Evolution of separate predation- and defence-evoked venoms in carnivorous cone snails

Sébastien Dutertre1,2, Ai-Hua Jin1, Irina Vetter1,3, Brett Hamilton4, Kartik Sunagar5,6, Vincent Lavergne1, Valentin Dutertre1, Bryan G. Fry1,7, Agostinho Antunes5,6, Deon J. Venter4,8, Paul F. Alewood1 & Richard J. Lewis1

Venomous animals are thought to inject the same combination of toxins for both predation and defence, presumably exploiting conserved target pharmacology across prey and predators. Remarkably, cone snails can rapidly switch between distinct venoms in response to predatory or defensive stimuli. Here, we show that the defence-evoked venom of Conus geographus contains high levels of paralytic toxins that potently block neuromuscular receptors, consistent with its lethal effects on humans. In contrast, C. geographus predation-evoked venom contains prey-specific toxins mostly inactive at human targets. Predation- and defence-evoked venoms originate from the distal and proximal regions of the venom duct, respectively, explaining how different stimuli can generate two distinct venoms. A specialized defensive envenomation strategy is widely evolved across worm, mollusk and fish-hunting cone snails. We propose that defensive toxins, originally evolved in ancestral worm-hunting cone snails to protect against cephalopod and fish predation, have been repurposed in predatory venoms to facilitate diversification to fish and mollusk diets.

1 Institute for Molecular Bioscience, The University of Queensland, Brisbane, 4072 Queensland, Australia. 2 Institut des Biomolécules Max Mousseron, UMR 5247, Université Montpellier 2—CNRS, Place Eugène Bataillon, Montpellier Cedex 5 34095, France. 3 School of Pharmacy, The University of Queensland, Brisbane, 4102 Queensland, Australia. 4 Pathology Department, and Mater Research Institute, Mater Health Services, South Brisbane, 4101 Queensland, Australia. 5 CIMAR/CIIMAR, Centro Interdisciplinar de Investigação Marinha e Ambiental, Universidade do Porto, Rua dos Bragas, 177, Porto 4050-123, Portugal. 6 Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre, Porto 4169-007, Portugal. 7 Venom Evolution Lab, School of Biological Sciences, The University of Queensland, Brisbane, 4072 Queensland, Australia. 8 Department of Medicine, The University of Queensland, Brisbane, 4072 Queensland, Australia. Correspondence and requests for materials should be addressed to R.J.L. (email: r.lewis@imb.uq.edu.au).
Venomous marine gastropods of the genus *Conus* have evolved one of the most sophisticated envenomation strategies known, allowing these slow animals to capture worms, mollusks and even fish. Utilizing a hollow, harpoon-like radula, cone snails inject a complex cocktail of potent venom peptides (conotoxins) to rapidly immobilize the prey. This strategy is underpinned by a remarkable diversity of conotoxins that target a wide range of membrane proteins, including the FDA-approved Ca,2,2 inhibitor α-MVIIA (Prialt) used to treat intractable pain. To maximize venom potency, cone snails deploy synergistic groups of conotoxins, known as ‘cabals’.

For example, the ‘lightning-strike cabal’ comprises potassium channel blocking γ-conotoxins and excitatory sodium channel modifying δ-conotoxins that produce immediate tetanic paralysis in fish. In contrast, the ‘motor cabal’ developed in particular by *C. geographus* comprises inhibitory α-, μ- and κ-conotoxins that target neuromuscular receptors and produce flaccid paralysis in fish. However, the role of the paralytic motor cabal in *C. geographus* predation is unclear, since it mainly uses an alternate ‘nirvana cabal’ to sedate fish prior to capture using a net strategy (see Supplementary Fig. 1; Supplementary Movie 1).

Molecular and phylogenetic studies have demonstrated that the evolution of envenomation strategies is typically a predatory rather than a defensive adaptation, despite the critical importance of defence for animal survival. While a shell can serve as the first line of defence, repair marks commonly observed in many *Conus* species indicate they can survive physically damaging attacks from predators such as octopus or fish (see Supplementary Fig. 2), possibly by using their venom defensively (see Supplementary Movie 2). The defensive use of venom can also result in human injuries, with *Conus geographus* stings producing confirmed fatalities. Such deleterious effects are currently explained by a unique venom that acts on targets with conserved pharmacology across prey and predator, and a separately evolved defensive strategy to deter aggressors has not been investigated previously.

