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Intraspecific variation of venom injected by fish-hunting *Conus* snails

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Summary

Venom peptides from two species of fish-hunting cone snails (*Conus striatus* and *Conus catus*) were characterized using microbore liquid chromatography coupled with matrix-assisted laser desorption/ionization-time of flight-mass spectrometry and electrospray ionization-ion trap-mass spectrometry. Both crude venom isolated from the venom duct and injected venom obtained by milking were studied. Based on analysis of injected venom samples from individual snails, significant intraspecific variation (i.e. between individuals) in the peptide complement is observed. The mixture of peptides in injected venom is simpler than that in the crude duct venom from the same snail, and the composition of crude venom is more consistent from snail to snail. While there is animal-to-animal variation in the peptides present in the injected venom, the composition of any individual's injected venom remains relatively constant over time in captivity. Most of the *Conus striatus* individuals tested injected predominantly a combination of two neuroexcitatory peptides (s4a and s4b), while a few individuals had unique injected-venom profiles consisting of a combination of peptides, including several previously characterized from the venom duct of this species. Seven novel peptides were also putatively identified based on matches of their empirically derived masses to those predicted by published cDNA sequences. Profiling injected venom of *Conus catus* individuals using matrix-assisted laser desorption/ionization-time of flight-mass spectrometry demonstrates that intraspecific variation in the mixture of peptides extends to other species of piscivorous cone snails. The results of this study imply that novel regulatory mechanisms exist to select specific venom peptides for injection into prey.

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Key words: *Conus striatus*, *Conus catus*, conotoxin, injected venom, duct venom, intraspecific variation, liquid chromatography, mass spectrometry.

Introduction

More than 500 species of marine snails comprise the genus *Conus*, and all are venomous predators. The venom of a typical cone snail contains small post-translationally modified peptides, many of which are directed towards voltage- and ligand-gated ion channels and receptors with exquisite specificity (Olivera, 1997). A complex mixture of peptide toxins is produced in a long tubular venom duct containing secretory cells. Venom is ultimately discharged into prey by injection through a highly modified radular tooth, resembling a barbed hypodermic needle, which is gripped near the tip of an extensible proboscis. Mechanisms for transferring toxins from the venom duct into the proboscis for injection are not well understood, but in at least one species, *C. californicus*, peptides appear to be packaged into the radular teeth themselves (Marshall et al., 2002). In the fish-hunting species *C. catus*, and presumably in other species as well, the radular tooth is explosively propelled in a ballistic fashion into the prey prior to venom expulsion through the tooth (Schulz et al., 2004). Venoms of both *C. striatus* and *C. catus* induce an immediate tetanic paralysis of the harpooned fish, thereby enabling prey capture (Kohn, 1956).

Isolation of venom peptides from *Conus* snails in the vast majority of cases has employed crude venom obtained from dissected venom ducts (duct venom, DV), with material being pooled from multiple animals. The use of DV enables peptide characterization based on conventional analytical and sequencing methods that require fairly large amounts of material. However, it is not clear how peptides in DV are related to those actually injected during prey capture. In several

studies, venom expelled through the radular tooth during feeding behavior was collected by a simple 'milking' procedure (Bingham et al., 2005, 1996; Hopkins et al., 1995; Martinez et al., 1995; Shon et al., 1997, 1995; Teichert et al., 2004; Walker et al., 1999), and analysis indicated that this injected venom (IV) was not identical to DV from the same species (Bingham et al., 1996; Martinez et al., 1995). Venom milking is thus advantageous because it provides the biologically relevant peptides used to subdue prey and does not require sacrificing the snail. However, there have been no detailed comparisons of the complement of peptides in DV with that in IV.

Additionally, prior research has not addressed variation in the IV composition from snail to snail within a given species. In studies employing IV (Bingham et al., 2005, 1996; Hopkins et al., 1995; Martinez et al., 1995; Shon et al., 1997, 1995; Teichert et al., 2004; Walker et al., 1999), the samples were pooled from multiple snails, and therefore the degree of intraspecific variation could not be assessed. Intraspecific variation has been examined using DV from individual snails; however these studies have produced conflicting results. Variation in the peptide profiles of DV between *C. textile* individuals from the same reef has been reported (Bingham et al., 1996; Jones et al., 1996), whereas DV of *C. regius* was found to be consistent from snail to snail, regardless of the gender or size of the animals or season of collection (Vianna Braga et al., 2005).

Genes encoding *Conus* toxins have evolved by a mechanism of strong positive selection (Conticello et al., 2001; Duda and Palumbi, 2004, 1999, 2000; Espiritu et al., 2001), and diversification of these peptide toxins may be responsible for success in the acquisition of new feeding behaviors and niche expansion leading to speciation within the genus. Although the biological relevance of these peptides is widely recognized, it is not known how the expression, maturation and delivery of these toxins are regulated. Thus, a detailed comparison of the peptides in DV and IV from individual snails represents a first step in elucidating the mechanisms involved in the delivery of toxins for prey capture.

In this study, peptides in both IV and DV were collected from individual snails and characterized using microbore high-performance liquid chromatography (HPLC) coupled with matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-MS) and electrospray ionization-ion trap-mass spectrometry (ESI-MS). In many cases, these sensitive methods permit analysis of samples smaller than an IV sample from a single milking. MS is also information-rich and selective, providing a more complete profile of peptides than is possible with ultraviolet/visible (UV/Vis)-absorbance detection alone.

Here, we report the first significant intraspecific differences in the composition of venom peptides injected by individual piscivorous snails, *C. striatus* and *C. catus*. In addition, we find that the profile of peptides in IV, including known and putative novel peptides, is far less complex than the profile observed for DV.

Materials and methods

Chemicals

Trifluoroacetic acid (TFA) and glacial acetic acid were obtained from Sigma Chemical Co. (St Louis, MO, USA). HPLC-grade acetonitrile and methanol were obtained from Fisher Scientific (Pittsburgh, PA, USA). MilliQ water was used for HPLC solvents, and Burdick and Jackson water (Muskegon, MI, USA) was used for DV sample preparation and peptide-trap rinsing. Bovine insulin and α-cyano-4-hydroxycinnamic acid (CHCA) were purchased from Aldrich. *Aplysia californica* peptides (acidic peptide and α-bag cell peptide 1-9) were obtained from American Peptide Company (Sunnyvale, CA, USA).

Venom collection

Conus striatus L. (6.5–7 cm shell length: Tutuila Island, American Samoa) and *Conus catus* Hwass in Bruguière 1792 (1.8–4.3 cm shell length: Kauai, Hawaii) were collected from limited areas of coral reef-flats and subsequently maintained at Hopkins Marine Station in closed tanks at 27°C under equivalent environmental conditions. All snails were fed live fish about once per week. *Conus striatus* were fed commercially procured goldfish and *C. catus* were fed small marine fish, generally sculpins (*Clinocottus* spp. and *Oligocottus* spp.), collected from local intertidal areas. Captive snails were milked to obtain IV samples as described elsewhere (Hopkins et al., 1995). Briefly, a snail was induced to inject venom into a 0.5 or 1.5 ml centrifuge tube covered by a latex membrane with a fish fin exposed on top. This procedure was periodically carried out with identified *C. striatus* and *C. catus* individuals for several months, after which (in the case of the *C. striatus*) the animals were killed, and venom ducts were dissected. DV samples were prepared by manually rolling out the duct contents with further treatments as described below.

