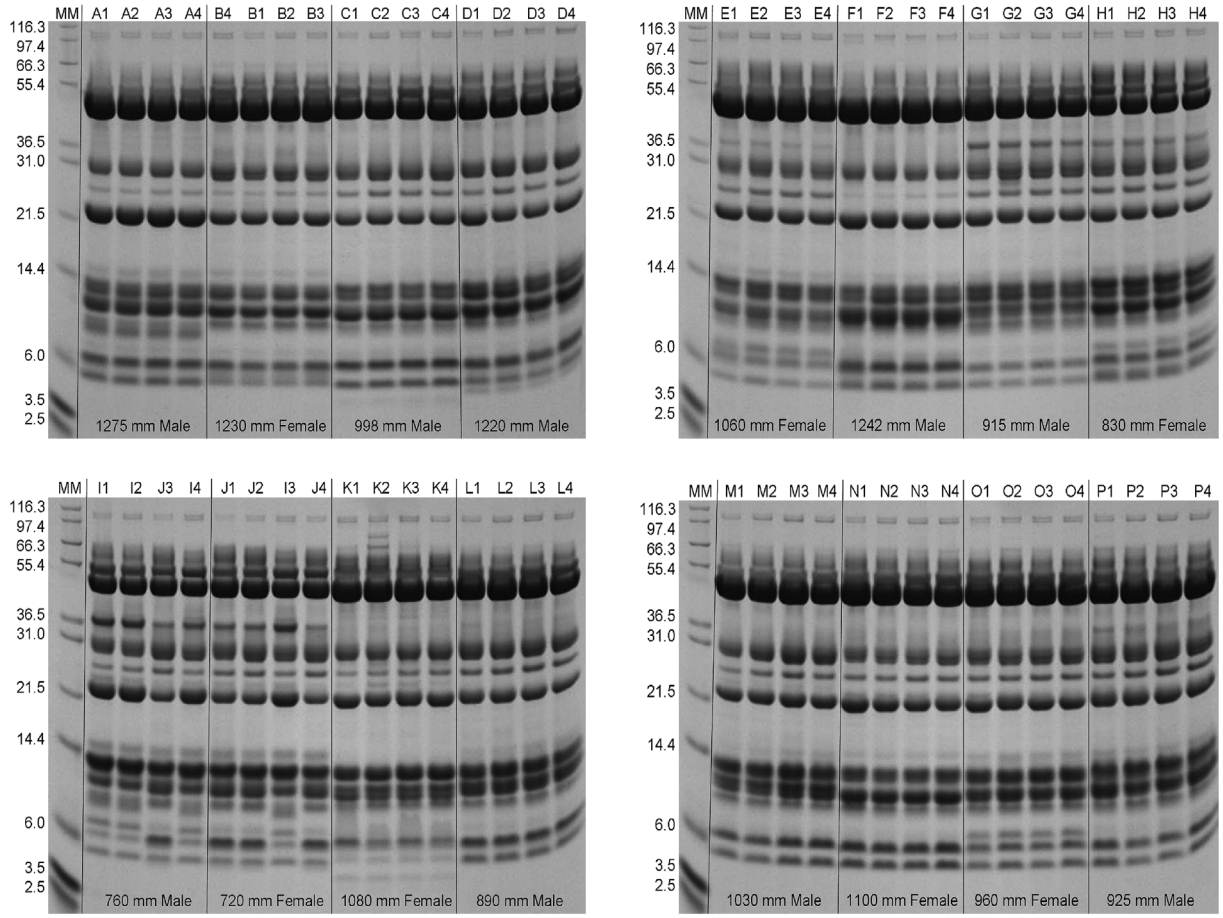

Fig. 3. 1-D SDS-PAGE of venoms from four consecutive extractions from all 16 *C. atrox*; minor differences between individuals are apparent, but within individual variation is not. Lane 1 (MM) in each gel is the protein standard, labeled in kDa. Individual snakes are labeled with a letter (A-P), while extractions (collected at 2 month intervals) are labeled with numbers (1 for the first extraction and 4 for the last extraction). Snake initial total length (mm) and gender are listed below each set of four venom extractions. Note that samples I3 and J3 were inadvertently loaded into the opposite lanes; minor differences between these two individual snakes are clearly observed in intensity of bands at approximately 37, 23, 10 and 7.5 kDa.

