Danger in the reef

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Danger in the reef: Proteome, toxicity, and neutralization of the venom of the olive sea snake, *Aipysurus laevis*

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Highlights

- The venom proteome of the olive sea snake, *Aipysurus laevis*, is presented
- Most abundant venom components are phospholipases A_2 and short neurotoxins
- Lethality screening coupled to abundance estimation identified the medically relevant toxins
- BioCSL Sea Snake Antivenom neutralizes venom lethality
- ICP Anti-Coral Antivenom cross-recognizes phospholipases A_2 from *A. laevis* venom

Abstract

Four specimens of the olive sea snake, *Aipysurus laevis*, were collected off the coast of Western Australia, and the venom proteome was characterized and quantitatively estimated by RP-HPLC, SDS-PAGE, and MALDI-TOF-TOF analyses. *A. laevis* venom is remarkably simple and consists of phospholipases A_2 (71.2%), three-finger toxins (3FTx; 25.3%), cysteine-rich secretory proteins (CRISP; 2.5%), and traces of a complement control module protein (CCM; 0.2%). Using a Toxicity Score, the most lethal components were determined to be short neurotoxins. Whole venom had an intravenous LD_{50} of 0.07 mg/kg in mice and showed a high phospholipase A_2 activity, but no proteinase activity *in vitro*. Preclinical assessment of neutralization and ELISA immunoprofiling showed that BioCSL Sea Snake Antivenom was effective in cross-neutralizing *A. laevis* venom with an ED_{50} of 821 µg venom per mL antivenom, with a binding preference towards short neurotoxins, due to the high degree of conservation between short neurotoxins from *A. laevis* and *Enhydrina schistosa* venom. Our results point towards the possibility of developing recombinant antibodies or synthetic inhibitors against *A. laevis* venom due to its simplicity.

(176 words)

1. Introduction

The viviparous sea snakes are a diverse clade of more than 60 species that are phylogenetically nested within the front-fanged Australo-Melanesian terrestrial elapids (Hydrophiinae) (Rasmussen et al., 2011). They are highly aquatic and occupy most shallow-marine habitats throughout the tropical and subtropical Indo-West Pacific, yet are estimated to share a common ancestor dated at only 6–8 million years ago (Sanders et al., 2008; Lukoschek et al., 2012). The amphibious sea kraits (Hydrophiinae: Laticauda) represent an independently aquatic and earlier diverging lineage that is the sister to terrestrial and viviparous marine hydrophiines (Keogh, 1998; Scanlon and Lee, 2004; Sanders et al., 2008). Two major clades are recognised within the viviparous marine group: An 'Aipysurus' lineage comprising ten species found primarily in the Australo-Papuan region, and a 'Hydrophis' lineage containing at least 50 species distributed throughout the Indo-West Pacific (Rasmussen et al., 2011).

In the *Aipysurus* group, the olive sea snake, *Aipysurus laevis*, has a large muscular head and is the most robustly built and longest species recorded, reaching more than 170 cm in total length (Smith, 1926; Cogger, 1975). *A. laevis* has been recorded from Aru Archipelago and Kai Islands (Indonesia) in the west and from the northern coast of Australia and southern coast of New Guinea (Timor Sea and Arafura Sea) to New Caledonia in the east (Coral Sea) (Cogger, 1975; Ineich and Rasmussen, 1997; Sanders et al., 2014). *A. laevis* is found in shallow marine habitats – coral reefs as well as sandy, rocky, and mud-bottom habitats, and is often one of the most abundant species throughout its range (Cogger, 1975; Lukoschek et al., 2007; Sanders et al., 2014). It hunts primarily in crevices on the sea floor, and the following fish families have been found as prey items in *A. laevis*: Acanthuridae, Apogonidae, Carangidae, Clupeidae, Engraulidae, Labridae, Lutjanidae, Pempheridae, Pomacentridae, Scaridae,

Scorpaenidae and Serranidae (McCosker, 1975; Voris and Voris, 1983). Fish eggs, crabs, shrimp and pelecypod (Limidae) have also been found in stomach content (McCosker, 1975; Voris and Voris, 1983).

During mating season *A. laevis* is more prone to defensive attacks than at other times of the year (Heatwole, 1975). However, normally *A. laevis* will ignore a diver even if the diver approaches quite close (Heatwole, 1975). *A. laevis* has up to at least 5 mm long fangs and the venom is known for being extremely toxic (Limpus, 1978; Minton, 1983; Mackessy and Tu, 1993; Greer, 1997). *A. laevis* is commonly caught as by-catch, and commercial trawler fishers and recreational fishers handling nets are therefore the typical bite victims of *A. laevis*.

The venoms of sea snakes, typically containing α-neurotoxins and phospholipases A_2 (PLA_2s), are known to be generally more potent than the venoms from terrestrial snakes in terms of lethality (Minton, 1983; Takasaki, 1998). In contrast to the latter, however, only few studies have been focused on determining the comprehensive composition of sea snake venoms by means of proteomic analyses, i.e. venomics. The venom of *A. laevis* has been shown to be neurotoxic, nephrotoxic, and myotoxic in mice, causing acute renal tubular degeneration, proliferative glomerulonephritis, local muscle degeneration, necrosis, enlarged spleen, inflammation, and lymphadenopathy (Zimmerman et al., 1992a, 1992c; Ryan and Yong, 1997, 2002). Regarding the venom components of *A. laevis*, a total of four short-chain neurotoxin isoforms with minor amino acid sequence variations (P19958, P19959, P19960, and P32879) and one PLA_2 (P08872) have been fully sequenced (Maeda and Nobuo, 1976; Ducancel et al., 1988, 1990). The short α-neurotoxins display a high affinity towards the acetylcholine receptor (Ishikawa et al., 1977), which is in agreement with the very low LD_{50} observed for the whole venom (Tamiya, 1973; Maeda and Nobuo, 1976).

Toxicity of the venom has additionally been tested in different fish species, showing variations in responses (Berman, 1983; Zimmerman et al., 1990, 1992a, 1992c). It has been suggested that several components of the venom may act in a synergistic manner to potentiate toxic effects (Ryan and Yong, 1997). Finally, antivenoms raised against tiger snake (*Notechis scutatus*) or common sea snake (*Enhydrina schistosa*) venoms have been shown to have some cross-reactivity towards the venom of *A. laevis*, although the efficacies of these antivenoms are lower than against the venoms of homologous species (Baxter and Gallichio, 1974).

Aiming to further develop understanding of sea snake venoms and to expand knowledge of venom intra-species variability, this study presents the proteomic analysis of the venom of *A. laevis*, together with an assessment of variability in three different specimens, and of toxicity of all its main protein components in mice. In addition, the ability to cross-recognize and neutralize *A. laevis* venom was evaluated for two antivenoms against coral snakes and sea snakes.

2. Materials and Methods

2.1 Snake venom

Aipysurus laevis venom was obtained from four specimens ("Mifisto", "Medusa", "His", and "Nessi") kept at the National Aquarium, Den Blå Planet, Denmark. All specimens were collected at night by Kate L. Sanders from a boat using spotlights and dip nets. The boat was operating at shallow water close to Broome, Australia. The venom, collected in the National Aquarium of Denmark, was immediately frozen, lyophilized, and kept at -20 °C. In order to assess individual variability, a small sample of venom from each snake was kept separated, while the remaining material was pooled.

134

135 *2.2 Venom separation by reverse-phase HPLC and SDS-PAGE*

136 The pooled venom of *A. laevis* was fractionated by sequential RP-HPLC and
137 SDS-PAGE separation steps, following the 'snake venomics' analytical strategy
138 (Calvete, 2011) under conditions described previously (Lomonte et al., 2014). Venom
139 load for the RP-HPLC step on C_{18} (4.6 x 250 mm column, 5 μm particle diameter;
140 Teknokroma) was 2 mg. Protein fractions were monitored at 215 nm, manually
141 collected, dried by vacuum centrifugation, and electrophoretically separated under
142 reducing conditions. Resulting bands were stained with colloidal Coomassie blue G-
143 250, and digitally recorded on a ChemiDoc® imager using ImageLab® software (Bio-
144 Rad).

145

146 *2.3 Protein identification by tandem mass spectrometry of tryptic peptides*

147 Protein bands were excised from gels, destained with 50% acetonitrile in 25 mM
148 ammonium bicarbonate, and then subjected to reduction (10 mM dithiothreitol),
149 alkylation (50 mM iodoacetamide), and overnight in-gel digestion with sequencing
150 grade trypsin (Sigma), in 50 mM ammonium bicarbonate at 37°C. The resulting tryptic
151 peptides were extracted with 50% acetonitrile containing 1% trifluoroacetic acid (TFA),
152 and analyzed by MALDI-TOF-TOF on an AB4800-Plus Proteomics Analyzer (Applied
153 Biosystems), under conditions previously described (Lomonte et al., 2014). In each run,
154 CalMix® standards (ABSciex) spotted onto the same plate were used as external
155 calibrants. Resulting spectra were searched against the UniProt/SwissProt database
156 using ProteinPilot® v.4 and the Paragon® algorithm (ABSciex) for protein identification
157 at ≥ 95% score confidence, or manually interpreted. Few peptide sequences with lower
158 confidence scores were manually searched using BLAST (http://blast.ncbi.nlm.nih.gov)

159 for protein similarity and assignment to protein families.

