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Comparative Venomics Reveals the Complex Prey Capture Strategy of the Piscivorous Cone Snail *Conus catus*

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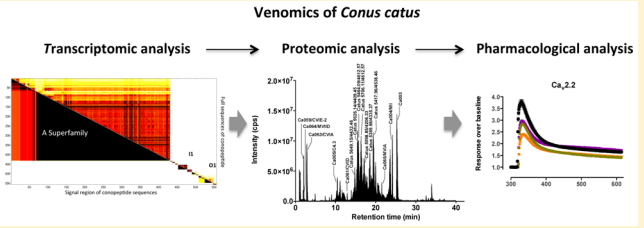
Supporting Information

ABSTRACT: Venomous marine cone snails produce a unique and remarkably diverse range of venom peptides (conotoxins and conopeptides) that have proven to be invaluable as pharmacological probes and leads to new therapies. *Conus catus* is a hook-and-line fish hunter from clade I, with ∼20 conotoxins identified, including the analgesic ω-conotoxin CVID (AM336). The current study unravels the venom composition of *C. catus* with tandem mass spectrometry and 454 sequencing data. From the venom gland transcriptome, 104 precursors were recovered from 11 superfamilies, with superfamily A (especially κA-) conotoxins dominating (77%) their venom. Proteomic analysis confirmed that κA-conotoxins dominated the predation-evoked milked venom of each of six *C. catus* analyzed and revealed remarkable intraspecific variation in both the intensity and type of conotoxins. High-throughput FLIPR assays revealed that the predation-evoked venom contained a range of conotoxins targeting the nAChR, Ca_v, and Na_v ion channels, consistent with α- and ω-conotoxins being used for predation by *C. catus*. However, the κA-conotoxins did not act at these targets but induced potent and rapid immobilization followed by bursts of activity and finally paralysis when injected intramuscularly in zebrafish. Our venomics approach revealed the complexity of the envenomation strategy used by *C. catus*, which contains a mix of both excitatory and inhibitory venom peptides.



KEYWORDS: *Conus catus, transcriptome, κA-conotoxin, ω-conotoxin, glycosylation, predatory-evoked venom, intraspecific variation*

INTRODUCTION

Conus represent the largest genus of marine invertebrates, with more than 750 species described to date.[1] The early evolution of venom has been a key innovation contributing to their explosive radiation into vermivorous (worm hunters), molluscivorous (mollusk hunters), or piscivorous (fish hunters) species. The venom typically comprises a complex cocktail of potent peptides known as conotoxins or conopeptides that modulate a diverse array of key physiological targets.[2,3] Each species of cone snail has evolved its own repertoire of venom peptides[4] that are adaptively fine-tuned for effective prey capture[5] and defense.[6] In addition to providing unique pharmacological tools, these small bioactive peptides are also potential therapeutics, exemplified by the FDA-approved $Ca_v2.2$ channel blocker ω-MVIIA (Prialt) marketed for unmanageable pain[7,8] and χ-conotoxin MrIA (Xen2174) with potential in cancer pain.[9]

Recent advances in the field of venomics,[10] which were facilitated by drastic reductions in the cost of second-generation sequencing and the enhanced sensitivity of tandem mass spectrometry (MS/MS), has facilitated a comprehensive analysis of the conotoxins in *Conus geographus*,[6,11] *Conus bullatus*,[12] *Conus consors*,[13] *Conus victoriae*,[14] *Conus pulicarius*,[15] *Conus mamoreus*[16] and *Conus miles*.[17] *Conus catus* is an Indo-Pacific hook-and-line fish hunter in the phylogenetic clade *Pionoconus* (clade I) that is closely related to *Conus magus*, *C. consors*, *Conus striatus* and *C. striolatus*.[18] The prey-capture strategy of these species is thought to involve an initial rigid immobilization associated with the "lightning strike cabal" followed by a slower flaccid paralysis associated with the "motor cabal".[19−21] Currently, only seven ω-conotoxins CVIA−CVIF[22−24] and catus-C2,[5,18] δ-conotoxin CVIE,[25] and μ-conotoxin CIIIA[26] have been pharmacologically characterized, whereas three κA-like conotoxins C4.1/C4.1b/C4.2, eight ω-like conotoxins (C6.1−8), and one α-like peptide (C4.3)[18] have also been identified from the venom of *C. catus* (Supporting Information Table S1).

Using an integrated venomics approach, this study revealed that the predation-evoked venom of *C. catus* is largely dominated by the glycosylated κA-conotoxins. To further explore the origins of conotoxin diversity, the predation-evoked milked venom of six *C. catus* specimens was analyzed both qualitatively and quantitatively by tandem mass spectrometry,

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revealing significant intraspecies variation among the peptides present. While κA-conotoxin precursors have been discovered in clade I cone snails, *C. catus* contains the largest pool of glycosylated peptides yet reported. Interestingly, the predation-evoked injected venom from *C. catus* also contained significant amount of vertebrate-active and paralytic α-, ω-, and μ-conotoxins, in contrast to the predatory venom of *C. geographus* and another clade I cone snail, *C. consors*.[6,27]

EXPERIMENTAL PROCEDURES

RNA Extraction, cDNA Library, 454 Sequencing, and Assembly

Six adult *C. catus* specimens collected from One Tree Island on the Great Barrier Reef (Queensland, Australia) were sacrificed and dissected on ice. To extract total RNA, the venom ducts were removed and placed in a 1.5 mL tube containing 1 mL of TRIzol reagent (Invitrogen). RNA extraction from the six venom ducts was carried out according to the manufacturer's instructions, with mRNA being further purified using an Oligotex mRNA mini kit (Qiagen, Valencia, CA). Extracted mRNA was submitted to the Australian Genomic Research Facility (AGRF), where cDNA library construction and sequencing were carried out using a Roche GS FLX Titanium sequencer from one-eighth of a plate. Finally, the sequence assembly was performed using Newbler 2.3 (Life Science, Frederick, CO).