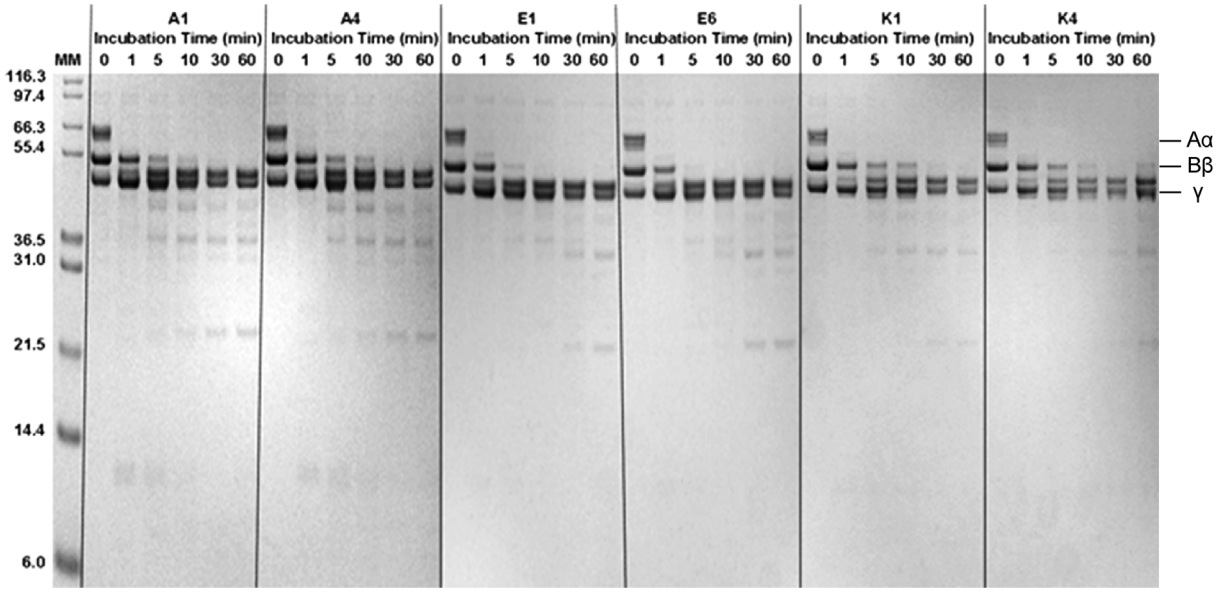

Fig. 4. 1-D SDS-PAGE of the fibrinogen degradation assay for three *C. atrox* (snakes A, E, and K), for the first (1) and last (4 or 6) venom extractions. Lane 1 is the protein standard (MM), and bands are labeled in kDa. Time 0 is the control, showing the three fibrinogen subunits as indicated on the right side. Note that the degradation pattern is identical for all extractions, even though sample E6 was obtained 27 months after the initial extraction. The image is a composite of three gels (one for each snake).

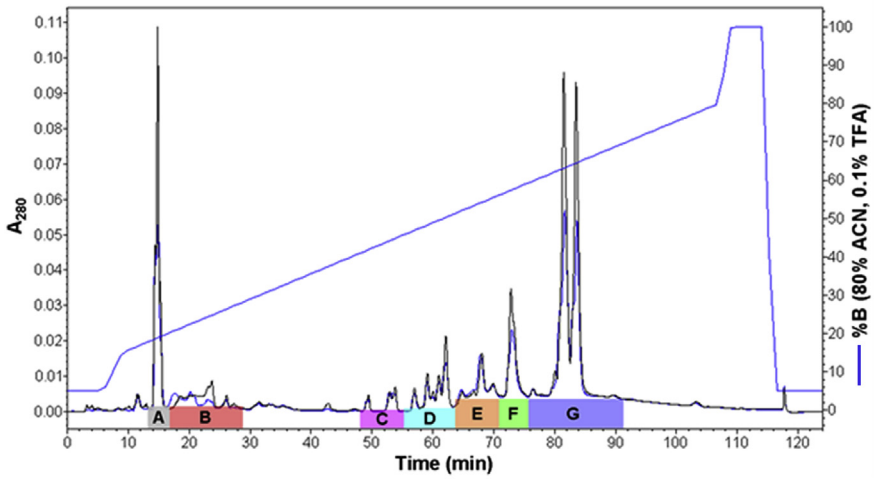

Fig. 5. HPLC chromatogram of the first (black) and fourth (blue) venom extractions of *C. atrox* A. Note that peak elution times are identical for both samples. The most abundant proteins in each peak are given by letters and different colored regions: A (gray) – peptides (including BPPs and tripeptide inhibitors); B (red) – disintegrins; C (purple) – PLA_2, CRiSP; D (cyan) – SPs; E (orange) – PLA_2, C-type lectins; F (green) – P-I MPr; G (blue) – P-III MPr, LAAO. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

presence/absence of venom proteins or peptides (in the range of 4000–84000 m/z) between first and last extractions, but again, minor differences between individual snakes were observed (Fig. 7).