160

161 *2.4 Relative protein abundance estimations*

162 Areas of the RP-HPLC chromatographic peaks at 215 nm were integrated using
163 ChemStation® (Agilent) in order to estimate relative protein abundances (Calvete,
164 2011). For peaks containing several electrophoretic bands, percentage distributions were
165 assigned by densitometry, using ImageLab® (Bio-Rad).

166

167 *2.5 Phospholipase A_2 and proteolytic enzyme activities*

168 Enzymatic activities of *A. laevis* venom were tested comparatively with samples
169 obtained from other elapid snakes (*Dendroaspis polylepis*, *Naja kaouthia*; obtained
170 from Latoxan, France; and *Micrurus nigrocinctus*, obtained from Instituto Clodomiro
171 Picado) or the viperid *Bothrops asper* (Instituto Clodomiro Picado). PLA_2 activity was
172 assayed on the chromogenic 4-nitro-3-octanoyloxybenzoic acid (NOBA) synthetic
173 substrate, as described (Lomonte et al., 2015). Venoms (20 µg, dissolved in 25 µL of
174 10 mM Tris, 10 mM $CaCl_2$, 0.1 M NaCl, pH 8.0, buffer) were mixed with 200 µL of the
175 same buffer and 25 µL of NOBA to achieve a final substrate concentration of 0.32 mM.
176 Plates were incubated for 60 min at 37 °C, and absorbance was recorded at 405 nm in a
177 microplate reader. Proteolytic activity was determined on azocasein, according to Wang
178 et al. (2004). Venoms (40 µg, dissolved in 50 mM Tris–HCl, 0.15 M NaCl, 5 mM $CaCl_2$
179 buffer, pH 8.0) were added to 100 µL of azocasein (10 mg/mL in the same buffer), and
180 incubated for 90 min at 37 °C. The reaction was stopped by addition of 200 µL of 5%
181 trichloroacetic acid, and after centrifugation (5 min, 13,000 rpm), 150 µL of
182 supernatants were mixed with 100 µL of 0.5 M NaOH, and absorbance was recorded at
183 450 nm. All samples in these assays were run in triplicate wells, and controls of solvents

184 without venoms were included.

185

186 *2.7 Lethality screening*

187 Lethality assays were conducted in CD-1 mice, supplied by Instituto Clodomiro
188 Picado, following protocols approved by the Institutional Committee for the Use and
189 Care of Animals (CICUA), University of Costa Rica. The lethality of the whole venom
190 and fractions or isolated toxins was tested by intravenous (i.v.) injection in groups of
191 four mice (18–20 g body weight). Various amounts of venom or fractions/toxins were
192 dissolved in phosphate-buffered saline (PBS; 0.12 M NaCl, 0.04 M sodium phosphate
193 buffer, pH 7.2), and injected in the caudal vein, using a volume of 100 µL. Deaths
194 occurring within 24 h were recorded, and the LD_{50} values were calculated by probits
195 (Finney, 1971), using the BioStat® software (AnalySoft).

196 The toxicity of venom fractions was initially screened by selecting a dose based on
197 fraction abundance. The dose was selected to assess whether the fraction would score
198 above or below 1 according to the Toxicity Score defined by Laustsen et al. (2015a) as
199 the toxin abundance (%) divided by its LD_{50}. Fractions that were not lethal at this dose
200 (yielding a Toxicity Score <1) were considered as having insignificant toxicity, whereas
201 fractions, which did kill mice at this level, were further evaluated, and LD_{50} values were
202 determined for them.

203

204 *2.8 Myotoxicity of phospholipases A_2*

205 A pool of all the PLA_2 fractions was prepared, and doses of 30 µg, dissolved in
206 50 µL PBS, were injected intramuscularly, either in the right gastrocnemius, the thigh or
207 the soleus, to groups of five mice (18-20 g). In another experiment, mice received 30 µg
208 of the PLA_2 fractions in the soleus muscle. Injection of PBS was used for the control

group. Blood was collected after 3 h from the tip of the tail into heparinized capillaries. Plasma creatine kinase (CK) activity was determined using an UV kinetic assay (CK-Nac®, Analyticon). After blood collection, mice were sacrificed by CO_2 inhalation and a sample of muscles were obtained and immediately fixed in 10% formalin solution. After routine processing, tissues were embedded in paraffin, sectioned, and stained with hematoxylin-eosin for histological observation. In addition, in order to assess the acidic or basic nature of the various PLA_2s of the venom, chromatographic peaks 9-18 were analyzed by zone electrophoresis under native conditions, using a 1% agarose gel dissolved in 0.1 M Tris, 0.3 M glycine, pH 8.6 buffer. The gel was run at 75 V for 90 min, and protein migration was detected by Coomassie R-250 staining.

2.9 Antivenom neutralization studies

Two antivenoms were used: (a) BioCSL Sea Snake Antivenom, manufactured by BioCSL Limited (Melbourne, Victoria, Australia) (batch 054908201; expiry date: 04/2015); (b) Monospecific *Micrurus nigrocinctus* Anticoral Antivenom from Instituto Clodomiro Picado (batch 5310713ACLQ, expiry date 07/2016), for comparison. Mixtures containing a fixed amount of venom and several dilutions of antivenoms were prepared using PBS as diluent, and incubated at 37 ºC for 30 min. Controls included venom incubated with PBS instead of antivenom. Aliquots of 100 µL of the solutions, containing 4×LD_{50} of venom (11.2 µg/mouse) were then injected i.v. into groups of four mice (18-20 g). Deaths occurring within 24 h were recorded for assessing the neutralizing capacity of antivenoms. Neutralization was expressed as the Median Effective Dose (ED_{50}) of antivenom, defined as the ratio µg venom/mL antivenom at which 50% of the injected mice were protected. ED_{50}s were estimated by probits, as described in Section 2.7.

234

235 *2.10 Antivenom immunoprofiling by ELISA*

236 Wells in MaxiSorp plates (NUNC, Roskilde, Denmark) were coated overnight
237 with 0.6 µg of each HPLC venom fraction, dissolved in 100 µL PBS. Then, wells were
238 blocked by adding 100 µL PBS containing 2% (w:v) bovine serum albumin (BSA,
239 Sigma) at room temperature for 1 h, and washed five times with PBS. A dilution of each
240 antivenom in PBS containing 2% BSA was prepared such that the protein concentration
241 was 86 µg/mL (as measured by their absorbance at 280 nm on a NanoDrop® 2000c
242 instrument, Thermo Scientific), and 100 µL were added to the wells for 2 h. After five
243 washings with PBS, 100 µL of a 1:2000 dilution of rabbit anti-horse IgG (whole
244 molecule)-alkaline phosphatase conjugated antibodies (Sigma A6063, in PBS, 2% BSA)
245 was added to each well for 2 h, and then washed five times with FALC buffer (0.05 M
246 Tris, 0.15 M NaCl, 20 µM $ZnCl_2$, 1 mM $MgCl_2$, pH 7.4). Color was developed by
247 adding 100 µL of *p*-nitrophenyl phosphate (1 mg/mL in 9.7% v/v diethanolamine
248 buffer, pH 9.8), and the absorbances at 405 nm were recorded at several time intervals
249 in a microplate reader (Multiskan FC, Thermo Scientific).

250

251

252 **3.0 Results and Discussion**

253 *3.1 Venomics*

254 A detailed proteomics characterization was performed on the pooled venom
255 from *A. laevis*. From 20 fractions resolved by RP-HPLC, 35 peptidic bands were
256 obtained after SDS-PAGE separation (Fig.1). By in-gel digestion and MALDI-TOF-
257 TOF analysis, 99.2% of the protein bands could be assigned to toxin families. As shown
258 in Fig.2, the predominant family of proteins in this venom corresponds to PLA_2s

(71.2%), followed by a significant proportion of three-finger toxins (3FTx; 25.3%). A small amount of cysteine-rich secretory proteins (CRISP; 2.5%) and traces of a complement control module protein (CCM; 0.2%) were also detected. These results highlight the simple protein family composition of *A. laevis* venome, which essentially relies on a relatively small group of PLA_2 and 3FTx isoforms to exert its trophic role. Also, these findings are in agreement with the trend emerging from recent proteomic studies on sea snake venoms, which have revealed that their venoms are much simpler than their terrestrial elapid counterparts in terms of the number of dominant protein families and diversification of isoforms, typically within the PLA_2 and 3FTx families (Fry et al., 2003; Li et al., 2005). Thus far, sea snake venom proteomes have been deciphered for *Hydrophis cyanocinctus* (Calvete et al., 2012) and *Pelamis platura* (Lomonte et al., 2014). Similar to these, *A. laevis* venom contains few toxin families. However, in contrast to the venoms of *P. platura* and *H. cyanocinctus*, where the main toxin families are three-finger toxins (50% and 81% of all venom proteins, respectively) followed by PLA_2s (33% and 19% of all venom proteins, respectively), *A. laevis* venom displays the opposite relative venom composition, being dominated by PLA_2s (71.2%) followed by 3FTxs (25.3%). Furthermore, whereas the three-finger toxins of *A. laevis* venom are all short neurotoxins, *P. platura* and *H. cyanocinctus* venoms contain both short and long neurotoxins. The current findings on *A. laevis* venom composition differ from a previous study, where three of the short neurotoxins were reported to represent 22%, 33%, and 21% of the venom (76% altogether), respectively (Maeda and Tamiya, 1976). The reasons behind these discrepant results are difficult to determine, although they may reflect possible intraspecies differences in venom composition in specimens collected in different geographical locations: Maeda and Tamiya (1976) used *A. laevis* collected from Ashmore Reef, which is separated from our collection localities near