Conopeptide Sequence Analysis

Raw cDNA reads and Newbler-2.3-assembled isotigs were searched using the default parameters in ConoSorter[28] and sorted using SignalP and ConoPrec to identify the conserved signal sequences, cysteine frameworks, and cleavage sites.[29] During this process, precursors less than 50 amino acid in length, signal sequence hydrophobicity less than 40%, and repeated sequences were manually removed. Considering the published variations in signal conservation within superfamilies, the cutoff value used to assign a gene superfamily was set as 53.3%.[28]

Alignment, Phylogenetic Tree, and Similarity Matrix Construction

Multiple sequence alignments for all conopeptides were performed using MUSCLE, v. 3.8.31, followed by manual adjustment using Geneious software, v. 7.1.7. Prior to phylogeny analysis, poorly aligned positions and ambiguous blocks were removed using GBlocks, v. 0.91b.[30,31] Phylogenetic trees were constructed by Genious, v. 7.1.7, using statistical method UPGMA, genetic distance model Jukes-Cantor, and bootstrapped 1000 times with a topology threshold of 50%. In order to build the similarity matrix, aligned sequences were ordered vertically and horizontally according to their phylogenetic rank order. Similarity scores (%) were then given to all sequences based on their evolutionary distances using MATLAB software, v. R2013a, and plotted as a heat map.[31] Homologues sequences were retrieved from the matrix and categorized into gene superfamilies using ConoPrec.

Venom Sample Preparation for Proteomics

Six adult specimens of *C. catus* were milked in predatory mode as described previously.[16] The collected milked venom (5−10 μL/specimen) contained insoluble white granules that were removed prior to analysis by centrifuging at 1000*g*. The predation-evoked venoms were separately analyzed for intra-specific variation and then pooled for further comparative proteomic and pharmacological studies.

Reduction−Alkylation and Enzyme Digestion

The aliquots of venom were lyophilized and reconstituted in 50 μL of freshly prepared 100 mM NH_4HCO_3 in 30% acetonitrile at pH 8 prior to reduction and alkylation using the previously described triethylphosphine/iodoethanol protocol.[16] Sigma proteomics sequencing grade trypsin and endoproteinase Glu-C were used for enzyme digestion of reduced and alkylated peptides, as described.[17]

Mass Spectrometry and Proteomic Analysis

Liquid chromatography−electrospray mass spectrometry (LC−ESI−MS) was performed on an AB Sciex QSTAR Pulsar or TripleTOF 5600 instrument, as previously described.[16] Two alternating scanning methods allowed selective detection of glycopeptides on the TripleTOF 5600, with full scan data (100−2400 m/z) obtained using an in-source CE of 8, and a CE of 35 used to increase the ion velocity between capillary and skimmer regions in the ion source to induce sugar dissociation. Information-dependent acquisition was performed on the reduced, reduced/alkylated, and enzymatically digested venom samples. ProteinPilot 4.0 software was used for peak list generation and sequence identification by searching the LC−ESI−MS/MS spectra against the sorted conopeptide sequences using previously described parameters.[17] The post-translational modifications commonly found in conopeptides (amidation, deamidation, hydroxylation of proline and valine, oxidation of methionine, carboxylation of glutamic acid, cyclization of N-terminal glutamine (pyroglutamate), bromination of tryptophan, and sulfation of tyrosine) were considered with a mass tolerance set at ±0.05 Da for precursor ions and ±0.1 Da for fragment ions. Tandem mass spectra were acquired only on 2 to 5 charged ions, with information-dependent acquisition settings set to exclude former target ions for 8 s and exclude isotopes within 4 Da. The threshold score for accepting individual peptide spectra was set as 99%.

Preparative HPLC Fractionation

A 1 mg aliquot of pooled predatory-evoked injected venom was fractionated using a Thermo C_{18} 4.6 × 150 mm column (5 μm) fitted to a Shimadzu Prominence HPLC system with 0.043% trifluoroacetic acid/90% acetonitrile(aq) as elution buffer B and 0.05% trifluoroacetic acid(aq) as buffer A. A linear gradient of 1% B min^{-1} was maintained at a flow rate of 1 mL min^{-1} for 80 min. The eluent was monitored at 214 and 280 nm, with 214 nm used to guide sample collection.

Bioassay for Ca_v, Na_v, and nAChR Activity

The biological activity of the HPLC fractions were assessed using high-throughput Ca^{2+} imaging assays in human SH-SY5Y cells, as previously described.[6,32] Cells were plated into 384-well black-walled imaging plates at a density of 35 000 to 50 000 cells per well and cultured for 48 h. Fluorescent responses (excitation, 470−495 nm; emission, 515−575 nm) were assessed using the FLIPR^{Tetra} fluorescent plate reader (Molecular Devices) after a 30 min incubation with a fluorescent Ca^{2+} dye (Calcium 4 No Wash dye, Molecular Devices) diluted in physiological salt solution (composition in mM: NaCl 140, glucose 11.5, KCl 5.9, $MgCl_2$ 1.4, NaH_2PO_4 1.2, $NaHCO_3$ 5, $CaCl_2$ 1.8, HEPES 10). The equivalent of 50 μg crude venom was then added 5 min prior to stimulation of endogenously expressed Na_v, Ca_v, and nAChR isoforms. To assess activity at Na_v isoforms endogenously expressed in SH-

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SY5Y cells (Na_v 1.2 and Na_v 1.7), SH-SY5Y cells were stimulated with veratridine (50 μM); activity at $Ca_v2.2$ channels was assessed in the presence of nifedipine (10 μM) after stimulation with KCl (90 mM) and $CaCl_2$ (5 mM). Nicotine (30 μM) was used to activate endogenously expressed human $\alpha3\beta2$ and $\alpha3\beta4$ nAChR, and endogenously expressed human $\alpha7$ nAChR was activated using the $\alpha7$ nAChR agonist choline (30 μM) in the presence of the allosteric modulator PNU120596 (10 μM, Sigma-Aldrich). Responses were normalized to baseline using ScreenWorks 3.2.0.14 (Molecular Devices).

Matrix-Assisted Laser Desorption Ionization-MS

Matrix-assisted laser desorption ionization (MALDI)-MS analyses were conducted using an AB SCIEX (Framingham, MA, USA) 4700 TOF-TOF to identify masses associated with the active fractions determined above. A 0.5 μL aliquot of each fraction were diluted 1:1 with matrix comprising 10 mg mL^{-1} α-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile/0.1% formic acid(aq). MALDI-TOF spectra were acquired in reflector positive operating mode with source voltage set to 20 kV and Grid1 voltage at 12 kV, mass range 1000−8000 Da, and a focus mass of 3500 Da. Each plate was calibrated using Calmix (4700 Proteomics analyzer calibration mixture) from Applied Biosystems (Foster City, CA).

Fish Assay

Zebrafish were maintained using standard husbandry procedures and used following the approved guidelines of the Animal Ethics Committee at the University of Queensland. Venoms fractions were tested in adult wild-type zebrafish (500−600 mg). Intramuscular (i.m.) injections (5 μL) were performed using a Hamilton syringe with a cemented point style 2 26s gauge needle (Sigma-Aldrich no. 20795-U). Injected animals were placed in 1 L tanks under ambient light conditions, and their swimming tracks were recorded using the zebrabox revolution (Viewpoint) imaging platform.

