Danger in the reef

proteome, toxicity, and neutralization of the venom of the olive sea snake, *Aipysurus laevis*

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

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31 **Highlights**

32

- 33 • The venom proteome of the olive sea snake, *Aipysurus laevis*, is presented
- 34 • Most abundant venom components are phospholipases A₂ and short neurotoxins
- 35 • Lethality screening coupled to abundance estimation identified the medically
- 36 relevant toxins
- 37 • BioCSL Sea Snake Antivenom neutralizes venom lethality
- 38 • ICP Anti-Coral Antivenom cross-recognizes phospholipases A₂ from *A. laevis*
- 39 venom

40

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 A₂ (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 LD₅₀ of 0.07 mg/kg in mice and showed a high phospholipase A₂ 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 ED₅₀ 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)

59 1. Introduction

60 The viviparous sea snakes are a diverse clade of more than 60 species that are
 61 phylogenetically nested within the front-fanged Australo-Melanesian terrestrial elapids
 62 (Hydrophiinae) (Rasmussen et al., 2011). They are highly aquatic and occupy most
 63 shallow-marine habitats throughout the tropical and subtropical Indo-West Pacific, yet
 64 are estimated to share a common ancestor dated at only 6–8 million years ago (Sanders
 65 et al., 2008; Lukoschek et al., 2012). The amphibious sea kraits (Hydrophiinae:
 66 Laticauda) represent an independently aquatic and earlier diverging lineage that is the
 67 sister to terrestrial and viviparous marine hydrophiines (Keogh, 1998; Scanlon and Lee,
 68 2004; Sanders et al., 2008). Two major clades are recognised within the viviparous
 69 marine group: An ‘Aipysurus’ lineage comprising ten species found primarily in the
 70 Australo-Papuan region, and a ‘Hydrophis’ lineage containing at least 50 species
 71 distributed throughout the Indo-West Pacific (Rasmussen et al., 2011).

72 In the *Aipysurus* group, the olive sea snake, *Aipysurus laevis*, has a large
 73 muscular head and is the most robustly built and longest species recorded, reaching
 74 more than 170 cm in total length (Smith, 1926; Cogger, 1975). *A. laevis* has been
 75 recorded from Aru Archipelago and Kai Islands (Indonesia) in the west and from the
 76 northern coast of Australia and southern coast of New Guinea (Timor Sea and Arafura
 77 Sea) to New Caledonia in the east (Coral Sea) (Cogger, 1975; Ineich and Rasmussen,
 78 1997; Sanders et al., 2014). *A. laevis* is found in shallow marine habitats – coral reefs as
 79 well as sandy, rocky, and mud-bottom habitats, and is often one of the most abundant
 80 species throughout its range (Cogger, 1975; Lukoschek et al., 2007; Sanders et al.,
 81 2014). It hunts primarily in crevices on the sea floor, and the following fish families
 82 have been found as prey items in *A. laevis*: Acanthuridae, Apogonidae, Carangidae,
 83 Clupeidae, Engraulidae, Labridae, Lutjanidae, Pempheridae, Pomacentridae, Scaridae,

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

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

94 The venoms of sea snakes, typically containing α -neurotoxins and
95 phospholipases A₂ (PLA₂s), are known to be generally more potent than the venoms
96 from terrestrial snakes in terms of lethality (Minton, 1983; Takasaki, 1998). In contrast
97 to the latter, however, only few studies have been focused on determining the
98 comprehensive composition of sea snake venoms by means of proteomic analyses, i.e.
99 venomics. The venom of *A. laevis* has been shown to be neurotoxic, nephrotoxic, and
100 myotoxic in mice, causing acute renal tubular degeneration, proliferative
101 glomerulonephritis, local muscle degeneration, necrosis, enlarged spleen, inflammation,
102 and lymphadenopathy (Zimmerman et al., 1992a, 1992c; Ryan and Yong, 1997, 2002).
103 Regarding the venom components of *A. laevis*, a total of four short-chain neurotoxin
104 isoforms with minor amino acid sequence variations (P19958, P19959, P19960, and
105 P32879) and one PLA₂ (P08872) have been fully sequenced (Maeda and Nobuo, 1976;
106 Ducancel et al., 1988, 1990). The short α -neurotoxins display a high affinity towards
107 the acetylcholine receptor (Ishikawa et al., 1977), which is in agreement with the very
108 low LD₅₀ 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.