284 Broome by more than 600 km of mostly unsuitable (deep water) habitat. Potentially, the
285 observed differences could also be explained by interspecific hybridization, which has
286 previously been observed for *A. laevis* and closely related *A. fuscus* on Ashmore Reef,
287 where hybrid individuals closely resemble *A. laevis* in phenotype (Sanders et al., 2014).
288 In similarity with the predominance of PLA_2s over 3FTxs herein reported for *A. laevis*
289 venom, a transcriptomic study on the venom glands of *Aipysurus eydouxii* revealed the
290 existence of as many as sixteen unique PLA_2 transcripts, in contrast to a single
291 transcript corresponding to a 3FTx (Li et al., 2005). This could suggest that both
292 *Aipysurus* species share the same venom compositional predominance. However,
293 assessment of this possibility would require a direct examination of the *A. eydouxii*
294 venom proteins, in addition to its venom gland transcripts.

295 Individual variations of toxin expression in snake venoms are not uncommon
296 (Chippaux et al., 1991). To investigate the possible individual variability in *A. laevis*,
297 samples from three specimens ("Mifisto", "Medusa", and "Nessi") were compared by
298 RP-HPLC (Fig.3). This analysis revealed that some qualitative variation in toxin
299 expression was indeed present, although most fractions did not show significant
300 deviation in abundance between specimens or pooled venom.

301 Unlike several terrestrial elapids (Aird, 2002; Laustsen et al., 2015a),
302 nucleosides were not detected in *A. laevis* venom. On the other hand, its high content of
303 PLA_2s suggests that this venom might induce myotoxicity, as previously shown in
304 experimental studies (Zimmerman et al., 1992c; Ryan and Yong, 1997, 2002). Systemic
305 myotoxicity, i.e. rhabdomyolysis, with myoglobinuria characterizes envenomings by
306 some species of sea snakes in humans (Reid, 1961), and is responsible for acute kidney
307 injury. However, when a pool of PLA_2 fractions of *A. laevis* venom was tested for
308 myotoxicity in mice, only a mild effect was observed, as judged by increments in

309 plasma CK activity. Mice receiving PBS had CK activity of 215 ± 10 U/L, and mice
310 injected in the gastrocnemius, thigh, or soleus muscles with 30 µg of the PLA_2 fraction
311 pool had plasma CK activities of 926 ± 160 U/L, 1196 ± 119 U/L, and 764 ± 182 U/L
312 (mean ± SEM), respectively. Increments in CK were significant only when the PLA_2
313 fraction pool was injected in the gastrocnemius and thigh muscles ($p<0.05$). Thus, *A.*
314 *laevis* PLA_2s only induced a mild myotoxic effect. In agreement, histological analysis of
315 the soleus muscle 3 h after injection of PLA_2 fraction pool showed only few scattered
316 necrotic fibers (Fig.4). These observations contrast with the prominent increment in
317 plasma CK activity described for other elapid venoms, such as that of *Micrurus*
318 *nigrocinctus* (Fernández et al., 2011). By using native zone electrophoresis, it was
319 observed that all PLA_2 fractions (peaks 9-18) migrated towards the anode, indicating
320 that they were of acidic nature (not shown). This observation could explain the low
321 myotoxic effect of the PLA_2 pool tested, since commonly PLA_2s having potent
322 myotoxic effects are of a basic nature (Montecucco et al., 2008). Our results suggest
323 that myotoxicity is unlikely to be a significant effect in envenomings by *A. laevis*.

324 In agreement with its proteomic composition showing an abundance of PLA_2s,
325 high PLA_2 activity of the venom was confirmed *in vitro* (Fig.5A), whereas no
326 proteinase activity was detected (Fig.5B), in line with the absence of these enzymes in
327 the venom proteome. Three-finger toxins were shown to represent the second major
328 group of venom proteins in terms of abundance (25.3%), and all of them were identified
329 as short neurotoxin isoforms (Table 1), previously characterized by Maeda and Tamiya
330 (1976) and Ducancel et al. (1990). These short neurotoxins have been shown to bind
331 with high affinity to nicotinic receptors at the motor end-plate of muscle fibers, leading
332 to flaccid paralysis, which may result in respiratory failure and death (Maeda and
333 Nobuo, 1976; Ducancel et al., 1990).

All venom fractions were examined for acute toxicity in CD1 mice, and LD_{50} values were determined for most of those having a Toxicity Score below 1 (Table 2). All fractions containing short neurotoxins (fractions 1-4) and some fractions containing PLA_2s (fractions 5-18) induced lethality in mice, although the LD_{50} values of the short neurotoxins were 10-40 fold lower than those of the PLA_2s. Evaluated on the basis of their Toxicity Score, the short neurotoxins of *A. laevis* venom are the most relevant toxins to target in order to counteract the main clinical manifestations of the venom. The venom of *A. laevis* is remarkably simple compared to terrestrial elapids, such as *Dendroaspis polylepis* (Laustsen et al., 2015a) and *Naja kaouthia* (Laustsen et al., 2015b), which display a more diverse arsenal of toxins, although also being dominated by only two main toxin families.

The concept of a Toxicity Score for acute toxicity was presented for the first time in Laustsen et al. (2015a), and this score can be used to rank the importance of the individual toxins for acute toxicity in the given *in vivo* model (typically rodents). By examining the difference between the Toxicity Score of whole venom and the Accumulated Toxicity Score for all venom components (the sum of the Toxicity Scores for all the for the individual venom components), an indication of how the toxins in whole venom interact can be deduced. For a venom displaying synergism, the Toxicity Score for whole venom will be higher that the sum of the Toxicity Score for the individual components, since the synergistic effects between toxins will lead to an increased potency of the venom. For *A. laevis* there seems to be an indication that the Toxicity Score of whole venom (TS = 676) is almost the double of the Accumulated Toxicity Score of the venom components (ATS = 357) (Table 2), indicating that synergistic effects may exist. This observation is supported by previous studies indicating the presence of synergism (Ryan and Yong, 1997), which is quite fascinating

given the simplicity of this venom, being dominated by only a few very similar isoforms of short neurotoxins responsible for the main neurotoxic effects.

3.2 Venom neutralization and antivenom profiling

The ability of BioCSL Sea Snake Antivenom and ICP Anti-Coral Antivenom to neutralize *A. laevis* venom was investigated in CD-1 mice. The BioCSL Sea Snake Antivenom was effective in neutralizing lethality with an ED_{50} of 821 μg venom per mL antivenom (95% confidence limits: 478–1439 μg/mL), whereas no neutralization was observed for ICP Anti-Coral Antivenom at a level of 100 μg venom per mL. Our observations are in agreement with previous findings on the ability of BioCSLSea Snake Antivenom to neutralize the neuromuscular blocking activity of *A. laevis* and other sea snake venoms (Chetty et al., 2004). To further investigate the antivenoms, both BioCSL Sea Snake Antivenom and ICP Anti-Coral Antivenom were profiled by ELISA to determine the extent of binding between antivenom antibodies and toxins in *A. laevis* venom (Fig.6). Two general trends present themselves: BioCSL Sea Snake Antivenom displays significantly higher binding to fractions containing short neurotoxins (fractions 1-4), whereas the ICP Anti-Coral Antivenom displays either similar or even increased binding against PLA_2 containing fractions (fractions 5-18). This finding further supports that the short neurotoxins are responsible for the main toxic effects of *A. laevis* venom.

The underlying reason for the differences in binding preference between the two antivenoms may be explained by the venom compositions of *Micrurus nigrocinctus* and *Enhydrina schistosa*, which are used in the immunization mixtures of BioCSL Sea Snake Antivenom and ICP Anti-Coral Antivenom, respectively (Fig.7A). It must be noted, however, that it is not unlikely that horses hyper-immunized with several

384 different snake venoms were used for production of BioCSL Sea Snake Antivenom, and
385 that the "monovalence" of this antivenom is primarily due to the horses being boosted
386 with *E. schistosa* venom immediately before bleeding (Chetty et al., 2004; O'Leary and
387 Isbister, 2009; Herrera et al., 2014). Therefore, unexpected cross-reactivity is not an
388 unlikely event. *E. schistosa* venom has a high abundance of 3FTxs with a high degree of
389 conservation relative to the short neurotoxins found in *A. laevis* (Fig.7B), and it is
390 therefore not surprising that the BioCSL Sea Snake Antivenom has a strong preference
391 for fractions 1-4, containing short neurotoxins from *A. laevis*. In comparison, the PLA_2s
392 found in *M. nigrocinctus* venom are not more similar to the PLA_2s found in *A. laevis*
393 venom than the PLA_2 reported for *E. schistosa* venom (Fig.7C) (Fohlman and Eaker,
394 1977). However, it is speculated that the much higher abundance of PLA_2s in the
395 immunization mixture used for producing ICP Anti-Coral Antivenom in itself drives the
396 immunological response towards a stronger recognition against PLA_2s in general. Given
397 that BioCSL Sea Snake Antivenom readily cross-recognizes the neurotoxic components
398 having the highest Toxicity Scores, and since this antivenom was shown to neutralize
399 whole venom in rodents, BioCSL Sea Snake Antivenom should be useful for treating
400 human snakebite accidents inflicted by *A. laevis*.