In this article, we report for the first time the remarkable ability of cone snails to rapidly and reversibly switch between functionally and structurally distinct venoms in response to predatory or threatening stimuli. The defence-evoked venom typically comprises paralytic toxins, previously thought to participate in prey capture, that explain the symptoms associated with human envenomation. In contrast, the predation-evoked venom appears largely devoid of these paralytic toxins. The venom duct shows a corresponding regionalization of toxin production, with high levels of defence-evoked and predation-evoked venoms in the proximal and distal sections, respectively. Finally, molecular evolution analyses revealed that both predatory and defensive toxins are evolving under strong positive selection. Together, these data suggest that ancestral defensive toxins originally evolved to protect against fish and cephalopod predators facilitated a shift from worm-hunting to fish- and mollusk-hunting strategies.

**Results**

**Distinct predation- and defence-evoked venoms in cone snails.** Fish-hunting *C. geographus* possesses one of the most fragile shells (Supplementary Fig. 3) and produces arguably the most potent venom, suggesting that reduced protection may have co-evolved with a highly developed defensive strategy in cone snails. To investigate the evolution of predatory and defensive envenomation strategies in cone snails, we developed a new method that allowed the sequential collection of injected venom from individual *C. geographus* using alternating predatory and defensive stimuli (Supplementary Fig. 4). Surprisingly, the defence-evoked venom was significantly more complex than predation-evoked venom (Fig. 1a–c), with limited overlap in peptide composition (<50%), indicating that defence- and predation-evoked venoms are produced by distinct and independently controlled mechanisms (Fig. 1d, Supplementary Fig. 5). The predation-evoked venom, which was injected only when the proboscis came in close proximity to appropriate prey tissue, lacked most of the paralytic peptides thought to enable prey capture but instead contained high levels of the fish-specific sodium channel inhibitor μ-conotoxin GS and non-paralytic peptides, including the vasopressin receptor agonist conopressin-G and the NMDA receptor antagonist conantokin G (Supplementary Fig. 6). In contrast, paralytic peptides dominated the defence-evoked venom, which was injected immediately when the proboscis contacted a solid surface (Supplementary Movie 3).

Similar predation- and defence-evoked venom profiles were obtained for several *C. geographus* specimens (Supplementary Figs 5–7). To examine how broadly a separate defensive strategy has evolved in *Conus* species, we extended these studies to holothuricivorous and vermicivorous cone snail species. The molluscivorous *Conus marmoreus* can inject its prey multiple times during a single feeding event, allowing predation- and defence-evoked venoms to be collected over short time-intervals (Fig. 1e–g). The predatory- and defence-evoked venoms of *C. marmoreus* were again distinct, with the first and third injections (both predatory) being identical (Fig. 1e,g), despite an intervening defensive sting being collected minutes earlier (Fig. 1f). The occurrence of only trace amounts of major predatory toxins (for example, Mr1e) detected in the defence-evoked venom confirmed that there was minimal venom carryover between stings (Fig. 1b). As observed for *C. geographus*, the predation-evoked venom of *C. marmoreus* was relatively simple compared with its defence-evoked venom, which contained several known vertebrate-active neurotoxins (Fig. 1e). Since *C. marmoreus* is not known to prey on vertebrates, these results suggest that these vertebrate-active toxins have specifically evolved for defence. In contrast, neurotoxins previously thought to participate in prey capture are absent from the predation-evoked venom, including the μO-conotoxins that inhibit mollusk and vertebrate sodium channels.

Confirming this is a widely evolved strategy on cone snails, complex defence-evoked venoms were also obtained from other fish- (*Conus obscureus*), mollusk- (*Conus victoriae*) and worm-hunting species (*Conus planorbis* and *Conus coronatus*) (Supplementary Figs 8–10).

**Pharmacological profiles of predation- and defence-evoked venoms.** To further investigate the biological significance of separate envenomation strategies, we compared the biological activity of predation- and defence-evoked *C. geographus* venoms across human sodium and calcium channels, and nicotinic acetylcholine receptors. Our results confirmed that the defence-evoked venom contained high levels of paralytic peptides acting at mammalian ion channels (Fig. 2a–h), which likely account for the human fatalities associated with *C. geographus* defensive stings (up to ~6 mg venom injected per strike). In contrast, the predation-evoked venom of *C. geographus* was inactive at these human targets, except for calcium channel activity associated with trace amounts of the highly potent calcium channel blockers α-GVIA and α-GVIIIA. Since *C. geographus* is a piscivorous species, a fish bioassay was used to determine the effective dose (ED₅₀) in vivo of both the predation- and defence-evoked venoms (Supplementary Fig. 11). Defence-evoked venom was 350-fold more potent (ED₅₀ = 10 µg kg⁻¹) than the predation-evoked venom at producing paralysis in fish, consistent with its role in deterring large predators, although sufficient predation-evoked...
Venom (~1 mg) was injected to rapidly paralyse fish up to 200 g. Surprisingly, one of the major and novel components of the predation-evoked venom (G117, Fig. 1a) did not induce paralysis when injected in fish, although a role for this peptide in the ‘nirvana cabal’ cannot be excluded.