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

The pooled venom of *A. laevis* was fractionated by sequential RP-HPLC and SDS-PAGE separation steps, following the ‘snake venomics’ analytical strategy (Calvete, 2011) under conditions described previously (Lomonte et al., 2014). Venom load for the RP-HPLC step on C₁₈ (4.6 x 250 mm column, 5 µm particle diameter; Teknokroma) was 2 mg. Protein fractions were monitored at 215 nm, manually collected, dried by vacuum centrifugation, and electrophoretically separated under reducing conditions. Resulting bands were stained with colloidal Coomassie blue G-250, and digitally recorded on a ChemiDoc[®] imager using ImageLab[®] software (Bio-Rad).

2.3 Protein identification by tandem mass spectrometry of tryptic peptides

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

for protein similarity and assignment to protein families.

2.4 Relative protein abundance estimations

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

2.5 Phospholipase A₂ and proteolytic enzyme activities

Enzymatic activities of *A. laevis* venom were tested comparatively with samples obtained from other elapid snakes (*Dendroaspis polylepis*, *Naja kaouthia*; obtained from Latoxan, France; and *Micrurus nigrocinctus*, obtained from Instituto Clodomiro Picado) or the viperid *Bothrops asper* (Instituto Clodomiro Picado). PLA₂ activity was assayed on the chromogenic 4-nitro-3-octanoyloxybenzoic acid (NOBA) synthetic substrate, as described (Lomonte et al., 2015). Venoms (20 µg, dissolved in 25 µL of 10 mM Tris, 10 mM CaCl₂, 0.1 M NaCl, pH 8.0, buffer) were mixed with 200 µL of the same buffer and 25 µL of NOBA to achieve a final substrate concentration of 0.32 mM. Plates were incubated for 60 min at 37 °C, and absorbance was recorded at 405 nm in a microplate reader. Proteolytic activity was determined on azocasein, according to Wang et al. (2004). Venoms (40 µg, dissolved in 50 mM Tris-HCl, 0.15 M NaCl, 5 mM CaCl₂ buffer, pH 8.0) were added to 100 µL of azocasein (10 mg/mL in the same buffer), and incubated for 90 min at 37 °C. The reaction was stopped by addition of 200 µL of 5% trichloroacetic acid, and after centrifugation (5 min, 13,000 rpm), 150 µL of supernatants were mixed with 100 µL of 0.5 M NaOH, and absorbance was recorded at 450 nm. All samples in these assays were run in triplicate wells, and controls of solvents

without venoms were included.

2.7 Lethality screening

Lethality assays were conducted in CD-1 mice, supplied by Instituto Clodomiro Picado, following protocols approved by the Institutional Committee for the Use and Care of Animals (CICUA), University of Costa Rica. The lethality of the whole venom and fractions or isolated toxins was tested by intravenous (i.v.) injection in groups of four mice (18–20 g body weight). Various amounts of venom or fractions/toxins were dissolved in phosphate-buffered saline (PBS; 0.12 M NaCl, 0.04 M sodium phosphate buffer, pH 7.2), and injected in the caudal vein, using a volume of 100 μ L. Deaths occurring within 24 h were recorded, and the LD₅₀ values were calculated by probits (Finney, 1971), using the BioStat[®] software (AnalySoft).

The toxicity of venom fractions was initially screened by selecting a dose based on fraction abundance. The dose was selected to assess whether the fraction would score above or below 1 according to the Toxicity Score defined by Laustsen et al. (2015a) as the toxin abundance (%) divided by its LD₅₀. Fractions that were not lethal at this dose (yielding a Toxicity Score <1) were considered as having insignificant toxicity, whereas fractions, which did kill mice at this level, were further evaluated, and LD₅₀ values were determined for them.

2.8 Myotoxicity of phospholipases A₂

A pool of all the PLA₂ fractions was prepared, and doses of 30 μ g, dissolved in 50 μ L PBS, were injected intramuscularly, either in the right gastrocnemius, the thigh or the soleus, to groups of five mice (18–20 g). In another experiment, mice received 30 μ g of the PLA₂ 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 CO₂ 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 PLA₂s 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 °C for 30 min. Controls included venom incubated with PBS instead of antivenom. Aliquots of 100 µL of the solutions, containing 4×LD₅₀ 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 (ED₅₀) of antivenom, defined as the ratio µg venom/mL antivenom at which 50% of the injected mice were protected. ED₅₀s were estimated by probits, as described in Section 2.7.