401 The venom of *A. laevis* is remarkably simple. It could therefore be feasible to
402 develop modern antivenoms based on human(ized) monoclonal antibodies or peptide-
403 based inhibitors against this venom, since it is likely that only few antibodies are needed
404 to obtain its full neutralization. The degree of conservation, especially in the clinically
405 relevant short neurotoxins is high, and it is therefore likely that a potent, cross-reactive
406 antibody or peptide-based inhibitor capable of neutralizing all of these components can
407 be developed.

408

4.0 Concluding remarks and outlook

A proteomic analysis and functional study of *A. laevis* venom was carried out, revealing that this venom is remarkably simple and dominated by PLA_2s (71.2% of venom protein content) followed by short neurotoxins of the three-finger toxin family (25.3% of venom protein content). Also, cysteine-rich secretory proteins (CRISP) and a complement control module (CCM) were detected. Based on thorough toxicity testing of the individual fractions obtained from whole venom, the most relevant toxins to target for an effective antivenom against acute toxicity are the short neurotoxins. Based on their Toxicity Scores, the toxins present in *A. laevis* venom seem to interact in a slightly synergistic manner, possibly due to the short neurotoxins all targeting the nicotinic receptors at the motor end-plate of muscle fibers. BioCSL Sea Snake Antivenom was capable of neutralizing *A. laevis* venom in CD-1 mice when venom and antivenom were preincubated and administered by i.v. injection. ELISA-based immunoprofiling indicated that the BioCSL Sea Snake Antivenom has a binding preference for short neurotoxins. Therefore, this antivenom should be of clinical use for treating bites inflicted by *A. laevis*. Finally, given the simplicity of *A. laevis* venom, a potential for developing a modern antivenom based on human(ized) monoclonal antibodies or peptide-based inhibitors may be a possibility in the future.

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Ethical statement

The authors declare that there are no conflicts of interest related to this study. J.M. Gutiérrez and B. Lomonte work at the Instituto Clodomiro Picado (Universidad de Costa Rica), where the anti-coral snake antivenom used in this study is produced. Sources that provided financial support were not involved in the collection, analysis, or interpretation of data, nor in writing the report and submitting it for publication.

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606

607 **Figure legends**

608

609 **Figure 1:** Separation of *A. laevis* **(A)** venom proteins by RP-HPLC **(C)**, followed by
610 SDS-PAGE **(B)**. Two mg of venom were fractionated on a C_{18} column and eluted with
611 an acetonitrile gradient (dashed line), as described in Methods**.** Fractions were further
612 separated by SDS-PAGE under reducing conditions. Molecular weight markers (Mw)
613 are indicated in kDa. Coomassie-stained bands were excised, in-gel digested with
614 trypsin, and subjected to MALDI-TOF/TOF analysis for assignment to protein families,
615 as shown in Table 1.

616

617 **Figure 2:** Composition of *A. laevis* venom according to protein families, expressed as
618 percentages of the total protein content. 3FTx: three-finger toxin; PLA_2: phospholipase
619 A_2; CRISP: cysteine-rich secretory protein; CCM: complement control module. (see
620 Table 1).

621

622 **Figure 3:** Comparison of the chromatographic profiles of the venoms from three
623 individual *A. laevis* speciments ("Mifisto", "Medusa", and "Nessi"). The patterns of the
624 individual venoms are similar, although some differences do occur in abundance for
625 certain fractions. Fraction peaks representing more than 1% of total venom protein,
626 which have an abundance deviating more than 50% from the pool, are marked with *.

627

628 **Figure 4**: Light micrographs of sections of mouse soleus muscles collected 3 h after
629 injection of either phosphate-buffered saline (PBS) solution **(A)** or 30 µg of a pool of all
630 the PLA_2 fractions of the venom of *A. laevis* **(B)** (see Methods for experimental details).
631 A normal histological pattern is observed in **(A)**, whereas few scattered necrotic muscle

632 fibers (arrows) are observed in **(B)**, thus evidencing the mild myotoxic activity of this
633 venom. Hematoxylin-eosin staining. Bar represents 100 μm.
634
635 **Figure 5: (A)** Comparison of the phospholipase A_2 activity between the venoms of
636 *Aipysurus laevis, Dendroaspis polylepis, Naja kaouthia,* and *Micrurus nigrocinctus*. *A*.
637 *laevis* displays high enzymatic activity, although lower than *M*. *nigrocinctus*. **(B)**
638 Comparison of the proteolytic activity between the venoms of *A*. *laevis, D*. *polylepis, N*.
639 *kaouthia, M*. *nigrocinctus*, and *Bpthrops asper*, evaluated on azocasein. *A*. *laevis* shows
640 negligible proteinase activity.
641
642 **Figure 6:** ELISA-based immunoprofiling of antivenoms (**CSL Sea Snake**: BioCSL Sea
643 Snake Antivenom from BioCSL Limited, **ICP Micrurus**: Monospecific *Micrurus*
644 *nigrocinctus* Anticoral Antivenom from Instituto Clodomiro Picado, for comparison,
645 and a negative control (**Horse negative:** normal serum from non-immunized horses
646 from Instituto Clodomiro Picado) to all fractions of *A*. *laevis* venom separated by RP-
647 HPLC (see Methods for details). For identification of venom fractions refer to Table 2.
648 Each bar represents mean ± SD of triplicate wells.
649
650 **Figure 7: (A)** Comparison between the venom profiles and similarities of toxins from
651 *Aipysurus laevis*, *Enhydrina schistosa* (*Hydrophis schistosa*), and *Micrurus*
652 *nigrocinctus*. *E*. *schistosa* venom is used for production of BioCSL Sea Snake
653 Antivenom, while *M*. *nigrocinctus* venom is used for production of ICP's Monospecific
654 *M*. *nigrocinctus* Anticoral Antivenom. *Venom composition is based on venomics
655 studies of *M*. *nigrocinctus* (Fernández et al., 2011) and the reported study of *Hydrophis*
656 *cyanocinctus* (Calvete et al., 2012), as such studies have not been performed on *E*.

657 *schistosa*. Notice that the PLA_2 content of the venoms differ. The green frames
658 highlight observations used in discussion of Fig.6 (see text). **(B)** Alignment of all
659 known three-finger toxins (3FTx) from *A*. *laevis* with the most similar toxins from *M*.
660 *nigrocinctus* and *E*. *schistosa*. A high degree of conservation exists between short
661 neurotoxins from *A*. *laevis* and the most similar *E*. *schistosa* toxin, possibly explaining
662 the observed cross-reactivity of BioCSL Sea Snake Antivenom. **(C)** Alignment of the
663 single known PLA_2 from *A*. *laevis* with the most similar toxins from *M*. *nigrocinctus*
664 and *E*. *schistosa*, showing only a limited degree of conservation.

Table 1: Assignment of the RP-HPLC isolated fractions of *Aipysurus laevis* venom to protein families by MALDI-TOF-TOF of selected peptide ions from in-gel trypsin-digested protein bands.

Peak	%	Mass (kDa)▼	Peptide ion		MS/MS-derived sequence *	Conf (%)	Sc	Protein family **	Related protein, code
			m/z	z					
1	21.8	10	1564.8	1	TTTDCADDSCYBK	99	9	3FTx	short neurotoxin D
			1450.8	1	XTCCNBBSSBPK	99	19		*Aipysurus laevis*, P19960
			1300.8	1	GCGCPBVBPGXK	99	18		
2a	0.3	15	1564.5	1	TTTDCADDSCYBK	99	8	3FTx	short neurotoxin D
			1450.6	1	XTCCNBBSSBPK	99	14		*Aipysurus laevis*, P19960
			1300.6	1	GCGCPBVBPGXK	99	16		
2b	0.3	10	1300.6	1	GCGCPBVBPGXK	99	9	3FTx	short neurotoxin D
									Aipysurus laevis, P19960
3a	1.0	15	1436.6	1	TTTDCADDSCYK	99	12	3FTx	short neurotoxin
			1564.7	1	TTTDCADDSCYBK	99	11		*Aipysurus laevis*, P19960
			1450.7	1	XTCCNBBSSBPK	99	18		
			1300.7	1	GCGCPBVBPGXK	99	17		
3b	1.0	10	1564.7	1	TTTDCADDSCYBK	99	9	3FTx	short neurotoxin
			1436.6	1	TTTDCADDSCYK	99	8		*Aipysurus laevis*, P19960
			1450.7	1	XTCCNBBSSBPK	99	13		
			1300.7	1	GCGCPBVBPGXK	99	14		
4a	0.5	15	1300.7	1	GCGCPBVBPGXK	99	11	3FTx	short neurotoxin
			1436.6	1	TTTDCADDSCYK	65.7	6		*Aipysurus laevis*, P19960
4b	0.4	10	1436.6	1	TTTDCADDSCYK	99	10	3FTx	short neurotoxin
			1450.7	1	XTCCNBBSSBPK	99	14		*Aipysurus laevis*, P19960
			1300.7	1	GCGCPBVBPGXK	99	13		
			1564.7	1	TTTDCADDSCYBK	96.8	8		
5a	0.1	29	1758.8	1	NXYBFDNMXBCANK	99	10	PLA_2	phospholipase A_2
			1895.8	1	AHDDCYGVAED(N^{da})GCSPK	99	26		*Aipysurus eydouxii*, ~Q5DNC9
			1774.8	1	NXYBFDN(M^{ox})XBCANK	99	11		
5b	0.3	18	1758.9	1	NXYBFDNMXBCANK	99	14	PLA_2	phospholipase A_2
			1870.9	1	CYCGWGGSGTPVDAXDR	99	14		*Aipysurus eydouxii*, ~Q5DNE1
			2995.5	1	ATWHYMDYGCYCGWGGSGTPVDAXDR	99	19		
			2337.1	1	MDYGCYCGWGGSGTPVDAXDR	99	19		

