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An analysis of venom ontogeny and prey-specific toxicity in the Monocled Cobra (*Naja kaouthia*)

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ABSTRACT

Venoms of snakes of the family Elapidae (cobras, kraits, mambas, and relatives) are predominantly composed of numerous phospholipases A_2 (PLA_2s) and three-finger toxins (3FTxs), some of which are lethal while others are not significantly toxic. Currently, the only identified prey-specific toxins are several nonconventional 3FTxs, and given the large diversity of 3FTxs within Monocled Cobra (*Naja kaouthia*) venom, it was hypothesized that several 3FTxs, previously found to be non-toxic or weakly toxic 3FTxs in murine models, could potentially be toxic towards non-murine prey. Additionally, it was hypothesized that ontogenetic dietary shifts will be correlated with observable changes in specific 3FTx isoform abundance. Adult and juvenile *N. kaouthia* venom composition was investigated using ion-exchange FPLC, 1D and 2D SDS-PAGE, mass spectrometry, and various enzymatic and LD_{50} assays. Alpha-cobratoxin (α-elapitoxin) was the only significantly toxic (LD_{50} < 1 μg/g) 3FTx found in *N. kaouthia* venom and was equally toxic toward both lizard and mouse models. The abundance and diversity of 3FTxs and most enzyme activities did not vary between adult and juvenile cobra venoms; however, total venom PLA_2 activity and specific PLA_2 isoforms did vary, with juveniles lacking several of the least acidic PLA_2s, and these differences could have both biological (related to predation) and clinical (antivenom efficacy) implications. Nevertheless, the ubiquitous presence of α-cobratoxin in both adult and juvenile cobra venoms, with high toxicity toward both reptiles and mammals, represents a venom compositional strategy wherein a single potent toxin effectively immobilizes a variety of prey types encountered across life history stages.

1. Introduction

The Monocled Cobra (*Naja kaouthia*) is the most abundant species of Asian cobra, with a range that includes India, Bangladesh, Nepal, Myanmar, southwestern China, and Thailand (Mukherjee and Maity, 2002; Reali et al., 2003). In Thailand, snakebite envenomations by *N. kaouthia* account for the highest number of human fatalities among all venomous snake species (Kulkeaw et al., 2007). Patients who have systemic envenoming by *N. kaouthia* usually develop neurotoxic symptoms, including ptosis, dysphagia, and increased salivation, followed by coma and death from respiratory paralysis in severe cases (Sells et al., 1994; Reali et al., 2003).

Because *N. kaouthia* is very common and is responsible for human morbidity and mortality, there have been many studies published characterizing specific venom proteins and describing overall venom composition (Hamako et al., 1998; Sakurai et al., 2001; Meng et al., 2002; Mukherjee and Maity, 2002; Doley and Mukherjee, 2003; Kulkeaw et al., 2007; Mordvintsev et al., 2007, 2009; Debnath et al., 2010), including two recent publications on the complete venomics profile of *N. kaouthia* (Laustsen et al., 2015; Tan et al., 2015). The venom of *N. kaouthia* is primarily composed of three-finger toxins (3FTxs; neurotoxic and cardiotoxic/cytotoxic) and phospholipase A_2 (PLA_2) isoforms (Namiranian and Hider, 1992; Kulkeaw et al., 2007; Laustsen et al., 2015; Tan et al., 2015). Geographic venom variation was also recently documented for *N. kaouthia* (Tan et al., 2015), but because pooled venoms were used in these studies, there is still a lack of information on individual intraspecific venom variation or ontogenetic venom compositional changes in *N. kaouthia*.

Venom variability has been documented at the family, genus,

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species and intraspecific levels (Chippaux et al., 1991; Mackessy, 2010b). Intraspecific venom variation may occur between individuals of different geographic locations, dietary habits, genders and age (Minton and Weinstein, 1986; Mackessy, 1988, 1993; Chippaux et al., 1991; Daltry et al., 1996a; Daltry et al., 1996b; Mackessy et al., 2003; Menezes et al., 2006; Alape-Giron et al., 2008). Ontogenetic venom variation studies have largely focused on the subfamily Crotalinae (pit vipers), *Crotalus* and *Bothrops* species in particular (Meier, 1986; Minton and Weinstein, 1986; Mackessy, 1988, 1993; Gutiérrez et al., 1991; Saldarriaga et al., 2003; Alape-Giron et al., 2008; Zelanis et al., 2009). A few studies have analyzed ontogenetic venom variation in the Elapidae (cobras, kraits, mambas, and relatives), but the conclusions of these studies varied (see below).

Several studies of Australian elapids found that there was no significant difference in the venom composition of juvenile and adult Coastal Taipans (*Oxyuranus scutellatus*), Inland Taipans (*Oxyuranus microlepidotus*), and Tiger Snakes (*Notechis scutatus*) (Tan et al., 1992, 1993a, b). Minton (1967) found that venom toxicity, hemorrhagic activity, indirect hemolysin, direct hemolysin, and hemagglutinin for *Naja naja* ssp. increased with age. Unfortunately, since this study was conducted before the more recent revisions of the genus *Naja* that revealed at least ten species of *Naja* throughout India and southeast Asia (Wüster, 1996), it is unknown which species was actually studied. Meier and Freyvogel (1980) found a decrease in venom toxicity with age for the Black-necked Spitting Cobra (*Naja nigricollis*). A more recent study on ontogenetic venom variation in *Naja atra* found higher phosphomonoesterase and L-amino acid oxidase activity and lower nucleotidase, PLA_2, hyaluronidase, and fibrinolytic activity within neonate *Naja atra* venom (He et al., 2014).

Ontogenetic changes in venom composition have been found to correlate with snake dietary shifts (Mackessy, 1988; Andrade and Abe, 1999; Mackessy et al., 2006; Zelanis et al., 2009). In Pacific Rattlesnakes (*Crotalus oreganus* [*viridis*]; Mackessy, 1988) and Jararacas (*Bothrops jararaca*; Andrade and Abe, 1999), an ontogenetic shift in diet from ectothermic prey (arthropods, lizards, and amphibians) as a juvenile to endothermic prey (mammals) as an adult was associated with a shift in venom toxicity, with juvenile venom more toxic to ectotherms and adult venom more toxic towards mammals. A similar pattern was noted for the Brown Treesnake (*Boiga irregularis*), with juvenile venoms more toxic than adult venoms towards geckos, suggestive that this trend is not just found in vipers (Mackessy et al., 2006).

In their first year, *N. kaouthia* feed primarily on frogs and newborn rats, and adult snakes feed on adult rats, snakes, lizards, fish, birds, and bird eggs (Chaitae, 2000). Therefore, as *N. kaouthia* ages, a wider diversity of prey is taken, most likely because juveniles are gape-limited to feed on smaller prey. Prey-specific toxins have not been identified in cobra venoms, even though 3FTxs that are weakly toxic towards murine models have been identified in cobra venoms, and 3FTxs are currently the only venom proteins to be directly linked to prey-specific toxicity (Pawlak et al., 2006, 2009; Heyborne and Mackessy, 2013). Non-conventional/weak 3FTxs exist in *N. kaouthia* venom, and this subclass of 3FTxs have been suggested to confer prey-specific toxicity in King Cobra (*Ophiophagus hannah*) venoms (Chang et al., 2013), but this hypothesis has not been tested.

Documentation of intraspecific variation in venoms also has important clinical and antiserum production applications. Intraspecific venom variation in the Spectacled Cobra (*Naja naja*) has resulted in the manifestation of different clinical envenomation symptoms and a lack of antivenom efficacy between India and Sri Lanka *N. naja* populations (Kularatne et al., 2009). The present study compares the biochemical composition of adult and juvenile *N. kaouthia* venoms and addresses the following questions: Are there differences in venom protein content and activity between adult and juvenile cobras? Is there a difference in the toxicity of adult and juvenile cobra venoms towards ectothermic (lizards) and endothermic (mammalian) prey? Are there components of *N. kaouthia* venom, such as nonconventional 3FTxs, that exhibit prey-specific toxicity? It was hypothesized that non-conventional 3FTxs in *N. kaouthia* venoms would exhibit selective toxicity toward non-mammalian prey.