2.10 Antivenom immunoprofiling by ELISA

Wells in MaxiSorp plates (NUNC, Roskilde, Denmark) were coated overnight with 0.6 µg of each HPLC venom fraction, dissolved in 100 µL PBS. Then, wells were blocked by adding 100 µL PBS containing 2% (w:v) bovine serum albumin (BSA, Sigma) at room temperature for 1 h, and washed five times with PBS. A dilution of each antivenom in PBS containing 2% BSA was prepared such that the protein concentration was 86 µg/mL (as measured by their absorbance at 280 nm on a NanoDrop® 2000c instrument, Thermo Scientific), and 100 µL were added to the wells for 2 h. After five washings with PBS, 100 µL of a 1:2000 dilution of rabbit anti-horse IgG (whole molecule)-alkaline phosphatase conjugated antibodies (Sigma A6063, in PBS, 2% BSA) was added to each well for 2 h, and then washed five times with FALC buffer (0.05 M Tris, 0.15 M NaCl, 20 µM ZnCl₂, 1 mM MgCl₂, pH 7.4). Color was developed by adding 100 µL of *p*-nitrophenyl phosphate (1 mg/mL in 9.7% v/v diethanolamine buffer, pH 9.8), and the absorbances at 405 nm were recorded at several time intervals in a microplate reader (Multiskan FC, Thermo Scientific).

3.0 Results and Discussion

3.1 Venomics

A detailed proteomics characterization was performed on the pooled venom from *A. laevis*. From 20 fractions resolved by RP-HPLC, 35 peptidic bands were obtained after SDS-PAGE separation (Fig.1). By in-gel digestion and MALDI-TOF-TOF analysis, 99.2% of the protein bands could be assigned to toxin families. As shown in Fig.2, the predominant family of proteins in this venom corresponds to PLA₂s

(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 PLA₂ 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 PLA₂ 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 PLA₂s (33% and 19% of all venom proteins, respectively), *A. laevis* venom displays the opposite relative venom composition, being dominated by PLA₂s (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

Broome by more than 600 km of mostly unsuitable (deep water) habitat. Potentially, the observed differences could also be explained by interspecific hybridization, which has previously been observed for *A. laevis* and closely related *A. fuscus* on Ashmore Reef, where hybrid individuals closely resemble *A. laevis* in phenotype (Sanders et al., 2014). In similarity with the predominance of PLA₂s over 3FTxs herein reported for *A. laevis* venom, a transcriptomic study on the venom glands of *Aipysurus eydouxii* revealed the existence of as many as sixteen unique PLA₂ transcripts, in contrast to a single transcript corresponding to a 3FTx (Li et al., 2005). This could suggest that both *Aipysurus* species share the same venom compositional predominance. However, assessment of this possibility would require a direct examination of the *A. eydouxii* venom proteins, in addition to its venom gland transcripts.

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

Unlike several terrestrial elapids (Aird, 2002; Laustsen et al., 2015a), nucleosides were not detected in *A. laevis* venom. On the other hand, its high content of PLA₂s suggests that this venom might induce myotoxicity, as previously shown in experimental studies (Zimmerman et al., 1992c; Ryan and Yong, 1997, 2002). Systemic myotoxicity, i.e. rhabdomyolysis, with myoglobinuria characterizes envenomings by some species of sea snakes in humans (Reid, 1961), and is responsible for acute kidney injury. However, when a pool of PLA₂ fractions of *A. laevis* venom was tested for myotoxicity in mice, only a mild effect was observed, as judged by increments in

plasma CK activity. Mice receiving PBS had CK activity of 215 ± 10 U/L, and mice injected in the gastrocnemius, thigh, or soleus muscles with 30 μ g of the PLA₂ fraction pool had plasma CK activities of 926 ± 160 U/L, 1196 ± 119 U/L, and 764 ± 182 U/L (mean $\pm$ SEM), respectively. Increments in CK were significant only when the PLA₂ fraction pool was injected in the gastrocnemius and thigh muscles ($p < 0.05$). Thus, *A. laevis* PLA₂s only induced a mild myotoxic effect. In agreement, histological analysis of the soleus muscle 3 h after injection of PLA₂ fraction pool showed only few scattered necrotic fibers (Fig.4). These observations contrast with the prominent increment in plasma CK activity described for other elapid venoms, such as that of *Micrurus nigrocinctus* (Fernández et al., 2011). By using native zone electrophoresis, it was observed that all PLA₂ fractions (peaks 9-18) migrated towards the anode, indicating that they were of acidic nature (not shown). This observation could explain the low myotoxic effect of the PLA₂ pool tested, since commonly PLA₂s having potent myotoxic effects are of a basic nature (Montecucco et al., 2008). Our results suggest that myotoxicity is unlikely to be a significant effect in envenomings by *A. laevis*.