RESULTS

Conopeptide Content in *C. catus* Venom Duct Transcriptome

454 sequencing generated 136 495 cDNA reads with an average length of 409 bp after trimming and cleaning. The raw cDNA reads were searched using the default parameters in our in-house program ConoSorter, and retrieved peptides were filtered through a SignalP and ConoServer programs to identify 557 putative conopeptide sequences. The cDNA read numbers of the precursors and isoforms of the transcriptome were unevenly distributed, with only 8% of the precursors having more than 10 cDNA read. In contrast, 70% were single reads (see Supporting Information Figure S1) recently reported as "transcriptomic messiness".[17] By evaluating the mutations generating these rare transcripts at both amino acid and nucleotide levels, they were categorized into three groups: (i) transcripts with a single mutation (29.8%), (ii) transcripts with premature stop codons (16%), and (iii) transcripts with frame shifts leading to unusual C-terminal elongations (54%). Given their high frequency (>7% for each type), these single read variants cannot be explained by the inherent error rate of the 454 sequencing (<1.07%).[33] However, given that these single read variants were all associated with single amino acid changes around a few dominant sequences and to avoid analyzing possible false sequences, these rare isoforms were excluded from further analysis, and the remaining 104 precursors were named Ca001−Ca104 (see Supporting Information Figure S2).

The resulting 104 putative conopeptides were categorized into 11 gene superfamilies using ConoPrec (Figure 1),

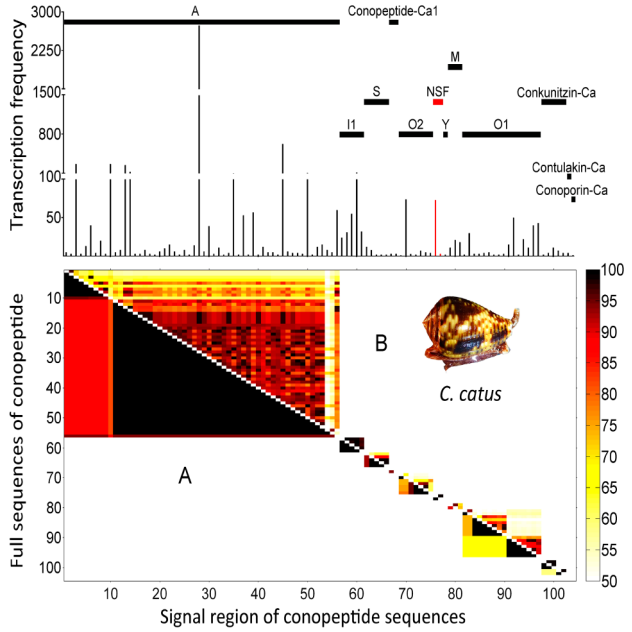

Figure 1. Visualization of the transcriptome of *C. catus*. The heat-map matrix (bottom panel) displays sequence clusters and similarity of conopeptide transcripts in *C. catus*. Sequences in these plots are ranked according to the phylogenetic tree, and the order is the same in both the horizontal and vertical directions. The panels A and B show correlations among conserved signal regions and full-length sequences, respectively. Sequence clusters are represented by squares along the diagonal. Sequences in clusters are colored according to their evolutionary distance in such a way that when one sequence in the horizontal direction matches the other sequences in the vertical direction (or vice versa), the area will be filled with the color representing the similarity with a threshold of 53.3%. The top panel of the graph shows the frequency of precursor conopeptides in the venom duct transcriptome of *C. catus*. Red represents novel superfamily, and black lines represent known superfamilies.

including the cysteine-rich gene superfamilies A, O1, O2, I1, M, S, Y and conkunitzin and the linear contryphans, contulakins, and conoporins. Using a signal peptide similarity cutoff of 53.3%,[28] only one new superfamily, Cat-NSF1, was identified from the *C. catus* transcriptome. The signal peptides and the frameworks corresponding to the 11 identified superfamilies are listed in Table 1. Interestingly, apart from A and O1 superfamily peptides, the other nine superfamilies had not been reported previously from *C. catus* venom. In contrast, only 10 out of 23 previously reported precursors (Supporting Information Table S1) were found in our transcriptome of six pooled specimens, indicative of significant intraspecific variation in this species.

Proteomics of *C. catus* Predation-Evoked Venom

LC−ESI−MS and LC−ESI−MS/MS were used to unravel the venom components of the predation-evoked injected venom of *C. catus*. The LC−ESI−MS spectrum of the pooled predatory-evoked milked venom of six specimens is shown in Figure 2, with 7 of the 10 previously reported peptides found in the transcriptome identified as major ions (LC−ESI−MS/MS spectrum is shown in Supporting Information Figure S3).

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Table 1. Characteristics of Superfamilies Identified in the *C. catus* Venom Duct Transcriptome

family	no. precursors	no. reads	signal peptide	main framework	probable pharmacology
A	56	5012	MGMRMMFTVFLLVVLATTVVS	IV, I	κA, α
			MFTVFLLVVLATTVVS		
			MFTVFLLVVLATTA		
			MFTVFLLVGLATTLVSIPSDG		
			MFTVFLLVGLATTLVSILQMVHLMA		
O1	16	219	MKLTCVLIIAVLFLTAITA	VI/VII	ω, δ
			MKLTCVVIVAVLLLTACQLITA		
I1	5	255	MKTVAVFLVVALAVAYG	XXII	
O2/Contryphan	7	96	MEKLTILLLVAAVLMSTQALIQG	VI/VII	
M	3	50	MMSKLGVLLTICLVLLPLTA	III	μ
			MMSKLGVLLTICLLLFPLTVLS		
			MLKMPVLLLAILLLLPLATA		
S	5	26	MFVLLLLFTLASSQQ	VIII	σ
			MMSKMGAMFVLLLLFTLASVQQ		
Y	1	2	MLKMPVLLLAILLLLPLATA	VI/VII	
Cat NSF1	2	76	MGALMLVHFVASIQDSAA	VI/VII	δ
Conkunitzin-Ca	5	28	MEGRRFAAVLILTICMLAPGTG		
			MEGRRLAVVLIVTSCLSALTVG		
Conopeptide-Ca	2	6	MLRLIIAAAVFVSACLA		
Contulakin-Ca	1	4	MQTAYWVMVMMMVWITAPLSEG		
Conoporin-Ca	1	1	MEVPFPALKTMVTVFLLLMGNMSPVVM		

Interestingly, two of these contoxins (MII and MVIA) were initially identified from the clade I species *C. magus*. In the extracted ion profile of the milked venom, carbohydrate marker ions corresponding to $HexNAc^+$ (m/z 204) and $Hex\text{-}HexNAc^+$ (m/z 366) were abundant from 9 to 17.5 min (Supporting Information Figure S4), while minor peaks corresponding to $[NeuAc - H_2O]^+$ (m/z 274) and $NeuAc^+$ (m/z 292) were also observed (Supporting Information Figure S4). As observed in a proteomic study of *C. consors* venom, we predicted that these glycosylated regions are derived from the abundant κA-conotoxins present in *C. catus* venom.[13] Unfortunately, the ConoMass tool in Conoserver does not support the addition of glycosylation as a possible PTM; therefore, κA-like conotoxins could not be characterized with high precision. Excluding the κA-conotoxins, 51 out of 56 conopeptides (>90%) were identified at the LC−MS level with a precision of ±0.25 Da.

