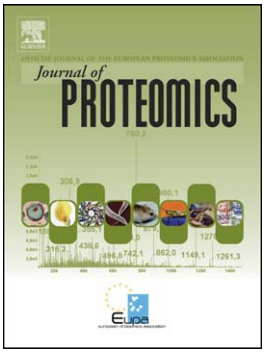

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Two color morphs of the pelagic yellow-bellied sea snake, *Pelamis platura*, from different locations of Costa Rica: snake venomics, toxicity, and neutralization by antivenom

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ABSTRACT

The yellow-bellied sea snake, *Pelamis platura*, is the most broadly distributed snake species. Despite being endowed with a highly lethal venom, a proteomic analysis of its toxin composition was unavailable. The venoms of specimens collected in Golfo de Papagayo and Golfo Dulce (Costa Rica), where two distinctive color morphs occur, were chromatographically compared. The latter inhabits a fjord-like gulf where the transit of oceanic sea snakes into and from the basin is restricted, thus possibly affecting gene flow. RP-HPLC evidenced a conserved venom protein profile in both populations, despite their divergent color phenotypes. Following a trend observed in other sea snakes, *P. platura* venom is relatively simple, being composed of proteins of the three-finger toxin (3FTx), phospholipase A_2 (PLA_2), cysteine-rich secretory protein (CRISP), 5′-nucleotidase, and metalloproteinase families. The first three groups represent 49.9%, 32.9%, and 9.1% of total venom protein, respectively. The most abundant component (~26%) is pelamitoxin (P62388), a short-chain 3FTx, followed by a major basic PLA_2 (~20%) and a group of three isoforms of CRISPs (~9%). Whereas isolated pelamitoxin was highly lethal to mice, neither the PLA_2 nor the CRISP fraction caused death. However, the PLA_2 rapidly increased plasma creatine kinase activity after intramuscular injection, indicating its myotoxic action. Differing from myotoxic PLA_2s of viperids, this PLA_2 was not cytolytic to murine myogenic cells *in vitro*, suggesting possible differences in its mechanism of action. The median lethal dose (LD_{50}) estimates for *P. platura* crude venom in mice and in three species of fishes did not differ significantly. The sea snake antivenom manufactured by CSL Ltd. (Australia), which uses *Enhydrina schistosa* as immunogen, cross-recognized the three major components of *P. platura* venom and, accordingly, neutralized the lethal

activity of crude venom and pelamitoxin, therefore being of potential usefulness in the treatment of envenomations by this species. (297 words)

1. INTRODUCTION

Snake venoms are complex mixtures of bioactive molecules, including enzymes, non-enzymatic proteins, and several inorganic components [1]. In the last fifteen years, the application of proteomic methods to assess the compositional diversity of venoms has revolutionized our knowledge of toxic secretions in advanced snakes, leading to a deeper understanding of their biological functions and medical implications [2-6]. Not surprisingly, most studies in 'venomics' have been conducted in species of great medical importance, although the description of venoms from other species continues. These studies convey that an integral catalog of the secreted toxins is needed in order to fully understand the pathophysiology of envenomations and to improve their treatments [7]. In addition, assessing venom proteomes provides insights into the evolution of toxins and the contexts in which they evolved [8]. In some cases, phylogenetically related snake species resemble each other in venom activity and composition [9,10], and thus venom makeup could result from the evolutionary history of those species [11]. However, both substantial divergences and convergences in toxin constituents and/or their proportions have also been noticed between closely related species as well as between phylogenetically distant taxa [12-14]. Since venom denotes a key adaptation to subdue and digest prey, this situation implies that venom variation also results from natural selection acting upon differences in the identity or type of prey [13-15].

Regardless of the causes that shape it, venom variation endows snakes with the ability to adapt to different ecological niches and to diversify within them. One group that has recently done so is comprised by the true sea snakes (Hydrophiinae: Hydrophiini), a clade of nearly sixty species of elapids that diverged from a common ancestor and experienced rapid radiation in the last 1.5-3 million years [16]. The Hydrophiini evolved

the ability to persist in the severe conditions imposed by marine environments: high levels of solar radiation and salinity, extreme temperatures, and underwater shelters. They present a characteristic paddle-like tail that enhances their swimming ability, and are ovoviviparous, spending most of their lives in their aquatic habitats [17,18]. Most sea snakes are benthic predators that exhibit great diet specialization and are constantly looking for prey in crevices of rocks and corals, or on the muddy bottom of their habitats [19]. A notable exception to these modes is the yellow-bellied sea snake *Pelamis platura* (recently proposed to change to *Hydrophis platura*; [16]), the only truly pelagic species that completes its lifecycle in the water column [20].

The yellow-bellied sea snake is an abundant species throughout most of its range, and can be often observed floating on the surface of slicks, i.e. long smooth lines in the surface where sea currents converge [21,22]. Unlike other sea snakes [23], *P. platura* feeds on a variety of small fishes on the water surface [21,24-26]. Its venom contains pelamitoxin, a three-finger toxin (3FTx) acting as potent postsynaptic blocker with high lethality to experimental animals [27,28]. Despite *P. platura* being a widespread sea snake species with a highly lethal venom, a comprehensive account of its venom constituents was still lacking, perhaps as a consequence of its low medical impact.

Along its broad distribution in tropical and subtropical waters of the Indo-Australian region and the Pacific coast of the American continent [29], the largest for any snake species, two types of populations have been described for *P. platura* [30]: those restricted to particular areas in warm temperature waters (i.e. resident populations); and those composed of individuals that drift in currents throughout a wider region (i.e. waif populations, often in cooler waters). A resident population of *P. platura* restricted to the Golfo Dulce, in southwestern of Costa Rica, was reported recently [31]. In this locality,

most individuals are bright yellow (Fig.1D), but this morph is extremely rare in other localities of the species distribution [21,22,24]. Dominance of the yellow-morph in this locality might reflect gene flow restrictions. Biogeographically, Golfo Dulce is a fjord-type gulf, where the sea water enters slowly [32,33] and restricts the movement of oceanic sea snakes into and from the basin [31,34]. In contrast, the population off the Pacific coast of Costa Rica exhibits a typical bicolored or tricolored pattern (Fig.1A) that prevails elsewhere along the species distribution [24,25].

The apparent restriction of gene flow in these distinct color-morph populations is of great interest [35] because it could lead to differences in the expression of venom constituents, and in turn could reveal differences in the evolutionary forces that underlie venom variation in this sea snake, as observed in other wide-ranging venomous snakes [36,37]. Therefore, the present study first compared the chromatographic profiles of the venoms of these two morphologically distinctive populations of *P. platura* from Costa Rica, and then, since they showed identical chromatograms, the venom composition of *P. platura* from Golfo Dulce was analyzed using venomics and the assessment of several *in vitro* and *in vivo* activities. In addition, the neutralization of this venom by a sea snake antivenom from Australia was evaluated.

2. MATERIALS AND METHODS

2.1 Venom samples

Adult individuals of *P. platura* were collected at approximately 2 Km from the shore, at two locations along the Pacific coast of Costa Rica (Fig.1B): Golfo de Papagayo

(~10°34′30″N, 85°43′19″ W; Guanacaste province; 47 individuals), and Golfo Dulce (~8°35′36″ N, 83°16′09″ W; Puntarenas province, 84 individuals). At the first location, all individuals exhibited the bicolored or tricolored pattern of the yellow-bellied sea snake, whereas all individuals from the second locality were yellow morphs. Venom was extracted directly into non-heparinized microhematocrit tubes, and specimens were thereafter released near their collecting sites. Venoms from each of the two populations were pooled, and finally conserved by freeze-drying and storage at -20°C.

2.2 Reverse-phase HPLC comparative profiling

The venoms (~2 mg) of *P. platura* from Golfo de Papagayo or Golfo Dulce, respectively, were dissolved in 200 µL of water containing 0.1% trifluoroacetic acid (TFA), centrifuged at 15,000 x*g* for 5 min to remove debris, and fractionated by RP-HPLC on a C_{18} column (Teknokroma; 4.6 x 250 mm, 5 µm particle) using an Agilent 1200 chromatograph. Elution was performed at 1 mL/min by applying a gradient towards solution B (acetonitrile, containing 0.1% TFA), as follows: 0% B for 5 min, 0-15% B over 10 min, 15-45% B over 60 min, 45–70% B over 10 min, and 70% B over 9 min. Absorbance of the eluent was recorded at 215 nm and chromatograms were compared using the ChemStation B.04.01 software (Agilent).