In agreement with its proteomic composition showing an abundance of PLA₂s, high PLA₂ activity of the venom was confirmed *in vitro* (Fig.5A), whereas no proteinase activity was detected (Fig.5B), in line with the absence of these enzymes in the venom proteome. Three-finger toxins were shown to represent the second major group of venom proteins in terms of abundance (25.3%), and all of them were identified as short neurotoxin isoforms (Table 1), previously characterized by Maeda and Tamiya (1976) and Ducancel et al. (1990). These short neurotoxins have been shown to bind with high affinity to nicotinic receptors at the motor end-plate of muscle fibers, leading to flaccid paralysis, which may result in respiratory failure and death (Maeda and Nobuo, 1976; Ducancel et al., 1990).

All venom fractions were examined for acute toxicity in CD1 mice, and LD₅₀ 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 PLA₂s (fractions 5-18) induced lethality in mice, although the LD₅₀ values of the short neurotoxins were 10-40 fold lower than those of the PLA₂s. 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 than 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 ED₅₀ 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 BioCSL Sea 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 PLA₂ 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

different snake venoms were used for production of BioCSL Sea Snake Antivenom, and that the “monovalence” of this antivenom is primarily due to the horses being boosted with *E. schistosa* venom immediately before bleeding (Chetty et al., 2004; O’Leary and Isbister, 2009; Herrera et al., 2014). Therefore, unexpected cross-reactivity is not an unlikely event. *E. schistosa* venom has a high abundance of 3FTxs with a high degree of conservation relative to the short neurotoxins found in *A. laevis* (Fig.7B), and it is therefore not surprising that the BioCSL Sea Snake Antivenom has a strong preference for fractions 1-4, containing short neurotoxins from *A. laevis*. In comparison, the PLA₂s found in *M. nigrocinctus* venom are not more similar to the PLA₂s found in *A. laevis* venom than the PLA₂ reported for *E. schistosa* venom (Fig.7C) (Fohlman and Eaker, 1977). However, it is speculated that the much higher abundance of PLA₂s in the immunization mixture used for producing ICP Anti-Coral Antivenom in itself drives the immunological response towards a stronger recognition against PLA₂s in general. Given that BioCSL Sea Snake Antivenom readily cross-recognizes the neurotoxic components having the highest Toxicity Scores, and since this antivenom was shown to neutralize whole venom in rodents, BioCSL Sea Snake Antivenom should be useful for treating human snakebite accidents inflicted by *A. laevis*.

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

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 PLA₂s (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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Figure legends

Figure 1: Separation of *A. laevis* (A) venom proteins by RP-HPLC (C), followed by SDS-PAGE (B). Two mg of venom were fractionated on a C₁₈ column and eluted with an acetonitrile gradient (dashed line), as described in Methods. Fractions were further separated by SDS-PAGE under reducing conditions. Molecular weight markers (Mw) are indicated in kDa. Coomassie-stained bands were excised, in-gel digested with trypsin, and subjected to MALDI-TOF/TOF analysis for assignment to protein families, as shown in Table 1.

Figure 2: Composition of *A. laevis* venom according to protein families, expressed as percentages of the total protein content. 3FTx: three-finger toxin; PLA₂: phospholipase A₂; CRISP: cysteine-rich secretory protein; CCM: complement control module. (see Table 1).

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

Figure 4: Light micrographs of sections of mouse soleus muscles collected 3 h after injection of either phosphate-buffered saline (PBS) solution (A) or 30 µg of a pool of all the PLA₂ fractions of the venom of *A. laevis* (B) (see Methods for experimental details). A normal histological pattern is observed in (A), whereas few scattered necrotic muscle

fibers (arrows) are observed in **(B)**, thus evidencing the mild myotoxic activity of this venom. Hematoxylin-eosin staining. Bar represents 100 μ m.