Conus striatus *injected venom fractionation and mass spectrometry*

In Fig. 1, single IV samples from *C. striatus* (~25–50 µl) were fractionated on an LKB Bromma HPLC system (Sweden) fitted with a Microsorb™ (Rainin) column (C_{18}, 4.6 mm×150 mm, 5 µm particle diameter, 10 nm pore size). Separations utilized a uniform flow rate of 0.5 ml min^{-1} with a solvent B gradient of 5% to 50% over 36 min and 50%–80% B for an additional 9 min [Solvent A: H_2O, 0.1% TFA (v/v); Solvent B: acetonitrile, 0.08% TFA (v/v)]. Fractions were analyzed by MALDI-MS in linear and reflectron modes as described below.

For the work described in Fig. 2A,C, single *C. striatus* IV samples were loaded into a peptide trap (inline with the injection loop), rinsed with pure water, and fractionated using a microbore HPLC system (Magic 2002, Michrom BioResources, Auburn, CA, USA) equipped with a Vydac Pepmap column (C_{18}, 1 mm×150 mm, 3 µm particle diameter, 10 nm pore size). A gradient separation was performed at

30 μl min^{-1} using solvents A and B [A: 95% H_2O, 5% acetonitrile, 0.1% acetic acid and 0.02% TFA (v/v); B: 90% acetonitrile, 10% H_2O, 0.08% acetic acid and 0.014% TFA, (v/v)]. Sample fractions were eluted with a solvent B gradient of 5% to 15% over 10 min, 15% to 45% over an additional 30 min, and finally 45% to 80% over 10 min. In this case, the UV/Vis flow-cell was bypassed, and the flow was directed to the inlet port of an LCQ Deca electrospray ionization-ion trap-mass spectrometer (Thermo, San Jose, CA, USA).

For the ESI-MS experiment, a spray voltage of 4.3 kV, a capillary temperature of 220°C, a capillary voltage of 21 V and a tube lens offset of 10 V were employed. A triple play [full scan from 50 to 2000 m/z, ZoomScan, collisionally induced dissociation (CID) scan at 35% collisional energy] data-dependent acquisition method with dynamic exclusion was developed using the Xcalibur software. Spectra were processed using the base peak ion chromatogram and a seven-point boxcar smoothing calculation. The multiply charged peaks detected in the ESI-MS experiments were manually deconvoluted based on the high-resolution ZoomScans to obtain peptide masses.

For data presented in Fig. 3D,E,F, two *C. striatus* IV samples were pooled for each snail, and the entire volume was fractionated using the same microbore HPLC system and solvents as for Fig. 2A,C. A separation at a uniform flow rate of 100 μl min^{-1} using a Vydac (Hesperia, CA, USA) Reverse Phase Polymer column (2.1 mm×150 mm) with 5 μm particles and 30 nm pore size was performed. The solvent gradient began by increasing solvent B from 5% to 15% over 5 min and was completed by a gradient up to 65% B over the next 60 min. Detection was performed *via* a dual UV/Vis detector set at 220 nm and 280 nm as well as with online ESI-MS as described above.

Conus striatus *duct venom fractionation and mass spectrometry*

For results depicted in Fig. 2B,D, crude DV was removed from 1 cm sections of the anterior (near the proboscis) and posterior (near the venom bulb) regions of the venom duct and combined. Samples (including insoluble material) were then diluted with 20 μl of water, sonicated for 2 min and then directly used for HPLC separation. In the case of Fig. 3B,D,F, the content of the entire venom duct was sampled, and the insoluble material was removed by centrifugation (10 000 ***g*** for 2 min) such that only the clear supernatant was used for injection into the HPLC column. The pellet was resuspended in water, and the centrifugation and supernatant collection were repeated. A sample of DV or DV supernatant (85% of the total volume) was loaded into a peptide trap, rinsed with pure water and subjected to the same HPLC separation as described above for Fig. 2A,C. In this case, fractions were collected on a Gilson FC 203B fraction collector (Middletown, WI, USA) and subjected to MALDI-MS (see below).

The remaining volume (15%) of each DV sample was subjected to the same online HPLC-ESI-MS experiment as described above for the IV samples. Manual deconvolution of the ESI-MS data yielded peptide masses consistent with those obtained by MALDI-MS. Dual, complementary mass-spectrometric techniques were performed to aid in the proper mass assignment of peaks.

In order to avoid carryover of peptides from sample to sample, an extensive flushing procedure was performed between each venom sample.

MALDI mass spectrometry of venom fractions

As noted above, IV and DV fractions from *C. striatus* were subjected to MALDI-MS following an HPLC separation. For the *C. catus* samples, IV was collected and directly subjected to MALDI-MS. A 0.5 μl aliquot of each sample (*C. striatus* venom fraction or unpurified *C. catus* IV) was spotted onto a gold-plated target along with 0.5 μl of a matrix (15 mg CHCA, 600 μl acetonitrile, 400 μl water, 3 μl TFA). Positive-ion mass spectra were acquired using linear and reflectron modes on a Voyager DE STR (Applied Biosystems, Foster City, CA, USA) time-of-flight mass-spectrometer equipped with delayed ion extraction. A pulsed nitrogen laser (337 nm) was used as a desorption/ionization source. External mass calibration was performed using a peptide standard mixture containing 60 μmol l^{-1} of α-bag cell peptide 1–9, 120 μmol l^{-1} of acidic peptide, and 120 μmol l^{-1} of bovine insulin.

Peptide nomenclature

In the present work we use an existing scheme for naming conotoxins as detailed elsewhere (Walker et al., 1999). In order to be consistent with this nomenclature, the previously reported peptide κA-SIVA (Craig et al., 1998) and the reported sequence SIVB (Santos et al., 2004) are referred to as s4a and s4b, respectively, because the high-affinity targets of these venom isolated peptides have not been determined (W. P. Kelley, J. R. Schulz, J. A. Jakubowski, W. F. Gilly and J. V. Sweedler, unpublished). The known *C. striatus* and *C. catus* α- and ω-conotoxins identified in the present study also follow the established nomenclature. According to this naming scheme, sequence SO5 (Lu et al., 1999) should be named S6.5, corresponding to venom-isolated peptide s6e; however, a different amino acid sequence already exists that is named S6.5 that corresponds to conotoxin δ-SVIE (Bulaj et al., 2001). Therefore, we retain the SO5 name for both the sequence and the putative venom-isolated peptide (and for consistency do the same for SO4).

Results

Variation in the composition of injected venom in Conus striatus

Ten *C. striatus* were marked for identification purposes and periodically milked at intervals ranging between 1 and 6 months while in captivity. The IV samples from a single milking of each individual were analyzed by reverse-phase HPLC. Representative data are illustrated in Fig. 1 for three snails. In the case of each individual, the two stacked