2. Materials and methods

2.1. Reagents

Reagents for protein concentration assays were purchased from BioRad Inc. (San Diego, CA, U.S.A). Precast NuPAGE 12% Bis-Tris mini gels, Novex Mark 12 unstained molecular mass standards, LDS sample buffer and MES running buffer were purchased from Life Technologies (Grand Island, NY, U.S.A). Two-dimensional gel electrophoresis supplies, including DeStreak rehydration solution, IPG pH 3–11 buffer and Immobiline DryStrip pH 3–11, were purchased from GE Healthcare (Pittsburgh, PA, U.S.A). Phospholipase A_2 assay kit was purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A). Azocasein, DTT, DTNB, acetylthiocholine iodide, bis-p-nitrophenylphosphate, L-kynurenine, human fibrinogen and all other reagents (analytical grade or better) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A).

2.2. Venoms and animals

Pooled and lyophilized adult (over three years in age) and 10 individual juvenile (less than three months in age) *N. kaouthia* venoms were donated by the Kentucky Reptile Zoo (Slade, KY, U.S.A). All *N. kaouthia* were of Thailand origin and kept under the same husbandry conditions. The pooled adult venom included parents of the juveniles to reduce the potential effects of venom variation due to genetic variability. Venoms were stored frozen at −20 °C until needed. Venom protein concentration was determined using bovine gamma globulin standard and the method of Bradford (1976) as modified by BioRad Inc. The calculated protein concentrations for each crude venom sample were used in all other analyses and enzymatic activity calculations. NSA mice (*Mus musculus*) were bred in the University of Northern Colorado (UNC) Animal Resource Facility and House Geckos (*Hemidactylus frenatus*) were obtained from Bushmaster Reptiles (Longmont, CO, USA); all procedures were reviewed and approved by the UNC IACUC (protocol 1504D-SM-SMLBirds-18).

2.3. One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE on NuPage 12% Bis-Tris mini gels (Life Technologies, Inc., U.S.A) was performed to compare the relative molecular masses of venom components of adult and juvenile *N. kaouthia*. Samples and buffers were prepared under reducing conditions according to the manufacturer. Venom samples were run at 10 μg and 20 μg per lane, with 5 μL of Novex Mark 12 unstained mass standard in one lane for estimation of molecular masses. The gel was run at 180 V, stained with 0.1% Coomassie Brilliant Blue R250 overnight, destained (50/40/10, v/v, ddH_2O:methanol:glacial acetic acid) for two hours, and imaged.

2.4. Two-dimensional polyacrylamide gel electrophoresis (2D SDS-PAGE)

Two-dimensional gel electrophoresis was performed to evaluate protein composition differences between adult and juvenile *N. kaouthia* venoms. Venom (170 μg) was added to 135 μL DeStreak rehydration solution containing 18 mM DTT and 0.5% IPG pH 3–11 buffer (GE Healthcare, Pittsburgh, PA, U.S.A). This solution was added to the ceramic holder, overlain with a 7 cm IPG pH 3–11 strip and then mineral oil, and placed on an IPGphor Electrofocusing System (GE Healthcare, Pittsburgh, PA, U.S.A). The following isoelectric focusing steps were performed: rehydration for 12 h, 500 V for 30 min, 1000 V for 30 min, 5000 V for 3 h, and a 40 V hold. For the second dimension electrophoresis, the focused IPG strip was equilibrated in a reduction buffer (l mL 4× LDS buffer, 400 μL 150 mM DTT [final concentration 15 mM], and 2.6 mL ddH_2o) at ambient temperature for 10 min and then equilibrated in an alkylation buffer (1 mL 4× LDS, 3 mL ddH_2O, and a final concentration of 60 mM iodoacetamide) at ambient temperature for 10 min. SDS-PAGE was carried out using NuPage 12% Bis-Tris 2D well gels following the same procedure as described above for 1D SDS-PAGE.

2.5. Enzyme assays

Metalloproteinase activity of adult and juvenile crude venoms (80 μg) were determined using azocasin as a substrate (Aird and da Silva, 1991), and activity was expressed as $\Delta A_{342\ nm}$/min/mg venom protein. L-amino acid oxidase activity was assayed according to Weissbach et al. (1961) with 80 μg of venom, and the activity was expressed as nmol product formed/min/mg protein. Phospholipase A_2 activity was determined using a commercially available kit (Cayman Chemical Co., Michigan, USA) as described by the manufacturer using 2 μg venom in 200 μL total volume. Absorbance was measured at 414 nm every minute for five minutes at 37 °C and the linear portion of the curve was used to express activity as μmol product formed/min/mg protein. Phosphodiesterase activity was assayed with 80 μg venom using 1.0 mM bis-p-nitrophenylphosphate as a substrate, following the protocol of Laskowski (1980); activity was reported as $\Delta A_{400\ nm}$/min/mg protein. Acetylcholinesterase activity was determined using venom (5 μg) incubated with the 7.5 mM acetylthiocholine iodide substrate (21.67 mg/mL) in a cuvette at 37 °C (Ellman et al., 1961). Absorbance at 412 nm was recorded every 10 s for five minutes. The linear portion of the graph produced was used to calculate specific activity as μmol of thiocholine produced/min/mg venom protein. Fibrinogenase activity was determined using 20 μg of venom incubated with human fibrinogen (final concentration 0.5 mg/mL in 100 mM Tris-HCl, pH 8.0) in a total volume of 200 μL for periods of 0, 1, 5, 10, 30 and 60 min (Ouyang and Huang, 1979). Twenty μL of this reaction mixture was removed at each time point, mixed with an equal volume of 4% SDS and 5% 2-mercaptoethanol, and then heated in boiling water for 10 min. Five μL aliquots were combined with 2× LDS buffer, electrophoresed on a 12% NuPAGE Bis-Tris gel and processed as above. All assays were performed in duplicate for the pooled adult *N. kaouthia* venom and for each individual juvenile venom sample.

2.6. Cation-exchange FPLC (fast protein liquid chromatography)

Cation-exchange FPLC was also used to compare venom compositional differences between adult and juvenile *N. kaouthia* venoms. Crude venom (3 mg) in a volume of 500 μL of buffer (20 mM MES, pH 6.0) was injected onto a GE Healthcare Tricorn Mono S 5/50 GL column (5 × 50 mm) equilibrated with 20 mM MES, pH 6.0. The elution was performed at a flow rate of 1 mL/min at ambient temperature with a linear NaCl gradient from 0 to 0.4 M over 60 min and then 1 M NaCl for 5 min. Eluted proteins were monitored at 280 nm. The protein composition of each peak was evaluated by SDS-PAGE and MALDI-TOF MS.

2.7. MALDI-TOF MS (matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry)

MALDI-TOF MS was used to determine the molecular masses of all venom proteins found in the FPLC peaks. Fractions from all major Mono S FPLC peaks were analyzed with a Bruker Ultraflex MALDI-TOF mass spectrometer (Proteomics and Metabolomics Facility, Colorado State University, Fort Collins, CO, U.S.A) operating in linear mode. Approximately 1 μg of protein (1.0 μL) from each peak was spotted onto a sinapinic acid matrix (10 mg/mL in 50% acetonitrile, 0.1% trifluoroacetic acid (TFA); 1.0 μL), an isopropanol wash (2.5 μL) was used to remove salts, and the spectra were acquired in the mass range of 3.0–25 kDa. These protein masses were then matched to previously identified *N. kaouthia* venom proteins (Kulkeaw et al., 2007; Laustsen et al., 2015).

2.8. RP-HPLC (reverse-phase high performance liquid chromatography)

A Waters RP-HPLC (Empower software) was used to purify proteins from adult *N. kaouthia* venom that were used in toxicity assays. Fractions (2 mL total) from all Mono S FPLC major peaks were injected onto a Phenomenex Jupiter 5 μm C18 (250 × 4.60 mm) column connected to Waters 515 HPLC pumps. The column was equilibrated with 0.1% TFA (buffer A) at a flow rate of 1 mL/min. Elution was carried out with a linear gradient of 0–35% buffer B (80% acetonitrile containing 0.01% TFA) over 5 min, then a linear gradient of 35%–60% buffer B over 30 min, and 100% buffer B for the remaining 10 min at room temperature (~23 °C). The flow rate was 1 mL/min and protein elution was continuously monitored at 280 and 220 nm with a Waters 2487 Dual λ absorbance detector. Fractions associated with the most abundant protein observed in each chromatogram were lyophilized, assayed for protein concentration (Bradford, 1976, as modified by BioRad Inc.) and purity evaluated by SDS-PAGE analysis.