3.6. Summary of qualitative analyses

Minimal differences between venom extractions within individual snakes and minor variation between individuals were observed (Table 1). There were no detectable differences in qualitative venom profiles between males and females or in relation to the time of the year (season) that the venom samples were collected.

3.7. Protein concentration

Protein concentrations for the first through the fourth extractions were statistically identical ($p = 0.678$; Table 1), and male and female venom protein concentrations were similarly indistinguishable ($p > 0.529$). There was no correlation between total length and venom protein concentration for extractions one and two ($r^2 = 0.00$; $p = 0.815$, 0.507), a weak positive correlation for extraction three ($r^2 = 0.18$, $p = 0.056$) and a moderate positive correlation for extraction four ($r^2 = 0.52$, $p = 0.001$). There was a weak positive correlation between total length and the overall change in protein concentration from the first to last extractions ($r^2 = 0.22$, $p = 0.037$).

3.8. Enzyme assays

3.8.1. Azocasein metalloproteinase activity

All 16 snakes were averaged for each extraction time point and subjected to a one-way ANOVA and a Tukey's HSD test to analyze whether any of the extraction means were significantly different. The same process was utilized for each of the other quantitative enzyme assays. Azocasein metalloproteinase activity did not vary significantly (Table 1) between extractions one (0.632 ± 0.085), two (0.637 ± 0.093), three (0.629 ± 0.095), or four (0.617 ± 0.080; $p = 0.932$). There was no correlation between total length and AZO MPr activity for any of the extractions ($r^2 = 0.00$, $p = 0.906$). There was also no correlation between total length and the overall change in AZO MPr activity from the first to last extractions ($r^2 = 0.00$, $p = 0.712$). Females (0.655 ± 0.096) possessed statistically significant

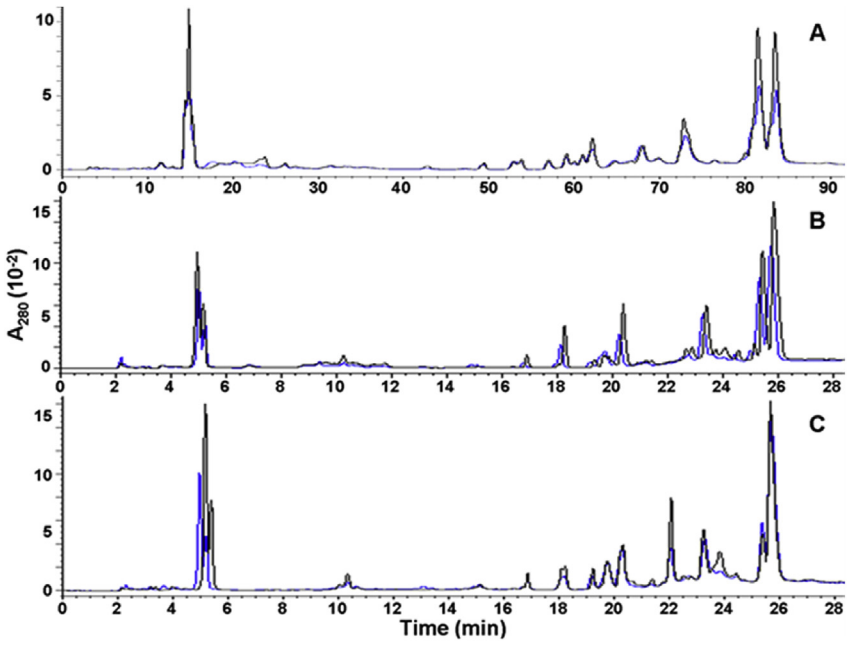

Fig. 6. Comparison of HPLC chromatograms from the first (black) and last (blue) venom extractions for three adult *C. atrox*. A. Snake A, using a longer run time and a Vydac C4 column; venom extractions were 8 months apart (A1 and A4). B. Snake E, using a shorter run time and a Phenomenex C4 column; venom extractions were 27 months apart (E1 and E6). C. Snake K, same conditions as B, but venom samples were collected 8 months apart (K1 and K4). Slight shifts in retention times seen in B and C are due to thermal effects. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