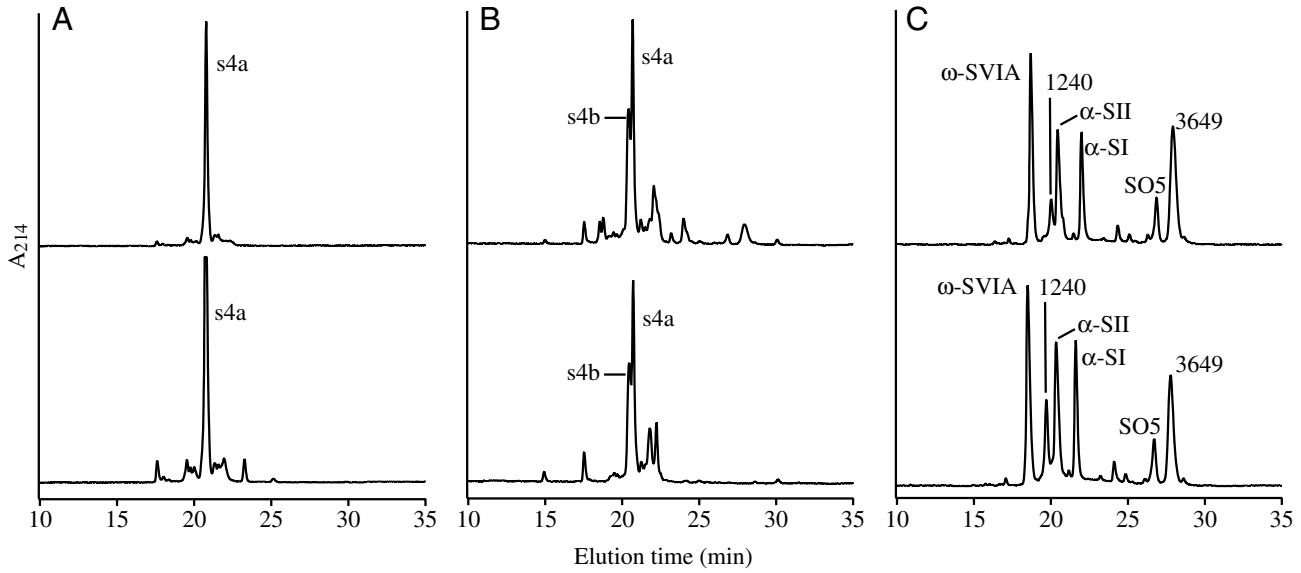

Fig. 1. Comparison of IV profiles by HPLC. (A–C) UV chromatograms of the three types of *C. striatus* IV patterns observed, demonstrating the reproducibility of the profile within individuals for samples taken at different times in captivity. For the profiles shown, sampling intervals were 6 (A), 3 (B) and 4 months (C). Peptides are labeled by protonated monoisotopic mass or, where appropriate, known *C. striatus* toxins as determined by off-line MALDI-MS.

chromatograms show that the profile of the major peaks present in IV samples changed little over time in captivity within this range of milking intervals. While there is variation of the low abundance peptides, the overall profile of the primary toxins remains constant within each individual. Furthermore, the extent of these small changes does not appear to correlate with the milking interval. More importantly, the mixture of injected peptides varied considerably among individual snails, and three general categories of profiles were observed. Three of the ten snails injected predominantly peptide s4a (Fig. 1A), whereas five snails injected a combination of peptides s4a and s4b (Fig. 1B). Peptide s4a is identical in sequence to the neuroexcitatory toxin observed in the DV of *C. striatus* that was previously designated as κA-SIVA (Craig et al., 1998). Peptide s4b is a homologous neuroexcitatory peptide (W. P. Kelley, J. R. Schulz, J. A. Jakubowski, W. F. Gilly and J. V. Sweedler, unpublished).

Two *C. striatus* individuals exhibited a radically different type of IV peptide-profile (Fig. 1C). These IV samples contained peptides that were previously characterized from the DV of this species, including the calcium-channel blocker ω-SVIA (Ramilo et al., 1992) and nicotinic acetylcholine-receptor blockers α-SI and α-SII (Ramilo et al., 1992; Zafaralla et al., 1988), as confirmed by MALDI-MS. Interestingly, neither s4a nor s4b were observed components of the IV from these snails.

Comparison of duct venom and injected venom samples in Conus striatus

In order to assess whether similar variations exist in the peptide complement of DV, samples of DV from two *C. striatus* individuals with distinct IV profiles were analyzed using HPLC-ESI-MS. Fig. 2A,C show the IV profiles; the corresponding DV profiles are in Fig. 2B,D, respectively. The base peak chromatograms presented closely resemble the UV chromatogram profiles and, therefore, have been interpreted similarly. In this case, one snail injected predominantly ω-SVIA, α-SI and α-SII (Fig. 2A), similar to the pattern in Fig. 1C, whereas the other individual showed the most commonly observed profile of mainly s4a and s4b (Fig. 2C). Peak splitting of s4a and s4b was observed (Fig. 2C), possibly due to *cis–trans* isomerization of the hydroxyproline residues (Watson and Kenney, 1992). According to MS analysis, components within each peak doublet are the same mass. The prominent peptides labeled in Figs 1C and 2A are similar except for the last chromatographic peak, which contains peptides of masses 3649 Da and 9433 Da, respectively. Co-elution of the putative 9433 Da and 3649 Da peptides was likely based on the ESI-MS results; however the MALDI-MS analyses of this fraction for Fig. 1C did not assess peptides above 6000 m/z.

The DV samples from these same individuals contain many hydrophobic peptides in addition to the peptides observed in the IV samples. Most notably, peptides s4a, ω-SVIA and α-SI as well as several putative toxins (SO5 and masses 2556, 3431, 3335 and 2049 Da) were all present in the DV of both snails despite the distinct nature of the IV components. It is possible that these hydrophobic components represent precursors of mature conotoxins; however, there are also known classes of hydrophobic conotoxins (e.g. δ-conotoxins; Bulaj et al., 2001). Without reduction and alkylation of the disulfide bonds, reliable sequence information was not obtained from the MS/MS scans of these masses. While a global reduction–alkylation online MS/MS procedure in conjunction

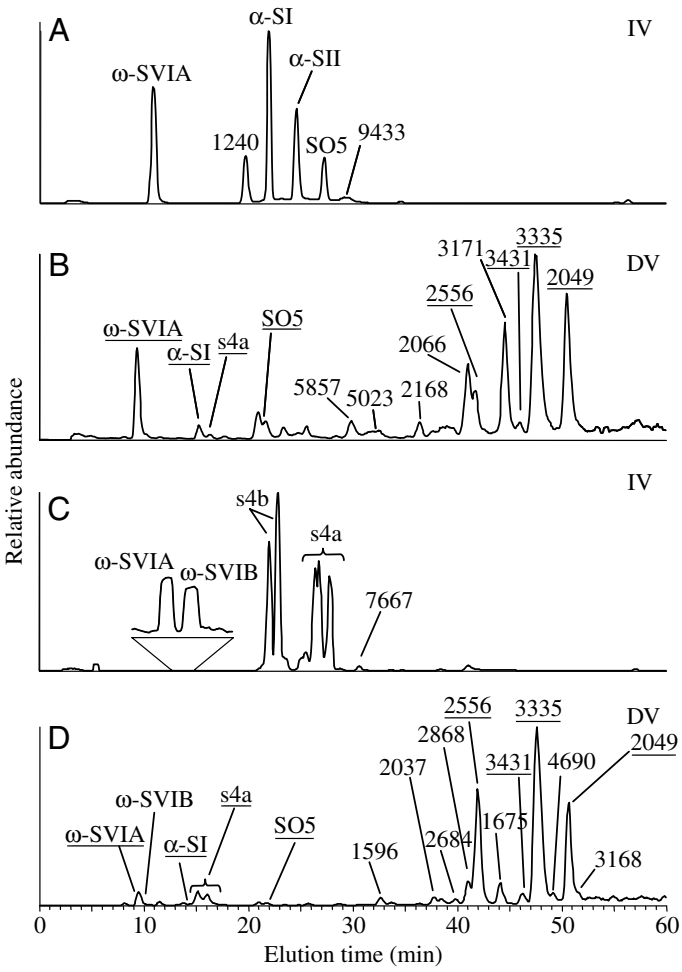

Fig. 2. Comparison of DV and IV using HPLC-ESI-MS. The base peak ion chromatograms show the peptide profiles for the IV of two snails (A,C), and the corresponding DV from the same individuals (B,D, respectively). Abundant peptides are labeled by protonated monoisotopic mass (except 9433 Da, an average mass) or, where appropriate, by known or putative *C. striatus* toxins. Masses or peptides common to both DV samples are underlined.

with cDNA sequencing would enable sequence determination (Jakubowski and Sweedler, 2004), conotoxin sequencing has not been the focus of this comparative study.