2.9. LC-MS/MS (liquid chromatography-tandem mass spectrometry)

To determine the identities of toxins used for LD_{50} assays, each RP-HPLC purified protein peak was analyzed by LC-MS/MS, performed at the Florida State University College of Medicine Translational Science Laboratory (Tallahassee, FL, U.S.A). Samples were digested using the Calbiochem ProteoExtract All-in-one Trypsin Digestion kit (Merck, Darmstadt, Germany) with LC/MS grade solvents according to the manufacturer's instructions. The LC-MS/MS analyses were performed using an LTQ Orbitrap Velos equipped with a Nanospray Flex ion source and interfaced to an Easy nanoLC II HPLC (Thermo Scientific). Peptide fragments were separated using a vented column configuration consisting of a 0.1 × 20 mm, 3 μm C18 trap column and a 0.075 × 100 mm, 3 μm C18 analytical column (SC001 and SC200 Easy Column respectively, Thermo Scientific). The elution gradient consisted of 5% buffer B (0.1% formic acid in HPLC grade acetonitrile) and 95% buffer A (0.1% formic acid) at the run start, to 35% B at 60 min, to 98% B from 63 to 78 min with a flow rate of 600 nl/min from 64 to 78 min, and 5% B at 300 nl/min at 79 min. The mass spectrometer was operated in positive mode nanoelectrospray with a spray voltage of +2300 V. A "Top 9" method was used with precursor ion scans in the Orbitrap at 60 K

resolving power and fragment ion scans in the linear ion trap. Precursor ion selection using MIPS was enabled for charge states of 2+, 3 + and 4+. Dynamic exclusion was applied for 60 s at 10 ppm. ITMS scans were performed using collision-induced dissociation (CID) at 35% normalized collision energy. MS/MS peptide spectra produced were interpreted using Mascot (Matrix Science, London, UK; version 1.4.0.288), Sequest (Thermo Fisher Scientific, San Jose, CA, U.S.A; version 1.4.0.288), and X! Tandem (thegpm.org; version CYCLONE 2010.12.01.1), assuming a trypsin digestion. The Mascot5_Trembl_bony vertebrate database, and the Sequest and X! Tandem Uniprot Serpentes (A8570) databases, were used for homology searches. Sequest and X! Tandem were searched with a fragment ion mass tolerance set to 0.6 Da and a parent ion tolerance of 10 ppm. Mascot was searched with a fragment ion mass tolerance of 0.8 Da and a parent ion tolerance of 10 ppm. Glu → pyro-Glu of the N-terminus, ammonia loss of the N-terminus, Gln → pyro-Glu of the N-terminus, carbamidomethylation of cysteines and carboxymethylation of cysteines were specified as variable post-translational modifications within X! Tandem. Oxidations of methionine, carbamidomethyl cysteine, and carboxymethyl cysteine were specified as variable post-translational modifications within Mascot and Sequest. Results were viewed and validated within Scaffold (Proteome Software Inc., Portland, OR, U.S.A; version 4.4.6), and protein identities were accepted if they could be established at >99.9% probability and contained at least one identified peptide. Given the sensitivity of the MS/MS instrument, purified protein identities were also restricted to those proteins that consisted of the largest number of fraction spectra, after normalization to protein molecular mass as determined by MALDI-TOF MS.

2.10. Lethal toxicity (LD_{50}) assays

Comparative toxicity of adult and juvenile *N. kaouthia* venoms was evaluated (LD_{50} assays) using a non-model species, House Gecko (*Hemidactylus frenatus*), and a standard model species, NSA mouse (*Mus musculus*). In addition, LD_{50} assays were also performed for RP-HPLC purified venom proteins from adult *N. kaouthia* venom to determine if any toxin exhibited prey-specific toxicity. Doses used for crude venoms were 0.2, 0.6, 0.8, 1.0, 1.2 and 1.5 μg/g (in 50 μL) for lizards and 0.2, 0.4, 0.5, 0.6, 0.8 and 1.0 μg/g (in 100 μL) for mice. Doses for individual purified proteins were 0.1, 0.5, 1, and 5 μg/g in lizards; toxicities toward mice were taken from the literature (Karlsson, 1973; Fryklund and Eaker, 1975; Joubert and Taljaard, 1980a, 1980b,c; Laustsen et al., 2015). All doses were injected intraperitoneally in sterile PBS, and three animals per dose were used; three mice and three lizards were injected with only PBS as controls. Doses were adjusted to individual animal body masses, with lethality expressed as micrograms of venom per gram body mass (μg/g) producing 50% mortality after 24 h (Reed and Muench, 1938). All procedures were reviewed and approved by the UNC-IACUC (protocol 1504D-SM-SMLBirds-18).

2.11. Data analysis

A series of single-sample t-tests were performed in R (version 3.1.1) for each crude venom enzyme activity to determine if there was a significant difference between adult and juvenile *N. kaouthia* venoms. Single-sample t-tests were chosen instead of independent t-tests because there was only one pooled sample for the adult *N. kaouthia* venom ($n = 1$). Therefore, the adult sample was used as the reference mean for the comparison between the adult and juvenile samples. For the LD_{50} assays, the nonparametric Spearman-Karber method was used for both LD_{50} value estimations and determination of 95% confidence intervals (Finney, 1978). If confidence intervals overlapped, it was determined that there was not a significant difference between the values being compared.

3. Results and discussion

3.1. One-dimensional gel electrophoresis (SDS-PAGE)

There were several qualitative and quantitative differences in protein composition noted between adult and juvenile *N. kaouthia* reduced SDS-PAGE venom profiles. Approximately 18 protein bands between 4 and 100 kDa were observed in *N. kaouthia* venoms (Fig. 1). Bands around 31–36.5 kDa were present in several juvenile venoms but were completely absent in the pooled adult *N. kaouthia* venom. There were also two bands in juvenile venoms around 25 kDa (typically CRiSPs), and only one was apparent in the adult venoms. A striking difference between adult and juvenile venoms was the low abundance in juvenile venoms of bands around 13 kDa (typically PLA_2s) (Doley and Mukherjee, 2003; Mukherjee, 2007). These data indicate that juvenile venoms differ in several respects from adult *N. kaouthia* venoms.

Recently, Tan et al. (2015) completed proteomes for venoms of *N. kaouthia* from three different localities using a venomics methodology (Calvete, 2013). Venoms from *N. kaouthia* originating from Thailand were included in the Tan et al. (2015) study; therefore, this publication was used as an additional reference for the identification of reduced SDS-PAGE protein bands for the present study (Fig. 1).

The bands at 31–36.5 kDa observed in some juvenile venoms and absent from the adult venom are potentially cobra venom factor γ-chains (Kulkeaw et al., 2007; Tan et al., 2015). Cysteine-rich secretory proteins (CRiSPs) were present at a mass of approx. 25 kDa for Thailand *N. kaouthia* (Tan et al., 2015); these proteins frequently occur at this mass range in many reptile venoms (Mackessy, 2010b). The two bands in this range for juveniles indicate that some juvenile venoms have two abundant CRiSP isoforms, whereas the adult venoms have only one. The low abundance in juvenile venoms of bands at approx.13 kDa are in the mass range expected for PLA_2s (Doley and Mukherjee, 2003; Doley et al., 2004; Mackessy, 2010b; Tan et al., 2015), indicating a lower abundance to near complete absence of PLA_2 enzymes in some juvenile *N. kaouthia* venoms (Fig. 1).

3.2. Two-dimensional polyacrylamide gel electrophoresis (2D SDS-PAGE)

A 2D gel electrophoretic profile of pooled adult *N. kaouthia* venom is shown in comparison to one juvenile venom (Fig. 2), and there were five major differences between the gel profiles. First, there is a larger abundance of high molecular mass proteins (approximately around 67 kDa) within the juvenile venom. There are also proteins present (approx. 36.5 kDa) in the juveniles that are absent in the adults, and two proteins (at approx. 14 kDa and 6 kDa) are absent from the juvenile venom but present in abundance in the adult *N. kaouthia* venom. Lastly, there are differences in abundances of basic low molecular mass proteins (most likely 3FTxs) clustered on the bottom left (basic) edge of the gels.