**Origin of predation- and defence-evoked venoms.** The long, convoluted venom duct is the dominant toxin secretory organ in cone snails, but it is unclear if other embryologically related organs might also participate in venom production. Analyses of the transcriptomes and proteomes of the interconnected venom gland, salivary gland and radular sac of C. geographus unequivocally demonstrated that both predation- and defence-evoked venoms arise from the venom duct and not these other associated tissues (Supplementary Figs 12 and 13), with 127 conotoxin sequences recovered from the venom duct, including 43 confirmed by tandem mass spectrometry (MS/MS) (Supplementary Table 1). In contrast, no conotoxin sequences were found in the salivary gland, and only three rare conotoxin transcripts were identified in the radula sac transcriptome, although these were not detected in the injected venom. To understand how a single gland can rapidly and reversibly produce two distinct venoms, we analysed the venom peptide composition along the venom duct of C. geographus (Fig. 3a–c). Unexpectedly, the paralytic toxins found in the defence-evoked venom were abundant in the proximal duct (sections 7–12), whereas the major toxins found in the predation-evoked venom dominated the distal sections (sections 1–6 close to the pharynx) (Fig. 3d, Supplementary Fig. 14). In addition, structural differences within the venom duct support the distinct partition of the gland producing toxins (Fig. 3e). These results suggest that stimulus-dependent spatiotemporal release of toxins from different segments of the venom duct can generate functionally and biochemically distinct predation- and defence-evoked venoms (Fig. 3f).

**Defence as a major evolutionary force driving cone snail venom evolution.** To assess the influence of natural selection on conotoxins, we determined the non-synonymous to synonymous nucleotide substitution rate ratio (o) and identified sites evolving under episodic bursts of positive Darwinian selection (Supplementary Tables 2 and 3). Indeed, since the distinct distribution of toxins along a single venom gland is a unique evolutionary innovation, it could facilitate the separate evolution of specialized predatory and defensive venoms, which likely contributed to the rapid speciation observed in the genus Conus over the last 33 million years.

Our analyses detected a large number of positively selected sites (o > 1) in all toxin superfamilies examined, indicating that both predatory and defensive conotoxins are rapidly evolving under the influence of positive selection.
channels explains the lethal effect of the active fractions. The potent block of these key physiological ion channels is observed in both predation- and defence-evoked venoms (Fig. 4b), with most of the characterized defensive toxins being found in a distinct clade separate from the predatory toxins.

**Discussion**

In this study, we have discovered that the carnivorous gastropods of the genus *Conus* were able to rapidly and reversibly alternate between two distinct venoms in response to predatory or defensive stimuli. Surprisingly, defence-evoked venoms obtained from the deadly *C. geographus* contained high levels of paralytic conotoxins of the motor cabal, suggesting this cabal has evolved for defence and not for prey capture as previously suggested. Consistent with a prominent defensive role for the motor cabal, the pharmacology of the defence-evoked venom correlates with the symptomatology following *C. geographus* envenomation in humans, with death typically resulting from respiratory paralysis. In contrast, the predation-evoked venom contains prey-specific toxins that show low activity on human ion channels, indicating predation- and defence-evolved venoms have separately evolved for different functions. Expanding our study on the feeding mode of molluscivorous cone snails that routinely inject prey multiple times to achieve full paralysis, we have investigated the most behaviourally relevant milking sequence, where predatory use of venom is occasionally interrupted by deployment of defensive venom. The intervening defensive sting does not alter the composition and quantity of venom injected next, as the two separate predatory stings were strictly identical both qualitatively and quantitatively, with no evidence of depletion. Complex defence-evoked venom could also be obtained from other fish-hunting species, as well as from mollusk- and worm-hunting species, demonstrating that a specialized defensive behaviour and associated defensive venom has evolved widely across the genus *Conus*.

