Insights into the origins of fish hunting in venomous cone snails from studies of *Conus tessulatus*

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Prey shifts in carnivorous predators are events that can initiate the accelerated generation of new biodiversity. However, it is seldom possible to reconstruct how the change in prey preference occurred. Here we describe an evolutionary "smoking gun" that illuminates the transition from worm hunting to fish hunting among marine cone snails, resulting in the adaptive radiation of fish-hunting lineages comprising ∼100 piscivorous *Conus* species. This smoking gun is δ-conotoxin TsVIA, a peptide from the venom of *Conus tessulatus* that delays inactivation of vertebrate voltage-gated sodium channels. *C. tessulatus* is a species in a worm-hunting clade, which is phylogenetically closely related to the fish-hunting cone snail specialists. The discovery of a δ-conotoxin that potently acts on vertebrate sodium channels in the venom of a worm-hunting cone snail suggests that a closely related ancestral toxin enabled the transition from worm hunting to fish hunting, as δ-conotoxins are highly conserved among fish hunters and critical to their mechanism of prey capture; this peptide, δ-conotoxin TsVIA, has striking sequence similarity to these δ-conotoxins from piscivorous cone snail venoms. Calcium-imaging studies on dissociated dorsal root ganglion (DRG) neurons revealed the peptide's putative molecular target (voltage-gated sodium channels) and mechanism of action (inhibition of channel inactivation). The results were confirmed by electrophysiology. This work demonstrates how elucidating the specific interactions between toxins and receptors from phylogenetically well-defined lineages can uncover molecular mechanisms that underlie significant evolutionary transitions.

evolution | prey preference | cone snails | conotoxin

Among the key evolutionary events that can lead to the rapid generation of new biodiversity are shifts in food resource utilization. For biodiverse lineages, such events can trigger adaptive radiations leading to many new species. Such events may be responsible for much of the total biodiversity on Earth. For example, the bees (Hymenoptera, Apoidea, Apiformes) are a successful and ecologically important radiation with more than 16,000 named species (1). They are derived from the sphecoid wasps (Apoidea, Spheciformes), which feed arthropod prey to their developing larvae. The bees, which feed pollen to their offspring, radiated with the angiosperms. In most respects, most bee species retain sphecoid ways of life, and many are often mistaken for wasps by casual observers. The shift from larval carnivory to larval vegetarianism in bees entailed many behavioral and physiological adaptations, but these evolved so long ago that there is no practical way to reconstruct the sequence of critical events.

However, more recent shifts in food resource utilization provide opportunities to study accompanying molecular changes. For example, ruminant mammals and birds have recruited originally defensive lysozymes and ribonucleases to new roles as digestive enzymes, with similar patterns of amino acid sequence changes in several independent cases (2–7). As herbivores, bees and ruminants face metabolic and other physiological challenges involved in the utilization of their food sources, which are easy to find and to harvest. By contrast, predators that shift onto a new kind of prey may often find the new resource easy to assimilate but initially very challenging to capture.

The detailed mechanisms that accompany a prey shift and subsequently lead to an adaptive radiation are generally difficult to reconstruct. Molecular data from diverse species provide an opportunity to reevaluate potential mechanisms that have resulted in these pivotal events in evolution and make it possible to attempt more detailed reconstructions. In this article, we focus on an unusual and dramatic prey shift among the marine snails that hunt fish (8) and provide evidence for an evolutionary "smoking gun" supporting a specific mechanism (9). It would seem a priori a very unlikely evolutionary outcome for a relatively slow moving snail, which is unable to swim, to successfully specialize in hunting fish as prey. Nevertheless, there appear to be more than 100 species of predatory gastropod molluscs belonging to the genus *Conus* (cone snails) that prey on teleost fish (10–16).