Integrated Transcriptomic and Proteomic Analysis

To confirm expression of identified transcriptomic sequences, LC−ESI−MS/MS was performed on the reduced, alkylated, and trypsin/GluC digested predatory venom, and full and partial mature sequences with a confidence value over 99% were identified (Table 2). The abundant glycosylated κA-conotoxins interfered with LC−MS and MS/MS analysis. Despite of this limitation, 4 full mature sequences and fragments from 30 other sequences were retrieved from the ProteinPilot 4.0 data guided by the final set of 104 precursors. The most prominent feature of *C. catus* venom duct transcriptome was the high level of expression of A superfamily conotoxins, which comprised 77.2% of total transcriptomic reads (Figure 1). When the precursors of A superfamily were clustered according to mature sequence and framework, it was evident that the A superfamily was dominated by κA-conotoxins (framework IV, 72% of A superfamily) and α-conotoxins (framework I, 10.5% of A superfamily).

κA-Conotoxins. κA-conotoxins with cysteine framework $CC(X_7)C(X_2)CXC(X_3)C$ dominated both the *C. catus* transcriptome and proteome. The precursors C4.1 and C4.1b were the most abundant members of the transcriptome, with 2747 and 627 cDNA reads and 29 and 11 variants with >2 cDNA reads, respectively. All κA-conotoxins found could be divided into two groups following the conserved structural features described previously.[34] The C4.1 and C4.1b variants had sequence similarity to previously reported O-glycosylated conotoxins CcTx from *C. consors* (82%)[35,36] and SIVA from *C. striatus* (73%),[37,38] including the conserved serine at position 7, where the glycan moiety is O-linked. Peptides Ca009−Ca015 (including C4.2) showed sequence similarity to MVIA from *C. magus* (79%), including conserved threonines at positions 7 and 9, where two glycan moieties are O-linked.[39] Apart from the consensus cysteines, both of these groups also share a highly conserved glycine at position 14, leucine at position 4, valine at position 5, and threonine at position 10, which are conserved across all κA-conotoxin sequences reported to-date.

On the basis of the above structural similarities and our observations on the presence of carbohydrate marker ions, we attempted to identify the glycan moiety and the glycosylation pattern in κA-conotoxins from *C. catus*. Unfortunately, using collisional fragmentation of glycopeptides at high and low collision energies (Figure 2), most of the glycan moieties appeared as oligomers that could not be precisely characterized. Furthermore, despite all major peaks in the protein profile of *C. catus* venom being identified as glycosylated κA-conotoxins, assigning sequences to these identified masses was confounded by their sequence length (>38 amino acids), the diversity of possible PTMs, and likely heterogeneity of glycosylation. For these reasons, transcriptomic sequences for the κA-conotoxins could be confidently matched to the MS-identified masses. Therefore, these detected masses were labeled using the corresponding molecular mass and retention time.

Several unusual A superfamily conotoxins were also identified. Four C4.1 (Ca028, Ca031, Ca036, Ca040) and two C4.1b variants (Ca047, Ca051) had extended mature sequences that formed a novel elongated framework $CC(X_7)C(X_2)CXC(X_3)C(X_{26})C(X_2)C$ that contained eight cysteines. However, despite having high cDNA read numbers (Supporting Information Figure S2), these elongated sequence were not

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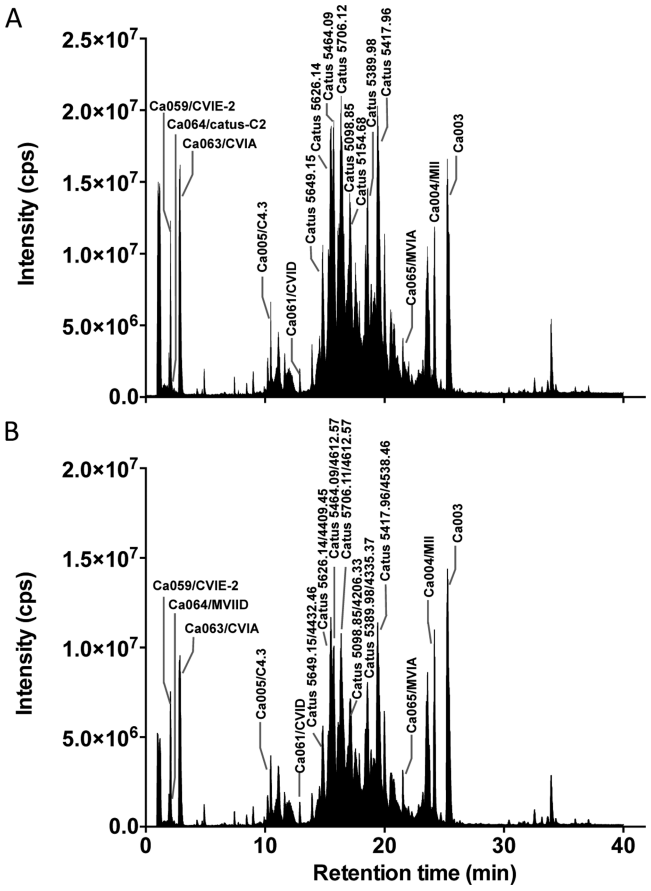

Figure 2. Total ion current trace of the pooled predatory venom of *C. catus* at low collision energy (A) and high collision energy (B). LC–MS run on the TripleTOF-5600 at intelligent multiple collision energy mode reveals the presence of a glycan moiety attached to the κA subfamily of conotoxins. At higher collision energy, the glycan gets separated from the mature peptide sequence, and at lower collision energy, the glycan remains intact on the peptide. The peaks were labeled using corresponding molecular mass for a specific retention time. The masses present in the venom profile were identified using the Analyst 1.6 program.