2.2 Venomic characterization

Since the comparison by RP-HPLC of the venoms of *P. platura* from Golfo de Papagayo and Golfo Dulce did not reveal differences (Fig.2), only the venom from Golfo Dulce was subsequently analyzed following the 'snake venomics' strategy [4,38], with

slight modifications [10]. Briefly, HPLC fractions were collected manually, dried in a vacuum centrifuge, re-dissolved in water, and further separated by SDS-PAGE under reducing conditions (5% 2-mercaptoethanol at 100°C for 5 min) in 12% gels. After Coomassie blue R-250 staining, gel images were recorded using ChemiDoc/ImageLab (Bio-Rad) for densitometry analysis, which was combined with the integration of chromatographic peak areas to obtain estimates of protein relative abundances. Protein bands were then excised and subjected to in-gel reduction (10 mM dithiothreitol), alkylation (50 mM iodacetamide), and overnight digestion with trypsin in a ProGest processor (Digilab), following manufacturer's recommendations. The resulting peptides were extracted and characterized by tandem mass spectrometry, as described below.

2.3 Mass spectrometry

Tryptic digests were dried *in vacuo*, redissolved in 10-40 µl of 0.1% formic acid, depending on band size, and submitted to nano-UPLC-ESI-MS/MS mass spectrometry. Toward this end, tryptic peptides were separated by nano-Acquity UltraPerformance LC® (UPLC®) using a BEH130 C_{18} column (100 µm x 100 mm, 1.7µm particle) on-line with a Waters SYNAPT G2 High Definition Mass Spectrometry System. The flow rate was set to 0.6 µl/min and the column was developed with a linear gradient of 0.1% formic acid in water (solution A) and 0.1% formic acid in acetonitrile (solution B), isocratically 1% B for 1 min, followed by 1-12% B for 1min, 12-40% B for 15min, 40-85% B for 2min. Doubly- and triply-charged ions were selected for collision-induced dissociation (CID) MS/MS. Fragmentation spectra were interpreted manually, processed in ProteinLynx Global SERVER 2013 version 2.5.2., or by using the online form of the Mascot program at http://www.matrixscience.com against the NCBI non-redundant database. MS/MS mass

tolerance was set to ± 0.6 Da. Carbamidomethyl cysteine, and propionamide cysteine and oxidation of methionine were fixed and variable modifications, respectively. Some tryptic peptides were analyzed by MALDI-TOF-TOF on a 4800 Plus Proteomics Analyzer (Applied Biosystems) using α-cyano-hydroxycinnamic acid as matrix, under previously described conditions [10].

2.4 Venom lethality

Animal experiments were conducted in CD-1 mice of either sex, following protocols approved by the Institutional Committee for the Use and Care of Animals (CICUA), University of Costa Rica. Mice were housed in cages for groups of 4-6, and provided food and water *ad libitum*. The lethal potency of the venom was tested by intraperitoneal (i.p.) and intravenous (i.v.) routes, respectively, in groups of five mice of 16-18 g body weight. Deaths induced by the injection of varying doses of venom (1.25-10 μg), dissolved in 0.12 M NaCl, 0.04 M sodium phosphate buffer (PBS; pH 7.2), were recorded after 48 hr. The median lethal dose (LD_{50}) was estimated by probits. In addition, the lethal potency of the crude venom was tested in three species of fish: spotted snapper *Lutjanus guttatus* (Lutjanidae), scissortail damselfish *Chromis atrilobata* (Pomacentridae), and sleeper goby *Dormitator maculatus* (Eleotridae), after permits issued by the Ministry of Environment of Costa Rica SINAC-ACAT 069-2012. The first two are common species off the Pacific coast of Costa Rica and thus are sympatric with both populations of *P. platura*, and at least *C. atrilobata* has been observed by one of the authors (M. Sasa) to be a prey for this snake. The sleeper goby is a freshwater species, abundant in permanent creeks along the Pacific coast of Central America. Venom was injected by i.p. route in groups of five

fishes of similar weight (variation coefficient <10%), kept in 20 L aquaria. Water (marine or freshwater) temperature was maintained at 22 °C, and varying doses of venom (0.02-1.50 μg), dissolved in distilled water, were adjusted for the mean weight of the group. Deaths were recorded after 48 hours, and the LD_{50} was estimated by probits.

2.5 Myotoxic activity

Groups of four mice (18-20 g body weight) received an intramuscular (i.m.) venom injection of 1 or 2 μg, respectively, in 50 μL of PBS, into their right gastrocnemius. A control group was treated with PBS alone. After three hours, blood samples were obtained from the tip of the tail and collected into heparinized capillary tubes; then, after centrifugation, the plasma creatine kinase (CK) activity was determined using a UV kinetic assay (CK-Nac, Biocon Diagnostik) [39]. CK activity was expressed in units/L. Mice were euthanized by CO_2 inhalation 6 h after venom injection, and samples of the injected muscle tissue were obtained, fixed in 10% formalin, embedded in paraffin, and processed for histologic evaluation of hematoxylin/eosin-stained sections.

2.6 Cytotoxic activity

The cytotoxic effect of the venom was assayed on the murine myogenic cell line C2C12 (ATCC CRL-1772), as described [40]. Varying amounts of venom, diluted in 150 μL of Dulbecco's Modified Eagle's Medium supplemented with 1% fetal calf serum (assay medium; DMEM, 1% FCS), were added to near-confluent cell monolayers in 96-well plates, after removal of growth medium (DMEM, 10% FCS), in triplicate wells. Cells were tested both in their myoblast stage and after differentiation to multinucleated myotubes,

obtained 4-6 days after confluence, grown in the presence of 1% FCS. Controls for 0 and 100% toxicity consisted of assay medium, and 0.1% Triton X-100 diluted in assay medium, respectively. After 3 h at 37 °C, cell supernatants were collected to determine the lactic dehydrogenase (LDH) activity released from damaged cells, using a UV kinetic assay (LDH-P, Wiener Laboratories). Venom of the Central American coral snake, *Micrurus nigrocinctus* (Elapidae), was included as a comparative control.

2.7 Phospholipase A_2 activity

Venom phospholipase A_2 (PLA_2) activity was determined on the monodisperse synthetic substrate 4-nitro-3-octanoyl-benzoic acid (NOBA) [41]. Varying amounts of venom, dissolved in 25 µL of water, were added to 200 µL of 10 mM Tris, 10 mM $CaCl_2$, 0.1 M NaCl, pH 8.0, in triplicate wells of a microplate. After mixing, 25 µL of NOBA (1 mg/mL in acetonitrile) were added, to achieve a final substrate concentration of 0.32 mM. The mixtures were incubated for 60 min at 37 ºC, and absorbances at 405 nm were recorded. PLA_2 activity was expressed as the change in absorbance x 1000. Venom of the Central American coral snake, *Micrurus nigrocinctus* (Elapidae), was included as a comparative control.

2.8 Neutralization of lethality by antivenom

The ability of the equine CSL Ltd. sea snake antivenom (Victoria, Australia; batch 054908201, expiry date 04-2015; 167 g protein/L) to neutralize the lethal effect of *P. platura* venom was tested in mice. Mixtures were prepared containing a fixed venom/antivenom ratio (0.2 mg/mL), and incubated at 37 ºC for 30 min. This single ratio was chosend on the basis of the authors' experience on the potency of antivenoms against

other elapidic venoms such as *Micrurus nigrocinctus*. Then, aliquots of the mixture, containing four LD_{50} of venom, were injected either i.p. or i.v. into CD-1 mice (16-18 g). Control mice received four LD_{50} of venom incubated with PBS instead of antivenom. Deaths occurring during 48 h were recorded. The neutralizing ability of this antivenom was additionally tested against the lethal effect of the isolated major neurotoxin of the venom (pelamitoxin). Control mice received an i.v. injection containing four LD_{50} of toxin alone, whereas a mixture of toxin and antivenom (0.2 mg toxin/ml antivenom), preincubated for 30 min at room temperature, was injected to another group of mice under otherwise identical conditions. In addition, the equine Central American coral snake antivenom (Elapidae) manufactured at Instituto Clodomiro Picado (University of Costa Rica; batch 5200313, expiry date March 2016; 30 g protein/L) was tested against the crude venom of *P. platura*, under identical conditions as for the CSL sea snake antivenom.