All cone snails, including those that hunt fish, use venom as the primary weapon for capturing their prey. Some fish-hunting cone snails have also evolved an anatomical specialization: a hollow harpoon-shaped radular tooth that allows them to tether the fish as venom is injected. In this article, we present evidence, both behavioral and molecular, that elucidates how the shift to

Significance

Only rarely is it possible to reconstruct molecular events that trigger the radiation of new lineages. Here we report key evidence that allows reconstruction of the transition from worm hunting to fish hunting among the species-rich family (Conidae) of marine cone snails (>700 species), which resulted in the emergence of multiple biodiverse piscivorous clades. A priori, the evolution of fish-hunting specialists would seem extremely improbable in a lineage of slowly moving snails that cannot swim, unlike their fish prey. The combination of results from molecular neuroscience, phylogenetic analysis, and chemical biology demonstrates that an ancestral cone snail venom peptide similar to δ-conotoxin TsVIA, a defensive venom component, preadapted a worm-hunting cone snail lineage, enabling the shift to a piscivorous lifestyle.

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The authors declare no conflict of interest.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database. For a list of accession numbers, see Table S1.

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fish hunting may have occurred in cone snails, a biodiverse lineage of molluscs comprising over 700 species.

Phylogenetic data of all types are consistent with the conclusion that the ancestral cone snails preyed on marine worms. The great majority of living cone snail species are believed to feed primarily on polychaetes, although a few species will feed on other types of marine worms (e.g., hemichordates, eunicids) (17). It has been suggested (9, 18, 19) that in the Miocene, there were at least two separate events that independently triggered the generation of fish-hunting cone snail lineages from worm-hunting ancestors (another significant prey shift also occurred, namely from worm hunting to mollusc hunting). The prey shifts from worm hunting to fish hunting and mollusc hunting led to a series of robust adaptive radiations that continues to the present. The fossil evidence (15, 16) suggests that there are probably a larger number of living fish-hunting and mollusc-hunting cone snail species than there have ever been in the geologic past.

Results and Discussion

Molecular Phylogeny of *Conus tessulatus*. In terms of geographic range, this is the most successful of all *Conus* species: It is found across the entire Indo-Pacific into the western Pacific (Panamic) marine province, from Mozambique to the Sea of Cortez in Mexico. Its presence in the latter site, where it has become the most common *Conus* species, has only been noted in recent decades. *C. tessulatus* feeds on polychaete worms (Fig. 1).

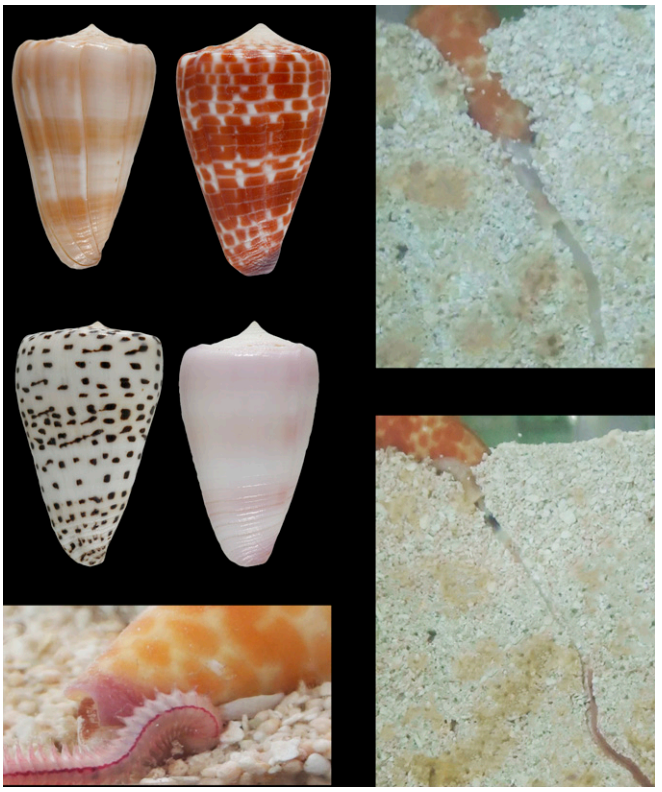

Fig. 1. *C. tessulatus* and related cone snails. (*Top Left*) Shells of the *Tesseliconus* clade are shown. Clockwise from top left: *C. sandwichensis* (Oahu, HI); *C. tessulatus* (Cocos Island, off Mexico); *C. suturatus* (Queensland, Australia); and *C. eburneus* (Cebu, Philippines). *C. tessulatus* is geographically the most widely distributed of all *Conus* species. In contrast, *C. sandwichensis* is an endemic species, restricted to the Hawaiian Islands. (*Bottom Left*) *C. tessulatus* engulfing its polychaete worm prey on the surface. (*Top Right*) Close-up view of *C. tessulatus* extending its proboscis. (*Bottom Right*) *C. tessulatus* attacking worm prey buried under the substrate.