			2091.0	1	YGCYCGWGGSGTPVDAXDR	99	17		
6	0.5	18	1758.9	1	NXYBFDNMXBCANK	99	13	PLA_2	phospholipase A_2
			1870.9	1	CYCGWGGSGTPVDAXDR	99	17		*Aipysurus eydouxii*, ~Q5DNE1
			2994.5	1	ATWHYMDYGCYCGWGGSGTPVDAXDR	99	21		
			2337.1	1	MDYGCYCGWGGSGTPVDAXDR	99	19		
			2091.0	1	YGCYCGWGGSGTPVDAXDR	99	21		
7	0.2	-	-	-	-	-	-	unknown	-
8	0.4	-	-	-	-	-	-	unknown	-
9	9.6	10	2091.0	1	DYGCYCGAGGSGTPVDAXDR	99	11	PLA_2	β-bungarotoxin chain A2
									Bungarus caeruleus, ~Q8QFW3
10	6.2	10	2337.1	1	MDYGCYCGWGGSGTPVDAXDR	99	11	PLA_2	phospholipase A_2
			2995.5	1	ATWHYMDYGCYCGWGGSGTPVDAXDR	99	18		*Aipysurus eydouxii*, ~Q5DNE1
			1710.9	1	NXYBFDN(M^{dt})XBCANK	99	14		
			2091.0	1	YGCYCGWGGSGTPVDAXDR	99	17		
			1870.9	1	CYCGWGGSGTPVDAXDR	91	9		
11a	3.1	23	1758.9	1	NXYBFDNMXBCANK	99	14	PLA_2	phospholipase A_2
									Aipysurus eydouxii, ~Q5DNE1
11b	9.1	15	2912.3	1	ATWHY(M^{ox})DYGCYCGSGGSGTPVDAXD	99	12	PLA_2	phospholipase A_2
			1998.8	1	R	98	8		*Aipysurus eydouxii*, ~Q5DND8
			2896.3	1	VHDDCYGVAEDNGCYPK	95.4	8		
			1774.8	1	ATWHYMDYGCYCGSGGSGTPVDAXDR NXYBFDN(M^{ox})XBCANK	67.2	7		
11c	7.2	10	2091.0	1	YGCYCGWGGSGTPVDAXDR	99	10	PLA_2	phospholipase A_2
			2895.4	1	ATWHYMDYGCYCGSGGSGTPVDAXDR	99	14		*Aipysurus eydouxii*, ~Q5DNE1
			1710.9	1	NXYBFDN(M^{dt})XBCANK	99	10		
12a	2.7	15	2896.3	1	ATWHYMDYGCYCGSGGSGTPVDAXDR	97.3	6	PLA_2	phospholipase A_2
			1774.8	1	NXYBFDN(M^{ox})XBCANK	95.3	6		*Aipysurus eydouxii*, ~Q5DNE1
12b	3.3	10	2835.4	1	WTXYSWBCTENVPTCNSESGCBK	99	15	PLA_2	phospholipase A_2
			2896.4	1	ATWHYMDYGCYCGSGGSGTPVDAXDR	99	23		*Aipysurus laevis*, P08872
			1213.6	1	CFAEAPYNNK	99	16		
			1758.9	1	NXYBFDNMXBCANK	99	18		
			1894.8	1	AHDDCYGVAEDNGCSPK	99	23		*Aipysurus eydouxii*, ~Q5DNE1
13a	1.7	20	1758.8	1	NXYBFDNMXBCANK	99	9	PLA_2	phospholipase A_2
			1213.6	1	CFAEAPYNNK	99	12		*Aipysurus laevis*, P08872
			2013.9	1	AHDDCYGVAEDNGCYPK	99	20		

13b	9.3	10	1758.9	1	NXYBFDNMXBCANK	99	16	PLA_2	phospholipase A_2
			2994.5	1	ATWHYMDYGCYCGWGGSGTPVDAXDR	99	22		*Aipysurus eydouxii*, ~Q5DNE1
			1870.9	1	CYCGWGGSGTPVDAXDR	99	19		
			2337.1	1	MDYGCYCGWGGSGTPVDAXDR	99	24		
			2091.0	1	YGCYCGWGGSGTPVDAXDR	99	24		
14a	0.6	20	2970.3	1	ATWHYTDYGCYCGBGGSGTPVDEXDR	99	12	PLA_2	phospholipase PLA-2
			1352.5	1	THDDCYGEAEK	99	17		*Notechis scutatus*, ~Q45Z32
14b	1.9	10	2994.4	1	ATWHYMDYGCYCGWGGSGTPVDAXDR	99	14	PLA_2	phospholipase A_2
									Aipysurus eydouxii, ~Q5DNE1
15a	0.2	15	1970.8	1	AHDDCYGVAEDNGCYPK	99	17	PLA_2	phospholipase A_2
									Aipysurus eydouxii, ~Q5DNE1
15b	0.1	10	1970.8	1	AHDDCYGVAEDNGCYPK	99	19	PLA_2	phospholipase A_2
			2995.4	1	ATWHYMDYGCYCGWGGSGTPVDAXDR	80	7		*Aipysurus eydouxii*, ~Q5DNE1
16a	0.1	15	-	-	-	-	-	unknown	-
16b	0.1	10	2050.0	1	YGCYCGSGGSGTPVDEXDR	99	12	PLA_2	PLA_2-Den-12
			1774.9	1	NXYBFDN(M^{ox})XBCANK	92.8	8		*Denisonia devisi*, ~R4G7G2
17	3.6	10	2905.5	1	SVWDFTNYGCYCGSGGSGTPVDEXDR	99	16	PLA_2	PLA_2-9
			2050.0	1	YGCYCGSGGSGTPVDEXDR	99	17		*Micrurus fulvius*, ~U3FYN8
18	11.6	10	2970.4	1	(S^{pa})VWDFTNYGCYCGSGGSGTPVDEXDR	99	16	PLA_2	PLA_2-9
			1352.6	1	(T^{fo})HDDCYGEAEK	99	21		*Micrurus fulvius*, ~U3FYN8
19a.i	0.4	37	1614.9	1	XGEEVTXGCNYGFR	99	11	CCM	complement decay-accelerating factor transmembrane isoform *Ophiophagus hannah*, ~V8NM67
19a.ii		37	1777.0	1	YXYVCBYCPAGNXR	99	15	CRISP	CRISP *Hydrophis hardwickii*, ~AAL54918
19b	1.6	20	1161.5	1	YNNDFSNCK	99	10	CRISP	CRISP-Aca-1
			1719.8	1	YXYVCBYCPAGNXR	99	13		*Acanthophis wellsi*, ~R4FJD0
19c	0.4	15	1776.8	1	YXYVCBYCPAGNXR	99	20	CRISP	CRISP *Hydrophis hardwickii*, ~AAL54918
19d	0.3	10	1776.8	1	YXYVCBYCPAGNXR	99	15	CRISP	CRISP *Hydrophis hardwickii*, ~AAL54918
20	0.1	-	-	-	-	-	-	unknown	-

* Cysteine residues are carbamidomethylated. Confidence (Conf) and Score (Sc) values are calculated by the Paragon® algorithm of ProteinPilot®. ▼: reduced SDS-PAGE mass estimations, in kDa. X: Leu/Ile; B: Lys/Gln; Z: pyroglutamate (2-oxo-pyrrolidone carboxylic acid). Possible, although unconfirmed/ambiguous amino acid modifications suggested by the automated identification software are shown in parentheses, with the following abbreviations: ^{ox}: oxidized; ^{da}: deamidated; ^{dt}: dethiomethyl; ^{pa}: propionamide; ^{fo}: formylated.

** Protein family abbreviations: 3FTx: three-finger toxin; PLA_2: phospholipase A_2; CRISP: cysteine-rich secretory protein; CCM: complement control module.