Our transcriptomic and proteomic investigation of embryologically related organs revealed that the venom duct produced all conotoxins found in both predation- and defence-evoked venoms, with only three rare conotoxin transcripts retrieved from the radular sac, and no conotoxin-like sequences found in the salivary gland. However, while these rare transcripts likely have no current functional role, we cannot exclude an ancestral role in conotoxin evolution given they have the canonical organization of conotoxin precursors (signal peptide, propeptide, mature toxin) found in the venom gland. Thus, the development of a specialized venom duct, where different toxin types are regionally produced, was a key functional innovation to allow separate venoms to be injected for predation and defence. At this stage, it is unclear if venom duct specialization arose from migration of specialized secretory cells from one section of the venom duct to another, or whether varying transcriptomic regulation explains the distinct venom peptide expression profiles in different duct regions. While proximal–distal heterogeneity in toxin production along the venom duct of *Conus textile* has been reported previously, its role was not identified. Our results now reveal that stimulus-dependent spatiotemporal release of toxins from different segments of the venom duct can generate functionally and biochemically distinct predation- and defence-evolved venoms that are presumably under separate neuronal control (see Fig. 3f). Stimulus-dependent release of venom likely explains the occasional ‘dry stings’, which correspond to injection of venom devoid of peptidic toxins. Regional specialization of toxin production also explains early observations that injection of distal duct venom had no effect on mice while extracts of proximal duct venom were lethal, and is supported by recent transcriptomic analysis that found distinct messenger RNA

![Figure 2](image_url)
**Figure 3 | Distribution of toxins in C. geographus venom duct and proposed mechanism for venom release.** (a) Twelve venom gland sections were spotted on a MALDI plate together with predation- and defence-evoked venom (O, oesophagus; P, proboscis; RS, radular sac; SG, salivary gland). (b) The resulting averaged spectrum is highly complex in the range 1,000–4,000 kDa corresponding to the size of most conotoxins (10–30 amino acids). (c) Gel view representation of MALDI results reveals distinct regionalization of many venom components along the duct. For example, the predatory toxin at 3,175 kDa and defensive toxin at 1,417 kDa show clear non-overlapping distribution along the duct. (d) Quantification of five major predatory (including conopressin-G at 1,035 kDa) and defensive (including α-GII at 1,417 kDa, μ-GIIIA at 2,610 kDa and ω-GVIIA at 3,316 kDa) toxins confirms this region-specific toxin production. (e) Histology (formaldehyde-fixed animal embedded in paraffin) reveals structural heterogeneity along the venom duct, including regions with a dense layer of secretory cells and a small lumen and others with a looser cell arrangement and a larger lumen, which could support such regional specialization. Gomori’s Trichrome stain shows muscle fibres in red, collagen in green and nuclei in blue/black (scale bar, 20 µm). (f) A simple hypothesis to explain the generation of separate stimulus-evoked venoms is proposed. An initial stimulus (predatory or defensive) is perceived by mechanical, visual and/or chemical (olfactory) sensors that transmit information to the cerebral ganglia surrounding the oesophagus (O) to activate two separate neuronal circuits. Predation-evoked stimuli activate neuronal circuit (blue) innervating the distal venom duct, causing the release of predatory venom peptides into the venom duct lumen. Similarly, threats including larger fish and cephalopods activate a separate defensive neuronal circuit (green) that innervates the proximal venom duct, causing the release of defensive toxins into the lumen. These lumen contents are then moved to the proboscis by a synchronized contraction of the muscular venom bulb to generate the injected ‘predation-evoked’ and ‘defence-evoked’ venoms. This key role of the venom bulb allows the rapid switch between the predation- and defence-evoked venoms observed. This mechanism of stimulus-dependent release of toxins from different sections of the venom duct explains how distinct predation- and defence-evoked venoms are generated.
expression patterns in distal and proximal C. geographus venom ducts.

While venom diversification in cone snails has only been associated with dietary specialization, our study reveals that both predatory and defensive strategies contribute to venom evolution by rapidly accumulating variations under the influence of positive Darwinian selection, often in an episodic manner. From recent studies on the evolutionary relationships of Conus species, it is now generally accepted that vermivory was the ancestral feeding mode from which specialized diets (that is, molluscivory and piscivory) arose. Evidence includes phylogenetic analyses of conotoxins expressed by piscivorous species that were likely derived from a set of loci that was present in the ancestral vermivorous lineages and the observation that some were likely derived from a set of loci that was present in the ancestral vermivorous lineages.