supported by MS/MS. Similar, long CcTx isoforms were also detected in the *C. consors* transcriptome[13] without proteomic confirmation. Finally, two closely related sequences (Ca055, Ca056) with only two cysteines in their mature sequence were identified in the transcriptome but not the proteome. These two sequences showed 55% similarity to superfamily A peptide G056 found in the *C. geographus* transcriptome.[6]

α-Conotoxins. Of the 6 identified α-conotoxins, 4 belonged to the neuronal AChR α4/7 subgroup with cysteine framework $CC(X_4)C(X_7)C$ (Ca001–Ca004/MII), one belonged to the muscle AChR α3/5 subgroup with cysteine framework $CC(X_3)C(X_5)C$ (Ca005/C4.3), and one belonged to the α4/4 subgroup with cysteine framework $CC(X_4)C(X_4)C$ (Ca006). All six identified α-conotoxins were found in the proteome, and MS identified masses matched to the peptides with N-terminal amidation and five peptides were validated by MS/MS (Table 2).

O1 Superfamily

Sixteen unique sequences (Ca057–Ca072) belonging to O1 superfamily were retrieved from the *C. catus* transcriptome after filtering for rare transcripts. However, only 6 of the 16 previously identified ω-conotoxins were present in our pooled transcriptome, along with 10 novel sequences. Indeed, out of previously characterized ω-conotoxins CVIA–CVIF,[22−24] only CVIA, CVID, CVIE-2, and catus-C2 were found in this transcriptome. The O1 conotoxin framework $C(X_6)C(X_{5-6})$-$CC(X_{2-3})C(X_{3-5})$ was found in 9 of the sequences, including ω-conotoxins CVIA, CVID, and CVIE-2, catus-C2, and δ-conotoxin MVIA, and four novel sequences Ca058, Ca060, Ca062, and Ca066 that were also found at the protein level. Among these novel precursors, Ca066 is most similar to CVIE-2 (92%), with a tyrosine at position 13, suggesting biological activity at vertebrate calcium channels. Six closely related novel peptides (Ca067–Ca072) shared the $C(X_6)C(X_4)CC(X_4)C$-(X_8) framework and showed 60–80% sequence similarity to the O1 superfamily peptide Bu9 from the *C. bullatus* transcriptome.[12]

Other Superfamilies

Nine additional superfamilies were identified in our *C. catus* transcriptome. These included three unique M superfamily peptides (Ca085–Ca087) with the μ-conotoxin framework CC-C-C-CC that were also identified in the proteome. Ca085 and Ca086 were 79 and 95% similar to CnIIID from the *C. consors*,[13] whereas Ca079 peptide had 91% similarity to μ-conotoxin SxIIIA from *C. striolatus*.[40] Seven O2 superfamily sequences Ca073 and Ca074 were identified as contryphans, whereas four sequences shared the $CX_6CX_5CCX_3CX_4C$ framework commonly found in O superfamily conotoxins. Five S superfamily peptides (Ca080–Ca084) with the framework VIII, five I1 superfamily peptides having the XXII framework (Ca088–Ca092), five conkunitzins with frameworks XIV (Ca093, Ca094) and IX (Ca095–Ca097), and a single sequence from Y superfamily peptide (Ca100) were also retrieved as cysteine-rich conotoxins. Cysteine-poor conopeptide families identified in the transcriptome include the contulakin (one sequence), two linear conopeptides (Ca098, Ca099) and a conoporin (Ca102). However, although corresponding masses for these minor superfamilies were detected by LC–MS, these peptides were not seen as prominent components of the venom. The other reason for not detecting the peaks corresponding to these peptides could be that the whole proteome is dominated by the κA-, α-, and ω-conotoxin-like peptides and the minor peaks were suppressed.

Unlike *C. marmoreus* and *C. miles* transcriptomes, only one new superfamily was found in the *C. catus* transcriptome (Cat-NSF1) comprising two sequences, Ca103 and Ca104, both with the VI/VII framework. Interestingly, the mature toxin of highly expressed Ca103 (74 cDNA reads) showed 94% similarity to the δ-conotoxin CnVIA found in *C. consors*.[25,40] However, this peptide was not detected in the proteome.

Intraspecific Variation of the Injected Venom of *C. catus*

Intraspecific variation is a common characteristic of cone snail venom.[27,41] To understand the extent of this variability in *C. catus*, we analyzed the predation-evoked venom profiles of six specimens individually by LC–MS on a QSTAR Pulsar (see Supporting Information Figure S5). The masses corresponding to the major peaks were identified and matched to sequences identified in the transcriptome. Collectively, the venom profiles followed a unique pattern, where O1 superfamily peptides and C4.3 eluted early, followed by glycosylated κA-conotoxins and the M superfamily peptide Ca086. Two peaks eluting at ∼41 min contained α-conotoxin MII and/or Ca003 peptide. However, the specific O1 and κA-conotoxins varied across

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Table 2. MS/MS Coverage of Abundant Transcriptomic Sequences[a]

name	MS/MS fragment	disulfides	PTM	precursor m/z	z
Ca001	ASGADTCCSNPACQVQHSDLC	Y		762.34	3
Ca002	ASGADTCCSNPVCQMQHHDLC	Y		788.36	3
Ca003	GCCSNPVCHLEHPNAC	Y	NH_2	929.90	2
Ca004/MII	CSNPVCHLEHSNLC	Y		827.86	2
Ca005/C4.3	NGRCCHPACGKHFS	Y		413.18	4
Ca011/C4.2	YSCPHQKKKRP	Y		723.90	2
Ca013	CMCTYSCPHQQK	Y		780.82	2
Ca014	CMCTYSCPHQQR	Y		802.88	2
Ca029/C4.1	CMCDNTCPPKKK	Y	1Hyp	780.35	2
Ca057	RCCPDLICIGGHVGR	Y		577.62	3
Ca058	CATYGKPCGIQNDCCNTCDPAR	Y		851.70	3
Ca061/CVID	LMYDCCSGSCSGTVGR	Y		885.87	2
Ca063/CVIA	RTSYDCCTGSCRSG	Y	NH_2	779.31	2
Ca064/catus-C2	KTMYNCCSGSCNR	Y		799.82	2
Ca065/MVIA	DGCYNAGTFCGIR	Y		732.82	2
Ca066	YGCSNAGAFCGIHPGLCCSE	Y	1Hyp	727.63	3
Ca067	GRVCQYPSRHSFGI	Y		550.94	3
Ca069	ASEGCGEIGAPCR	Y	Gln	668.81	2
Ca070	RRCCPDLRCVDGPVGRACQE	Y		669.29	2
Ca071	AGGVCRYPYN	Y		572.27	2
Ca076	TWNAPCSFTSQCCFGR	Y		970.42	2
Ca077	LTSQCCFGR	Y		551.75	2
Ca080	CTLVNNCQQNGACNGDCSCEGQICKCGYR	Y		996.41	3
Ca081	QYPLGCPGTCGK	Y	Pyr	647.80	2
Ca083	QYPLGCPGTCGKGGKCVGTCE	Y	Pyr; 1Hyp	745.02	3
Ca086	MCCRWPCPRQIDGE	Y		609.28	3
Ca088	ESCFGDKVPVTDYNCK	Y		631.62	3
Ca091	TMATTATCMQSKKDKSYSYACGYCGKKKE	Y		669.73	5
Ca092	NIVNPCVGPAL	Y		570.83	2
Ca093	DRPSYCNLPADSGSGTNRE	Y	1Hyp	700.33	3
Ca094	DRPSYCNLPADSGSGTNR	Y		651.97	3
Ca095	RFIYGGCGGNGNR	Y		708.32	2
Ca097	SAYAAWQCFLPK	Y		714.86	2
Ca102	VHMAFGVITTNPTAIEPGKR	N		713.75	3