Table 2: LD_{50} values of *Aipysurus laevis* venom and the RP-HPLC isolated fractions

Peak	%	Protein family	LD_{50} (95% C.L.)	Toxicity Score[1] % / LD_{50} (kg/mg)
Whole venom	100		0.15 (0.08-0.25)	676
1	21.8	3FTx: short neurotoxin	0.07 (0.04-0.15)	334
2	0.6	3FTx: short neurotoxin	0.18 (0.01-0.69)	3.3
3	2.0	3FTx: short neurotoxin	0.13 (0.09-0.44)	15.3
4	0.9	3FTx: short neurotoxin	0.28 (0.14-0.99)	3.2
5	0.3	PLA_2: Phospholipase A_2	>0.3	<1
6	0.5	PLA_2: Phospholipase A_2	>0.5	<1
7	0.2	Unknown	>0.25	<1
8	0.4	Unknown	>0.5	<1
9	9.6	PLA_2: Phospholipase A_2	>10	<1
10	6.3	PLA_2: Phospholipase A_2	>7.5	<1
11	19.4	PLA_2: Phospholipase A_2	>20	<1
12	6.0	PLA_2: Phospholipase A_2	>6	<1

13	11.0	PLA_2: Phospholipase A_2	>10	<1.1
14	2.5	PLA_2: Phospholipase A_2	>2.5	<1
15	0.3	PLA_2: Phospholipase A_2	>0.3	<1
16	0.2	PLA_2: Phospholipase A_2	>0.25	<1
17	3.6	PLA_2: Phospholipase A_2	3.05 (1.92-4.67)	1.2
18	11.6	CCM	>>2.5	<5
19	2.7	CRISP	>0.5	<1
20	0.1	CRISP	N.t.	N.t.

*http://snakedatabase.org/pages/LD50.php#legendAndDefinitions

[1]Toxicity Score was defined as the ratio of protein fraction abundance (%) in the venom divided by its estimated median lethal dose (LD_{50}) for CD-1 mice by i.v. injection.

[2]Mix indicates that the fraction did not contain a pure, isolated toxin, but instead a mixture of 2-4 different toxins in variable ratios indicted in the table.

N.t. : not tested

Figure 1

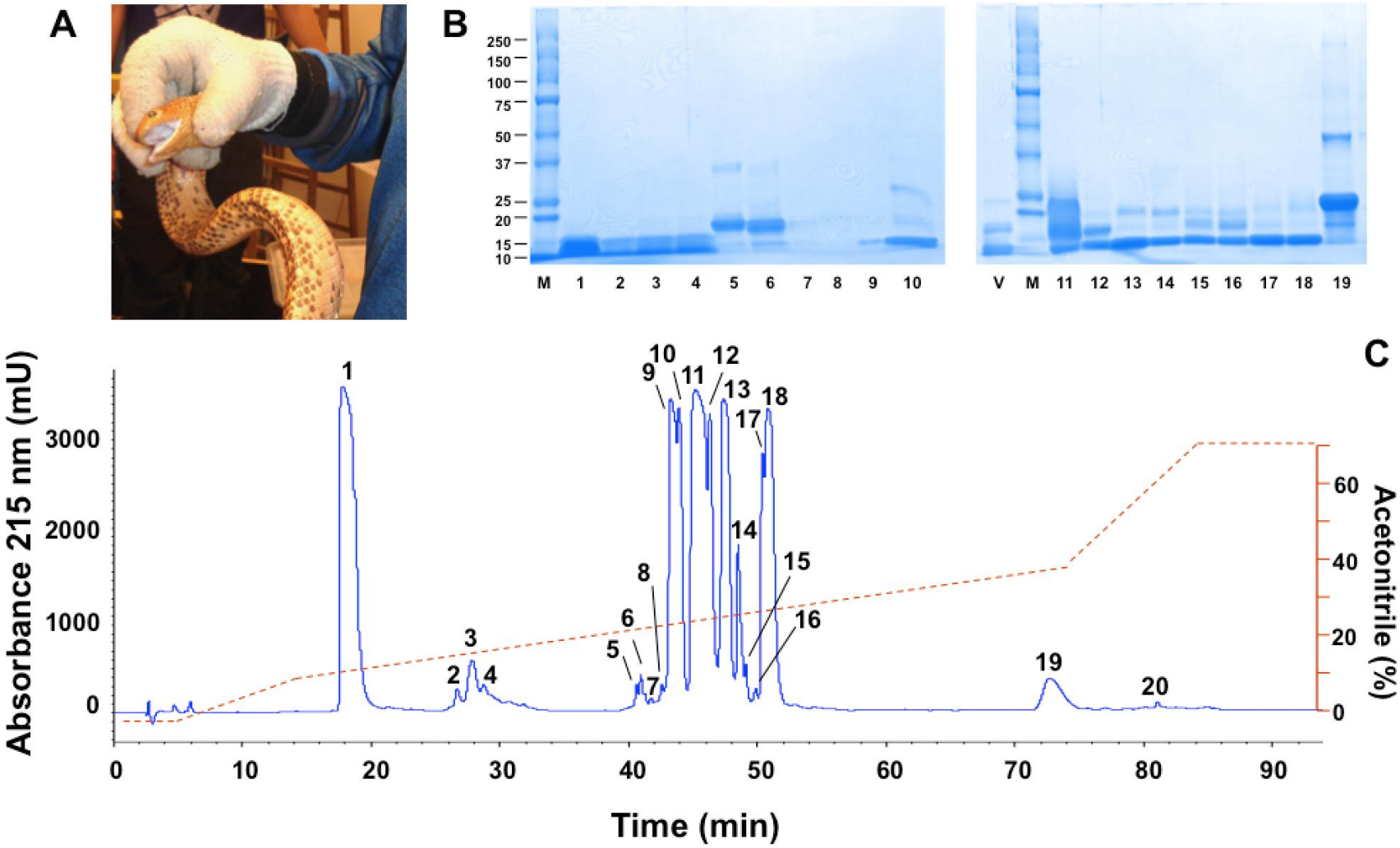

A
B
250
150
100
75
50
37
25
20
15
10
M 1 2 3 4 5 6 7 8 9 10
V M 11 12 13 14 15 16 17 18 19
C
Absorbance 215 nm (mU)
3000
2000
1000
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
0 10 20 30 40 50 60 70 80 90
Time (min)
Acetonitrile (%)
60
40
20
0