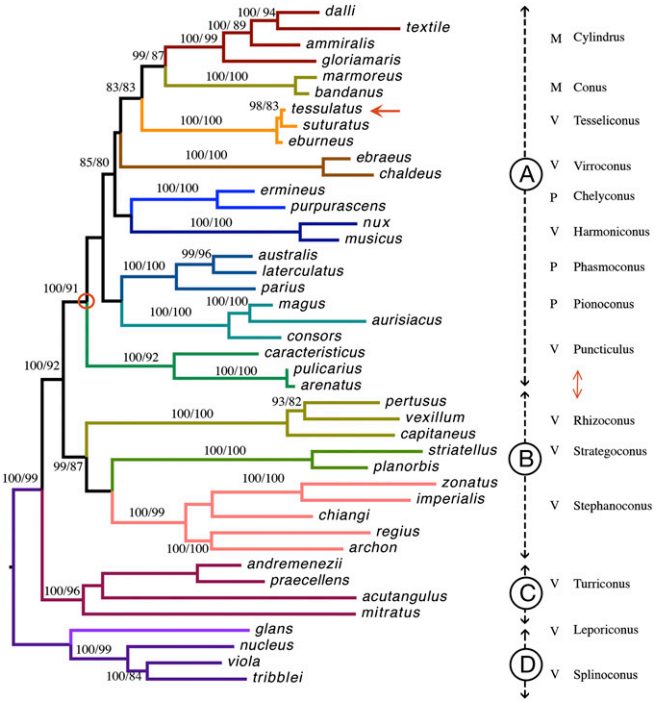

Fig. 2. Phylogeny of *C. tessulatus*. Maximum-likelihood tree inferred from 12S rRNA, 16S rRNA, and cytochrome oxidase subunit I sequences showing the placement of *C. tessulatus* (arrow) among other *Conus* species. Support values on the branches are Bayesian posterior probabilities (*Left*) expressed as percentages and approximate likelihood ratio statistics (*Right*). Individual clades (subgenera) are identified by different colors, and the primary prey known for that clade is indicated (M, molluscivorous; P, piscivorous; V, vermivorous). The red circle indicates the divergence between clades that are strictly vermivorous (*B–D*) and a major clade (*A*) with all of the lineages that use molluscivorous and piscivorous prey-capture strategies.

The phylogenetic relationship of *C. tessulatus* to its congeners (10, 20–23) is illustrated in Fig. 2. Shown are three fish-hunting cone snail clades, a subset of worm-hunting clades, and two mollusc-hunting clades. The names of clades (subgenera) shown in Fig. 2, *Right* were previously reported (10). Some worm-hunting clades are more closely related to the fish hunters than to most of the other worm-hunting clades within the genus: The phylogenetic trees indicate that the worm-hunting lineages in clade A shared a common ancestor with the fish-hunting species. Thus, when fish hunting originated, leading to an explosive adaptive radiation of fish-hunting *Conus* species, several descendant, primarily worm-hunting lineages remained. Whereas the great majority of vermivorous *Conus* species belong to the worm-hunting clades that are phylogenetically distant from the fish-hunting cone snail species, *C. tessulatus* and three other worm-hunting species in the subgenus *Tesseliconus*, *C. eburneus*, *C. suturatus*, and *C. sandwichensis* (Fig. 1), are more closely related to fish hunters (19). The trees shown (Fig. 2 and Fig. S1) are based on standard mitochondrial markers (Table S1); the same conclusion regarding *C. tessulatus* belonging to one of the worm-hunting clades most closely related to fish hunters is supported by a phylogenetic tree constructed from a single nuclear marker (19) that has been particularly useful in resolving cone snail clades (Fig. S2 and Table S1).