The proteome of Thailand *N. kaouthia* venom was described previously using two-dimensional gel electrophoresis to separate all venom proteins by molecular mass and isoelectric point, and proteins were then identified using tandem mass spectrometry of tryptic peptides (Kulkeaw et al., 2007). This study found that cobra venom factor contains three subunits (α-, β-, γ-chains) that will appear on a 2D SDS-PAGE gel at apparent molecular masses of approximately 70, 50 and 30 kDa. Using Kulkeaw et al. (2007) as a

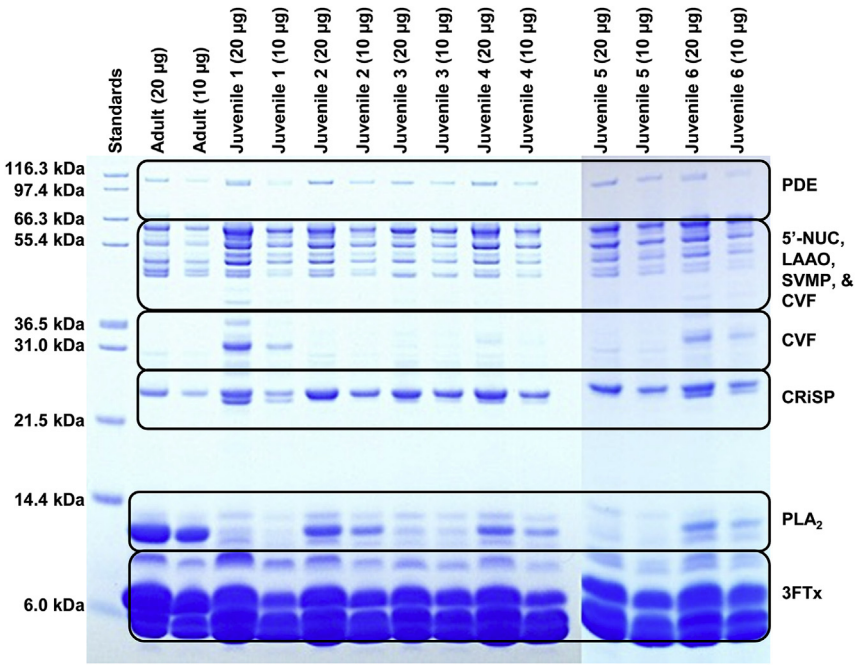

Fig. 1. SDS-PAGE comparison of *N. kaouthia* reduced venoms from adult (pooled) and individual juvenile snakes. Molecular mass standards are indicated in the far left lane. Protein families are identified on the far right and include PDE (phosphodiesterase), 5′-NUC (5′-nucleotidase), LAAO (L-amino acid oxidase), SVMP (snake venom metalloproteinase), CVF (cobra venom factor), CRiSP (cysteine-rich secretory protein), PLA_2 (phospholipases A_2) and 3FTx (three-finger toxin). Identities are based on previously published work (Mackessy, 2010a; Tan et al., 2015).

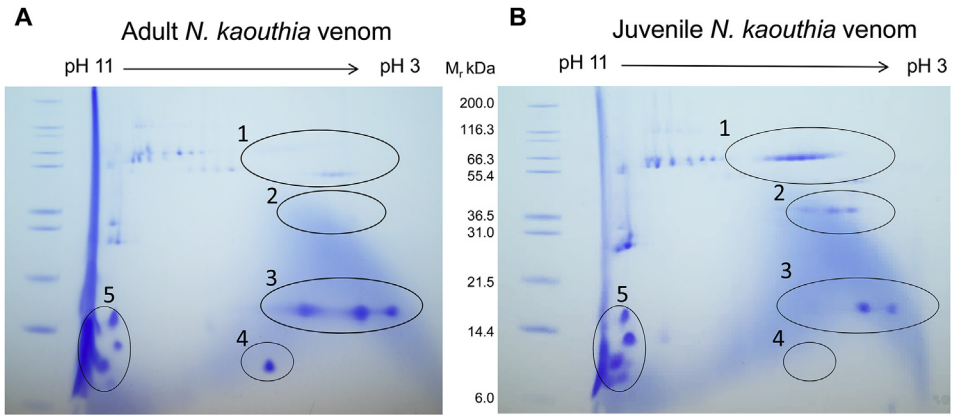

Fig. 2. Comparison of 2D gel electrophoresis profiles of adult and juvenile *Naja kaouthia* venoms. Venoms (pooled; 170 µg) from adult (A) and from one juvenile (NK-J1; 170 µg) (B) *N. kaouthia* were profiled under the same conditions (pH 3–11, 12% gel); these samples were also subjected to FPLC/HPLC fractionation (see below). Circled areas highlight differences in abundance of 1) cobra venom factors, 2) unidentified proteins, 3) PLA_2s, 4) a potential 3FTx, and 5) a cluster of 3FTxs (spot identities based on Kulkeaw et al., 2007).

reference, the larger mass proteins that are most abundant in the juvenile venoms are likely cobra venom factor (Fig. 2). This supposition is also corroborated by results observed with 1D SDS-PAGE gels in the present study.

Proteins of approx. 36.5 kDa were not identified in Kulkeaw et al. (2007); these proteins (spot 2, Fig. 2B) occur in juvenile *N. kaouthia* venom but are lacking in adult venom (spot 2, Fig. 2A). Proteins with masses of approx. 13–14 kDa were identified as PLA_2s (Kulkeaw et al., 2007), and it is evident that juvenile *N. kaouthia* venom lacks the distinct PLA_2 cluster (specifically the most basic isoform) that is abundant in adult *N. kaouthia* venoms. In general, there is also a lower abundance of PLA_2s within the juvenile venom profile, also corresponding with differences in adult and juvenile venoms observed on 1D SDS-PAGE. The last difference is the presence of a lower molecular mass, somewhat acidic protein (6–7 kDa) in adult venom and absent from juvenile venom (spot 4, Fig 2); differences in abundances of basic proteins within this mass range (spot 5, Fig 2), most likely 3FTxs (Tan et al., 2015) and PLA_2-interacting weak neurotoxins (Mukherjee, 2008), also exist between juvenile and adult venoms.

3.3. Enzyme assays

L-amino acid oxidase and metalloproteinase activities were not significantly different between adult and juvenile venoms ($p = 0.18$ and 0.14, respectively; Table 1). Metalloproteinase activity has previously been reported to be very low within elapid venoms in comparison to vipers, and the metalloproteinase activity reported in this study (using azocasein substrate) is similar to previously reported lower values using casein substrates (Aird and da Silva,

Table 1
Enzyme activity of adult and juvenile *Naja kaouthia* venoms.

	Adult *Naja kaouthia* venom[b]	Juvenile *Naja kaouthia* venom[c]
Metalloproteinase activity ($\Delta A_{342\ nm}$/min/mg)	0.004	0.005 ± 0.002
L-amino acid oxidase activity (nmol product/min/mg)	4.395	5.621 ± 2.668
Phospholipase A_2 activity (μmol product/min/mg)	2.216[a]	0.643[a] ± 0.742
Phosphodiesterase activity ($\Delta A_{400\ nm}$/min/mg)	0.337[a]	0.566[a] ± 0.123
Acetylcholinesterase activity (μmol product/min/mg)	7.202[a]	4.682[a] ± 2.485

[a] Significantly different by single sample *t*-test ($p < 0.05$).
[b] Pooled venom.
[c] $n = 10$.

1991; Das et al., 2013). Metalloproteinases in *N. kaouthia* venom have also been found to cleave platelet von Wilebrand factor (Hamako et al., 1998; Wijeyewickrema et al., 2007).

Phospholipase A_2, phosphodiesterase and acetylcholinesterase activities were significantly different between adult and juvenile venoms ($p = 0.00005$, 0.0002 and 0.01, respectively; Table 1). Consistent with the results observed in this study, significantly lower acetylcholinesterase and phospholipase A_2 activity has been reported in neonate Chinese Cobras (*Naja atra*) when compared to adults (He et al., 2014). PLA_2 activity in adult *N. kaouthia* originating from India (Das et al., 2013) was slightly higher than the value reported in the present study; however, PLA_2 activity appears to be quite variable among cobra venoms (Tan and Tan, 1988), and the relative abundance of PLA_2 enzymes within *N. kaouthia* venom proteomes from different localities varied from 12.2% to 23.5% (Tan et al., 2015). Within these same *N. kaouthia* venom proteomes, phosphodiesterases accounted for only 0.3–0.4% of *N. kaouthia* venom, and acetylcholinesterases were not present or were at levels below detection (Tan et al., 2015). However, some low abundance enzymes are difficult to detect using proteomic methodologies, but are detected using enzyme assays. It is possible that higher acetylcholinesterase activity within adult *N. kaouthia* venom could contribute to differential clinical symptoms.