2.9 Immunochemical analyses

The cross-recognition of *P. platura* venom and its three major components (peaks 1--2, 9, and 16; see Results) by the CSL antivenom was analyzed by enzyme-immunoassay (EIA) [42] and gel immunodiffusion (GID) [43]. In EIA, whole venom (1 μg/well) or fractions (0.2 μg/well) were adsorbed onto 96-well microplates in 100 μL of 0.1 M Tris, 0.15 M NaCl, pH 9.0 buffer by overnight incubation at room temperature, in duplicate. After five washings with PBS, non-specific binding sites were blocked with 100 μL of PBS containing 2% bovine serum albumin (BSA) for 30 min. Varying dilutions of CSL antivenom, prepared in 0.2% BSA-PBS, were then incubated for 2 h, followed by five washings with PBS. Bound antibodies were detected by addition of an anti-horse

immunoglobulin-alkaline phosphatase conjugate (Sigma), diluted 1:3000 in 0.2% BSA-PBS, and further incubation for 2 h. After four washings with PBS, and a final wash with 0.05 M Tris, 0.15 M NaCl, 20 μM $ZnCl_2$, 1 mM $MgCl_2$, pH 7.4 buffer, color was developed with *p*-nitrophenylphosphate (1 mg/ml in diethanolamine buffer, pH 9.8) for 30 min and measured in a microplate reader (Thermo) at 405 nm. Normal equine serum from a non-immunized animal was included as a control for background signal.

GID was performed in 1% agarose dissolved in PBS, pH 7.2. Wells were punched and filled with 40 μL of either CSL antivenom, whole venom, or each of the three major venom components described above. After 24 h of diffusion at room temperature, precipitation lines were recorded using the ChemiDoc/ImageLab software.

2.10 Statistical tests

Results of *in vitro* assays are presented as mean ± SD. Comparisons between means of two groups were performed with the Student's *t*-test, and values of $p<0.05$ were considered significant. Venom LD_{50} estimations for each *in vivo* model (mice or fishes) were conducted under a probit model. The goodness of fit of each data set to the model was evaluated using Chi^2 tests. The LD_{50} values for each species were compared using the doses ratio test [44] implemented in POLO-Plus (LeOra Software, version 2.0). This test computes the ratio of one lethal dose to another and estimates 95% confidence intervals for each ratio using the parameters of the probit function. The ratio test is a more powerful method of comparison than the classical analysis of confidence interval overlap [45].

3. RESULTS AND DISCUSSION

As reported in the literature [31,34,46], obvious color pattern differences were found in the two populations of *P. platura* sampled (Golfo de Papagayo and Golfo Dulce). Individuals from the latter location exhibited a mostly patternless, yellow body coloration phenotype (Fig.1D), differing from the black dorsum/yellow-bellied morphology (Fig.1A) most commonly reported for this species in the Golfo de Papagayo. Due to the conspicuous phenotypic differences, and considering that the population inhabiting Golfo Dulce, a fjord-like gulf protected from strong surface currents, might have been genetically isolated from the oceanic coastal populations [31,43,35], venom variation between the two color morphs was assessed. As shown in Fig.2, a comparison of their venom RP-HPLC profiles revealed a conserved phenotype, and thus the distinctive color pattern variation between these two populations is not associated with any significant differences in their expression of venom proteins.

The chromatographic profile of *P. platura* venom appears relatively simple (Fig.3), and shows similarities to those of venoms from some terrestrial elapids analyzed in our laboratories using the same methodology, such as the African spitting cobras [47] and some coral snakes [48,49], or the marine elapid *Hydrophis cyanocinctus* [50]. The venom of *P. platura* was separated into twenty fractions (Fig.3), and 32 out of the 36 protein bands obtained after electrophoretic separation of the chromatographic peaks could be assigned to known protein families following in-gel digestion and tandem mass spectrometry (Table 1). The scarcity of venom precluded pursuing further the identification of few minor components that remained unknown (altogether <1.5% of the protein content). As represented in Fig.1C, half of the protein constituents of *P. platura* venom belong to the three-finger toxin (3FTx) family (49.9%), whereas one-third correspond to PLA_2s (32.9%).

Cysteine-rich secretory proteins (CRISP) also represent a considerable proportion of this venom (9.1%), but only a few other types of commonly found snake venom components were detected, including 5′-nucleotidases (5′-NU) and metalloproteinases (SVMP), both below 1% in relative protein abundance (Fig.1C). In agreement with the colorless visual aspect of this venom, L-amino acid oxidase (which provides yellow color to snake venoms owing to its typical adsorption maxima at 465 and 380 nm due to the flavide adenine dinucleotide cofactor [51]) was not detected. Altogether, these findings follow the general trend observed for sea snake venoms, which biochemically tend to be simpler than those of terrestrial venomous snakes [17]. Unexpectedly, low amounts of hemoglobins α and β (2.1%), albumin (1.1%), and transferrin (1.8%) were identified in the venom (Fig.1C), but their presence could be explained by mild contamination with blood, possibly due to slight trauma during the difficult venom extraction procedure for this snake species. In addition, transferrin has previously been reported in the proteomic analysis of at least one snake venom, that of *Pseudechis australis* [52], therefore the possibility of its presence representing a true product of venom gland secretion in *P. platura* cannot be ruled out.

Among the various 3FTx components of *P. platura* venom, internal peptide sequences characteristic of both "short-chain" (type I) and "long-chain" (type II) subgroups of this protein family were identified (Table 1), in agreement with the early origin of these two types of 3FTx in elapid snakes, before the division between terrestrial Australian elapids and sea snakes, and terrestrial African and Asian elapids [53]. 3FTxs often display α-neurotoxic activity by means of their high-affinity binding to nicotinic acetylcholine receptor in the motor end-plate at the neuromuscular junction of diverse groups of animals [54], therefore showing potent paralyzing and lethal effects. The most abundant component

of *P. platura* venom (~26%; peaks 1-2 of Table 1) was identified as "pelamitoxin a" (P62388), a short-chain 3FTx previously isolated and sequenced [27,28,55]. The existence of an isoform named "pelamitoxin b" was reported later [56], differing from the former by a single amino acid at the tenth residue (Q/E), and this might explain the close elution and undistingushable isotope-averaged molecular mass observed for the overlapping peaks 1-2 (Fig.3), which were pooled for further functional and immunochemical analyses. Expectedly, the various 3FTx peptide sequences obtained showed high similarity to proteins from phylogenetically-related marine elapid genera such as *Enhydrina*, *Hydrophis*, *Lapemis*, and from diverse terrestrial elapids (Table 1). Likewise, the PLA_2 peptide sequences obtained are closely related to those of basic enzymes previously reported in these snake genera [57-60].

The three most abundant components of *P. platura* venom (peaks 1-2: 3FTx pelamitoxin; peak 9: PLA_2; and peak 16: CRISP) were collected for further study, and their possible roles in the toxic activities of this venom were evaluated. The crude venom was highly lethal to mice. By the i.p. route, its estimated LD_{50} was 2.3 μg/mouse (95% confidence limits: 1.5-3.6 μg; 0.13 μg/g body weight), whereas by the i.v. route the LD_{50} was 3.9 μg/mouse (95% confidence limits: 0.8-6.4 μg; 0.23 μg/g body weight). These estimates agree with an earlier report on the venom of *P. platura* from the Guanacaste province in Costa Rica by Bolaños et al. [61], who reported it being the "*most fluid, the most rapidly absorbed, the most potent for mice, and the most homogeneous of the venoms of Costa Rican snakes*", as well as with other studies compiled by Mackessy and Tu [17]. In lethality experiments, mice showed signs of neurotoxicity few minutes after injection, with

evident difficulty for breathing, and died within the first 20 min. Mice that did not die within this short time span survived the rest of assay.