Analysis of *C. tessulatus* Venom. Bioactive components of *C. tessulatus* venom were screened using calcium imaging of native dorsal root ganglion (DRG) neurons (24–28). This enables the simultaneous evaluation of multiple biological activities of various venom components across a population of heterogeneous cell types. Application of crude *C. tessulatus* venom to dissociated mouse

DRG cells (Fig. 3) amplified the response to a depolarizing stimulus (high extracellular K^+ concentration) in the vast majority of DRG neuronal cell types. After the venom was fractionated, one pool of fractions (40–46) mimicked the enhancing activity of the crude venom (Fig. 3). Notably, other fraction pools elicited different phenotypic effects on the dissociated DRG neurons, and these will be described elsewhere. Individual fractions from the pool were tested, and the activity was found in fraction 44. Subfractionation of fraction 44 resulted in the purification of a single peptide that retained the biological activity (Fig. 3). The amino acid sequence

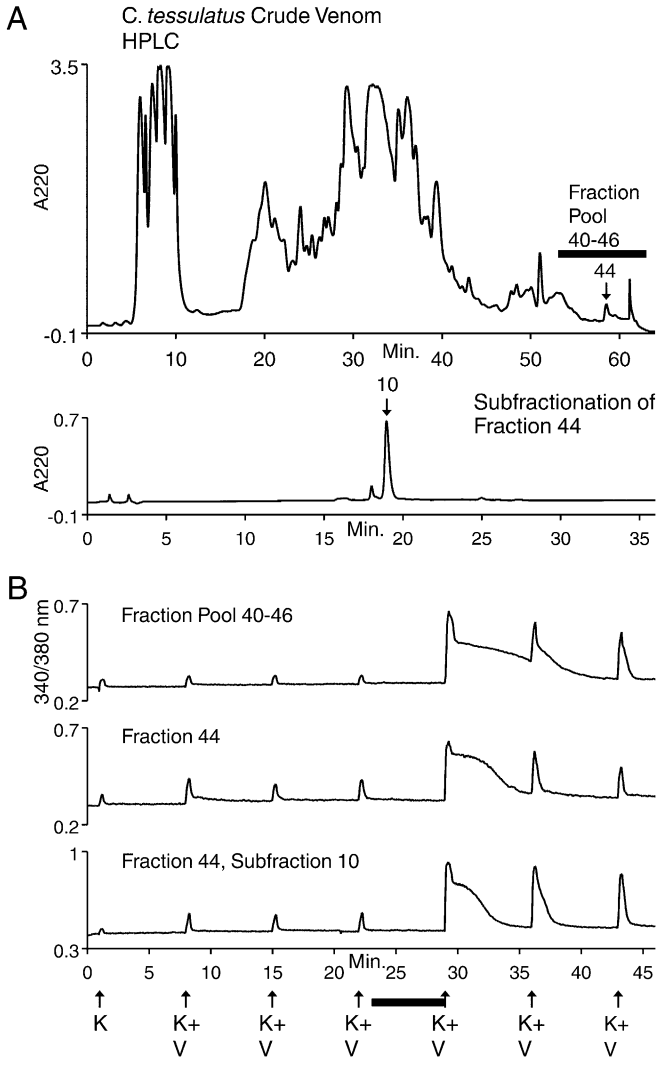

Fig. 3. Bioassay-guided purification of δ-conotoxin TsVIA from *C. tessulatus* venom. (*A*, *Top*) Reversed-phase HPLC chromatogram of *C. tessulatus* crude venom. The biological activity was first identified in fraction pool 40–46 and then in fraction 44. (*Bottom*) Subfractionation of fraction 44 resulted in one main peak (subfraction 10) containing a single peptide that retained the biological activity observed in fraction 44. (*B*) Calcium-imaging traces from selected DRG neurons. Each trace represents the responses of a single neuron. The experimental protocol shown under the *x* axis is as follows. Each arrow represents a 15-s application of either 20 mM K^+ (K) or 20 mM K^+ + 20 μM veratridine (K+V) to depolarize the neurons. Upward deflection of the trace represents an increase in cytosolic calcium concentration. K+V was used in screening venom fractions to facilitate the discovery of antagonists of voltage-gated Na channels. The horizontal bar indicates when the venom fraction was present in the bath solution. Each venom fraction caused a substantial enhancement of the amplitude and duration of the response to a depolarizing stimulus (K+V), which was slowly reversible.