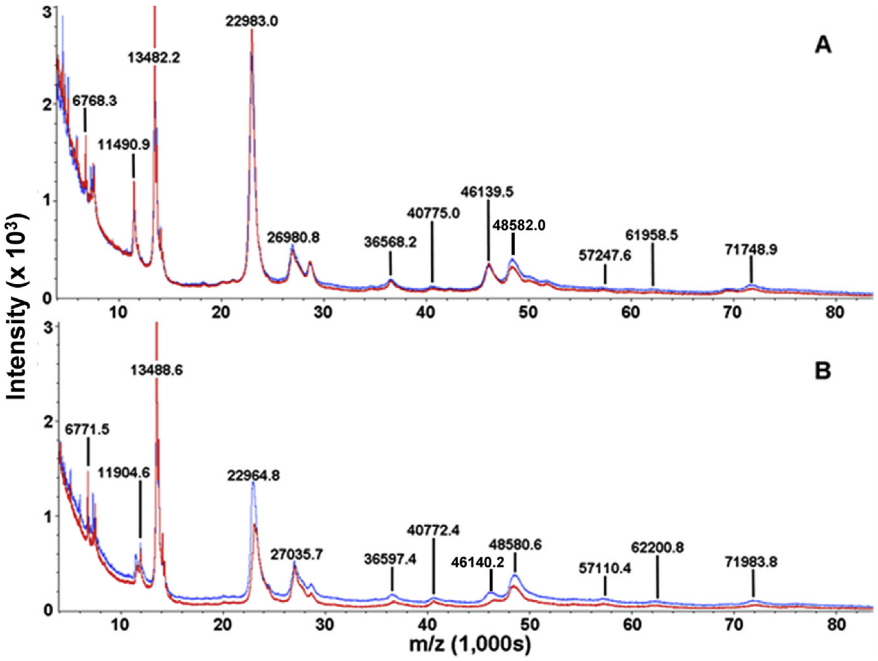

Fig. 7. Comparison of MALDI-TOF MS profiles from the first (blue: A1, E1) and last (red: A4, E6) venom extractions of *C. atrox* A (panel A) and E (panel B). Masses are labeled for most major peaks. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

higher AZO MPr activities than males (0.609 ± 0.073; $p < 0.049$).

3.8.2. L-amino acid oxidase activity

L-amino acid oxidase activity appeared to increase over time in captivity (Table 1), with extractions three (27.3 ± 7.3) and four (31.0 ± 7.5) having significantly higher activities than extractions one (23.4 ± 5.2) and two (24.5 ± 7.2; $p = 0.013$). There is an apparent negative correlation between total length and LAAO activity, which is absent for the second extraction but is present for all other extractions ($r^2 = 0.306, 0.000, 0.222, 0.398$; $p = 0.015, 0.516, 0.038, 0.005$). There was a slightly negative correlation between total length and the overall change in LAAO activity from the first to last extractions ($r^2 = 0.11$, $p = 0.114$). Males (25.2 ± 7.9) and females (28.3 ± 6.2) did not possess significantly different LAAO activities ($p > 0.062$).

3.8.3. Phosphodiesterase activity

Phosphodiesterase (PDE) activity appeared to increase over time in captivity (Table 1), with extraction four (0.241 ± 0.047) having significantly higher activities than extractions one (0.173 ± 0.036), two (0.219 ± 0.047), and three (0.2235 ± 0.042; $p < 0.001$). There was no correlation between total length and PDE activity ($r^2 = 0–0.05$, $p = 0.658–0.944$). There was also no correlation between total length and the overall change in PDE activity from the first to last extractions ($r^2 = 0.00$, $p = 0.712$). Males (0.218 ± 0.049) and females (0.209 ± 0.050) did not possess significantly different PDE activities ($p > 0.709$).

3.8.4. Kallikrein-like serine proteinase activity

Kallikrein-like serine proteinase (KLSP) activity did not change

Table 1
Summary of results from all qualitative and quantitative analyses showing effect, if any, of captivity/diet on *C. atrox* venom properties. Significant increases in enzyme activity, from first to last extractions, were seen only for LAAO and PDE.