PLA_2 activity, an important contributor to morbidities following envenomations by *N. kaouthia*, showed significant age-related differences (Table 1). The significantly lower PLA_2 activity of juvenile venoms also corresponds with the lower abundance and lack of PLA_2s observed in 1D and 2D SDS-PAGE juvenile venom profiles. Phospholipases A_2 in venoms often produce severe tissue damage as a result of cell membrane hydrolysis and release of autopharmacologically-active compounds such as arachidonic acid (Kini, 2003; Doley et al., 2004; Mukherjee, 2007). Results indicate that envenomations by juvenile *N. kaouthia* should result in significantly lower incidence of severe tissue damage compared to envenomations by adult cobras, which have a higher abundance of PLA_2 and therefore higher necrotizing potential.

Many snake venoms contain fibrinogenolytic enzymes, commonly serine proteinases, which superficially mimic the actions of thrombin on circulating fibrinogen (Mackessy, 1993, 2010c). Fibrinogen, composed of three subunits (Aα, Bβ, and γ), is typically converted by activated thrombin into fibrin during the formation of a blood clot via the selective cleavage of fibrinopeptides A and B from the α and β subunits, respectively. However, α-fibrinogenases in venoms hydrolyze the α subunit preferentially (sometimes exclusively), leading to consumptive coagulopathies in envenomated patients (Mackessy, 2010c). Both adult and juvenile *N. kaouthia* have venom α-fibrinogenases that hydrolyze the Aα subunit of fibrinogen, and both appear to catalyze hydrolysis at the same rate, with loss of the Aα-subunit within 30 min (Fig. 3). The β- and γ-subunits remained intact over the 60 min time course. These results are consistent with effects of *N. n. karachiensis* venom on aPTT, PT and TT with platelet-poor plasma (Asad et al., 2012). Das et al. (2013) have also reported the degradation of Aα-chain of fibrinogen and anticoagulant activity of venom of *N. kaouthia* from northeast India. A very recent report indicates that a 66 kDa metalloproteinase purified from *N. kaouthia* venom specifically hydrolyzed the Aα subunit of fibrinogen and also hydrolyzed fibrin (Chanda et al., 2015). It is also possible that serine proteases could also be involved (Mackessy, 1993).

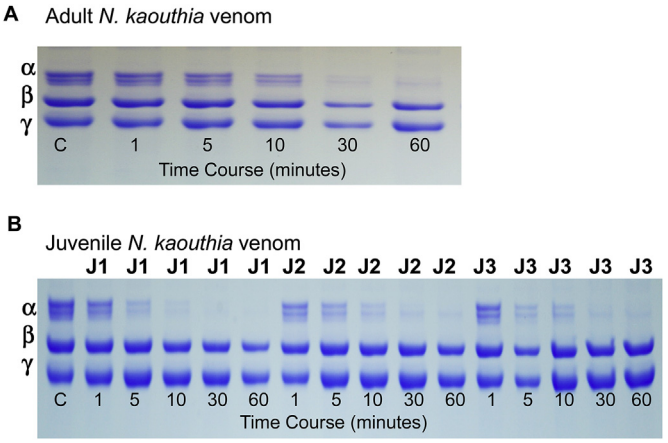

Fig. 3. Adult and juvenile *Naja kaouthia* venom fibrinogen digest assay. Adult (A) and three different juvenile (B; J1-J3) venoms incubated with fibrinogen over a 60 min time course. Fibrinogen subunits are labeled α, β, and γ. C, control: fibrinogen incubated in the absence of venom.

3.4. Chromatographic venom profiling, toxin purification, and mass spectrometry identifications

Enzyme assays, 1D, and 2D gels all demonstrated a difference in abundance and activity of PLA_2 enzymes in juvenile and adult *N. kaouthia* venoms. Cation-exchange FPLC venom profiles for adult and juvenile cobras were generated to observe relative abundances of individual PLA_2 isoforms. FPLC was also used as the first step in the purification of the major proteins within adult *N. kaouthia* venom to determine if any abundant components in *N. kaouthia* venom demonstrated significant toxicity towards a non-model organism (lizards).

FPLC chromatograms revealed several differences in protein abundance between adult and juvenile venoms. However, the number of peaks and elution time for each protein were essentially identical in both adult and juvenile *N. kaouthia* venoms (Fig. 4), indicating that the same proteins are present in both venoms but vary in abundance between adult and juvenile cobras; this type of intraspecific variation within cobras (variation in specific protein abundance between individuals) has been previously observed (Modahl et al., 2010).

Protein components in each FPLC peak were first identified by their relative SDS-PAGE masses (Fig. 4D), and then identified from

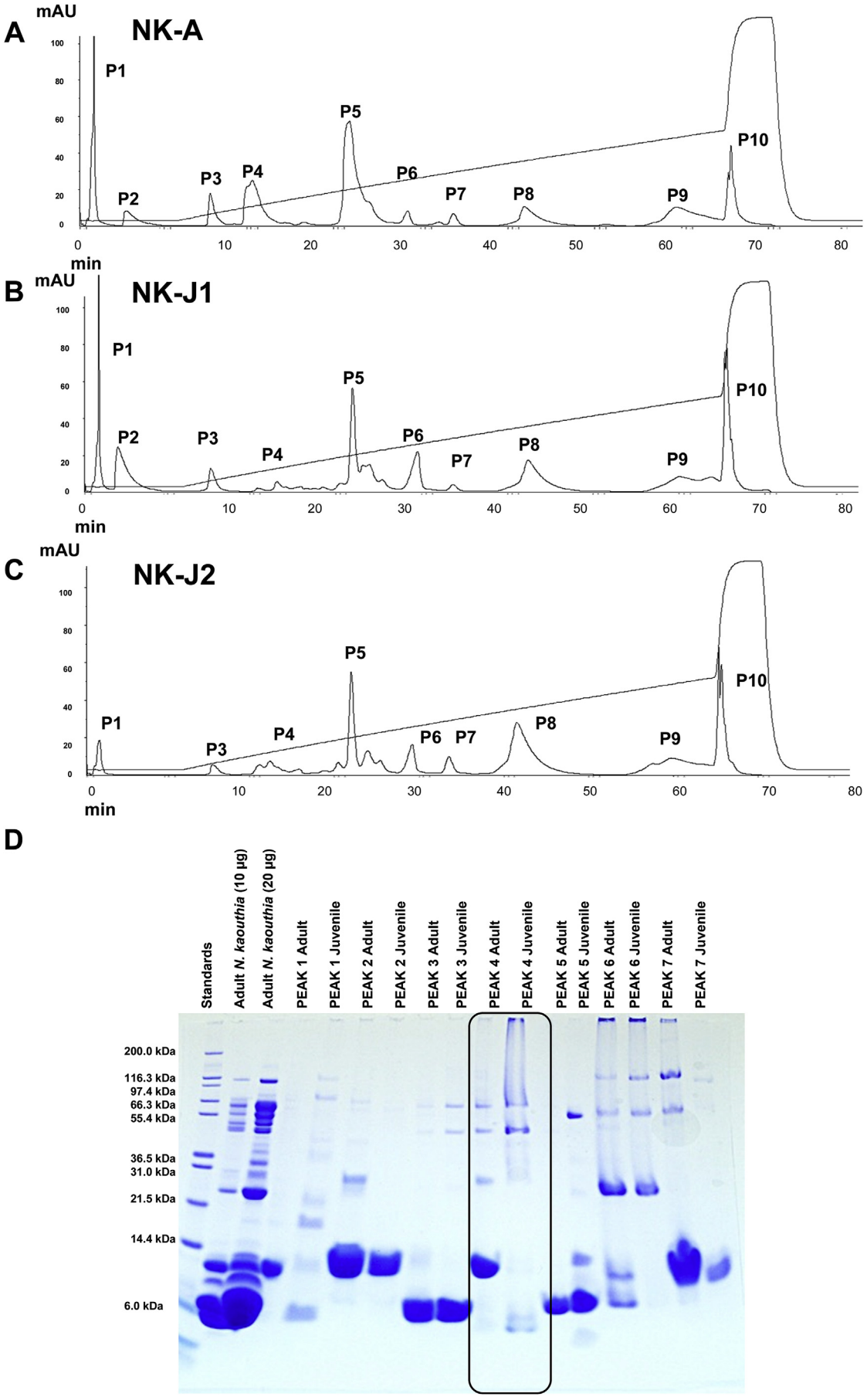

Fig. 4. Adult and juvenile *Naja kaouthia* venom fractionation via cation-exchange FPLC. Adult *N. kaouthia* venom (3 mg) was eluted with 20 mM MES and an NaCl gradient from 0 to 0.4 M over 70 min (A); the same methodology was used for several juvenile *N. kaouthia* venoms; two juvenile (NK-J1 and NK-J2) venom profiles shown (B and C, respectively). Peaks 1–7 from the adult and the NK-J1 juvenile venoms were subjected to reducing SDS-PAGE (Fig. 4D). Peak 4 (PLA_2) is highlighted within the chromatograms (adult fraction on left and juvenile fraction on right).