Figure 2

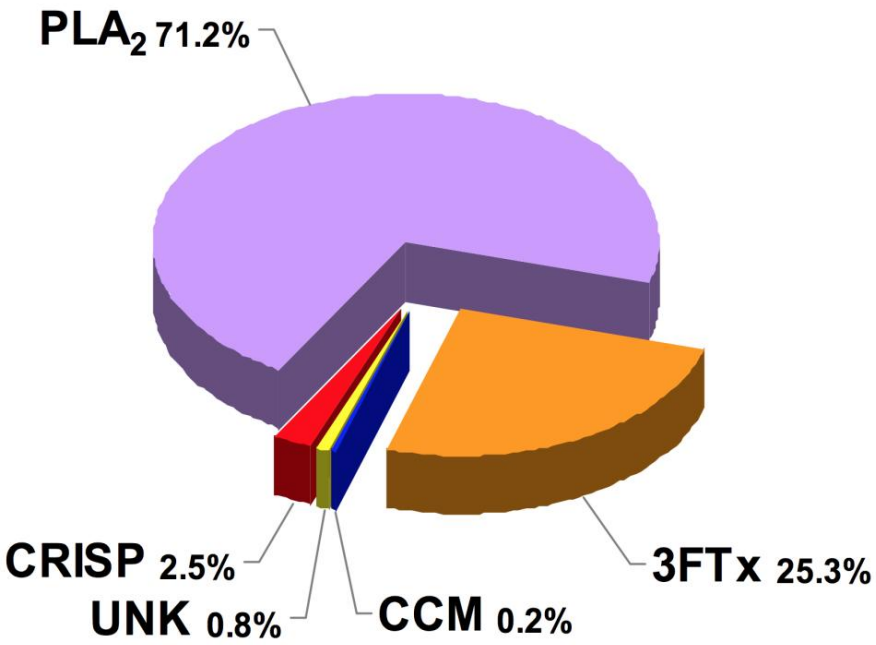


Figure 3

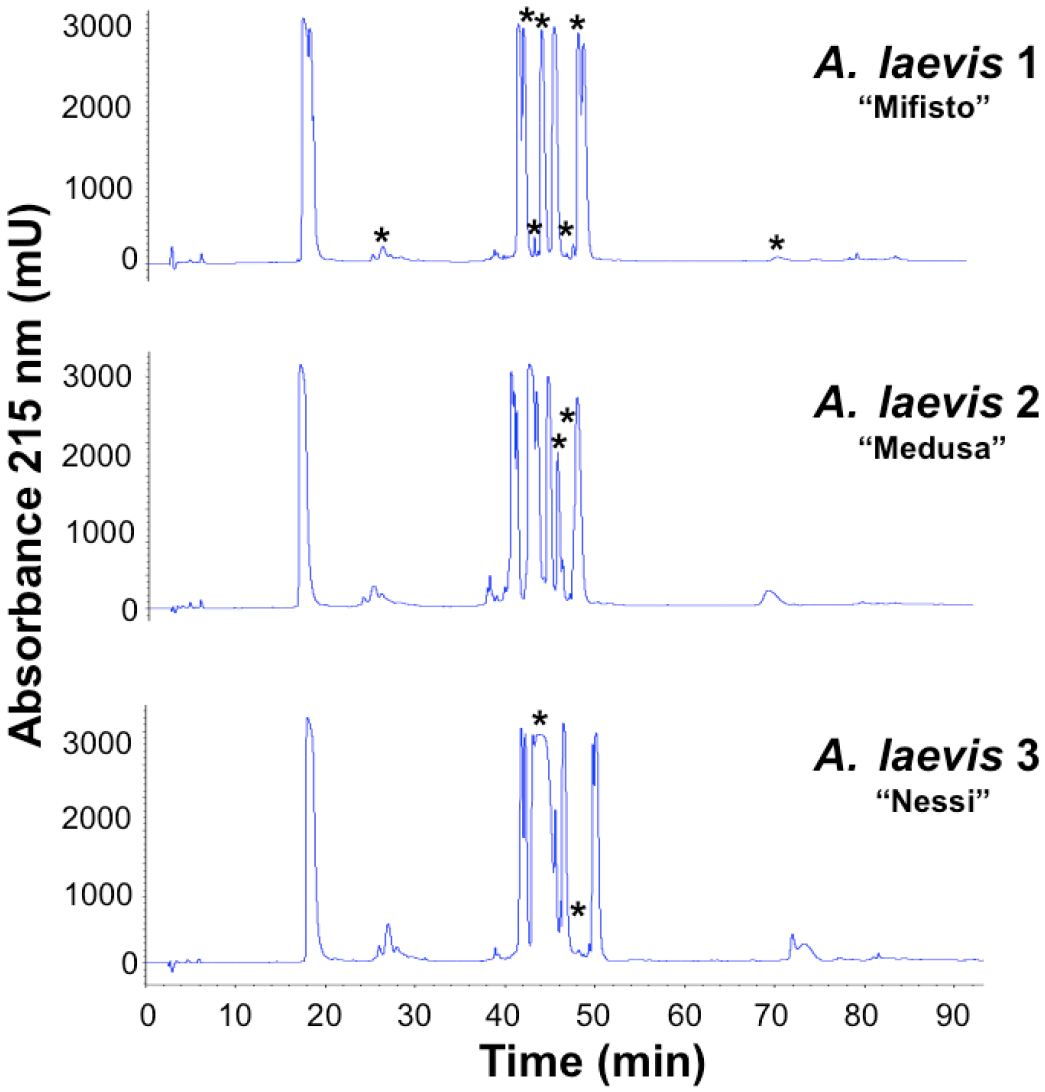

Absorbance 215 nm (mU)
3000
2000
1000
0
A. laevis 1
"Mifisto"
A. laevis 2
"Medusa"
A. laevis 3
"Nessi"
0
10
20
30
40
50
60
70
80
90
Time (min)