The separate defensive envenomation strategy employed by cone snails is a remarkable adaptation, changing our understanding of the biology, evolution and toxin diversification mechanisms in Conidae. This knowledge will also rationalize approaches to discover novel vertebrate-active conotoxins found preferentially in defence-evoked venom and proximal venom duct peptides, and prey-specific conotoxins found preferentially in predation-evoked venoms and distal venom duct peptides. Moreover, since the defensive use of venom is a general feature of
most venomous animals, its evolutionary impact on venom diversification remains to be assessed in phylogenetically unrelated groups. Indeed, snakes and spiders can control venom expenditure and produce pain-inducing toxins to deter predators29,30. Recently, regionalization of toxin production has been shown in sea anemone31, indicating that deployment of separate venoms might not be restricted to cone snails. Based on these observations, the evolution of specialized defensive venoms is predicted to be more important and widespread in other venomous animals than previously recognized, especially those with diversified diets. We propose that this specialization has allowed cone snails to repurpose conotoxins found in defensive venom to protect against fish and cephalopod threats to allow these predatory groups to become prey for piscivorous and molluscivorous cone snails.

Methods

Venom collection. All cone snails used in this study have been collected from Queensland coastal waters under a research permit issued from the Great Barrier Reef Marine Park (G10/33243.1). Cone snails were held in aquaria (5 weeks to 2 years) at a temperature maintained between 24–28 °C and a 12:12 light–dark cycle. Following each milking, the collecting tube was briefly centrifuged, lyophilized and stored at -20 °C until use.

Predation-evoked venom samples from the fish-hunting cone snails C. geographus and C. obscureus were obtained as previously described22. Briefly, a live fish is used to lure the cone snail and elicit a predatory behaviour with extension of the proboscis. A microcentrifuge tube covered with paraffin and a piece of fish tail is then presented at the tip of the proboscis, and upon contact with fish tissue, predation-evoked venom is forcefully injected through the hollow, harpoon-like radula into the collecting tube. To obtain defensively injected venom samples, a novel milking procedure was developed to engage the animal in a defensive mode. The procedure involved removing the cone snail from the tank and applying light pressure to the shell with long forceps until it was provoked to extend the proboscis. Once the proboscis was extended, a collecting tube covered with paraffin was presented to the tip of the proboscis until stinging occurred. Depending on the cone snail, several attempts were often required to trigger a stinging response and the delivery of venom into the tube. Owing to the serious health hazard associated with C. geographus envenomation, this milking procedure should only be attempted by persons fully aware of and protected from the risk of an accidental sting (for example, wearing thick gloves and carefully manipulating the snails with long forceps).

The predation-evoked venom from mollusk-hunting cone snails C. marmoratus and C. victoriae were collected as previously described14. Like other mollusk-hunting cone snails and C. victoriae usually inject their prey multiple times with venom during a single feeding event. Therefore, these species could be challenged in alternating predatory and defensive modes during a single milking session. The defence-evoked venom was obtained as outlined for C. geographus, except that repeated light poking of the foot of the animal was required to induce a similar defensive behaviour.

For the worm-hunting cone snails C. planorbis and C. coronatus, we used the predator C. marmoratus to trigger a defensive behaviour and initiate extension of the proboscis. The defence-evoked venom was then collected as described for C. geographus.

RNA extraction and transcriptome sequencing. The transcriptome of a pool of C. geographus venom ducts was recently published32. This study provides additional support to earlier proteomic and toxicity studies that suggested the presence of distinct venom-expression patterns along the duct, with disorientating and paralytic venoms expressed in different regions. To evaluate the possible contribution of different organs to conotoxin production, a single adult specimen of C. geographus from the Great Barrier Reef (Queensland, Australia) and paralytic venoms expressed in different regions. To evaluate the possible contribution of different organs to conotoxin production, a single adult specimen of C. geographus from the Great Barrier Reef (Queensland, Australia) and C. coronatus, were cut at 5 μm using a Leica RM2235 microtome. Gomori’s trichrome for 20 min. Finally, the slides were rinsed using Weigert haematoxylin for 10 min. Following this, the slides were stained using Weigert haematoxylin for 10 min. Following this, the slides were washed in running water, differentiated in acid alcohol and Scott’s tap water. The slides were then washed in running water, rinsed in distilled water and stained using Weigert haematoxylin for 10 min. Following this, the slides were washed in running water, differentiated in acid alcohol and Scott’s tap water. The slides were then washed in running water, rinsed in distilled water and stained with 4% paraformaldehyde, dehydrated using ethanol and processed using a Sakura Tissue-Tek VIP processor with a programme of 30 min xylene clear, and 4 × 30 min paraffin penetrations. The processed animals were then prepared into paraffin blocks (Shandon Histocentre 3) and cut at 5 μm using a Leica RM2235 microtome. Gomori’s trichrome for 20 min. The processed animals were then prepared into paraffin blocks (Shandon Histocentre 3) and cut at 5 μm using a Leica RM2235 microtome. Gomori’s trichrome for 20 min. Finally, the slides were rinsed with 0.1% TFA, and 2 μl of diluted matrix solution mixed with 1 μl sample and spotted onto a polished steel target. For all samples, 400 shots were acquired using a random walk function at a laser frequency of 200 Hz and saved, with 10 replicates of each sample averaged. Data were loaded into Clinprot Tools (Bruker Daltonics, Bremen, Germany) to visualize the 12 individual duct sections in ‘gel view’ using a colorimetric gradient to show the abundance of the components in respective fractions.