Crude *P. platura* venom was also highly toxic to sympatric marine (m) and allopatric freshwater (fw) fishes: estimated LD_{50} values by the i.p. route were 0.11 μg/g (95% confidence limits: 0.06-0.24 μg) for *Lutjanus guttatus* (m); 0.26 μg/g (0.04-0.31 μg) for *Chromis atrilobata* (m); and 0.089 μg/g (0.04-0.17 μg) for *Dormitator maculatus* (fw). At high doses (>0.2 μg/g) the swimming capabilities of fishes were reduced, and they became paralyzed in as little as 5 min after the injection. Evident dyspnoea, as inferred by labored breathing and more rapid gill movement, was also noticed. Unlike what happened in mice, all fishes that showed initial signs of envenomation eventually died. For all tested species, the values for goodness of fit were not large in fishes ($Chi^2 < 1.4$, df=3, $p>0.192$ in all cases), indicating that the data fits the probits model. In addition, the hypothesis of parallelism among the fishes and mice data sets was not rejected ($Chi^2= 3.23$, df=3, $p=0.357$) thus changes in toxicity per unit change in the doses are similar among the species. Ratios for LD_{50} values were not significantly different between the murine and the fish models (Fig.7).

In myotoxicity experiments performed in mice, venom doses of 2 μg or higher injected by the i.m. route resulted in rapid death, precluding blood sampling at an adequate time for assessing muscle damage. Therefore, this activity was tested at a maximal dose of 1 μg. At this low dose, no evidence of myonecrosis was recorded, since CK activity levels in the plasma of mice that received venom (158 ± 120 U/L) were not significantly different from those of mice that received only PBS (217 ± 60 U/L), three hours after injections

($p>0.05$). Thus, the search for myotoxic activity in the venom was followed by the analysis of the isolated, most abundant PLA_2.

The PLA_2 present in peak 9 (Table 1) showed an isotope-averaged mass of 13332 Da (Fig.5B). This protein was tested for lethality and myotoxicity in mice. No deaths were observed at doses of 20 µg, either by i.v. or i.m. routes of injection. Furthermore, a single mouse that received 40 µg i.v. also survived, thus indicating that this PLA_2 is not neurotoxic. However, when the dose of 20 µg was injected i.m., in the gastrocnemius, there was a clear increase in the plasma CK activity after three hours. Control mice receiving PBS alone showed a CK activity of 307 ± 232 U/L, whereas the activity of plasma of mice receiving 20 µg of the PLA_2 was 2992 ± 685 U/L ($p<0.05$), thus indicating that this PLA_2 is myotoxic. This was confirmed by histological observation of muscle tissue (results not shown). At this dose, no macroscopic evidence of myoglobinuria was observed.

In vitro, the whole venom of *P. platura* showed detectable, although weak PLA_2 activity (Fig.4A), which in comparison to the venom of the coral snake *M. nigrocinctus* (Elapidae), included as a control, was markedly lower. On the other hand, *P. platura* lacked cytotoxic effect upon C2C12 myoblasts in culture, also in sharp contrast to the venom of *M. nigrocinctus*, used as a control (Fig.4B). Since a subtype of elapid 3FTxs, the cytotoxins/cardiotoxins, typically display a potent *in vitro* cytolytic effect on many cell types [54], this result suggests that the abundant 3FTxs present in *P. platura* venom do not belong to such a subtype.

Differentiation of cultured myoblasts into myotubes has been shown to increase their susceptibility to certain myotoxins of snake venoms [40,62]. Exposure of differentiated myotubes to *P. platura* venom also led to negative results (data not shown), confirming its lack of cytotoxic activity. Interestingly, although its isolated PLA_2 induced

myotoxicity *in vivo*, it was not cytolytic when added to myotubes at 25 μg/well (165 μg/mL) (data not shown). The behavior of the newly isolated PLA_2 myotoxin from *P. platura* coincides with that of notexin, a potent myotoxic PLA_2 from the Australian terrestrial elapid *Notechis scutatus* [63,64] which was reported to lack cytotoxicity upon myogenic and other cell lines using the same method here tested [40,65]. This finding is in sharp contrast with PLA_2 myotoxins from viperid venoms, which induce a rapid cytolytic effect upon myogenic cells and other cell types [40,65]. Such functional difference suggests that myotoxicity of some elapid (group I) and viperid (group II) PLA_2s, despite leading to a similar pathological outcome, may involve distinct molecular mechanisms, in line with the convergent evolutionary processes that independently led to the acquisition of this toxic effect in these two groups of PLA_2s [66].

Peak 16 was identified as a CRISP (Table 1), which by nESI-MS analysis was resolved into three close molecular masses (24459, 24559, and 24661 Da; Fig.5C) that very likely correspond to isoforms. CRISPs represent a widely distributed protein family in viperid snake venoms [67,68]. Neurotoxic CRISPs have been identified and characterized from the Australian elapids *Pseudechis australis* and *P. porphyriacus*. These molecules target cyclic nucleotide-gated ion channels and inhibit smooth muscle contraction [69,70]. However, injecting fraction 16 into mice by the i.v. route, at a dose of 20 μg (1.05 μg/g), did not cause death or any evident behavioral alterations, suggesting that this relatively prominent CRISP component of the venom (~9%) does not play a role in its lethal activity, at least in mice. It would be of interest to assay the possible effects of this CRISP fraction in fish, being the natural prey of *P. platura*. We were only able to inject a single *Dormitator maculatus* fish of 4.3 g by i.p. route, using 0.29 μg/g body weight of the CRISP component,

resulting in no evident alterations or lethality. On the other hand, the major 3FTx, pelamitoxin (Fig.5A), was highly lethal to mice, with an LD_{50} of 3.9 μg/mouse by the i.p. route (95% confidence limits 1.7-8.2 μg; 0.23 μg/g body weight), in agreement with earlier studies of Liu et al. [55] on the characterization of pelamitoxin isolated from specimens collected in northern Taiwan. It is worth to note that an LD_{50} of 3.9 μg pelamitoxin/mouse would correspond to an LD_{50} of 15 μg crude venom/mouse, which is 6.5-fold higher than that determined above. This figure suggests that, at least in the mouse model, other venom components may contribute synergistically with pelamitoxin to venom lethality.

Altogether, the present data indicate that the potent lethal effect of the whole venom is mainly mediated by its abundant 3FTxs with α-neurotoxic activity, most notably the pelamitoxin component (~26%), which probably plays a key role in subduing the elusive fish that constitute this snake's diet. Although functional studies were conducted in mice, on the basis of its myotoxic activity and lack of neurotoxicity, we hypothesize that the major PLA_2 studied here (~20%) might contribute to the muscle digestion of fish prey, but would not appear to be involved in lethality. The role of the CRISP proteins in this venom, however, remains unknown.

Although very rarely described in the literature [17,71-73], potential human envenomations by *P. platura* can raise medical concern due to the unavailability of a specific antivenom in many regions of its distribution, as a consequence of the inherent difficulties to obtain sufficient venom for the immunization of large animals. Bolaños et al. [61] reported an average venom yield as low as 0.38 mg per individual, from specimens collected in Guanacaste, Costa Rica. In this regard, the cross-reactivity toward *P. platura* venom of the sea snake antivenom manufactured by the CSL in Australia, using the venom

of *Enhydrina schistosa* as immunogen, was evaluated. In agreement with studies reviewed by Mackessy and Tu [17] and Pickwell [74], a clear effectiveness of this antidote in the neutralization of the lethal effect of *P. platura* venom from Costa Rica was confirmed. Control mice receiving four LD_{50} of venom by either the i.v. or the i.p. routes died within 5 min. In contrast, when venom was incubated with this antivenom (at a ratio of 0.2 mg venom/mL antivenom) and then injected by either of these two routes, all the mice survived and showed no signs of neurotoxicity. On the other hand, as expected, the possible cross-neutralization of *P. platura* venom by the equine antivenom against *Micrurus nigrocinctus* manufactured at Instituto Clodomiro Picado (Costa Rica) was ruled out, since none of the mice receiving venom/antivenom mixtures (under the same experimental conditions as for the CSL sea snake antivenom) survived (data not shown). Although a full titration of the neutralizing potency of the CSL antivenom was not performed owing to the scarcity of *P. platura* venom, results obtained at the fixed level of 0.2 mg/mL clearly imply that significant antigenic similarities exist between proteins of *E. schistosa* and those of *P. platura* venom involved in its lethal effect. The ability of the CSL antivenom in neutralizing the toxicity of the venoms of true sea snakes as well as that of the taxonomically divergent yellow-lipped sea krait, *Laticauda colubrina* [75], is consistent with the parallel but independent streamlining of both marine lineages, and the low level of phylogenetic variation of their 3FTxs [53,76].