In every case examined, there were at least 50 putative peptides in the DV, but only the most abundant peptides are labeled in the figures. It is apparent both by the number of peaks that appear in the chromatograms, and the masses labeled, that IV is by no means identical to DV and contains a small subset of DV peptides, primarily less hydrophobic ones. Identifiable DV and IV peptides were fully processed from their propeptides and contained the known post-translational modifications such as carboxy-terminal amidation (s4a, ω-SVIB and α-SI) and proline hydroxylation (s4a and ω-SVIA; Craig et al., 1998; Ramilo et al., 1992; Zafaralla et al., 1988). Peptides s4a and s4b were also modified by O-glycosylation and pyroglutamylation (Craig et al., 1998). Thus, the apparent simplification of DV does not reflect differences in the modified states of the peptides.

Comparison of water-soluble duct venom and injected venom in Conus striatus

We noted that the DV is heterogeneous, containing a clear fluid as well as white, insoluble material. Similar observations were reported in *C. californicus* (Marshall et al., 2002). In order to further assess differences in DV and IV, IV samples from three *C. striatus* individuals with the most common venom profile (mainly s4a and s4b) were analyzed by HPLC-ESI-MS (Fig. 3A,C,E), and these data were compared to the profiles of the water-soluble peptides in the DV from the same individuals (Fig. 3B,D,F). In all three comparisons, the peptide mixture comprising IV was significantly simpler than the water-soluble components of DV, indicating that simply partitioning the water-soluble peptides from the insoluble matter in DV does not create IV. Although s4a was common to all DV samples (Fig. 3B,D,F), the amount of s4a relative to other peptides was significantly greater in the IV samples (Fig. 3A,C,E) *vs* the DV samples for each of these snails. In addition, the range of masses for the water-soluble peptides detected in the DV (~1000–5200 Da) was different than that for IV peptides from the same snail (~2700–7700 Da).

MALDI-MS profiling of Conus catus *injected venom samples*

Conus catus is another fish-hunting snail that employs the same prey capture strategy as *C. striatus*. In order to determine whether intraspecific variation in the peptide profile of IV extends to this species, IV samples from nine *C. catus* individuals were analyzed by linear MALDI-MS (see Table 1). Masses of all putative peptides detected in at least two individuals (for simplification) ranged from 1287 to 6910 Da. Variation between individuals clearly exists, and IV profiles ranged in complexity from quite low (snail A) to extreme (snail I). MALDI mass spectra of IV samples from snails A and I are presented in Fig. S1 in the supplementary material.

Samples of IV from *C. catus* individuals A, D and I were also analyzed by reverse-phase HPLC (data not shown) to confirm the varying complexity of the IV profiles. Multiple reverse-phase HPLC runs of the IV samples of snail I also confirmed a stable profile of the IV over time (data not shown) as demonstrated for *C. striatus* (Fig. 1), but examination of the *C. catus* DV for comparison has not yet been performed to permit continuation of IV collection with these animals.

Identification of putative conopeptides in Conus striatus *venom samples*

While the primary goals of this study were to compare venoms between individuals within one species, as well as within the venom apparatus of a single snail (IV *vs* DV), the information-rich MS techniques enabled us to determine mass matches to a number of identified and putative *C. striatus* peptides. Identification of well-known conopeptides by such a mass-matching approach has been used in another report (Craig et al., 1995). Since ω-SVIA, ω-SVIB (Ramilo et al.,

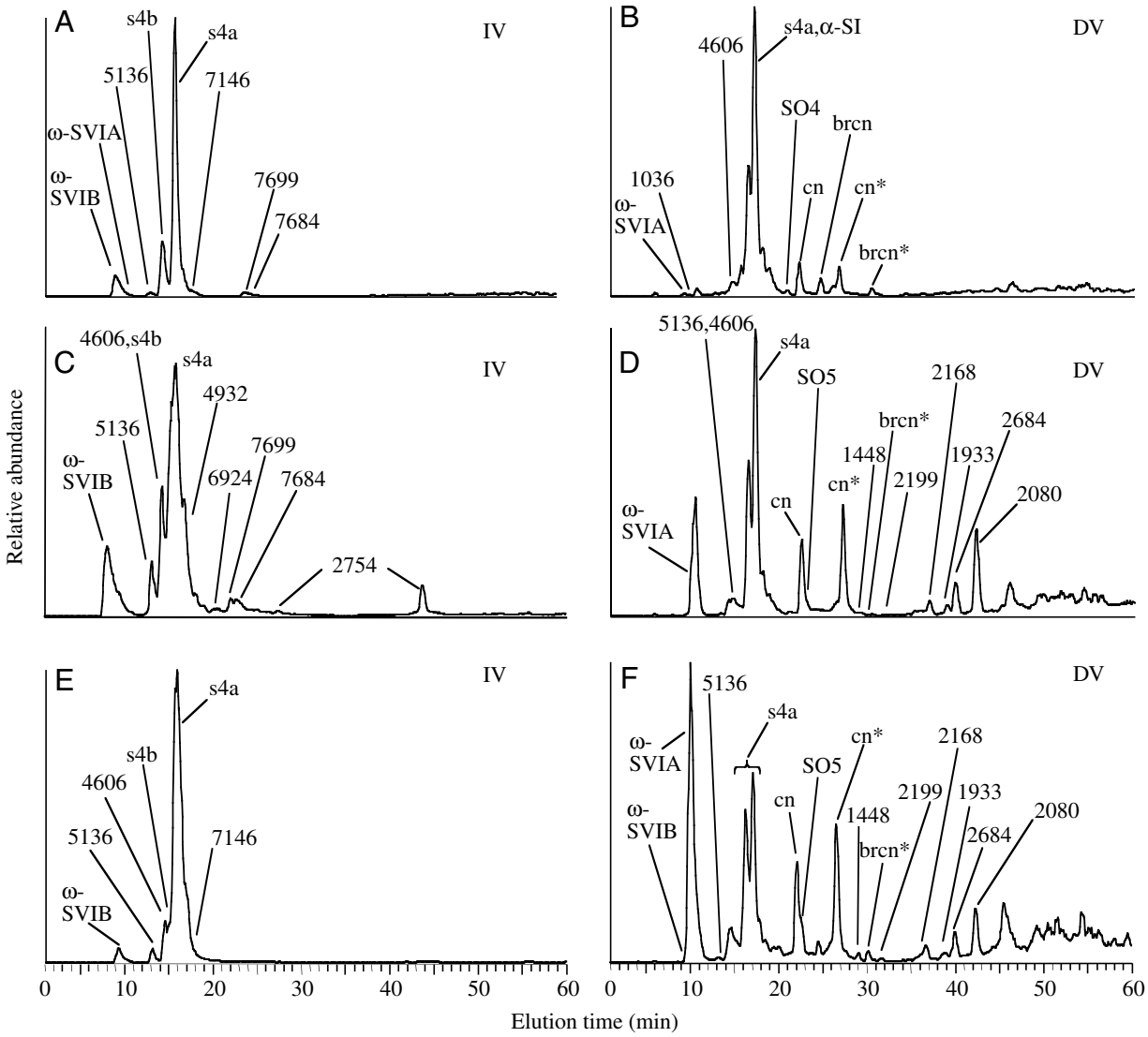

Fig. 3. Comparing water-soluble DV and IV using HPLC-ESI-MS. The base peak ion chromatograms show the peptide profiles for the IV of three snails (A,C,E), and the corresponding DV from the same individuals (B,D,F, respectively). Abundant peptides are labeled by protonated monoisotopic mass or, where appropriate, by known or putative *C. striatus* toxins. brcn, bromocontryphan; cn, contryphan; * indicates [des-Gly^1] (bromo)contryphan form.