Histology. Cone snails were fixed with paraformaldehyde, dehydrated using ethanol and processed using a Sakura Tissue-Tek VIP processor with a programme of 30 min xylene clear, and 4 × 30 min paraffin penetrations. The processed animals were then prepared into paraffin blocks (Shandon Histocentre 3) and cut at 5 μm using a Leica RM2235 microtome. Gomori’s trichrome for 20 min. Finally, the slides were rinsed with 0.1% TFA, and 2 μl of diluted matrix solution mixed with 1 μl sample and spotted onto a polished steel target. For all samples, 400 shots were acquired using a random walk function at a laser frequency of 200 Hz and saved, with 10 replicates of each sample averaged. Data were loaded into Clinprot Tools (Bruker Daltonics, Bremen, Germany) to visualize the 12 individual duct sections in ‘gel view’ using a colorimetric gradient to show the abundance of the components in respective fractions.

Bioassay at human Ca, Na, and nACHr in SH-SY5Y cells. Predation- and defence-evoked venoms (1 mg each) were separated on a C 18 analytical column (Grace Vydac) eluted at 1 ml min -1 with an UltiMate 3000 LC system (Dionex) and 1-min fractions collected using a FC 204 fraction collector (Gilson). The activity of each fraction was assessed using high-throughput Ca2+ imaging assays, as previously described31. In brief, SH-SY5Y cells (European Collection of Cell Cultures) were maintained in RPMI medium (Invitrogen, Australia) supplemented with 15% fetal bovine serum and 1-glutamine and passaged every 3–5 days using 0.25% trypsin/EDTA (Invitrogen). SH-SY5Y cells were plated at a density of 35,000–50,000 cells per well on 384-well black-walled imaging plates and cultured for 48 h. Fluorescent responses (excitation 470–495 nm; emission 515–575 nm) were monitored using the FLIP5 software (Perkin Elmer) at 1 Hz per well (4000 shots) after 30-min incubation with fluorescent Ca2+ dye (Calcium 4 No Wash dye, Molecular Devices) diluted in physiological salt solution (composition in mM: Ca2+, 115; Mg2+, 0.5; K+, 140; Na+, 5.4; HCO3–, 25; Cl–, 125).
NaCl 140, glucose 11.5, KCl 5.9, MgCl 2 1.4, NaH 2PO 4 1.2, NaHCO 3 5, CaCl 2 1.8, and nAChR isoforms to vary across sites within ‘n’ discrete categories,  m, which is used to identify amino acids under positive selection by calculating the posterior probabilities that a particular amino acid belongs to a given selection class (neutral, conserved or highly variable). Sites with greater posterior probability (PP ≥ 0.95) of belonging to the ‘o > 1’ class were inferred to be positively selected. Fast, Unconstrained Bayesian Approximation (FUBAR) implemented in HyPhy39 was used to detect sites evolving under the influence of pervasive diversifying and purifying selection. Mixed Effects Model Evolution (MEME)40 was also used to detect episodic burst of selection. To reveal the proportion of sites under different regimes of selection, an evolutionary fingerprint analysis was carried out using the evolutionary selection distance algorithm implemented in datamonkey41. We further utilized branch-site Random Effects Likelihood42 to identify lineages affected by episodic selection.