Cross-recognition of *P. platura* venom by the CSL antivenom was further characterized by EIA and GID analyses. The antivenom generated a clear precipitin line against whole venom by GID, together with an additional, although barely visible diffuse precipitate (Fig.6A), and a strong EIA signal in its titration against solid-phase adsorbed whole venom (Fig.6B). In addition, GID tests using the three major components of *P.*

platura venom resulted in the formation of three independent precipitation systems against the 3FTx, PLA_2, and CRISP, respectively, by their corresponding antibodies in CSL antivenom (Fig.6C). By solid-phase EIA, a higher signal in the recognition of PLA_2, followed by the 3FTx, and a weak recognition of CRISP were obtained (Fig.6D). Thus, the cross-recognition of *P. platura* venom by the CSL antivenom involves at least these three types of components, PLA_2 being more immunogenic than the other two according to EIA signals. Finally, in agreement with results of immunochemical tests, the CSL antivenom was able to neutralize the lethal effect of the isolated 3FTx, pelamitoxin, at a ratio of 0.2 mg toxin/mL antivenom: all mice receiving a lethal i.v. injection of the toxin previously incubated with the antivenom survived.

4. CONCLUDING REMARKS

Only 100 species in the snake lineages Acrochordidae, Laticaudinae, Homolopsidae, and Hydrophiini have independently colonized marine habitats [76]. The Hydrophiini, with 60 species in 19 genera, are the most diverse and widely distributed of all the lineages of marine snakes [20,77]. Unsurprisingly, sea snakes possess a number of morphological and biochemical adaptations to aquatic life [78]. Noteworthy, the specialized venoms of sea snakes tend to be biochemically simple despite being among the most toxic venoms known [79]. Lacking the high visual acuity or scent cues used by terrestrial snakes to prevent the escape of prey, which in the case of sea snakes consists largely of fish or fish eggs, and occassionally squid and cuttlefish, sea snakes have evolved venoms largely dependent on rapidly acting, highly toxic neurotoxins. However, sea snakes are not particularly aggressive, and human snakebite accidents are rare, occurring mainly among

marine fishermen while snakes are being removed from nets. It has been suggested that *P. platura*, with its small mouth and low venom yield, poses little threat to humans. Indeed, asymptomatic bites by *P. platura* have been reported and human deaths caused by this species remain unproven [71].

The venoms of *P. platura* from two localities of Costa Rica that differ strikingly in color phenotype do not vary in their protein profiles, as assessed by RP-HPLC. Proteomic analyses revealed that the venom of this widely distributed sea snake presents a relatively simple composition, including three major types of components that belong to the 3FTx, PLA_2, and CRISP protein families, respectively. This finding seems to be in concordance with the suggestion of Fry and colleagues of a strong functional association between the relatively simple venoms of sea snakes and their teleost-based diet of a single vertebrate class (fish) [80,81]. In contrast to some terrestrial elapid venoms that possess a complex protein pattern with a high number of isoforms, for example that of *Micrurus nigrocinctus* [82], the venom of *P. platura* might have evolved without a strong pressure for isoform diversification through gene duplication and divergence, or alternatively, might have undergone a secondary genetic simplification as part of its adaptation to the marine environment and a relatively specific type of prey, essentially consisting of small fish [24]. Future genomic analyses would be needed to discern between these possibilities. Regardless of the evolutionary history, and in line with observations on other marine snakes [17,50,81], *P. platura* presents a limited diversity in its toxic arsenal. An abundant 3FTx, pelamitoxin, is likely to play the main role in the paralyzing and lethal actions of this venom against its natural preys, while at least another major component, a PLA_2, is capable of damaging skeletal muscle, without causing neurotoxic effects, presumably contributing to the initiation of prey digestion. However, due to the scarcity of purified proteins the toxic

activity of 3FTxs and PLA_2s was only tested in mice, essentially because these experiments allowed comparison with reported studies using well-characterized homologous toxins. Thus, although the results indicated that the major PLA_2 eluted in peak 9 (which accounts for ~20% of the total venom proteins) was not lethal to mice, the possibility that this protein exhibits taxon-specific activity, as reported for other toxins [83-87], requires future detailed investigation. Finally, of relevance from a clinical standpoint, the sea snake antivenom manufactured by CSL in Australia using the venom of *E. schistosa* displays sufficient immunological cross-reactivity to provide protection from the lethal effects of *P. platura* venom in preclinical mouse assays, therefore being of potential usefulness in the event of envenomations by this species.

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CONFLICT OF INTEREST STATEMENT

The authors declare that no competing interests exist regarding this manuscript.

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LEGENDS FOR FIGURES

Figure 1: Two color morphs of *Pelamis platura* (**A**, **C**) from different geographical origins in Costa Rica (**B**), Golfo de Papagayo and Golfo Dulce, respectively, do not express differences in their venom protein profiles (see Fig.2). Relative abundances of venom protein families are shown as percentages of the total protein content in (**C**). 3FTx: three-finger toxins, PLA_2: phospholipases A_2; CRISP: cyteine-rich secretory proteins; 5'NU: 5'-nucleotidases; SVMP: metalloproteinases; TRF: transferrins; ALB: albumins; HBG: hemoglobins; UNK: unknown/unidentified.

Figure 2: Comparison of the protein elution patterns of the venoms of *Pelamis platura* collected in Golfo de Papagayo and Golfo Dulce, respectively, by reverse-phase HPLC fractionation on a C18 column, as described in Methods.

Figure 3: Elution profile of *Pelamis platura* (Golfo Dulce) venom proteins by reverse-phase HPLC. Venom (2.1 mg) was fractionated on a C18 column (4.6 x 250 mm) eluted with a gradient of acetonitrile containing 0.1% trifluoroacetic acid, as described in Methods. The gradient line is omitted for clarity. Venom fractions were analyzed by SDS-PAGE (top insert), under reducing conditions, in 12% gels, and stained with Coommassie blue R-250. Molecular weight markers (M) are indicated in kDa, at the right. The tryptic digests of the proteins were characterized by nESI-MS/MS or MALDI-TOF-TOF, as described in Methods. The three most abundant venom peaks (indicated by stars), were further characterized.

Figure 4: (**A**) Comparison of the phospholipase A_2 activity of *Pelamis platura* and *Micrurus nigrocinctus* venoms, upon the monodispersed synthetic substrate 4-nitro-3-octanoyl-benzoic acid (NOBA), as described in Methods. Each point represents mean ± SD of triplicate assays. (**B**) Comparison of the cytolytic activity of *P. platura* and *M. nigrocinctus* venoms on the myogenic nurine cell line C2C12 *in vitro*, estimated as the release of lactate dehydrogenase 3 hr after exposure of myoblasts to the venoms, as described in Methods. Each bars represents mean ± SD of triplicate assays.

Figure 5: Isotope-averaged molecular mass of the three major components (Fig.3) of the venom of *Pelamis platura* (**A**: peak 1-2, 3FTx; **B**: peak 9, PLA_2; **C**: peak 16, CRISP) as determined by direct infusion nESI-MS on a QTrap3200 instrument (ABSciex) operated in positive, enhanced multi-charge mode. Graphs show the corresponding deconvolutions of m/z peaks of the multicharged ion series of the intact proteins, using the Analyst v.1.5 software (ABSciex).

Figure 6: Immunochemical cross-reactivity of the CSL sea snake antivenom (αV) toward the whole venom of *Pelamis platura* (**A**, **B**) or three isolated components (**C**, **D**), evaluated by gel immunodiffusion (**A**,**C**) or enzyme-immunoassay (**B**,**D**). In **(C)**, the major precipitin line formed between the antivenom and the venom fuses with the 3FTx line, revealing its identity. Precipitin lines formed between antivenom and PLA_2 or CRISP components, respectively, show a non-identity pattern. In **(B)** and **(D)**, points or bars represent mean ± SD of duplicates, and all differences between antivenom and negative control values are significant ($p<0.05$).

Figure 7: Effect of *Pelamis platura* venom in *Lutjanus guttatus*. **(A)** The crude venom was injected by intraperitoneal route at a dose of 0.25 μg/g body weight. Death of the individual occurred approximately five hours after the injection. Notice the exposed gill filaments and arch (arrow) and the wide opening of the mouth, in comparison to the normal control individual in **(B)**.