1992), α-SI, α-SII (Ramilo et al., 1992; Zafaralla et al., 1988) and s4a (Craig et al., 1998) are well-characterized *C. striatus* peptides, an observed mass within 0.5 Da of the predicted value is sufficient to identify the peptides, and based on this criterion, these peptides were identified. We also determined mass matches based on ESI-MS and MALDI-MS data to within 0.5 Da for three putative peptides by using the calculated molecular masses with expected post-translational modifications predicted from published cDNA sequences. These peptides have yet to be isolated and characterized from venom extracts and include the predicted O-superfamily members SO4 (Lu et al., 1999) with three disulfide bonds and two hydroxyproline residues (Fig. 3B) and SO5 (Lu et al., 1999) with three disulfide bonds (Figs 1C, 2A,B,D, 3D,F), as well as the propeller peptide (mass 9433 Da; Ellison, 2003), which may exist as a dimer (Fig. 2A). The targets of these putative peptides are thus far unknown, although bioactivity of the propeller peptide has been reported (Ellison, 2003). Putative matches to the y_4 fragment ion in an SO5 MS/MS spectrum and the b_5 fragment ion in an s4a MS/MS spectrum (data not shown) provide further confirmation for these peptide designations. In all cases, further studies are required to verify these putative assignments.

Additionally, in Fig. 3B,D,F, several different forms of putative *C. striatus* contryphans were observed. Contryphan-Vn, identified in *C. ventricosus*, modulates certain voltage-gated and Ca^{2+}-dependent K^+ channels (Massilia et al., 2003), and a homologous peptide, contryphan-R, occurs in *C. radiatus* (Jimenez et al., 1997). In the present case, isotopic distribution characteristic of bromination with M and M+2 peaks in

Table 1. *Intraspecific variation in IV profiles of* Conus catus *as determined by MALDI-MS*

Individual	Peptide masses (Da) detected in injected venom samples																		
	1287	1311	1502	1616	1655	1679	1702	1718	1734	1737	2330	2696	2763	5085	5157	5184	5392	5451	5613	5634	5756	6910
A												✓		✓		✓		✓	✓	✓		
B		✓		✓			✓	✓	✓					✓	✓				✓			
C											✓	✓		✓			✓	✓	✓	✓	✓	✓
D	✓		✓	✓	✓	✓	✓								✓		✓		✓	✓		
E	✓		✓	✓		✓	✓				✓			✓				✓	✓	✓		
F			✓	✓		✓	✓				✓			✓	✓	✓		✓	✓	✓		✓
G		✓		✓			✓	✓	✓	✓				✓			✓		✓	✓	✓	
H	✓		✓	✓		✓	✓	✓			✓		✓	✓		✓	✓	✓	✓	✓		
I			✓	✓	✓	✓	✓	✓	✓	✓	✓		✓			✓	✓		✓		✓	

IV samples were analyzed from nine snails (individuals A–I).

All masses of putative peptides that were common to two or more snails are tabulated. Masses that differed by about 22 Da likely indicate the presence of Na^+ adducts and are therefore not listed. Masses that differ by 16 Da (1702/1718 Da) are possibly due to oxidation of proline or methionine residues.

approximately a 1:1 intensity ratio were observed for peptides with protonated monoisotopic masses of 1011 and 1068 Da. These MS data, as well as preliminary fragmentation studies (data not shown), are consistent with these peptides having the same sequences as [des-Gly^1] bromocontryphan-R and bromocontryphan-R, respectively, from *C. radiatus* (Jimenez et al., 1997). We have also identified a cDNA sequence encoding a putative contryphan in *C. striatus* (data not shown) that is identical to the contryphan-R cDNA sequence from *C. radiatus* (Jimenez et al., 1997), thus supporting this assignment. Also, peptides with protonated masses 933 and 990 Da were detected that correspond to these putative contryphan peptides without the bromine (Jimenez et al., 1996). In these cases, the M+2 peaks are less than 20% of the M peak intensity due to the loss of the bromine.

Although putative contryphans are present in the DV samples from *C. striatus* (Fig. 3B,D,F), they are not likely to be used for prey immobilization, at least in the snails analyzed, because they were not detected in the corresponding IV samples (Fig. 3A,C,E). Further analysis is required to determine the target of the contryphan forms of *C. striatus*. Table 2 summarizes the known and putative conotoxin matches proposed in this work.

Discussion

This study focused on the composition of venom employed by two species of piscivorous cone snails, *Conus striatus* and *C. catus*, and addresses two major questions. First, how consistent is the composition of venom that is injected into prey (IV) within a given species? Second, how does the peptide complement of IV for an individual snail compare to that for crude venom isolated from the venom duct (DV)? In order to address these questions, we employed HPLC purification followed by MALDI-MS or a combination of MALDI-MS and ESI-MS. The excellent selectivity and dynamic range of the MS techniques were essential for establishing the detailed mass profiles of peptides, including co-eluting peptides that would have been obscured if only UV/Vis absorbance detection were employed. These information-rich detection schemes allowed us to confidently compare and contrast venom samples both within the venom apparatus and between individual snails.

Intraspecific variation of the peptides in injected venom

Our results revealed significant intraspecific variation in the peptide composition of IV for both snail species. In the case of *C. catus*, some snails displayed a relatively simple profile (six predominant peptides in snail A; Table 1), whereas others employed up to 14 abundant peptides (snail I; Table 1). In *C. striatus*, which was studied in more detail, we found three qualitatively different types of IV (Fig. 1A–C). One IV profile consisted predominantly of the neuroexcitatory peptide s4a, and a second profile type contained primarily s4a and the related peptide, s4b. Surprisingly, the third IV type lacked s4a and s4b, but displayed the Ca^{2+}-channel blockers, ω-SVIA and

Table 2. *Identification of known and novel* Conus striatus *conotoxins by mass matching*

Peptide	Sequence	Calculated mass $(M+H)^+$ (Da)	Observed mass $(M+H)^+$ (Da)	Reference
α-SI	ICCNPACGPKYSCa	1353.5	1353.3±0.3	1
α-SII	GCCCNPACGPNYGCGTSCS	1790.5	1790.5	2
ω-SVIA	CRSSGSOCGVTSICCGRCYRGKCT	2494.0	2493.8	3, 2
ω-SVIB	CKLKGQSCRKTSYDCCSGSCGRSGKCa	2738.2	2738.0±0.1	3, 2
s4a	qKSLVPSVITTCCGYDOGTMCOOCRCTNSCa	4080.6	4080.3±0.2	4
SO4	ATDCIEAGNYCGPTVMKICCGFCSPYSKICMNYPKN (with 2 hyp)	3950.6	3950.3	3
SO5	STSCMEAGSYCGSTTRICCGYCAYFGKKCIDYPSN	3763.5	3763.4±0.4	3
Propeller	SGPADCCRMKECCTDRVNECLQRYSGREDKFVSFCYQEATVTC-GSFNEIVGCCYGYQMCMIRVVKPNSLSGAHEACKTVSCGNPCA	9432.8**	9433.0**	5
brcn	GCOdWEPWCa	1068.3	1068.1	6, 7
cn	GCOdWEPWCa	990.4	990.1±0.1	6, 7
brcn*	COdWEPWCa	1011.3	1011.1	6, 7
cn*	COdWEPWCa	933.3	933.1±0.1	6, 7

The α-, ω-conotoxins and s4a have all been previously isolated from the DV and characterized.