Analysis	Changes over captivity
1-D SDS-PAGE	~none
Fibrinogen Digest	~none
RP-HPLC	~none
MALDI-TOF MS	~none
Protein Conc.	none ($p = 0.678$)
AZO MPr	none ($p = 0.932$)
LAAO	**increase ($p = 0.013$)**
PDE	**increase ($p < 0.001$)**
KLSP	none ($p = 0.917$)
TLSP	none ($p = 0.956$)

significantly (Table 1) between venom extractions one (311.1 ± 59.4), two (315.5 ± 50.9), three (312.3 ± 53.5), and four (323.6 ± 56.4; $p = 0.917$). There was no correlation between total length and KLSP activity at the first and second extractions ($r^2 = 0.001, 0.00$; $p = 0.288, 0.541$), but there was a low negative correlation at the third extraction ($r^2 = 0.24$, $p = 0.033$) and a moderate negative correlation by the fourth extraction ($r^2 = 0.48$, $p = 0.002$). This may be due to a size/age-related response to captivity, where venoms from longer snakes tended to show lower KLSP activity and shorter snakes tended to show increased KLSP activity ($r^2 = 0.20$, $p = 0.048$). Males (319.6 ± 48.1) and females (310.5 ± 61.3) did not possess significantly different KLSP activity ($p > 0.621$).

3.8.5. Thrombin-like serine proteinase assay

Thrombin-like serine proteinase (TLSP) activity did not change significantly (Table 1) between venom extractions one (189.3 ± 48.6), two (195.8 ± 47.9), three (188.5 ± 35.7), and four (193.49 ± 36.2; $p = 0.956$). There was a weakly positive correlation between total length and TLSP activity ($r^2 = 0.22, 0.24, 0.15, 0.18$; $p = 0.040, 0.031, 0.077, 0.057$). There was no correlation between total length and the overall change in TLSP activity from the first to last extractions ($r^2 = 0.01$, $p = 0.305$). Males (193.8 ± 37.1) and females (189.2 ± 47.5) did not possess significantly different TLSP activities ($p > 0.760$).

3.8.6. All quantitative assays

Boxplots illustrate the percent changes in enzyme activity and protein concentration for all 16 snakes between venom extractions one and four (Fig. 8). These boxplots illustrate. how little percent change occurred in AZO MPr (−1.5 ± 12.6), KLSP (5.4 ± 14.5), TLSP (4.7 ± 15.6), and protein concentrations (0.2 ± 13.3), but how marked the percent increases were for LAAO (32.9 ± 19.9) and PDE (42.8 ± 26.5) activities. For snake E, which was the only snake retained and sampled over a longer period, enzyme activities increased somewhat during. extractions five and six, while protein concentration decreased (Fig. 9). There did not appear to be any differences in venom enzyme activities or protein concentrations related to the time of the year (season) that the venom samples were collected.

4. Discussion

Snake venom consists of numerous proteins/peptides that have evolved as a chemical means of incapacitating prey (e.g., Kardong et al., 1997; Mackessy, 2008). Although venom composition is known to vary at phylogenetic, population and individual levels, the reasons for this variation (age, diet, environment, season, etc.) are often not as clearly defined. Various aspects of venom from *C. atrox* have been analyzed for variation at phylogenetic levels, between populations, within populations, through ontogeny, and with regard to season. The present study was initiated to evaluate potential changes in venom composition that could occur in *C. atrox* maintained in captivity for a period of eight months. This study design subjected each snake to controlled conditions (diet, temperature, light cycle, etc.), as opposed to the highly variable and often stressful conditions present in the wild. Our results indicate that composition of venoms from 16 adult *C. atrox* showed only minor changes in response to captivity/diet.

Numerous techniques (such as SDS-PAGE, HPLC, and MALDI-TOF-MS) provide a good overall picture of venom composition, but specific enzyme assays can provide information about the probable *in vivo* activity of the venom. In this sense, enzyme assays may be a more useful tool for relating venom properties to envenomation symptoms than the information collected by fingerprinting techniques. This study found that although no apparent differences between venom samples from individual snakes were observed following MALDI-TOF-MS, HPLC or SDS-PAGE, enzyme assays revealed some changes in venom activities due to captivity and/or diet. These results underscore the importance of using multifaceted analyses to assess the levels of similarities and differences between venom samples.