MALDI-TOF MS spectra. Most of the peaks contained proteins in the mass range of 6–8 kDa, which is consistent with SDS-PAGE (Fig. 1) and previously published *N. kaouthia* venom proteomes (Kulkeaw et al., 2007; Tan et al., 2015). This is the molecular mass range observed for 3FTxs, which make up a large majority of proteins in many elapid venoms, including *N. kaouthia* (Kulkeaw et al., 2007; Mackessy, 2010b; Laustsen et al., 2015; Tan et al., 2015).

Of particular interest were proteins of 13–14 kDa, which is the mass range that has been reported for PLA_2 proteins in *N. kaouthia* venoms (Doley and Mukherjee, 2003; Kulkeaw et al., 2007; Tan et al., 2015). It was observed that for peak four, there was an abundance of PLA_2s in the fractionated adult *N. kaouthia* venom, but the juvenile venom lacked this PLA_2 band (Fig. 4D). Peak four of the adult *N. kaouthia* FPLC chromatogram consisted of 11.4% of the total area of all peaks; for juvenile venoms, this peak consisted only of 1.4–4.9% of the total peak area. MALDI-TOF MS spectra revealed that the primary component of peak four for adult *N. kaouthia* venom is a 13,248 Da protein (PLA_2) and in the same peak in juvenile *N. kaouthia* venom there is instead a 6841 Da protein (3FTx) as the primary component (Fig. 5). To confirm the identity of the 13,248 Da protein, PLA_2 assays showed activity of 0.0452 μmol/min/mL for fractions from peak four from adult *N. kaouthia* venom and 0.001 μmol/min/mL for peak four from juvenile venom.

All major FPLC cation-exchange peaks from the adult *N. kaouthia* chromatogram above 20 mAU (except the first eluting peak of unbound proteins) were subjected to a second purification step using reversed-phase HPLC to purify the most abundant toxin within each major peak (Fig. 6). These FPLC peaks (peak four, five, eight, nine, and ten) comprised approx. 70% of the adult crude *N. kaouthia* venom composition (based on FPLC chromatogram calculated peak area). Of primary interest was the PLA_2 isoform(s) that was nearly absent from juvenile *N. kaouthia* venoms (peak four) and the most abundant weak non-conventional 3FTx from *N. kaouthia* venom.

Purified toxin from the fourth peak (adult venom) was a PLA_2 isoform with a molecular mass of 13,248 Da (Figs. 5A and 6). Approximately 97% of the total MS/MS spectra (after normalization of spectra to protein molecular mass) were peptides from PLA_2 isoforms; a PLA_2 similar to natratoxin (PA2A_NAJAT) from *Naja atra* venom had the most abundant hits (Table 2). The fifth Mono S peak (Fig. 6) was a long-chain 3FTx, alpha-cobratoxin (alpha-elapitoxin; 3L21_NAJKA), with a molecular mass of 7817 Da (94% of total spectra; Table 2). Cardiotoxic/cytotoxic 3FTxs (97%) were the most abundant toxins from the eighth peak, and cytotoxic 3FTxs (94%) were the most abundant toxins within the tenth peak (Fig. 6; Table 2). The ninth peak contained the most abundant weak non-conventional 3FTxs within *N. kaouthia* venom (58%). Peptide sequence coverage of each reported toxin ranged from 62 to 100%. The identities of the most abundant toxins within this study are also consistent with the previously published *N. kaouthia* (Thailand origin) venom proteome, in which the most abundant toxins were alpha-elapitoxin/long-chain neurotoxins (33.4%), cytotoxic/cardiotoxic 3FTxs (27.6%), PLA_2s (12.2%), weak neurotoxic 3FTxs (8.9%) and short-chain 3FTxs (7.7%); all other individual protein families accounted for less than 3% (Tan et al., 2015).

3.5. Lethal toxicity (LD_{50})

Within cobra venoms, there are several different toxicity levels of 3FTxs: the "conventional" neurotoxins, with low murine LD_{50} values (~0.04–0.3 μg/g), and the "non-conventional" toxins, with higher murine LD_{50}s (~5–80 μg/g); (Nirthanan et al., 2003). However, the toxicity of these 3FTxs have only been determined using murine models, and many elapids exhibit non-mammalian prey preferences, often feeding on a diversity of prey, including lizards and other ectotherms (Luiselli et al., 1997, 2002). The major non-conventional weak 3FTx within *N. kaouthia* venom was of particular interest because other non-conventional 3FTxs are currently

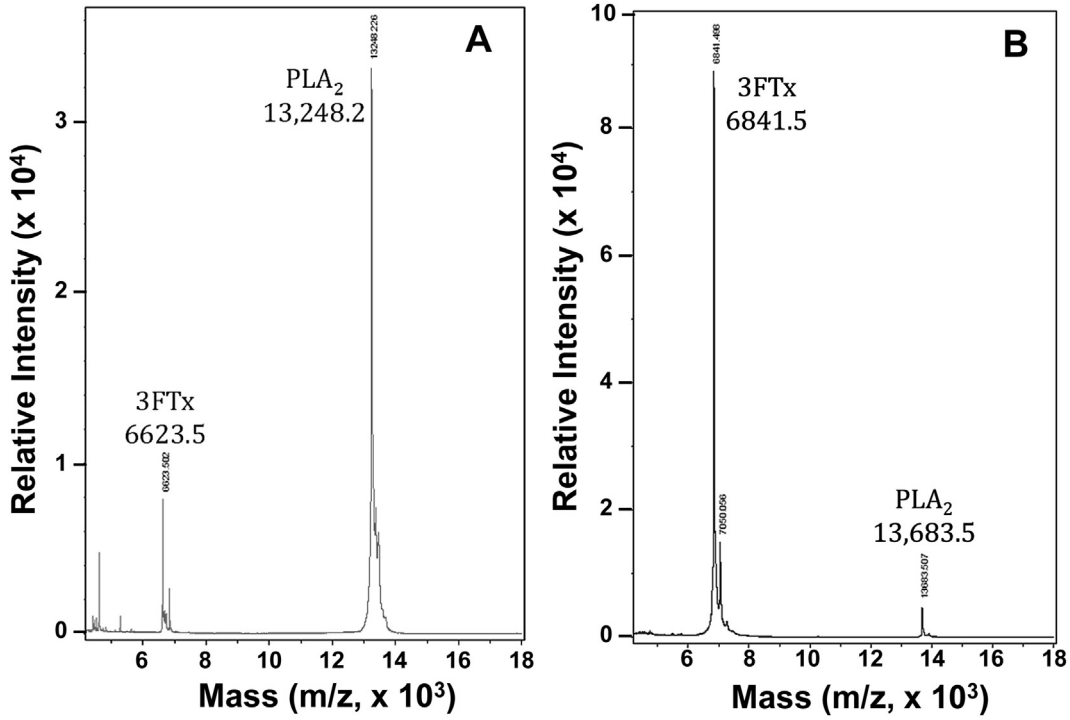

Fig. 5. MALDI-TOF spectra for Peak 4 of *Naja kaouthia* venom cation-exchange FPLC fractionations. MALDI-TOF spectra of Mono S peak 4 of adult (A) and juvenile *N. kaouthia* venoms (B).