Underlined peptides are those that are putatively identified in this work by mass matching to published cDNA sequences with predicted modifications.

Locations of PTMs are deduced by sequence homology. Sequence s4b is not included in this table since it will be the subject of a future manuscript (W. P. Kelley, J. R. Schulz, J. A. Jakubowski, W. F. Gilly and J. V. Sweedler, unpublished).

ESI-MS data is only included.

Values are means ± S.D. when multiple mass values were recorded.

**Average mass; all other masses are monoisotopic protonated values.

brcn, bromocontryphan; cn, contryphan; * indicates [des-Gly[1]] (bromo)contryphan form.

O, hydroxyproline (hyp); a, C-terminal amide; q, pyroglutamate; C, disulfide-bonded cysteine; S, O-linked glycosyl; W, 6-bromotryptophan; d, d-amino acid, by homology to contryphan-R.

[1]Zafaralla et al., 1988; [2]Ramilo et al., 1992; [3]Lu et al., 1999; [4]Craig et al., 1998; [5]Ellison, 2003; [6]Jimenez et al., 1996; [7]Jimenez et al., 1997.

ω-SVIB, in addition to the nicotinic acetylcholine-receptor blockers, α-SI and α-SII.

These chemically distinct venom types fall into two functional types as well, based on the predicted effects of the peptide complement on a fish victim. Due to the presence of excitatory toxins, s4a and s4b, the first two venom types would undoubtedly produce the strong, convulsive tetanic paralysis that is well-known for *C. striatus* (Craig et al., 1998). In contrast, these excitatory toxins are lacking in the third IV type, which contains α- and ω-conotoxins that are expected to block neuromuscular transmission (Olivera, 1997) and produce a flaccid paralysis of a fish. To our knowledge, paralysis of prey in this manner has not been reported for *C. striatus*. Observation of prey capture for snails that exhibit this third class of IV profile would certainly be valuable. In addition, further characterization of the putative propeller (Ellison, 2003) and SO5 (Lu et al., 1999) peptides observed in IV samples may reveal other prey capture strategies at the biochemical level within a single species.

Biological processes or physiological mechanisms underlying the variation in IV peptide profiles are not clear. In the present study, the prominent peptides in the IV compositions of individual snails of both species remained quite constant over time in captivity based on analysis of IV samples collected at 1–6 month intervals. Throughout captivity, all snails within each species were housed and fed in the same manner. Moreover, there was no obvious correlation of IV profile-type with any morphological features or collection locality. It is particularly striking that the two snails with disparate IV profiles analyzed in Fig. 2 were similar in size and shell pattern, and they were both collected at the same time and location. These observations suggest that genetic differences may contribute to the observed individuality in IV profiles, although environmental factors or ontogenetic differences cannot be ruled out.

To our knowledge, such a remarkable degree of intraspecific variation in the peptide composition of venom is novel. In the case of *Conus*, we are unaware of any reports of variation in the IV profile between individuals. Striking divergence of peptide complements when comparing venoms of different *Conus* species has been noted (Olivera, 1997). However, the existence of multiple distinct IV profiles within the same species reveals an even greater level of peptide diversity in *Conus* venoms than heretofore realized.

Significantly less dramatic variations in the peptide complement of injected venoms have been reported for several other organisms, including snakes (Chippaux et al., 1991; Creer et al., 2003; Daltry et al., 1996a,b; Francischetti et al., 2000; MacKessy et al., 2003; Monteiro et al., 1998a,b), spiders (Binford, 2001; Cristina de Oliveira et al., 1999; Escoubas et al., 2002), scorpions (El Hafny et al., 2002; Kalapothakis and Chavez-Olortegui, 1997; Pimenta et al., 2003) and bees (Lai

and Her, 2000). Variation of the peptide profiles of these other venomous animals has been associated with sex (Binford, 2001; Cristina de Oliveira et al., 1999; Escoubas et al., 2002), diet (Daltry et al., 1996b), age (Escoubas et al., 2002), geography (Binford, 2001; Creer et al., 2003), season (Monteiro et al., 1998b) and venom regeneration time (Pimenta et al., 2003). Studies controlling many of these factors have still observed venom variation, implying that intraspecific differences can be a result of genetic as well as environmental factors (Daltry et al., 1996a; Francischetti et al., 2000; Kalapothakis and Chavez-Olortegui, 1997; Monteiro et al., 1998a,b). Cellular and molecular mechanisms underlying and controlling such variation remain unknown.

Differences in composition of duct venom and injected venom

A consistent finding in this study, based on analysis of individual *C. striatus,* is that the peptide composition of DV is significantly more complex than that of IV, in agreement with previous studies (Bingham et al., 1996; Martinez et al., 1995). In general, IV appears to contain a substantially simplified subset of the DV peptides (Fig. 2). Furthermore, analysis of the water-soluble DV (Fig. 3) is not consistent with creation of IV by simply partitioning the water-soluble peptides from the insoluble matter in DV. These observations suggest that a more complex selection process exists by which certain peptides are transported from the venom duct and utilized for injection, whereas others are not. Moreover, many of the rejected forms are functional toxins that have previously been identified in DV.

Mechanisms underlying the evident simplification of DV for injection are unknown, although anatomical studies of *C. californicus* suggest that a specialized epithelial zone connecting the venom duct to the pharynx may be the site of such processing (Marshall et al., 2002). Additionally, the pronounced intraspecific variation observed in IV peptide profiles of both *C. striatus* and *C. catus* suggests that selection and delivery processes, which ultimately yield the biologically relevant venom used in prey capture, are likely to be complex and specifically utilized by individual snails to varying degrees. The intricacy of the venom preparation process is further exemplified by the distinct IV complements (Fig. 2A,C) that were created from relatively similar DV profiles (Fig. 2B,D). The reproducibility of DV profiles from snail to snail is in qualitative agreement with a recent study showing that the composition of DV in *Conus regius* is consistent even for snails of different gender and size or those collected in different seasons (Vianna Braga et al., 2005).

Our data generally support a theory of venom processing like that discussed above, although there are potentially additional facets of the venom preparation process. Notably, IV may contain peptides (e.g. ω-SVIB in Fig. 3) that cannot always be detected in DV from the same snail. The apparent lack of these peptides in the complex DV samples may be partially due to ion suppression effects. However, we cannot rule out some long-term changes in DV composition over time in captivity that are not reflected in IV samples obtained before the snails were killed. Alternatively, ω-SVIB (and other peptides) may be processed from larger precursors in the venom duct, or exported out of the duct into IV more efficiently than other peptides. Another possibility is that certain peptides may be synthesized in some component of the venom apparatus other than the venom duct, for example in the radular sac where the teeth are stored prior to use. Previous work on *C. californicus* revealed peptides inside the lumen of stored teeth (Marshall et al., 2002), but neither the identity of those peptides nor their route of delivery is known. Similar studies have not been performed for any other species. To further elucidate the mechanisms of conotoxin processing, it would be informative to compare cDNA libraries created from the radular sac and other parts of the venom apparatus with those created using venom duct tissue. Variation in gene expression is known to exist even within the venom duct, as shown by a recent analysis of *Conus textile* venom ducts (Garrett et al., 2005).