With one minor exception (seen in snake K), composition of the four venom extractions appeared to change little within each snake due to captivity/diet based on 1-D SDS-PAGE. These results largely agree with the results of several previous studies of viperids (Fiero et al., 1972; Gregory-Dwyer et al., 1986; Jones, 1976; Gubensek et al., 1974; Jimenez-Porras, 1964b; Johnson, 1987; Mackessy, 2010b; Schenberg, 1963) and *Alsophis portoricensis* (a rear-fanged dipsadid snake; Weldon and Mackessy, 2010); held in captivity for ≥3 months that also showed no change in electrophoretic profiles. Although this study did not attempt to simulate seasonal changes in photoperiod, temperature, and feeding, there did not appear to be season/time-related changes in any venom protein bands, in agreement with the lack of differences observed in the isoelectric focusing profiles of six *C. atrox* held in captivity for 20 months (Gregory-Dwyer et al., 1986). Three snakes, A, E, and K, showed no differences between their first and last venom extractions and only slight differences between individuals for the fibrinogen degradation assay.

Reversed-phase HPLC chromatograms for snakes A, E, and K were also very similar between the first and last extractions. This result indicated that the types and amounts of various compounds in the venom did not change by a substantial degree over the course of captivity. The MALDI-TOF MS profiles for snakes A and E from the first to last extractions were essentially identical, indicating that the types of proteins/peptides present in the venoms from snakes A and E did not appear to change from first to last extraction. These results illustrate that overall venom composition remains relatively constant in *C. atrox* in relation to captivity/diet. The use of more detailed proteomic/venomic analyses, such as those utilized by Calvete et al. (2009a) and Gibbs et al. (2011), could reveal more subtle changes in venom characteristics across extractions over the course of captivity than the venom chromatogram comparisons presented here, but it is unlikely that major venom constituents would show patterns different than those obtained.

Venom AZO MPr, KLSP, and TLSP activities remained stable over the duration of captivity, while LAAO and PDE activities increased significantly, indicating that only some classes of venom enzymes were affected, to varying degrees, by captivity/diet. Although it is possible that higher venom LAAO and PDE levels may be related to *C. atrox* ability to procure/digest murine prey, this seems unlikely, as neither is notably toxic to mice. Further, in a proteomic study of *C. atrox* venom, LAAO was found to comprise only 8% of total venom proteins, and PDE was not even detected (Calvete et al., 2009a). Alternatively, increases in LAAO and PDE activities may simply result from a highly stable captive environment (controlled temperature, humidity, light cycle, etc.) with regular access to food and water.

When four adult *A. mexicanus* were maintained in captivity for two-six months, their venoms went from colorless/slightly yellow to distinctly yellow in color, indicating a significant change in venom

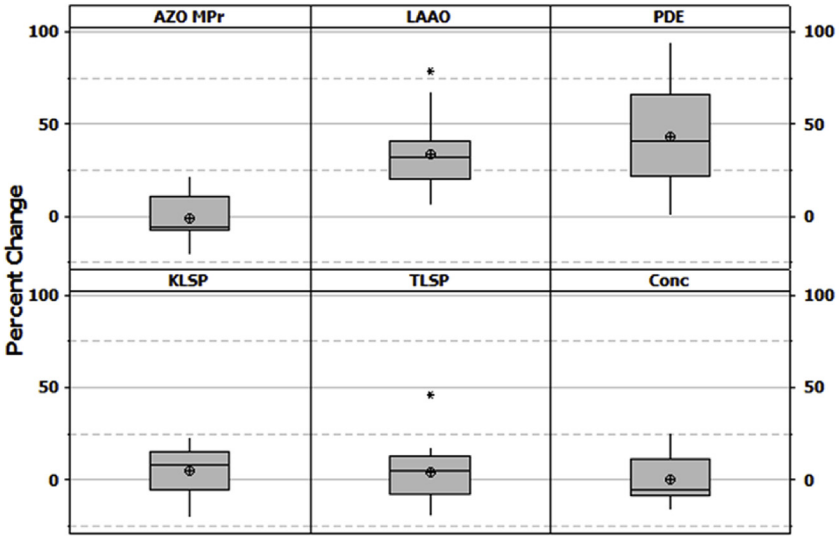

Fig. 8. Boxplots showing overall trends (percent increase/decrease between venom extractions one and four) in enzyme activity and protein concentration for all 16 *C. atrox*. While AZO MPr, KLSP, TLSP, and protein concentrations (Conc) show little change, LAAO and PDE show large positive increases in activity.