Figure 8: Comparison of the median lethal doses (LD_{50}) of *Pelamis platura* venom in the mouse and three species of fishes. Venom was injected by the intraperitoneal route and deaths were scored after 48 h. LD_{50} values (with 95% confidence intervals indicated by the error bars) were calculated by Probits. No significant differences in LD_{50} are found among the four species.

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

Peak	%	Mass [kDa]	Peptide ion		MS/MS-derived or N-terminal (Nt) sequence	Sco	Protein family; ~ related protein *
			m/z	z			
1	26.2	6680.4	748.8	2	MTCCNQQSSQPK	165[a]	short-chain α-neurotoxin (pelamitoxin a); P62388
			725.3	2	TTTNCAESSCYK		
			465.2	2	KTWSDHR		
			453.2	2	GCGCPQVK		
			918.3	2	SGIKLECCHTNECNN		
2	-	6704.1	748.8	2	MTCCNQQSSQPK	118[a]	short-chain α-neurotoxin (pelamitoxin a); P62388
			775.3	2	TTTNCAESSCYKK		
			652.3	2	IERGCGCPQVK		
			453.2	2	GCGCPQVK		
			918.3	2	SGIKLECCHTNECNN		
3	0.7	[7]	453.2	2	GCGCPQVK	*dn*	short-chain α-neurotoxin (pelamitoxin a); P62388
			465.2	2	KTWSDHR		
4a	1.0	[17]	777.4	2	MTCCNQQSSQPK	*dn*	short-chain α-neurotoxin (pelamitoxin a); P62388
			453.2	2	GCGCPQVK		
			465.2	2	KTWSDHR		
4b	6.8	[7]	734.8	2	MTCCNQQSSQPK	102[a]	short-chain α-neurotoxin (pelamitoxin a); P62388
			775.3	2	TTTNCAESSCYKK		
5a	0.4	[14]	620.3	2	MVCDCDVAAAK	115[a]	phospholipase A_2; ~ Q8UW08 *Hydrophis hardwickii*
			892.9	2	NAYNNANYNIDTNKR		
			902.4	2	M^{ox}VCDCDVAAAKCFAR		
5b	0.6	[8]	453.2	2	GCGCPQVK	*dn*	short-chain α-neurotoxin (pelamitoxin a); P62388
			481.8	2	(185.1)ELGCTAK	*dn*	long-chain α-neurotoxin; ~ Q8UW29

5c	2.1	[7]	481.8	2	(185.1)ELGCTAK	*dn*	long-chain α-neurotoxin; ~ Q8UW29
6a	1.5	[16]	601.8	2	GGSGTPVDELDR	40[a]	phospholipase A_2; ~ Q9PUI0
			675.8	2	IHDDCYGEAEK		
6b	1.6	[14]	892.9	2	NAYNNANYNIDTNKR	86[a]	phospholipase A_2; ~ Q8UW08
			1393.1	2	SSLDYADYGCYCGAGGSGTPVDELDR		
6c	7.0	7725.7	481.8	2	(185.1)ELGCTAK	*dn*	long-chain α-neurotoxin; ~ Q8UW29
			514.7	2	CDGFCSIR		
			1070.1	2	(312.3)YQPETCPPGQSLCYK		
7a	0.6	[14]	620.3	2	MVCDCDVAAAK	379[a]	phospholipase A_2; ~ Q8UW08
			595.6	3	NAYNNANYNIDTNKR		
			1393.1	2	SSLDYADYGCYCGAGGSGTPVDELDR		
			668.8	2	IHDDCYGEAEK	207[a]	phospholipase A_2; ~ Q8UW30
7b	0.3	[8]	453.2	2	GCGCPQVK		short-chain α-neurotoxin (pelamitoxin a); P62388
			481.8	2	(185.1)ELGCTAK		long-chain α-neurotoxin; ~ Q8UW29
			514.7	2	CDGFCSIR		
7c	1.3	[7]	1070.1	2	(312.3)YQPETCPPGQSLCYK	*dn*	long-chain α-neurotoxin; ~ Q8UW29
			514.7	2	CDGFCSIR		
			481.8	2	(185.1)ELGCTAK		
8a	1.0	[14]	620.3	2	MVCDCDVAAAK	223[a]	phospholipase A_2; ~ Q8UW08
			595.6	2	NAYNNANYNIDTNKR		
			1393.1	2	SSLDYADYGCYCGAGGSGTPVDELDR		
			690.6	4	IHDDCYGEAEKQGCYPK		phospholipase A_2; ~ Q8UW30
			601.8	2	GGSGTPVDELDR		phospholipase A_2; ~ Q9PUI0
8b	1.4	[7]	1070.1	2	(312.3)YQPETCPPGQSLCYK	*dn*	long-chain α-neurotoxin; ~ Q8UW29
			481.8	2	(185.1)ELGCTAK		
			507.7	2	CDGFCSIR		
			769.4	2	(216.4)TDNCNTVANWK		

			453.2	2	GCGCPQVK		short-chain α-neurotoxin (pelamitoxin a); P62388
9a	20.2	[14]	619.2	2	KVCDCDVAAAK	570[a]	phospholipase A_2; ~ Q8UW30
			668.8	2	IHDDCYGEAEK		
			690.6	3	IHDDCYGEAEKQGCYPK		
			1393.1	2	SSLDYADYGCYCGAGGSGTPVDELDR		
			1078.1	3	SSLDYADYGCYCGAGGSGTPVDELDRCCK		
			975.4	2	NLVQFSYVITCANHNR		phospholipase A_2; ~ P00610
			595.6	3	NAYNNANYNIDTNKR		phospholipase A_2; ~ Q8UW08
			601.8	2	GGSGTPVDELDR		phospholipase A_2; ~ Q9PUI0
9b	2.1	[7]	481.8	2	(185.1)ELGCTAK	*dn*	long-chain α-neurotoxin; ~ Q8UW29
			514.7	2	CDGFCSIR		
			1070.1	2	(312.3)YQPETCPPGQSLCYK		
10	3.7	[14]	620.3	2	MVCDCDVAAAK	270[a]	phospholipase A_2; ~ Q8UW08
			892.9	2	NAYNNANYNIDTNKR		
			1393.1	2	SSLDYADYGCYCGAGGSGTPVDELDR		
			668.8	2	IHDDCYGEAEK		phospholipase A_2; ~ Q8UW30
			601.8	2	GGSGTPVDELDR		phospholipase A_2; ~ Q9PUI0
11a	0.4	[14]	620.3	2	MVCDCDVAAAK	149[a]	phospholipase A_2; ~ Q8UW08
			892.9	2	NAYNNANYNIDTNKR		
			1393.1	2	SSLDYADYGCYCGAGGSGTPVDELDR		
			668.8	2	IHDDCYGEAEK		phospholipase A_2; ~ Q8UW30
11b	0.2	[8]	453.2	2	GCGCPQVK	*dn*	short-chain α-neurotoxin (pelamitoxin a); P62388
			481.8	2	(185.1)ELGCTAK		long-chain α-neurotoxin; ~ Q8UW29
11c	0.2	[7]	453.2	2	GCGCPQVK	*dn*	short-chain α-neurotoxin (pelamitoxin a); P62388

			481.8	2	(185.1)ELGCTAK		long-chain α-neurotoxin; ~ Q8UW29
12	3.5	[14]	620.3	2	MVCDCDVAAAK	212[a]	phospholipase A_2; ~ Q8UW08
			595.6	3	NAYNNANYNIDTNKR		
			1393.1	2	SSLDYADYGCYCGAGGSGTPVDELDR		
			668.8	2	IHDDCYGEAEK		phospholipase A_2; ~ Q8UW30
			601.8	2	GGSGTPVDELDR		phospholipase A_2; ~ Q9PUI0
13	1.6	[38]	579.3	2	QGEALNQLER	*dn*	unknown
			454.7	2	TLCAQCR		5'-nucleotidase; ~ ETE67245
			700.4	2	(244.1)LQLNSPFLPK		metalloproteinase; ~ AAR19274
14	0.5	[14]	634.7	2	(YG)WCNNNQGR	*dn*	unknown
			428.3	2	GPPNSDGSR		
15	0.6	-	-	-	no protein band	-	unknown
16a	0.9	[58]	433.7	2	SSIATPYK	149[a]	cystein-rich secretory protein; ~ Q8UW1
			529.3	2	SHLLGCASAK		
			597.8	2	EIVDKHNALR		
			671.8	2	CTFAHSPEHTR		
			888.9	2	YLYVCQYCPAGNIR		
16b	8.2	24449	433.7	2	SSIATPYK	190[a]	cystein-rich secretory protein; ~ Q8UW11
		24550	482.7	2	CQTEWIK		
		24649	529.3	2	SHLLGCASAK		
			597.8	2	EIVDKHNALR		
			671.8	2	CTFAHSPEHTR		
			888.9	2	YLYVCQYCPAGNIR		
			1342.5	3	SGPSCGDCPSACVNGLCTNPCEYE... ...DAYTNCNDLVK		
17	1.8	[92]	469.3	2	VASHAVVVR	*dn*	transferrin; ~ CAK18224