[a]Confidence value >99% including predicted PTMs. Cys-mod (Y), cysteine alkylation by maleimide; NH_2, C terminal amidation; Hyp, hydroxyproline; Pyr, N terminal pyroglutamate, Gln, glutamic acid substitution by glycine.

the six specimens, including masses for the dominant glycosylated κA-conotoxins, suggesting variation in either the glycan or the parent peptide sequence.

To visualize intraspecific variability, 20 of the most abundant masses present in the venom of each specimen (40 in total) were compared (Figure 3). κA-Conotoxins dominated the venom of all specimens except specimen 3, which was dominated by the α-conotoxin-like peptide Ca003. In addition, the specific κA-conotoxin present varied across specimens, with dominant peptides in one specimen undetectable in another. This could also be due to the large number of κA-conotoxins secreted in the venom duct (as seen in the transcriptome) in addition to the complex PTMs. The peptide counts obtained by reconstructing LC−MS chromatogram (QSTAR Pulsar) varied between specimens, where the corresponding peptide counts for specimens 1−6 were 281, 84, 73, 167, 45 and 30, respectively. Comparing venom complexity, specimen 1 was more complex, whereas specimens 5 and 6 were least complex. The well-known α-conotoxin MII was found only in specimen 1, whereas ω-conopeptides CVIA, CVID, CVIE-1, and catus-C2 were found across all specimens but at widely different intensities.

Pharmacological Profile of *C. catus* Predatory Venom

To further validate the biological significance of the *C. catus* venom, pooled predation-evoked venom (see Supporting Information Figure S6) was fractionated, and biological activity was determined across human sodium and calcium channels and nicotinic acetylcholine receptors. As expected from the results of our transcriptomic and proteomic analysis, mammalian $Ca_V2.2$ channel, α3β2 nAChR, and α7 nAChR blockers were present in the predatory venom of the *C. catus*, along with strong Na_V channel activators (Figure 4). Neuronal nAChR and $Ca_V2.2$ channel inhibitory activity could be directly related to the identified ω- and α-conotoxins in the transcriptome and proteome. To confirm this hypothesis, MALDI analysis was conducted to determine the molecular masses of the components present in each active fraction. Fraction 7 caused partial block of α3β2 nAChR, α7 nAChR, and $Ca_v2.2$ and contained a mass that matched the ω-conotoxin catus-C2. Fraction 20 also blocked the α3β2 nAChR and contained a mass that matched the α3/5 conotoxin C4.3. The α3β2 nAChR blocking fractions 30 and 31 contained α4/7 conotoxin Ca003, which is the most abundant α-conotoxin found in the *C. catus* transcriptome. Late-eluting fraction 58 activated Na_v channels and had a mass (3508.1 Da) that matched the O2 superfamily

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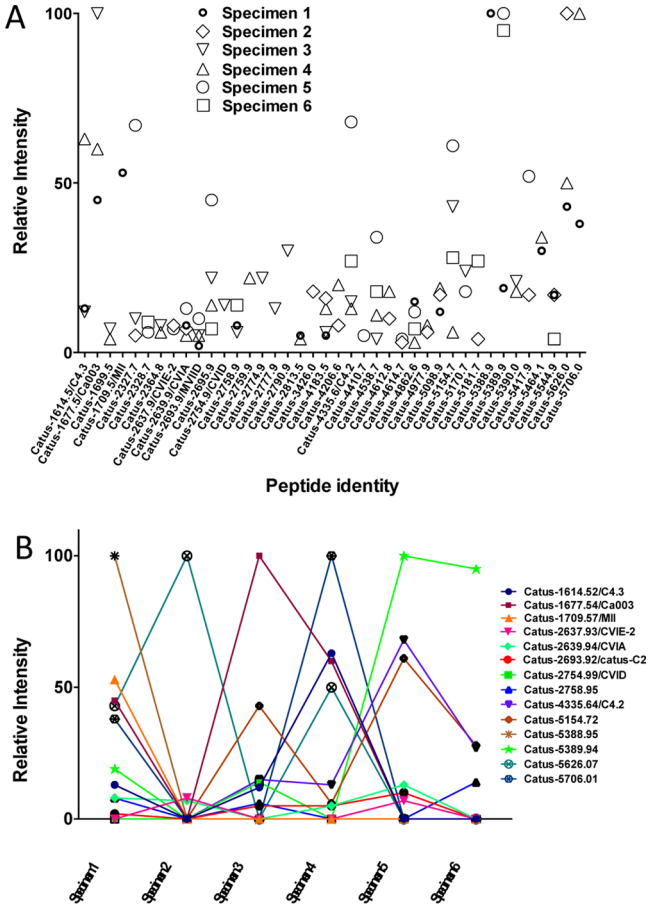

Figure 3. Variability of the *C. catus* predatory venom across six specimens as observed by LC−ESI−MS. Twenty most abundant masses present in the TIC profiles of each specimens were compared, and the resulting relative intensities of 40 peptides shared among these specimens ranked according to the molecular weight are graphically shown (A). The variability of the most abundant peptide in each species and known conotoxins was also compared (B).

peptide Ca075; however, confirmatory MS/MS support for this assignment could not be obtained. Activity for the κA-conotoxins was not identified in these screens, excluding human $Ca_V2.2$, $Na_v1.2$ and 1.7, or $\alpha3\beta2$ and $\alpha7$ nAChRs as potential targets for these peptides.