Studies of injected venom and conservation of Conus *resources*

Recently, concern over the potential over-exploitation of *Conus* snails for research purposes was discussed (Chivian et al., 2003; Duda et al., 2004). The present study demonstrates that the analysis of IV samples and a limited number of DV samples can yield a large data set from a small number of animals. Samples of IV can be obtained from the same snails over the course of several years for both chemical and physiological analyses. Furthermore, knowledge of a particular snail's IV profile can facilitate isolation and identification of peptides with novel or particularly high bioactivity, such as in the case of the snail displaying the s4a-type of profile in Fig. 1A. In the extreme case, analysis of IV samples by sensitive MS-based sequencing techniques can enable peptide discovery and characterization without the sacrifice of any animals whatsoever.

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List of abbreviations

DV	duct venom
IV	injected venom
HPLC	high performance liquid chromatography
MALDI-MS	matrix-assisted laser desorption/ionization-time of flight-mass spectrometry
ESI-MS	electrospray ionization-ion trap-mass spectrometry
UV/Vis	ultraviolet-visible
TFA	trifluoroacetic acid
CHCA	α-cyano-4-hydroxycinnamic acid.

References

Binford, G. J. (2001). An analysis of geographic and intersexual chemical variation in venoms of the spider *Tegenaria agrestis* (Agelenidae). *Toxicon* **39**, 955-968.

Bingham, J. P., Jones, A., Lewis, R. J., Andrew, P. R. and Alewood, P. F. (1996). *Conus* venom peptides (conopeptides): inter-species, intra-species and within individual variation revealed by ionspray mass spectrometry. In *Biomedical Aspects of Marine Pharmacology* (ed. E. Lazarovici, M. E. Spira and E. Zlotkin), pp. 13-27. Fort Collins, CO, USA: Alaken.

Bingham, J. P., Broxton, N. M., Livett, B. G., Down, J. G., Jones, A. and Moczydlowski, E. G. (2005). Optimizing the connectivity in disulfide-rich peptides: alpha-conotoxin SII as a case study. *Anal. Biochem.* **338**, 48-61.

Bulaj, G., DeLaCruz, R., Azimi-Zonooz, A., West, P., Watkins, M., Yoshikami, D. and Olivera, B. M. (2001). Delta-conotoxin structure/function through a cladistic analysis. *Biochemistry* **40**, 13201-13208.

Chippaux, J. P., Williams, V. and White, J. (1991). Snake venom variability: methods of study, results and interpretation. *Toxicon* **29**, 1279-1303.

Chivian, E., Roberts, C. M. and Bernstein, A. S. (2003). The threat to cone snails. *Science* **302**, 391.

Conticello, S. G., Gilad, Y., Avidan, N., Ben-Asher, E., Levy, Z. and Fainzilber, M. (2001). Mechanisms for evolving hypervariability: the case of conopeptides. *Mol. Biol. Evol.* **18**, 120-131.

Craig, A. G., Fischer, W. H., Rivier, J. E., McIntosh, J. M. and Gray, W. R. (1995). MS based scanning methodologies applied to *Conus* venom. In *Techniques in Protein Chemistry VI*. Vol. VI (ed. J. W. Crabb), pp. 31-37. San Diego: Academic Press.

Craig, A. G., Zafaralla, G., Cruz, L. J., Santos, A. D., Hillyard, D. R., Dykert, J., Rivier, J. E., Gray, W. R., Imperial, J., Delacruz, R. G. et al. (1998). An O-glycosylated neuroexcitatory *Conus* peptide. *Biochemistry* **37**, 16019-16025.

Creer, S., Malhotra, A., Thorpe, R. S., Stocklin, R., Favreau, P. and Chou, W. H. (2003). Genetic and ecological correlates of intraspecific variation in pitviper venom composition detected using matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS) and isoelectric focusing. *J. Mol. Evol.* **56**, 317-329.

Cristina de Oliveira, K., Goncalves de Andrade, R. M., Giusti, A. L., Dias da Silva, W. and Tambourgi, D. V. (1999). Sex-linked variation of *Loxosceles intermedia* spider venoms. *Toxicon* **37**, 217-221.

Daltry, J. C., Ponnudurai, G., Shin, C. K., Tan, N. H., Thorpe, R. S. and Wuster, W. (1996a). Electrophoretic profiles and biological activities: intraspecific variation in the venom of the Malayan pit viper (*Calloselasma rhodostoma*). *Toxicon* **34**, 67-79.

Daltry, J. C., Wuster, W. and Thorpe, R. S. (1996b). Diet and snake venom evolution. *Nature* **379**, 537-540.

Duda, T. F. and Palumbi, S. R. (1999). Molecular genetics of ecological diversification: duplication and rapid evolution of toxin genes of the venomous gastropod *Conus*. *Proc. Natl. Acad. Sci. USA* **96**, 6820-6823.

Duda, T. F. and Palumbi, S. R. (2000). Evolutionary diversification of multigene families: allelic selection of toxins in predatory cone snails. *Mol. Biol. Evol.* **17**, 1286-1293.

Duda, T. F., Jr and Palumbi, S. R. (2004). Gene expression and feeding ecology: evolution of piscivory in the venomous gastropod genus *Conus*. *Proc. R. Soc. Lond. B* **271**, 1165-1174.

Duda, T. F., Bingham, J. P., Livett, B. G., Kohn, A. J., Massilia, G. R., Schultz, J. R., Down, J., Sandall, D. and Sweedler, J. V. (2004). How much at risk are cone snails? *Science* **303**, 955.

El Hafny, B., Chgoury, F., Adil, N., Cohen, N. and Hassar, M. (2002). Intraspecific variability and pharmacokinetic characteristics of *Androctonus mauretanicus mauretanicus* scorpion venom. *Toxicon* **40**, 1609-1616.

Ellison, M. A. (2003). Novel natural and combinatorial alpha4,3 conotoxins as inhibitors of the alpha7 nicotinic acetylcholine receptor. PhD thesis, University of Utah, Salt Lake City, USA.

Escoubas, P., Corzo, G., Whiteley, B. J., Celerier, M. L. and Nakajima, T. (2002). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and high-performance liquid chromatography study of quantitative and qualitative variation in tarantula spider venoms. *Rapid Commun. Mass Spectr.* **16**, 403-413.

Espiritu, D. J. D., Watkins, M., Dia-Monje, V., Cartier, G. E., Cruz, L. J. and Olivera, B. M. (2001). Venomous cone snails: molecular phylogeny and the generation of toxin diversity. *Toxicon* **39**, 1899-1916.

Francischetti, I. M., Gombarovits, M. E., Valenzuela, J. G., Carlini, C. R. and Guimaraes, J. A. (2000). Intraspecific variation in the venoms of the South American rattlesnake (*Crotalus durissus terrificus*). *Comp. Biochem. Physiol.* **127C**, 23-36.

Garrett, J. E., Buczek, O., Watkins, M., Olivera, B. M. and Bulaj, G. (2005). Biochemical and gene expression analyses of conotoxins in *Conus textile* venom ducts. *Biochem. Biophys. Res. Commun.* **328**, 362-367.

Hopkins, C., Grilley, M., Miller, C., Shon, K. J., Cruz, L. J., Gray, W. R., Dykert, J., Rivier, J., Yoshikami, D. and Olivera, B. M. (1995). A new family of *Conus* peptides targeted to the nicotinic acetylcholine receptor. *J. Biol. Chem.* **270**, 22361-22367.