Figure 4

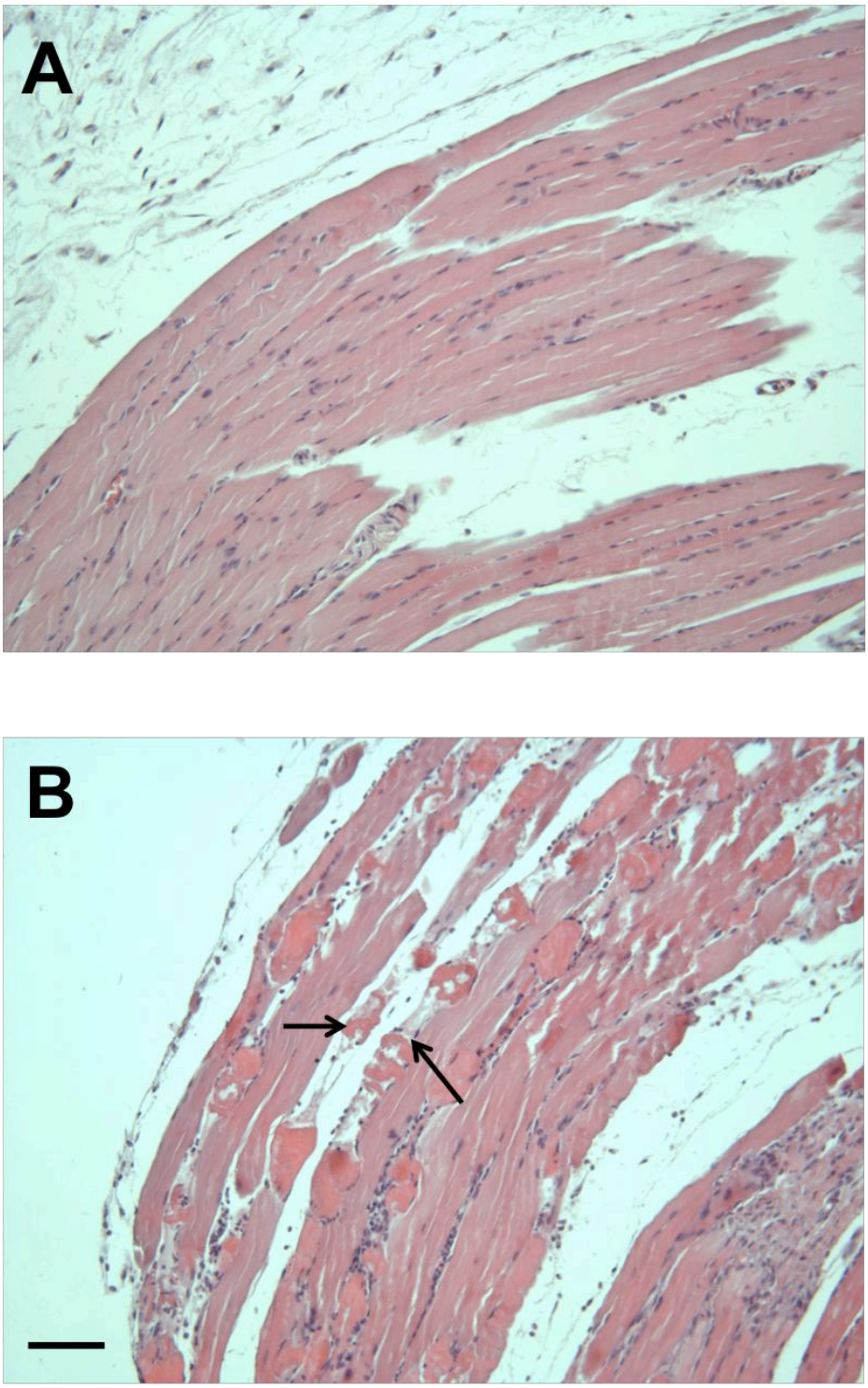


Figure 5

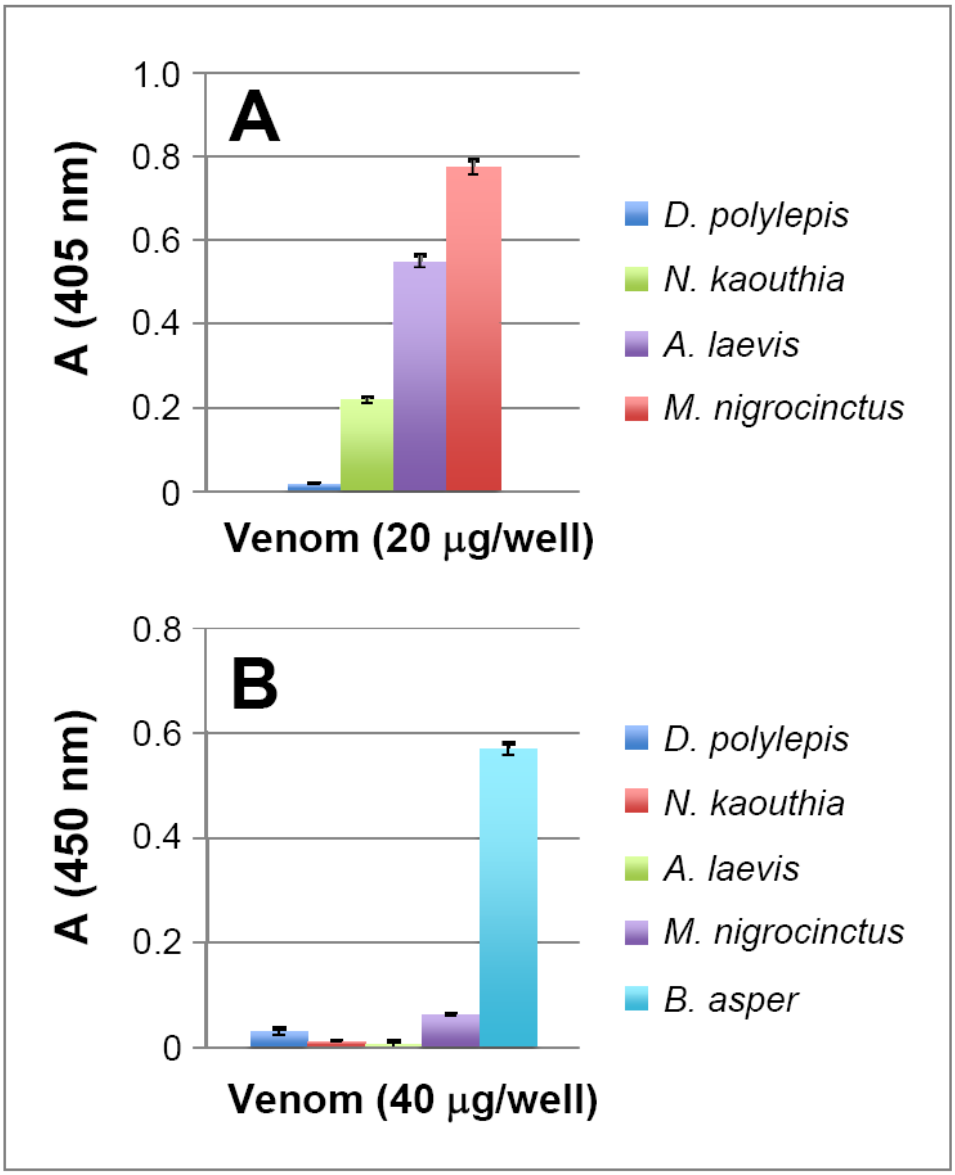

A
1.0
0.8
0.6
0.4
0.2
0
A (405 nm)
Venom (20 µg/well)
D. polylepis
N. kaouthia
A. laevis
M. nigrocinctus
B
0.8
0.6
0.4
0.2
0
A (450 nm)
Venom (40 µg/well)
D. polylepis
N. kaouthia
A. laevis
M. nigrocinctus
B. asper

Figure 6

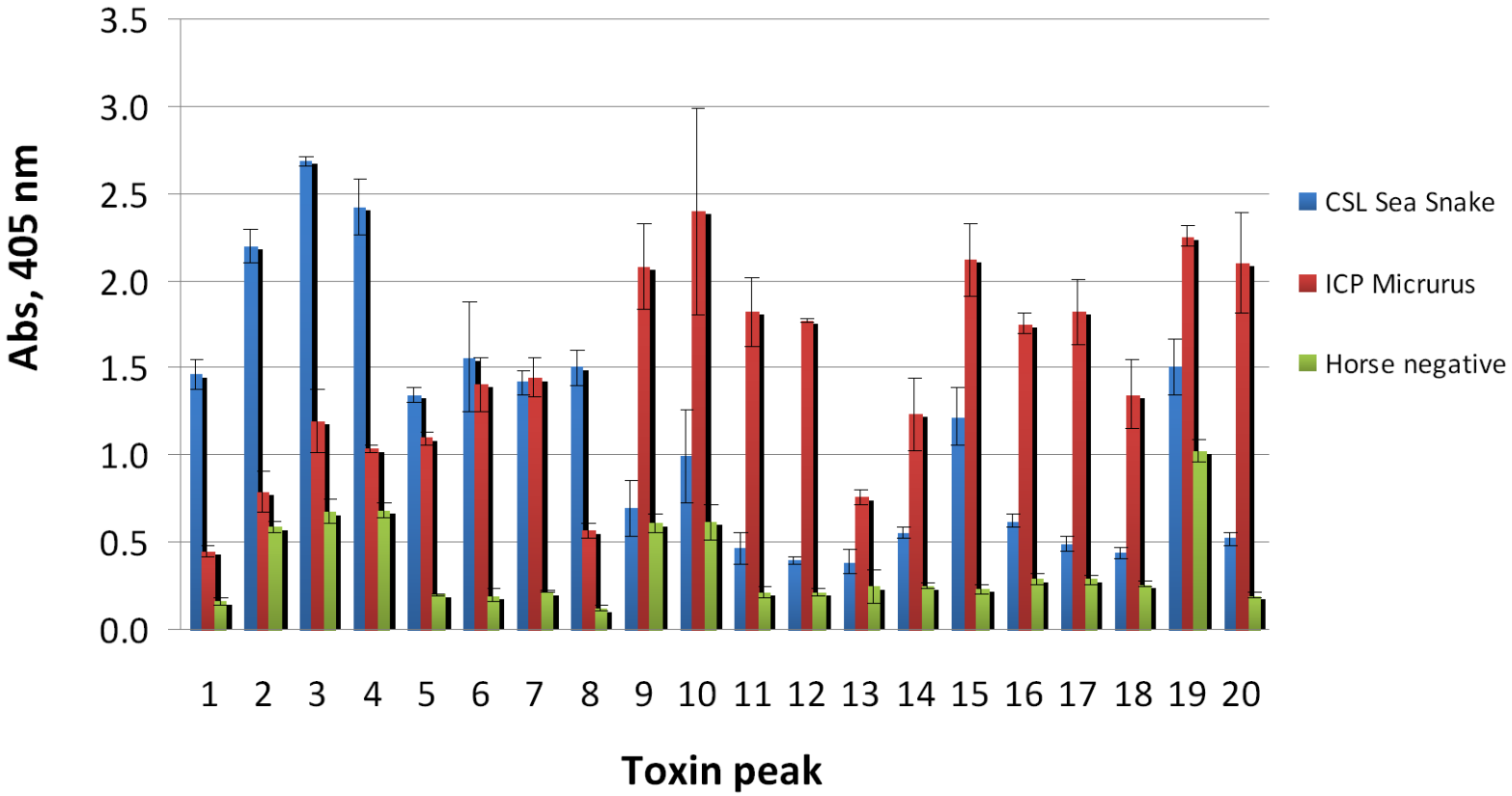
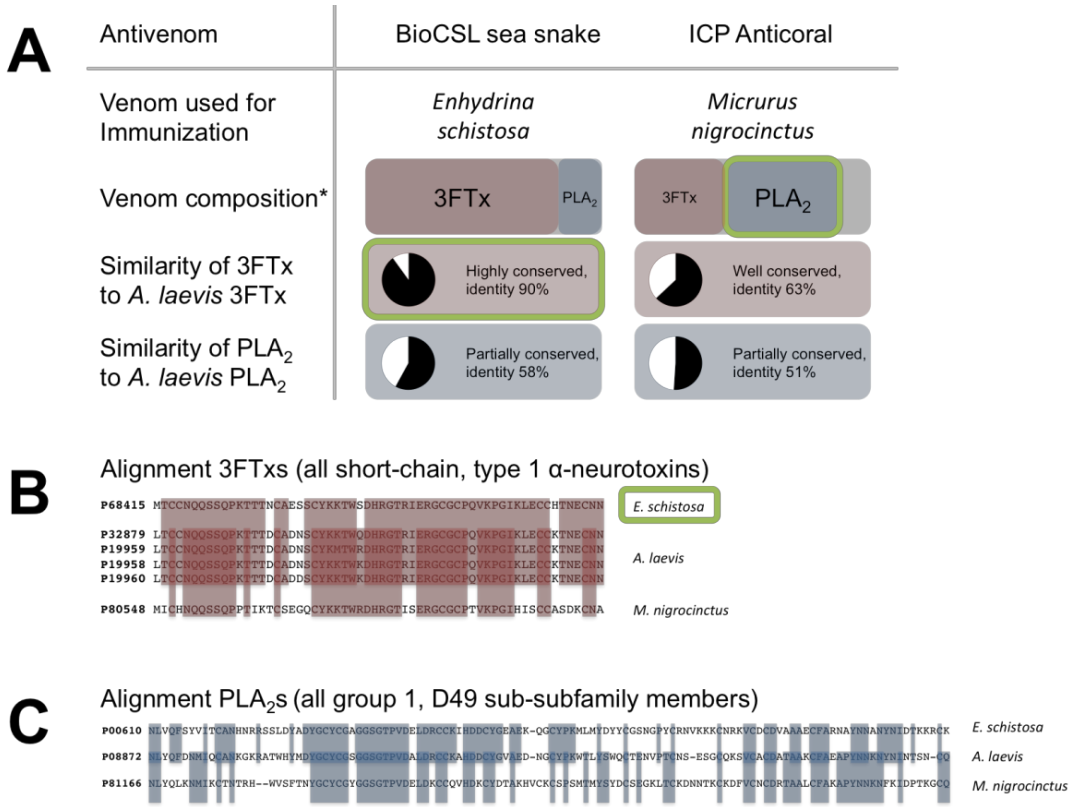

Abs, 405 nm
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Toxin peak
CSL Sea Snake
ICP Micrurus
Horse negative

Figure 7


A
Antivenom
BioCSL sea snake
ICP Anticoral
Venom used for Immunization
Enhydrina schistosa
Micrurus nigrocinctus
Venom composition*
3FTx
PLA2
3FTx
PLA2
Similarity of 3FTx to A. laevis 3FTx
Highly conserved, identity 90%
Well conserved, identity 63%
Similarity of PLA2 to A. laevis PLA2
Partially conserved, identity 58%
Partially conserved, identity 51%
B
Alignment 3FTxs (all short-chain, type 1 α-neurotoxins)
P68415
P32879
P19959
P19958
P19960
P80548
E. schistosa
A. laevis
M. nigrocinctus
C
Alignment PLA2s (all group 1, D49 sub-subfamily members)
P00610
P08872
P81166
E. schistosa
A. laevis
M. nigrocinctus

Graphical Abstract