Figure 5: **(A)** Comparison of the phospholipase A₂ activity between the venoms of *Aipysurus laevis*, *Dendroaspis polylepis*, *Naja kaouthia*, and *Micrurus nigrocinctus*. *A. laevis* displays high enzymatic activity, although lower than *M. nigrocinctus*. **(B)** Comparison of the proteolytic activity between the venoms of *A. laevis*, *D. polylepis*, *N. kaouthia*, *M. nigrocinctus*, and *Bpithrops asper*, evaluated on azocasein. *A. laevis* shows negligible proteinase activity.

Figure 6: ELISA-based immunoprofiling of antivenoms (**CSL Sea Snake**: BioCSL Sea Snake Antivenom from BioCSL Limited, **ICP Micrurus**: Monospecific *Micrurus nigrocinctus* Anticoral Antivenom from Instituto Clodomiro Picado, for comparison, and a negative control (**Horse negative**: normal serum from non-immunized horses from Instituto Clodomiro Picado) to all fractions of *A. laevis* venom separated by RP-HPLC (see Methods for details). For identification of venom fractions refer to [Table 2](#). Each bar represents mean $\pm$ SD of triplicate wells.

Figure 7: **(A)** Comparison between the venom profiles and similarities of toxins from *Aipysurus laevis*, *Enhydrina schistosa* (*Hydrophis schistosa*), and *Micrurus nigrocinctus*. *E. schistosa* venom is used for production of BioCSL Sea Snake Antivenom, while *M. nigrocinctus* venom is used for production of ICP's Monospecific *M. nigrocinctus* Anticoral Antivenom. *Venom composition is based on venomomics studies of *M. nigrocinctus* ([Fernández et al., 2011](#)) and the reported study of *Hydrophis cyanocinctus* ([Calvete et al., 2012](#)), as such studies have not been performed on *E.*

657 *schistosa*. Notice that the PLA₂ 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 PLA₂ 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	M/S/MS-derived sequence [*]	Conf (%)	Sc	Protein family ^{**}	Related protein, code	
1	21.8	10	m/z	z					
			1564.8	1	TTTDCADDSCYBK	99	9	3FTx	short neurotoxin D
			1450.8	1	XTCCNBSSBPK	99	19		<i>Aipysurus laevis</i> , P19960
			1300.8	1	GCGCPBVBPgXK	99	18		
2a	0.3	15	1564.5	1	TTTDCADDSCYBK	99	8	3FTx	short neurotoxin D
			1450.6	1	XTCCNBSSBPK	99	14		<i>Aipysurus laevis</i> , P19960
			1300.6	1	GCGCPBVBPgXK	99	16		
2b	0.3	10	1300.6	1	GCGCPBVBPgXK	99	9	3FTx	short neurotoxin D <i>Aipysurus laevis</i> , P19960
3a	1.0	15	1436.6	1	TTTDCADDSCYK	99	12	3FTx	short neurotoxin
			1564.7	1	TTTDCADDSCYBK	99	11		<i>Aipysurus laevis</i> , P19960
			1450.7	1	XTCCNBSSBPK	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		<i>Aipysurus laevis</i> , P19960
			1450.7	1	XTCCNBSSBPK	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		<i>Aipysurus laevis</i> , P19960
4b	0.4	10	1436.6	1	TTTDCADDSCYK	99	10	3FTx	short neurotoxin
			1450.7	1	XTCCNBSSBPK	99	14		<i>Aipysurus laevis</i> , P19960
			1300.7	1	GCGCPBVBPgXK	99	13		
			1564.7	1	TTTDCADDSCYBK	96.8	8		
5a	0.1	29	1758.8	1	NXYBFDNMXXBCANk	99	10	PLA ₂	phospholipase A ₂
			1895.8	1	AHDDCYGVAED(N ⁴⁹)GCSPK	99	26		<i>Aipysurus eydouxi</i> , ~Q5DNC9
			1774.8	1	NXYBFDN(M ⁶⁰)XBCANk	99	11		
5b	0.3	18	1758.9	1	NXYBFDNMXXBCANk	99	14	PLA ₂	phospholipase A ₂
			1870.9	1	CYCGWGGSGTPVDAXDR	99	14		<i>Aipysurus eydouxi</i> , ~Q5DNE1
			2995.5	1	ATWHYMDYGCYCGWGGSGTPVDAXDR	99	19		
			2337.1	1	MDYGCYCGWGGSGTPVDAXDR	99	19		