Jakubowski, J. A. and Sweedler, J. V. (2004). Sequencing and mass profiling highly modified conotoxins using global reduction/alkylation followed by mass spectrometry. *Anal. Chem.* **76**, 6541-6547.

Jimenez, E. C., Olivera, B. M., Gray, W. R. and Cruz, L. J. (1996). Contryphan is a D-tryptophan-containing *Conus* peptide. *J. Biol. Chem.* **271**, 28002-28005.

Jimenez, E. C., Craig, A. G., Watkins, M., Hillyard, D. R., Gray, W. R., Gulyas, J., Rivier, J. E., Cruz, L. J. and Olivera, B. M. (1997). Bromocontryphan – post-translational bromination of tryptophan. *Biochemistry* **36**, 989-994.

Jones, A., Bingham, J. P., Gehrmann, J., Bond, T., Loughnan, M., Atkins, A., Lewis, R. J. and Alewood, P. F. (1996). Isolation and characterization of conopeptides by high-performance liquid chromatography combined with mass spectrometry and tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **10**, 138-143.

Kalapothakis, E. and Chavez-Olortegui, C. (1997). Venom variability among several *Tityus serrulatus* specimens. *Toxicon* **35**, 1523-1529.

Kohn, A. J. (1956). Piscivorous gastropods of the genus *Conus*. *Proc. Natl. Acad. Sci. USA* **42**, 168-172.

Lai, C. C. and Her, G. R. (2000). Analysis of phospholipase A(2) glycosylation patterns from venom of individual bees by capillary electrophoresis/electrospray ionization mass spectrometry using an ion trap mass spectrometer. *Rapid Commun. Mass Spectr.* **14**, 2012-2018.

Lu, B. S., Yu, F., Zhao, D., Huang, P. T. and Huang, C. F. (1999). Conopeptides from *Conus striatus* and *Conus textile* by cDNA cloning. *Peptides* **20**, 1139-1144.

MacKessy, S. P., Williams, K. and Ashon, K. G. (2003). Ontogenetic variation in venom composition and diet of *Crotalus oreganus concolor*: A case of venom paedomorphosis? *Copeia* **4**, 769-782.

Marshall, J., Kelley, W. P., Rubakhin, S. S., Bingham, J. P., Sweedler, J. V. and Gilly, W. F. (2002). Anatomical correlates of venom production in *Conus californicus*. *Biol. Bull.* **203**, 27-41.

Martinez, J. S., Olivera, B. M., Gray, W. R., Craig, A. G., Groebe, D. R., Abramson, S. N. and McIntosh, J. M. (1995). Alpha-conotoxin EI, a new nicotinic acetylcholine receptor antagonist with novel selectivity. *Biochemistry* **34**, 14519-14526.

Massilia, G. R., Eliseo, T., Grolleau, F., Lapied, B., Barbier, J., Bournaud, R., Molgo, J., Cicero, D. O., Paci, M., Schinina, M. E. et al. (2003). Contryphan-Vn: a modulator of Ca^{2+}-dependent K^+ channels. *Biochem. Biophys. Res. Commun.* **303**, 238-246.

Monteiro, R. Q., Dutra, D. L. S., Machado, O. L. T., Carlini, C. R., Guimaraes, J. A., Bon, C. and Zingali, R. B. (1998a). *Bothrops jararaca* snakes produce several bothrojaracin isoforms following an individual pattern. *Comp. Biochem. Physiol.* **120B**, 791-798.

Monteiro, R. Q., Yamanouye, N., Carlini, C. R., Guimaraes, J. A., Bon, C. and Zingali, R. B. (1998b). Variability of bothrojaracin isoforms and other venom principles in individual jararaca (*Bothrops jararaca*) snakes maintained under seasonally invariant conditions. *Toxicon* **36**, 153-163.

Olivera, B. M. (1997). E. E. Just Lecture, 1996. *Conus* venom peptides, receptor and ion channel targets, and drug design – 50 million years of neuropharmacology. *Mol. Biol. Cell* **8**, 2101-2109.

Pimenta, A. M. C., Almeida, F. D., de Lima, M. E., Martin-Eauclaire, M. F. and Bougis, P. E. (2003). Individual variability in *Tityus serrulatus* (Scorpiones, Buthidae) venom elicited by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectr.* **17**, 413-418.

Ramilo, C. A., Zafaralla, G. C., Nadasdi, L., Hammerland, L. G., Yoshikami, D., Gray, W. R., Kristipati, R., Ramachandran, J., Miljanich, G. and Olivera, B. M. (1992). Novel alpha- and omega-conotoxins from *Conus striatus* venom. *Biochemistry* **31**, 9919-9926.

Santos, A. D., McIntosh, J. M., Hillyard, D. R., Cruz, L. J. and Olivera, B. M. (2004). The A-superfamily of conotoxins – structural and functional divergence. *J. Biol. Chem.* **279**, 17596-17606.

Schulz, J. R., Norton, A. G. and Gilly, W. F. (2004). The projectile tooth of a fish-hunting cone snail: *Conus catus* injects venom into fish prey using a high-speed ballistic mechanism. *Biol. Bull.* **207**, 77-79.

Shon, K. J., Grilley, M. M., Marsh, M., Yoshikami, D., Hall, A. R., Kurz,

B., Gray, W. R., Imperial, J. S., Hillyard, D. R. and Olivera, B. M. (1995). Purification, characterization, synthesis, and cloning of the lockjaw peptide from *Conus purpurascens* venom. *Biochemistry* **34**, 4913-4918.

Shon, K. J., Grilley, M., Jacobsen, R., Cartier, G. E., Hopkins, C., Gray, W. R., Watkins, M., Hillyard, D. R., Rivier, J., Torres, J. et al. (1997). A noncompetitive peptide inhibitor of the nicotinic acetylcholine receptor from *Conus purpurascens* venom. *Biochemistry* **36**, 9581-9587.

Teichert, R. W., Rivier, J., Dykert, J., Cervini, L., Gulyas, J., Bulaj, G., Ellison, M. and Olivera, B. M. (2004). AlphaA-Conotoxin OIVA defines a new alphaA-conotoxin subfamily of nicotinic acetylcholine receptor inhibitors. *Toxicon* **44**, 207-214.

Vianna Braga, M. C., Konno, K., Portaro, F. C., Carlos de Freitas, J., Yamane, T., Olivera, B. M. and Pimenta, D. C. (2005). Mass spectrometric and high performance liquid chromatography profiling of the venom of the Brazilian vermivorous mollusk *Conus regius*: feeding behavior and identification of one novel conotoxin. *Toxicon* **45**, 113-122.

Walker, C. S., Steel, D., Jacobsen, R. B., Lirazan, M. B., Cruz, L. J., Hooper, D., Shetty, R., DelaCruz, R. C., Nielsen, J. S., Zhou, L. M. et al. (1999). The T-superfamily of conotoxins. *J. Biol. Chem.* **274**, 30664-30671.

Watson, E. and Kenney, W. C. (1992). Multiple peak formation from reversed-phase liquid chromatography of recombinant human platelet-derived growth factor. *J. Chromatogr.* **606**, 165-170.

Zafaralla, G. C., Ramilo, C., Gray, W. R., Karlstrom, R., Olivera, B. M. and Cruz, L. J. (1988). Phylogenetic specificity of cholinergic ligands: alpha-conotoxin SI. *Biochemistry* **27**, 7102-7105.