			769.4	2	ESCAPGASLE(536.3)		
			574.8	2	DFPELICVR		
			829.9	2	NLLQWSGTESG(486.3)		
18a	1.1	[68]	452.2	2	FIMTHEK	*dn*	albumin; ~ S59517
			578.4	2	AXVVVLLEATK		
			462.8	2	LLPQAPASK		
			515.3	2	LVEQLQSGR		
18b	0.1	[62]	414.2	2	ETVLLPR	36[a]	metalloproteinase; ~ D6PXE8
18c	0.2	[28]	-	-	-	-	unknown
18d	0.1	[24]	444.7	2	LGEEEVGK	*dn*	unknown
18e	0.3	[16]	493.2	2	VHWSAEEK	47	hemoglobin β; ~ P22743
			532.8	2	VLTSFGEALK		
19	0.8	[15]	1331.7	1	CFQVSLATHLR	251	hemoglobin α; ~ Q7ZT83
			2128.2	1	VIDALTEAVNNLDDVAGALSK		
			1078.7	1	LFIVFPQSK		
			1500.8	1	TYFSHYNLSPGSK		
			1837.9	1	TYFPHFDLSPGSNDLK		
			1687.9	1	GTLSQLSDLHAYNLR		
			985.6	1	LFAAHPTTK		
20	1.0	[15]	1500.8	1	TYFSHYNLSPGSK	117	hemoglobin α, β; ~ P16418
			1837.9	1	TYFPHFDLSPGSNDLK		
			1054.6	1	VFTAFGDAVK		
			1687.9	1	GTLSQLSDLHAYNLR		
			1288.8	1	LLIVYPWTQR		

* Cysteine residues are carbamidomethylated, unless indicated by underlining, corresponding to propionamide. X: Leu/Ile; B: Lys/Gln; [ox]: oxidized; Sco: probability score values calculated by [a]Mascot (www.matrixscience.com); *dn*: manual *de novo* interpretation of spectra.

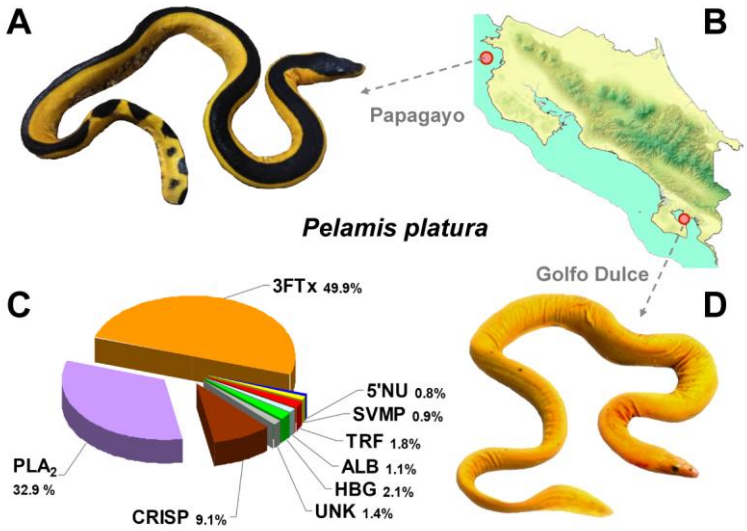
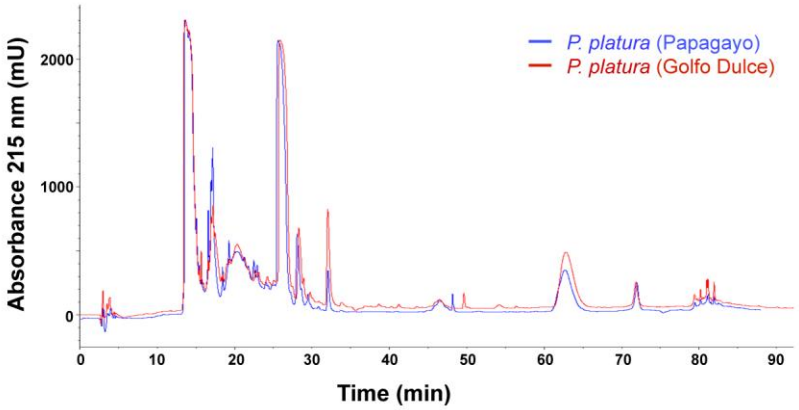


Figure1



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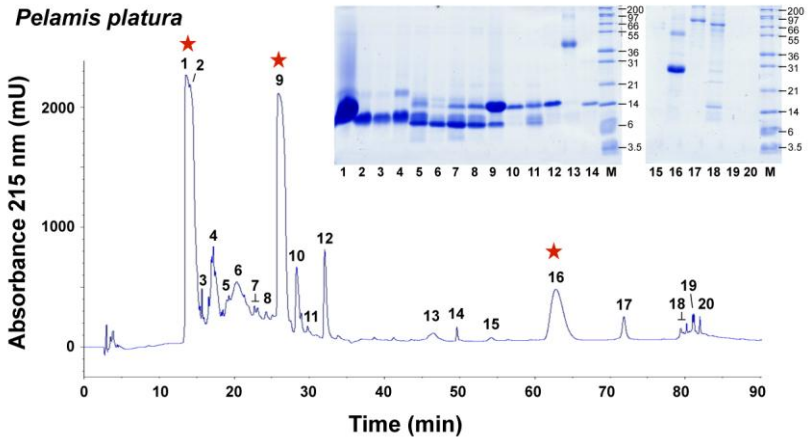

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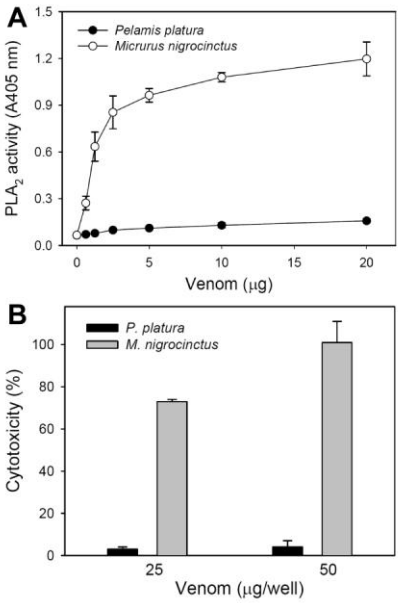

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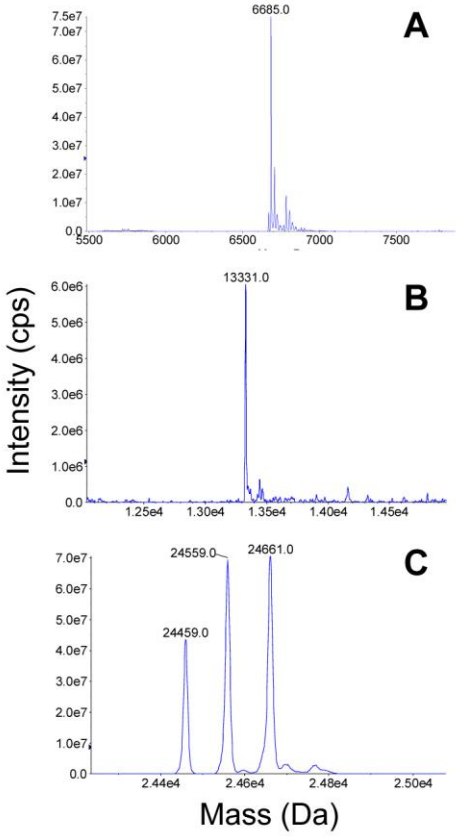