composition that was further supported by shifts in enzyme activities and gel profiles (Jimenez-Porras, 1964a). However, no such changes in venom color occurred in *C. atrox*, despite the fact that LAAO, which contains FAD as a cofactor and gives venom its yellow color, increased in activity. Gibbs et al. (2011) observed that differences in venom composition occurred to a greater degree in wild-caught adult *S. m. barbouri* fed different diets than in juveniles, with the greatest differences occurring in adults belonging to the mouse-fed group, suggesting that mice were more dissimilar to their native prey than frogs or lizards. Individual venom protein concentrations did not change significantly overall across the four extractions, but their correlation with size changed from none to a moderately strong positive correlation. Thus, the significant increases observed in *C. atrox* PDE and LAAO enzyme activity, along with the size-related shifts in protein concentration, LAAO, and KLSP activity, may be related to a diet consisting solely of inbred mice.

Body size in wild *C. atrox* has been reported to be male biased (Beaupre and Duvall, 1998; Taylor and Denardo, 2005), so there is a possibility that venom composition could also be influenced by sex. Females produced venoms with ~8% higher AZO MPr activities than males, suggesting a sex-based difference in this prominent venom protein family, but no other analyses revealed any significant differences between sexes. Hypothetically, slightly lower AZO MPr activity could be related to heightened testosterone levels (and/or the resulting higher metabolic rates) that male *C. atrox* experience in comparison to females (Taylor and Denardo, 2005). Venom from all but one female (snake J) showed a decrease in AZO MPr activity from first to last extraction (though these changes were not statistically significant). Sex-based differences in venom composition, if they occur in *C. atrox*, appear to be minimal.

The results found here, consistent with the findings of several other studies (Freitas-de-Sousa et al., 2015; Gibbs et al., 2011; Galizio et al., 2018; Lourenço et al., 2013; McCleary et al., 2016), suggest that captivity and/or a controlled diet impact venom composition in *C. atrox* to a minimal extent. Assuming that captive animals receive proper care, it is unlikely that venom of snakes raised in captivity for the purposes of

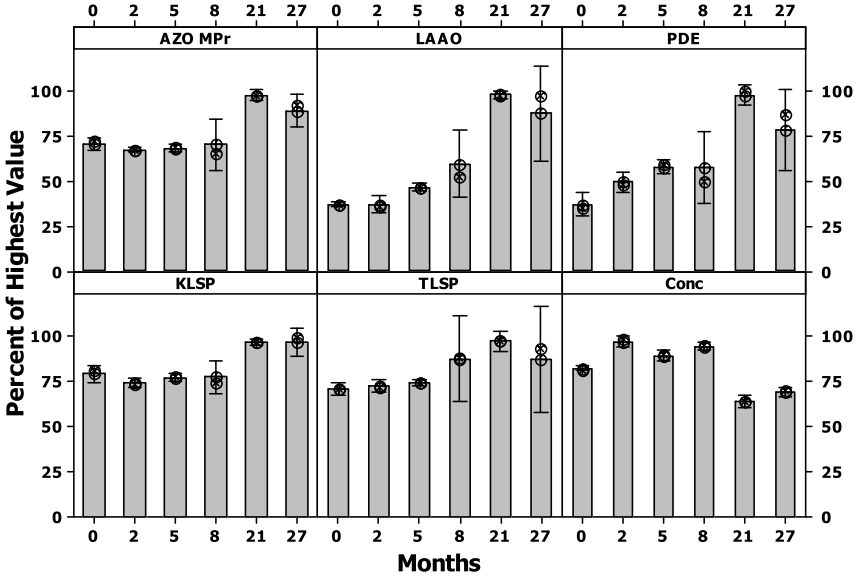

Fig. 9. Bar graphs showing the activity/concentration values for each *C. atrox* E venom extraction (displayed in months from capture) as percentages of the highest values. Activities increased during extractions five and six, while protein concentrations (Conc) decreased.

antivenom production or toxinological research will show significant differences from their wild-caught counterparts. However, possible changes in venom composition that occur due to captivity and/or diet have been evaluated for only a small number of species, so it is possible that different taxa could show differential effects of captivity.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2019.03.027.

Ethical statement

The authors hereby state that all procedures involving animals were conducted in a humane and ethical manner. All protocols were evaluated and approved (prior to initiating research) by the University of Northern Colorado Institutional Animal Care and Use Committee (UNC-IACUC).

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