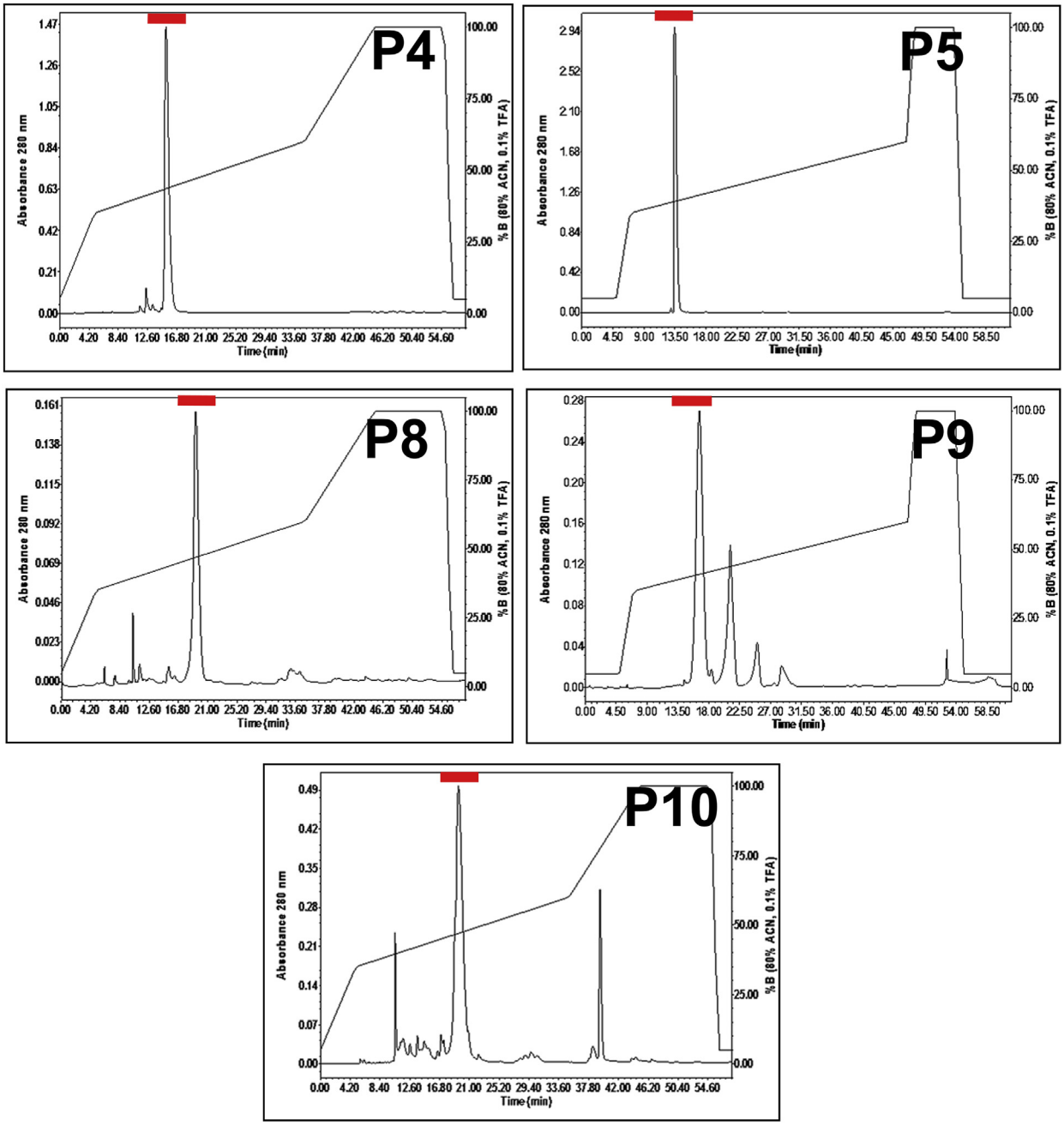

Fig. 6. Major protein peaks (P4, P5, P8, P9, and P10) from FPLC Mono S cation-exchange fractionation of adult *N. kaouthia* venom were subjected to a reversed-phase HPLC final purification step. The most abundant purified toxin from each chromatogram (red bar) was identified via HPLC-nESI-LIT-Orbitrap MS/MS and used for lethal toxicity (LD_{50}) assays. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the only identified prey-specific toxins (Pawlak et al., 2006, 2009; Heyborne and Mackessy, 2013). Non-conventional 3FTxs in rear-fanged snake venoms exhibit potent neurotoxicity towards non-murine species (lizards and birds; Pawlak et al., 2006; Pawlak et al., 2009; Heyborne and Mackessy, 2013). Because of these observations, all purified major non-conventional weak 3FTxs and other abundant toxins within *N. kaouthia* venom were tested for toxicity toward an ectothermic organism (*Hemidactylus frenatus*).

Adult and juvenile *N. kaouthia* crude venoms were evaluated using both lizards and mice to determine if a difference exists in toxicities towards non-model and model prey species. Differential venom toxicities towards different prey have been previously reported and linked to ontogenetic dietary shifts (Mackessy, 1988; Mackessy et al., 2006). The mouse i.p. LD_{50} value for adult *N. kaouthia* venom was 0.61 (95% confidence interval - 0.43–0.80) μg/g (Table 3), which is within the range of what has been previously reported (0.70 ± 0.09 μg/g) (Mukherjee and Maity, 2002); for juvenile *N. kaouthia* venom, the LD_{50} was 0.75 (95% CI - 0.60–0.91) μg/g. Because the lower and upper 95% confidence intervals for mouse LD_{50} values show extensive overlap, it was concluded that no difference in mammalian toxicity exists between adult and juvenile cobra venoms. However, the House Gecko i.p. LD_{50} value for adult *N. kaouthia* venom was 1.02 (0.88–1.16) μg/g and that for juvenile *N. kaouthia* venom was found to be 1.29 (1.26–1.32) μg/g

Table 2
Peptides and identities of purified toxins within major FPLC peaks.

Peak	%Total spectra	MS/MS-derived peptide sequences	NCBI/Uniprot accessions	Venom protein
P4	97%	(K)NmIQcTVPSR(S) (K)GGSGTPVDDLDR(C) (R)ccQVHDNcYNEAEK(I) (R)ccQVHDNcYNEAEKISGcWPYFK(T) (K)ISGcWPYFK(T) (K)TYSYEcSQGTLTcK(G) (K)GGNNAcAAAVcDcDR(L) (K)TYSYEcSQGTLTcK(G) SWWDFADYGcYcGR LAAIcFAGAPYNNNNYNIDLK (K)GGSGTPVDDLDR(C) GDNDAcAAAVcDcDR ccQVHDNcYNEAEKISR SWWDFADYGcYcGR (K)GGSGTPVDDLDR(C) SWWDFADYGcYcGR LAAIcFAGAPYNDNNYNIDLK (R)ccQVHDNcYNEAEK(I) GGNDAcAAAVcDcDR LAAIcFAGAPYNDNNYNIDLKAR NMIQcTVPNRSWWDFADYGcYcGR GGNNAcAAAVcDcDRLAAIcFAGAPYNDN-NYNIDLK GGSGTPVDDLDRccQIHDNcYNEAEK NMIQcTVPNR (K)GGNNAcAAAVcDcDR(L)	PA2A_NAJAT sp|P00596|PA2 A1_NAJKA Acidic phospholipase A2 sp|P15445|PA2 A2_NAJNA Acidic phospholipase A2 sp|Q9I900|PA2 AD_NAJSP Acidic phospholipase A2 sp|Q92084|PA2 NA_NAJSP Neutral phospholipase A2	PLA_2
P5	94%	IRcFITPDITSKDcPNGHVcYTK(T) (K)TWcDAFcSIRGK(R) (R)GKRVDLGcAATcPTVK(T) (R)VDLGcAATcPTVK(T) (K)DcPNGHVcYTK(T) (R)GRRVDLGcAATcPTVK(T) (K)TGVDIQCCSTDNcNPFPTRP	3L21_NAJKA 3L23_NAJNA E2IU01_NAJAT 3L22_NAJNA	3FTx (long-chain)
P8	97%	LKcNKLIPIASK(T) (K)LIPIASKTcPAGK(N) (K)MFmMSDLTIPVK(R) (R)GcIDVcPKNSLLVK(Y) (K)NSLLVKYVccNTDRcN IRcFITPDITSKDcPNGHVcYTK(T) (K)DcPNGHVcYTK(T) (K)TWcDAFcSIR(G) (R)GKRVDLGcAATcPTVK(T) LKcNKLIPLAYK(T) (K)TcPAGKNLcYK(M) (K)RGcIDVcPK(N)	3SA8_NAJKA 3L23_NAJNA 3SA1_NAJNA	3FTx (cyto-toxin)
P9	58%	EMIEccSTDKcNR LTcLNcPEMFcGK NGEKIcFKK (R)NGEKIcFK(K)	sp|P25679|3NO 29_NAJKA Weak toxin CM-9a 3NO2_NAJKA	3FTx (weak toxin)
P10	94%	(K)KFPLKIPIKR(G) (R)GcADNcPKNSALLK(Y) LKcHNTQLPFIYK(T) NSALLKYVccSTDKcN (K)YVccSTDKcN (T)LKcNKLVPLFYK(T) (K)LVPLFYKTcPAGK(N) (K)TcPAGKNLcYK(M) (K)MFMVATPKVPVKR(G) (K)RGcIDVcPK(S) (R)GcIDVcPKSSLLVK(Y) (K)SSLLVKYVccNTDRcN (K)YVccNTDRcN LKcNKLIPIASK(T) (K)LIPIASKTcPAGK(N) (K)MFmMSDLTIPVKR(G) (K)RGcIDVcPK(N) (K)NSLLVKYVccNTDRcN LKcNKLIPLAYK(T) (K)MFmVAAPK(V) (R)GcIDAcPK(N)	sp|P14541|3SO FH_NAJKA Cytotoxin homolog 3SA0_NAJSP 3SA8_NAJKA 3SA5_NAJKA 3SA1_NAJNA	3FTx (cyto-toxin)

(Table 3). In this case, the lower and upper 95% confidence intervals for House Gecko LD_{50} values do not overlap, indicating that adult *N. kaouthia* venoms are slightly more toxic toward lizards. Phospholipase enzymes, which are in greater abundance in adult *N. kaouthia* venoms, are among the more toxic and pharmacologically active components of snake venoms (Mackessy, 2010b). Higher PLA_2 enzyme concentrations, especially PLA_2s that covalently interact with low molecular mass weak-neurotoxin like

Table 3
Lethal toxicity (LD_{50}) of *Naja kaouthia* crude venom (adult and juvenile) using model and non-model organisms.