**References**

1. Koh, A. J. Piscivorous gastropods of the genus Conus. Proc. Natl Acad. Sci. USA 42, 168–171 (1956).
2. Terlau, H. et al. Strategy for rapid immobilization of prey by a fish-hunting marine snail. Nature 381, 148–151 (1996).
3. Lewis, R. J., Dutertre, S., Vetter, I. & Christie, M. J. Conus venom peptide pharmacology. Pharmacol. Rev. 64, 259–298 (2012).
4. Olivera, B. M. & Cruz, L. T. Conotoxins, in retrospect. Toxicon 39, 7–14 (2001).
5. Olivera, B. M. et al. Peptide neurotoxins from fish-hunting cone snails. Science 230, 1338–1343 (1985).
6. Olivera, B. M. Conus venom peptides: correlating chemistry and behavior. J. Comp. Physiol. A 185, 353–359 (1999).
7. Dalten, C. J., Wuster, W. & Thorpe, R. S. Diet and snake venom evolution. Nature 379, 537–540 (1996).
8. Fry, B. G. et al. The toxicogenic multimere: convergent recruitment of proteins into animal venoms. Annu. Rev. Genomics. Hum. Genet. 10, 483–511 (2009).
9. Agrawal, A. A., Laforsch, C. & Tollrian, R. Transgenerational induction of defences in animals and plants. Nature 401, 60–63 (1999).
10. Fainشمل, M. et al. New sodium channel-blocking conotoxins also affect calcium currents in Lymnaea neurons. Biochemistry 34, 5364–5371 (1995).
11. Daly, N. L. et al. Structures of muo-conotoxins from Conus marmoreus. Inhibitors of tetrodotoxin (TTX)-sensitive and TTX-resistant sodium channels in mammalian sensory neurons. J. Biol. Chem. 279, 25774–25782 (2004).
12. Page, L. Developmental modularity and phenotypic novelty within a biphasic life cycle: morphogenesis of a cone snail venom gland. Proc. Biol. Sci. 279, 77–83 (2012).
13. Dutertre, J. T. F. & Kohn, A. J. Species-level phylogeography and evolutionary history of the hyperdiverse marine gastropod genus Conus. Mol. Phylogenet. Evol. 34, 257–272 (2004).
14. Dutertre, S. et al. Deep venomics reveals the mechanism for expanded peptide diversity in cone snail venom. Mol. Cell Proteomics 12, 312–329 (2013).
15. Fegan, D. & Andresen, D. Conus geographus envenomation. Lancet 349, 1672 (1997).
16. Garrett, J. E., Buscek, O., Watkins, M., Olivera, B. M. & Bulaj, G. Biochemical and gene expression analyses of conotoxins in Conus textile venom ducts. Biochem. Biophys. Res. Commun. 328, 362–367 (2005).
17. Tayo, L. L., Lu, B., Cruz, L. J. & Yates, J. R. Proteomic analysis provides insights on venom processing in Conus textile. J. Proteomics. Res. 9, 2292–2301 (2010).
18. Dutertre, S., Biass, D., Stocklin, R. & Favreau, P. Dramatic intraspecific variations within the injected venom of Covus consors: an unexpected contribution to venom diversity. Toxicon 55, 1453–1462 (2010).
19. Endean, R. & Rudkin, C. Studies of the venoms of some Conidae. Toxicon 1, 49–64 (1963).
20. Hu, H., Bandyopadhyay, P. K., Olivera, B. M. & Yandell, M. Elucidation of the molecular envenomation strategy of the cone snail Conus geographus through transcriptome sequencing of its venom duct. BMC Genomics 13, 284 (2012).
21. Duda, J. T. F. Differentiation of venoms of predatory marine gastropods: divergence of orthologous toxin genes of closely related Conus species with different dietary specializations. J. Mol. Evol. 67, 315–321 (2008).
22. Duda, J. T. F. & Palumbi, S. R. Gene expression and feeding ecology: evolution of piscivory in the venomous gastropod genus Conus. Proc. Biol. Sci. 271, 1165–1174 (2004).
23. Nybakken, J. W. Ontogenetic change in the Conus radula, its form, distribution among the radula types, and significance in systematics and ecology. Malacologia 32, 35–54 (1990).
24. Darst, C. R., Cummings, M. E. & Cannatella, D. C. A mechanism for diversity in warning signals: conspecific use of a toxin in poison frogs. Proc. Natl Acad. Sci. USA 103, 5852–5857 (2006).
25. Boeve, J. L., Kuhn-Nentwig, L., Keller, S. & Nentwig, W. Quantity and quality of venom released by a species (C a n i n u s s a i l e s, Cenapidae). Toxicon 33, 1347–1357 (1995).
26. WY. H. Factors associated with the mass of venom expended by prairie rattlesnakes (Crotalus v. viridis) feeding on mice. Toxicon 30, 449–460 (1992).
27. Inceoglu, B. et al. One scorpion, two venoms: prevenom of Parabuthus transvaalicus acts as an alternative type of venom with distinct mechanism of action. Proc. Natl Acad. Sci. USA 100, 922–927 (2003).
28. Morgenstern, D. & King, G. F. The venom optimization hypothesis revisited. Toxicon 63, 120–128 (2013).
29. Rohlen, C. J. et al. A heteromeric Texas coral snake toxin targets acid-sensing ion channels to produce pain. Nature 479, 410–414 (2011).
30. Siemens, J. et al. Spider toxins achieve the capsacin receptor to produce inflammatory pain. Nature 444, 208–212 (2006).
31. Moran, Y. et al. Analysis of soluble protein contents from the nematocysts of a model sea anemone sheds light on venom evolution. Marine Biotechnol. 15, 329–339 (2013).
32. Hopkins, C. et al. A new family of Conus peptides targeted to the nicotinic acetylcholine receptor. J. Biol. Chem. 270, 22361–22367 (1995).
33. Lavergne, V. et al. Systematic interrogation of the Conus marmoreus venom duct transcriptome with Convertover reveals 15 novel conotoxins and 13 new gene superfamilies. BMC Genomics 14, 708 (2013).
34. Kaas, Q., Yu, R., Jin, A. H., Dutertre, S. & Craik, D. J. ConoServer: updated content, knowledge, and discovery tools in the conopeptide database. Nucleic Acids Res. 40, D325–D330 (2012).
35. Jin, A. H. et al. Transcriptomic messiness in the venom duct of Conus marmoreus correlates with venom diversity. Cell. Physiol. Biochem. 32, 384–383 (2013).
36. Sousa, S. R., Vetter, L., Ragnarsson, L. & Lewis, R. J. Expression and pharmacology of endogenous Cav channels in SH-SY5Y human neuroblastoma cells. PLoS One 8, e59293 (2013).
37. Delport, W., Poon, A. F., Frost, S. D. & Kosakovsky Pond, S. L. Datamonkey 2010: a suite of phylogenetic analysis tools for evolutionary biology. *Bioinformatics* **26**, 2455–2457 (2010).
38. Yang, Z. PAML 4: phylogenetic analysis by maximum likelihood. *Mol. Biol. Evol.* **24**, 1586–1591 (2007).
39. Murrell, B. *et al.* FUBAR: a fast, unconstrained bayesian approximation for inferring selection. *Mol. Biol. Evol.* **30**, 1196–1205 (2013).
40. Pond, S. L. & Frost, S. D. Datamonkey: rapid detection of selective pressure on individual sites of codon alignments. *Bioinformatics* **21**, 2531–2533 (2005).
41. Murrell, B. *et al.* Detecting individual sites subject to episodic diversifying selection. *PLoS Genet.* **8**, e1002764 (2012).
42. Pond, S. L., Scheffler, K., Gravenor, M. B., Poon, A. F. & Frost, S. D. Evolutionary fingerprinting of genes. *Mol. Biol. Evol.* **27**, 520–536 (2010).
43. Kosakovsky Pond, S. L. *et al.* A random effects branch-site model for detecting episodic diversifying selection. *Mol. Biol. Evol.* **28**, 3033–3043 (2011).