Effect of κA-Conotoxins on Zebrafish

To gain more insight into the mechanism of action of *C. catus* κA-conotoxins, we analyzed their effect using an *in vivo* behavioral assay in adult zebrafish. When a mix of purified κA-conotoxins (see Supporting Information Figure S7) at different concentrations (2.75−55 ng/mL) was injected i.m. into fish, it immediately decreased swimming movement and increased opercular movement. This effect on swimming was dose-dependent (EC_{50} of 34.74 ng/g) and lasted for 2−3 min. (Figure 5A). Interestingly, this immediate loss of activity was followed by a burst of rapid swimming lasting 1−2 min at doses above 11 ng/g and was mostly apparent at higher doses (55 and 22 ng/g). Figure 5B illustrates this behavior compared to the swimming pattern of saline-injected fish. This hyperactive stage was followed by rigid paralysis with stiff fibrillating fins.

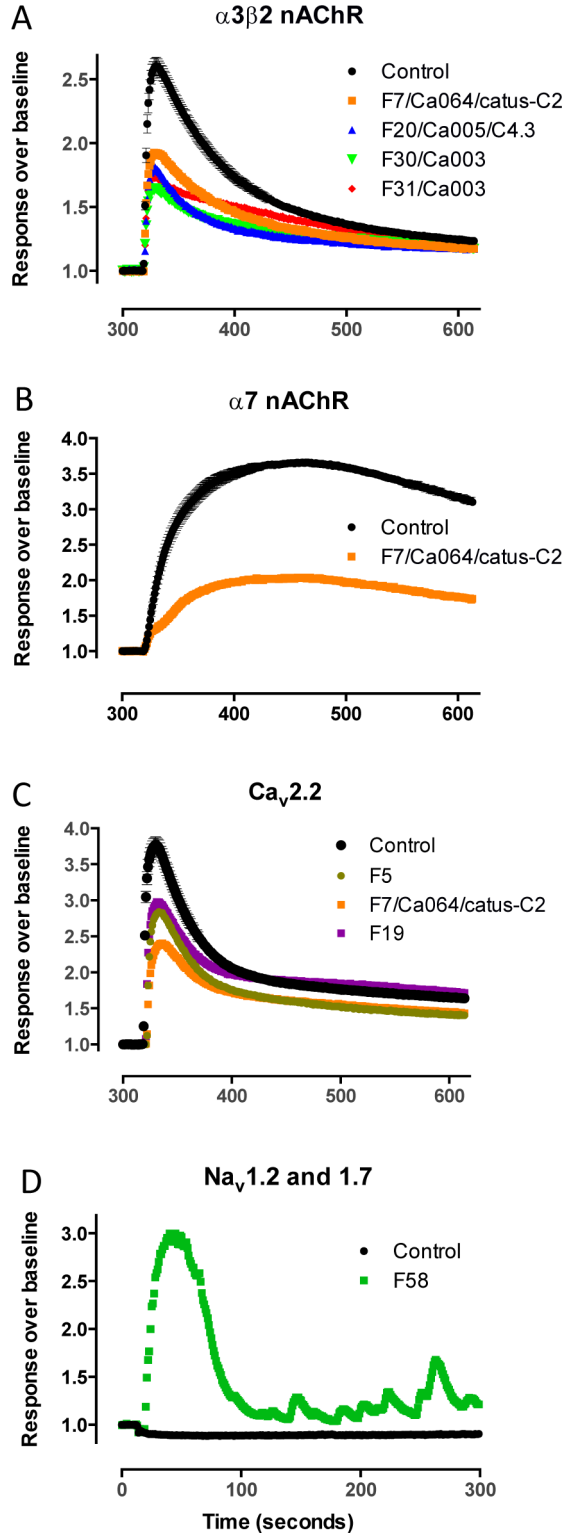

Figure 4. Activity of pooled *C. catus* venom fractions on the human receptors. One milligram of pooled venom from six specimens was separated on RP-HPLC, and 1 min fractions were collected. These resulting fractions were screened for their biological activity on human $\alpha3\beta2$ (A) and $\alpha7$ (B) nicotinic acetylcholine receptors, Ca2.2 voltage gated channel (C), and Na 1.2/1.7 voltage gated channels (D). Specific responses given by active fraction are graphically represented, and known toxins detected in these fractions are indicated. The venom components potently inhibited $\alpha3\beta2$ nAChR, $\alpha7$ nAChRs, and $Ca_V2.2$ channel, whereas fraction 58 activates voltage gated Na channels.

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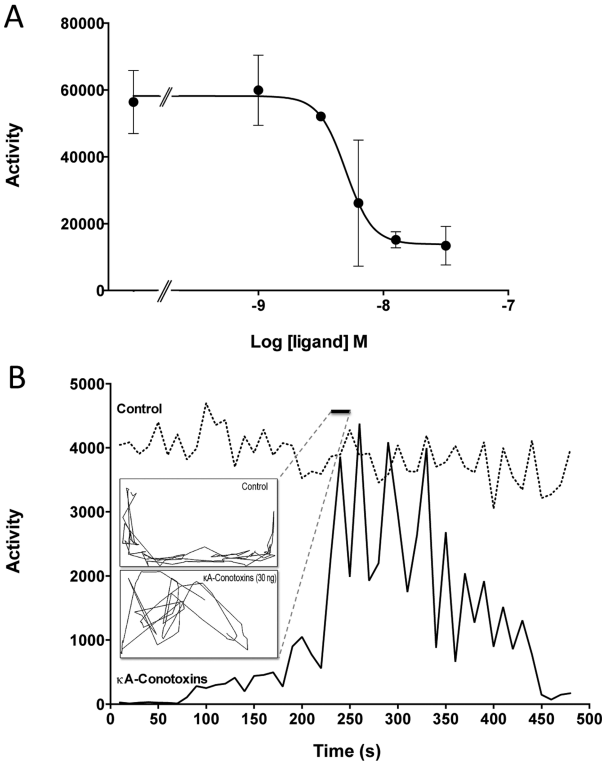

Figure 5. κA-Conotoxins-induced behavioral response in adult zebra fish. Adult zebra fish were injected with a pure fraction of κA-conotoxins, and their behavioral responses were measured qualitatively and quantitatively. (A) Dose−response curve showing the activity (measured by distance traveled) of zebra fish within 2 min after injection of known amounts (22, 11, 5.5, and 2.75 ng/g) of κA-conotoxins in comparison to the saline-only injections (n = 3). (B) Burst of activity observed after 2 min of 55 ng/g of κA-conotoxins injection (continuous line) compared to the activity of noninjected adult zebra fish (dotted line). The two boxes illustrate swimming tracks within the indicated time frame.