Figure5

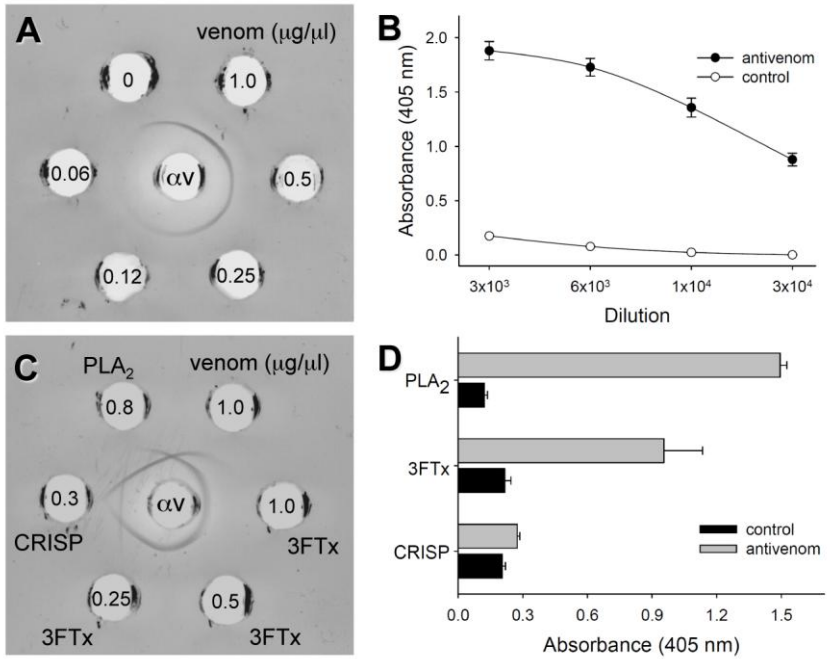
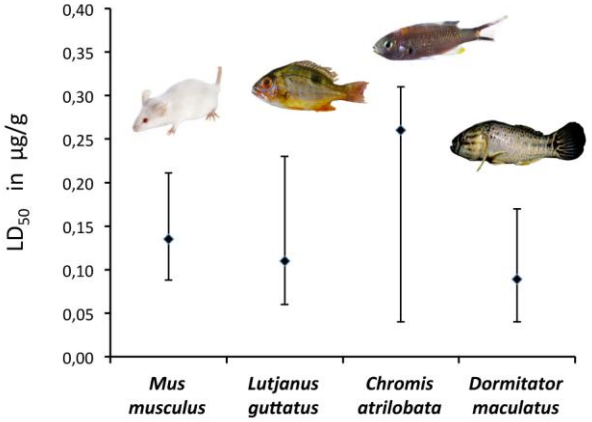


Figure6

Figure7



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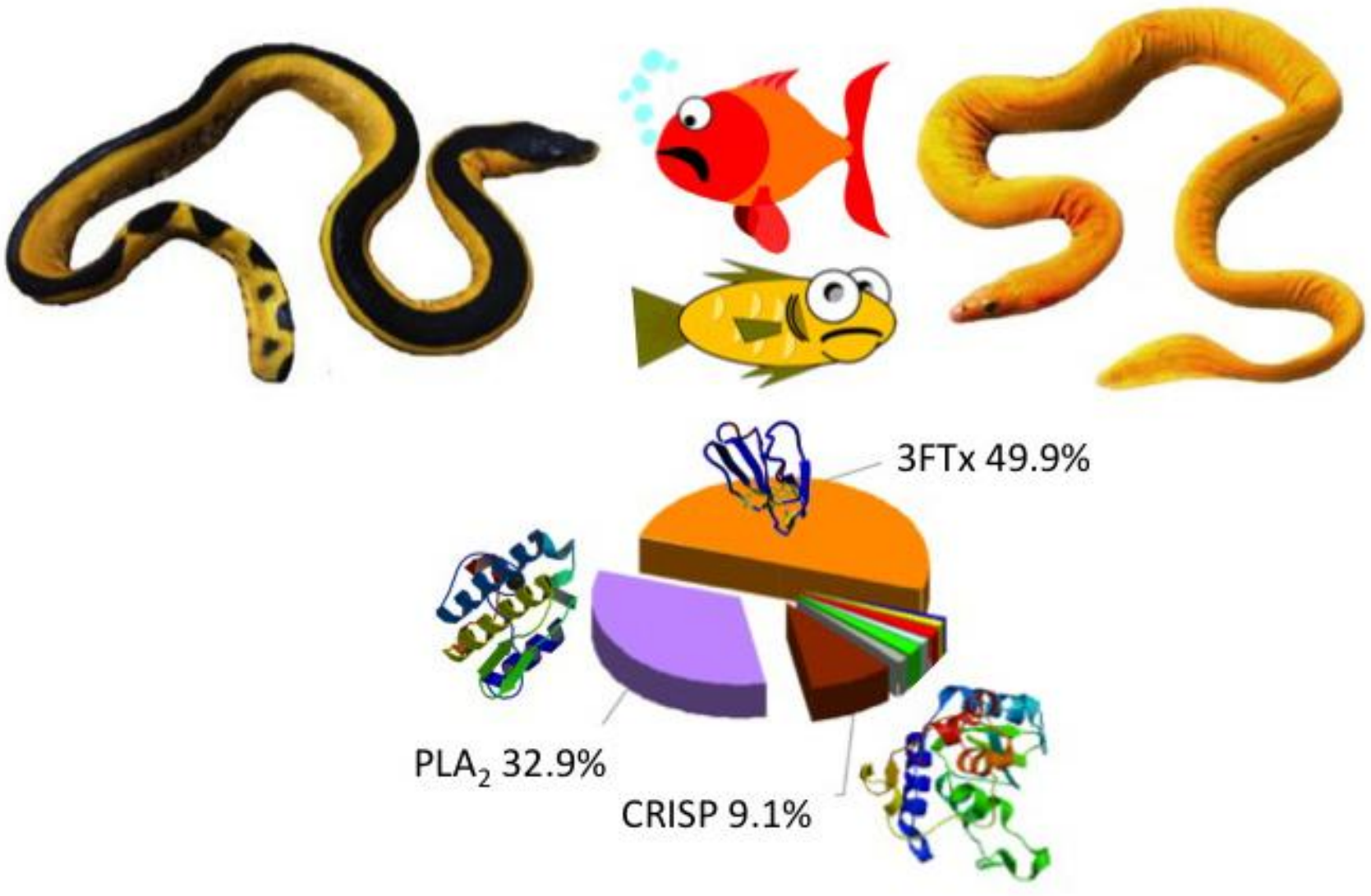

Graphical abstract

Highlights

- The yellow-bellied sea snake, *Pelamis platura*, is the most broadly distributed snake species
- Two distinct color morphs of this sea snake inhabit the Pacific coast of Costa Rica
- Venoms of both populations are conserved, despite their remarkable color phenotype divergence
- A proteomic and functional analysis of this highly lethal snake venom is presented
- Cross-neutralization of this venom by a sea snake antivenom is demonstrated

Biological significance

Integrative analyses of animal venoms that combine the power of proteomics (venomics) with the characterization of their functional and immunological properties are significantly expanding knowledge on these remarkable bioweapons, both from a basic and a medical perspective. Costa Rica harbors a unique population of the yellow-bellied sea snake, *Pelamis platura*, that is restricted to a fjord-like gulf (Golfo Dulce). This population differs markedly from oceanic populations found elsewhere along the Pacific coast of this country, by presenting a patternless bright yellow coloration, instead of the typical bicolored or tricolored pattern of this species. It has been suggested that the dominance of this yellow-morph in Golfo Dulce might reflect gene flow restrictions, caused by the oceanographic conditions at this location. The presents study demonstrates that the remarkable phenotypic variation between the two color morphs inhabiting Golfo Dulce and Golfo de Papagayo, respectively, is not associated with differences in the expression of venoms components, as shown by their conserved RP-HPLC profiles. Proteomic analysis revealed the relatively simple toxin composition of *P. platura* venom, which contains three predominant types of proteins: three-finger toxins (protein abundance: 49.9%), phospholipases A_2 (32.9%), and cysteine-rich secretory proteins (9.1%), together with few minor components. Further, the involvement of these most abundant proteins in the toxic effects of the venom, and their cross-recognition and neutralization by a sea snake antivenom produced against the venom of *Enhydrina schistosa*, were analyzed.