**Acknowledgements**

This research was supported by a NHMRC Program grant (R.J.L., P.F.A.), a UQ postdoctoral fellowship (S.D.), an IMB Postgraduate Award (V.L.), the Fundação para a Ciência e a Tecnologia (K.S., A.A.), NHMRC fellowships (R.J.L., I.V.) and ARC LEIF (FLIPR to R.J.L.) grants.

**Author contributions**

S.D. conceived the research, carried out transcriptomic and proteomic analyses, fish bioassays and wrote the manuscript; A.-H.J. carried out LC-MS/MS experiments and matched proteomic and transcriptomic data; I.V. carried out high-throughput Ca2+ imaging bioassays on human SH-SY5Y cells; B.H. and D.J.V. carried out MALDI experiments and performed histology of venom duct; K.S., A.A. and B.G.F. carried out the molecular evolution analyses; V.L. conceived the Conosorter program; V.D. developed the defensive milking method, collected predation- and defence-evoked venom samples and prepared figures; P.F.A. contributed to writing of the manuscript and provided funding and research facilities; and R.J.L. conceived the research, wrote the manuscript and provided funding and research facilities.

**Additional information**

Accession codes: cDNA sequences have been deposited in the DDBJ database under accession codes AB910766 to AB910895.

**Supplementary Information** accompanies this paper at http://www.nature.com/naturecommunications

**Competing financial interests:** The authors declare no competing financial interests.

**Reprints and permission** information is available online at http://npg.nature.com/reprintsandpermissions/

**How to cite this article:** Dutertre, S. *et al.* Evolution of separate predation- and defence-evoked venoms in carnivorous cone snails. *Nat. Commun.* **5**:3521 doi: 10.1038/ncomms4521 (2014).