DISCUSSION

Parallel developments in next-generation sequencing and state-of-the-art proteomics have facilitated an integrated approach to accelerate the discovery of conopeptides.[10] In the current study, we employed 454 pyrosequencing to obtain the complete venom duct transcriptome of *C. catus*, and a detailed proteomics analysis was conducted using advanced mass spectroscopic methods to reveal the complexity of the *C. catus* venom profile. Despite of its relatively small size (2.4−5.2 cm) and slow movement, *C. catus* can prey exclusively on fish using highly evolved envenomation machinery. Our results indicate that κA-conotoxins are the major element of *C. catus* predatory strategy that facilitate extremely rapid immobilization of the fish, along with paralytic ω- and α-conotoxins, indicating that the prey-capture strategy of *C. catus* is more complex than other fish hunting species studies to-date.

To investigate whether these predatory venom components affect human ion channels, we used bioassays measuring fluorescent changes in SH-SY5Y cells to reveal potent neuronal α3β2 nAChRs and $Ca_v2.2$ channel blockers, as well as Na_v channel activators present in the pooled predatory-evoked venom (Figure 4). It was surprising to see that a α3/5 conotoxin (Ca005/C4.3) blocked neuronal nAChRs since this subgroup of α-conotoxins is considered to be selective for muscle nAChRs.[42] α3β2 nAChR blocker Ca003 has not been characterized for its biological activity before. Interestingly, the ω-conotoxin catus-C2 also partially blocked α3β2 and α7 nAChR apart from expected $Ca_v2.2$ channel block. Therefore, further studies with the purified native peptides or synthetic peptides are necessary to determine the selectivity of these peptides.

Our previous study with *C. geographus* demonstrated that the predatory-evoked venom expressed in the distal section of the venom gland of this net-hunter contained a fish-specific cocktail of venom components that lacked paralytic neurotoxins, whereas the defensive venom generated from the proximal venom gland produced a more complex mix of paralytic neurotoxins active on human channels.[6] While functions of distal and proximal parts of the venom gland of *C. catus* are not fully resolved in this study, our results suggest that the venom profiles of distal and proximal ducts may not be as different as those found for *C. geographus*, since several paralytic conotoxins (CVIA, CVID, CVIE-2, MII, C4.3) identified in the venom duct transcriptome of *C. catus* were present in the predatory-evoked venom. However, it was clearly seen that the intensities of ω- and α-conotoxins in the predatory venom (except specimen 3 with relatively higher content of Ca003) were much less when compared to those of the κA-conotoxins (Figure 5 and Supporting Information Figure S3), suggesting that prey capture and immobilization are driven mainly by these glycosylated κA-conotoxins, as seen for other hook-and-line fish hunters, including *C. striatus* and *C. consors*.[34,38,43,44] *C. catus* predatory venom contains the largest pool of glycosylated κA-conotoxins described to date from cone snails, and efforts were taken to identify the activity of these toxins in prey capture. It was clearly seen that the κA-conotoxin injection induced rapid immobilization in fish followed by very short hyperactive phase and then spastic paralysis. Similar behavioral responses were seen for the κA-conotoxin identified from *C. striatus*, indicating the major role of κA-conotoxins in mediating the excitotoxic effect and paralysis seen in fish stung by these hook-and-line fish hunters.[38] It has been suggested that κA-conotoxins SIVA from *C. striatus* and CcTx from *C. consors* target tetrodotoxin-sensitive Na_v channels.[34,43] However, no activity was detected in our screen at human Na_v channels, and further studies are needed to explain the mechanism of action of *C. catus* derived κA-conotoxins.

The transcriptome of *C. catus* contained a large number of rare transcripts that likely to contribute to the biological messiness at the mRNA level identified previously (Supporting Information Figure S1).[16,17,45] Given the genes encoding conotoxins are some of the fastest evolving genes,[46] an increase in gene copy number encoding venom components creates opportunities for any beneficial mutations to be retained and optimized through positive selection for new uses.[41] Therefore, it was assumed that highly expressed transcripts are positively selected for key roles in prey capture or defense, whereas their variants provide a reservoir for further diversification and evolutionary selection.[47] Despite their dominance in all individuals analyzed, our mass spectrometric analysis revealed a remarkable difference between specific dominant κA-conotoxins present, suggesting either equivalent performance for various isoforms or that some particular variants provide a competitive advantage and hence are positively selected. Multiple genetic heterogeneity, differential peptide expression, and post-transitional processing likely contribute to this observed intraspecific complexity.[41] However, due to limitations in identification of the sequences corresponding to the

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observed masses, we were unable to determine if this diversity occurred at peptide primary sequence level or through variable processing of post-translational modifications. Further studies are required to deduce the complete structures of these glycosylated peptides using integrated mass spectrometry, chemical derivatization, sequential exoglycosidase digestion, and linkage analysis.[48]

Collectively, using this venomics approach we were able to decode the venom of *C. catus*. This study found that the κA-conotoxins dominated the predatory venom, which also contains significant levels of neurotoxic/paralytic α-, ω-, and μ-conotoxins previously attributed to defensive roles. Comparing the predatory venom of six individuals revealed remarkable intraspecific variation at the molecular level, allowing rapid adaptation to distinct evolutionary selection pressures. The significant variation in venom components likely continues to provide an evolutionary advantage for *C. catus* prey capture and survival strategies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.5b00630.

Transcription frequency distribution patterns; alignment of the conopeptide sequences retrieved from the *C. catus* transcriptome; total ion chromatogram of the pooled predatory venom from six *C. catus* specimens; evidence for the presence of carbohydrate marker ions in the TIC of *C. catus* predatory-evoked injected venom; LC−MS traces of the predatory venom milked from six specimens of *C. catus*; RP-HPLC trace of the *C. catus* predatory-evoked injected crude venom and MALDI/MS chromatogram of the *C. catus* κA-conotoxins fraction used for fish assay; table listing previously identified peptides from *C. catus* (PDF).

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

nAChR, nicotinic acetylcholine receptor; Ca_v, voltage gated calcium channels; Na_v, voltage gated sodium cannels; LC−ESI−MS, liquid chromatography−electrospray ionization−mass spectrometry; LC−ESI−MS/MS, liquid chromatography−electrospray ionization−tandem mass spectrometry; HPLC, high-performance liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; CE, collision energy; PTM, post-translational modifications

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