6	0.5	18	2091.0	1	YGCCYCGWGGSGTPVDAXDR	99	17		
			1758.9	1	NXYBFDNMXBCANK	99	13	PLA ₂	phospholipase A ₂
			1870.9	1	CYCGWGGSGTPVDAXDR	99	17		<i>Aipysurus eydouxi</i> , ~Q5DNE1
			2994.5	1	ATWHYMDYGCYCGWGGSGTPVDAXDR	99	21		
			2337.1	1	MDYGCYCGWGGSGTPVDAXDR	99	19		
			2091.0	1	YGCCYCGWGGSGTPVDAXDR	99	21		
7	0.2	-	-	-	-	-	-	unknown	-
8	0.4	-	-	-	-	-	-	unknown	-
9	9.6	10	2091.0	1	DYGCYCGAGSGTTPVDAXDR	99	11	PLA ₂	β -bungarotoxin chain A ₂ <i>Bungarus caeruleus</i> , ~Q8QFW3
10	6.2	10	2337.1	1	MDYGCYCGWGGSGTPVDAXDR	99	11	PLA ₂	phospholipase A ₂
			2995.5	1	ATWHYMDYGCYCGWGGSGTTPVDAXDR	99	18		<i>Aipysurus eydouxi</i> , ~Q5DNE1
			1710.9	1	NXYBFDN(M ⁴⁸)XBCANK	99	14		
			2091.0	1	YGCCYCGWGGSGTTPVDAXDR	99	17		
			1870.9	1	CYCGWGGSGTTPVDAXDR	91	9		
11a	3.1	23	1758.9	1	NXYBFDNMXBCANK	99	14	PLA ₂	phospholipase A ₂ <i>Aipysurus eydouxi</i> , ~Q5DNE1
11b	9.1	15	2912.3	1	ATWHY(M ⁸⁹)DYGCYCGSGSGTTPVDAXDR	99	12	PLA ₂	phospholipase A ₂
			1998.8	1	R	98	8		<i>Aipysurus eydouxi</i> , ~Q5DND8
			2896.3	1	VHDDCYGV AEDNGCYPK	95.4	8		
			1774.8	1	ATWHYMDYGCYCGSGSGTTPVDAXDR	67.2	7		
					NXYBFDN(M ⁹⁰)XBCANK				
11c	7.2	10	2091.0	1	YGCCYCGWGGSGTTPVDAXDR	99	10	PLA ₂	phospholipase A ₂
			2895.4	1	ATWHYMDYGCYCGSGSGTTPVDAXDR	99	14		<i>Aipysurus eydouxi</i> , ~Q5DNE1
			1710.9	1	NXYBFDN(M ⁹¹)XBCANK	99	10		
12a	2.7	15	2896.3	1	ATWHYMDYGCYCGSGSGTTPVDAXDR	97.3	6	PLA ₂	phospholipase A ₂ <i>Aipysurus eydouxi</i> , ~Q5DNE1
			1774.8	1	NXYBFDN(M ⁹³)XBCANK	95.3	6		
12b	3.3	10	2835.4	1	WTXYSWBCTENVPTCNSESGCBK	99	15	PLA ₂	phospholipase A ₂
			2896.4	1	ATWHYMDYGCYCGSGSGTTPVDAXDR	99	23		<i>Aipysurus laevis</i> , P08872
			1213.6	1	CFEAPYNNK	99	16		
			1758.9	1	NXYBFDNMXBCANK	99	18		
			1894.8	1	AHDDCYGV AEDNGCSPK	99	23		<i>Aipysurus eydouxi</i> , ~Q5DNE1
13a	1.7	20	1758.8	1	NXYBFDNMXBCANK	99	9	PLA ₂	phospholipase A ₂
			1213.6	1	CFEAPYNNK	99	12		<i>Aipysurus laevis</i> , P08872
			2013.9	1	AHDDCYGV AEDNGCYPK	99	20		

* 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; PLA₂: phospholipase A₂; CRISP: cysteine-rich secretory protein; CCM: complement control module.

Table 2: LD₅₀ values of *Aipysurus laevis* venom and the RP-HPLC isolated fractions

Peak	%	Protein family	LD ₅₀ (95% C.I.)	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>

¹Toxicity Score was defined as the ratio of protein fraction abundance (%) in the venom divided by its estimated median lethal dose (LD₅₀) for CD-1 mice by i.v. injection.

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

N.t. : not tested

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Graphical Abstract