Species	Adult *N. kaouthia* venom	Juvenile *N. kaouthia* venom
Mice (*Mus musculus*)	0.614 ± 0.182 μg/g i.p	0.754 ± 0.157 μg/g i.p
House Geckos (*Hemidactylus frenatus*)	1.023 ± 0.139 μg/g i.p	1.290 ± 0.032 μg/g i.p

i.p. = intraperitoneal.

peptides (kaouthiotoxins), are often related to increased toxicity (Mukherjee and Maity, 2002; Mukherjee, 2010). It is therefore possible that the greater abundance of PLA_2 enzymes within adult *N. kaouthia* venoms results in somewhat more toxic adult cobra venoms towards House Geckos.

Toxicity assays were also performed using HPLC-purified abundant venom proteins from adult *N. kaouthia* venom (Table 4) to determine if any toxins exhibited prey-specific toxicity. These toxins included PLA_2s, long-chain 3FTxs (alpha-cobratoxin), weak non-conventional 3FTxs, and the two primary cardiotoxic/cytotoxic 3FTxs. With the exception of the long-chain α-cobratoxin (alpha-elapitoxin; 3L21_NAJKA), intraperitoneal (i.p.) LD_{50} values were all above 5 μg/g (the highest dose tested) for lizards (*Hemidactylus frenatus*) (Table 4). Published toxicity values (LD_{50}s) observed in mice for the major cytotoxins within *N. kaouthia* venom range from 1.2 to 1.48 μg/g intravenous (i.v.) and 2.25 μg/g intraperitoneal (i.p.) (Joubert and Taljaard, 1980a; Ohkura et al., 1988). Therefore, *N. kaouthia* cytotoxins were less toxic towards lizards than toward mice (Table 4). The LD_{50} value of α-cobratoxin fell below 0.1 μg/g (the lowest dose tested), which is the same as previous reported values for this toxin in mice (Karlsson and Eaker, 1972). These results demonstrate that unlike venom from the rear-fanged Brown Treesnake (*Boiga irregularis*), which contains an abundant, prey-specific (lizards and birds), dimeric neurotoxic 3FTx (Pawlak et al., 2009), *N. kaouthia* venom has an abundant neurotoxic 3FTx that can bind effectively (and lethally) to the nicotinic acetylcholine receptors of a variety of prey types (both endothermic and ectothermic). As a generalist predator, *N. kaouthia* consume a variety of prey, and a "generalist" 3FTx that can incapacitate a large diversity of prey types is the main lethal toxin that has evolved in this venom. However, it is important to note that these results are only for one lizard species (*Hemidactylus frenatus*); other lizard species or other ectothermic species might respond differently to *N. kaouthia* venom toxins. The *H. frenatus* used for this study were chosen because they are a species that is sympatric with *N. kaouthia* and therefore a potential prey item; they were also commercially available in the numbers needed for toxicity determinations.

Because overall venom toxicity towards mice and lizards appears to result from α-cobratoxin, it is possible that the significant difference in PLA_2 activity between adult and juvenile *N. kaouthia* venoms primarily serves a pre-digestive role, rather than a major role in lethal toxicity. Snakes are gape-limited feeders, and juveniles are restricted to smaller prey. Larger prey are consumed by adult snakes, and the increase in PLA_2 enzymes in adult *N. kaouthia* venom may facilitate digestion of these larger prey by promoting myonecrosis. The biological roles of enzymatic venom proteins within viperids (particularly snake venom metalloproteinases) have been suggested to include aiding digestion (Lomonte et al., 2009; Mackessy, 2010a), and there may be functional convergence toward a pre-digestive role for some PLA_2s within elapid venoms.

4. Conclusions

Ontogenetic differences in composition of adult and juvenile *N. kaouthia* venoms were shown to exist, but these differences are not as pronounced as is typical of many viperid venoms. One- and two-dimensional SDS-PAGE results indicate a greater abundance of cobra venom factor and CRiSP isoforms in juvenile cobra venoms, and adult cobra venoms show a higher abundance of PLA_2 enzymes, including the least acidic PLA_2 isoforms that are completely absent from some juvenile venoms. Enzyme activities matched these results, with significantly lower PLA_2 activities observed in juvenile venoms. There was also a significant difference in phosphodiesterase and acetylcholinesterase activities between adult and juvenile venoms. Metalloproteinase, L-amino acid oxidase, and fibrinogen digest assays revealed no significant differences in activities between adult and juvenile venoms. Adult *N. kaouthia* crude venom does appear to be slightly more toxic towards House Geckos, but adult and juvenile crude *N. kaouthia* venoms were equally toxic towards mice.

Juvenile and adult cobras both consume endothermic and ectothermic prey and do not appear to shift in prey preferences (other than size) as they age (Chaitae, 2000). Although *N. kaouthia* also prey upon ectothermic prey (lizards and snakes), none of the non-conventional 3FTxs showed prey-specific effects, so our initial hypothesis was not supported. The main neurotoxic 3FTx within *N. kaouthia* venom, α-cobratoxin (α-elapitoxin), exhibits the same potent toxicity towards both mammalian and lizard prey. The presence of α-cobratoxin in both adult and juvenile cobra venoms represents a venom compositional strategy wherein a single potent toxin effectively immobilizes a variety of prey types encountered across snake life history stages. Future studies could evaluate possible ontogenetic venom variation in other cobra species that do have a significant age-related dietary shift. Similarly, investigation of non-conventional 3FTxs from cobra species which prey more extensively on non-mammalian species, such as *Ophiophagus hannah*, which is largely a snake specialist, could reveal novel prey-

Table 4
Lethal toxicity (LD_{50}) of purified *N. kaouthia* major venom toxins.

Peak/Toxin	Accession number	LD_{50} − lizards (*Hemidactylus frenatus*)	LD_{50} − mice (*Mus musculus*)
P4: Acidic PLA_2 similar to natratoxin	PA2A_NAJAT	LD_{50} > 5 μg/g i.p.	LD_{50} > 5 μg/g i.v. (Joubert and Taljaard, 1980b)
P5: α-elapitoxin	3L21_NAJKA	LD_{50} < 0.1 μg/g i.p.	LD_{50} < 0.1 μg/g i.v. (Karlsson, 1973)
P8: Cardio-cytotoxin	3SA8_NAJKA	LD_{50} > 5 μg/g i.p.	LD_{50} = 2.25 μg/g i.p. (Joubert and Taljaard, 1980a)
P9: Non-conventional weak 3FTx	3NO29_NAJKA	LD_{50} > 5 μg/g i.p.	LD_{50} > 5 μg/g i.v. (Joubert and Taljaard, 1980c)
P10: Cytotoxin	3SOFH_NAJKA	LD_{50} > 5 μg/g i.p.	LD_{50} = 2.25 μg/g i.p. (Joubert and Taljaard, 1980a)

i.p. = intraperitoneal; i.v. = intravenous.

specific toxins. Because venoms evolved as trophic adaptations that facilitate prey handling, it is important to explore intraspecific variation at several different levels, including overall venom protein composition, enzymatic activities, and toxicity to fundamentally different prey. Combined with proteomic and transcriptomic data, this functional data can provide deeper insight into the evolution of venom diversification and its relation to trophic habits of snakes.

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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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.toxicon.2016.04.049.

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