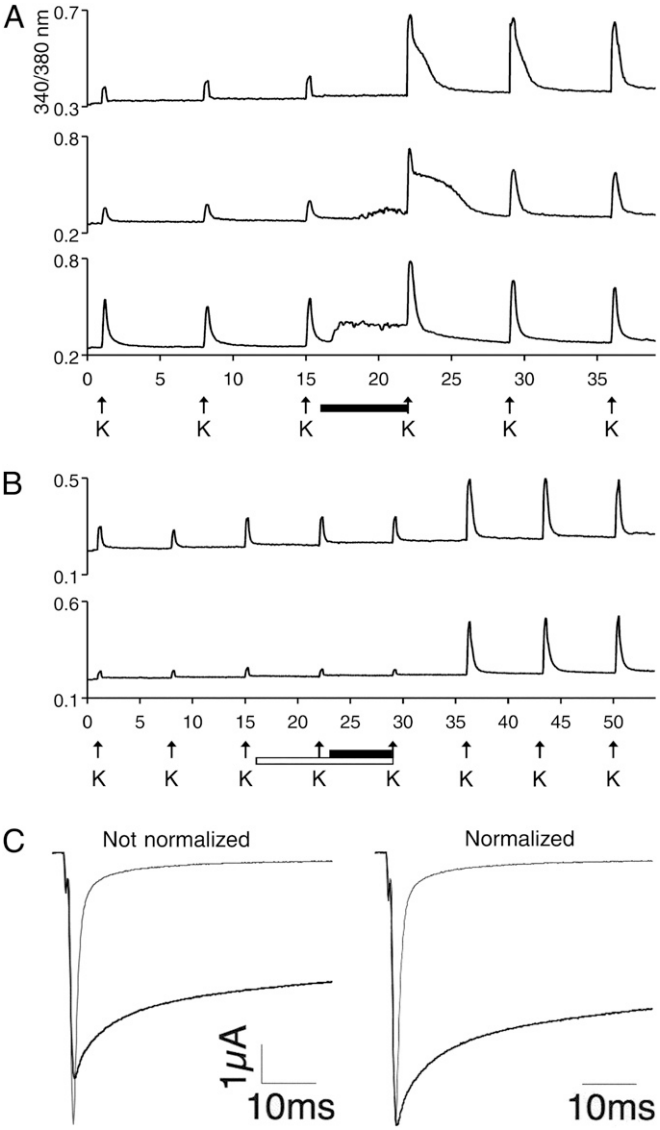

Fig. 4. Biological activity of δ-conotoxin TsVIA. (*A* and *B*) Calcium imaging. Each trace represents the responses of a single neuron. Each arrow represents a 15-s application of 20 mM K^+ to depolarize the neurons. Upward deflection of the trace represents a transient increase in cytosolic calcium concentration. (*A*) Calcium-imaging traces from selected DRG neurons in response to purified TsVIA. The horizontal bar indicates when ~1.6 μM purified TsVIA was present in the bath solution. In different DRG neurons, the peptide caused a substantial enhancement of the response to a depolarizing stimulus (K), with or without a direct increase in the baseline cytosolic calcium concentration. The enhancement of the response to the depolarizing stimulus was slowly reversible following removal of TsVIA. (*B*) The presence of 1 μM tetrodotoxin in the bath (open horizontal bar), to block voltage-gated Na channels, prevented all effects of TsVIA (closed horizontal bar), until unbound TTX and TsVIA were washed out of the bath. This result suggested that TsVIA acts on TTX-sensitive voltage-gated Na channels and does so with much slower reversibility than TTX. (*C*) Electrophysiology of voltage-gated sodium channels. The effect of TsVIA on voltage-clamped oocytes coexpressing mouse $Na_V1.6$ with rat $Na_V\beta1$ subunits. Shown are Na currents in the absence (thin traces) and presence (thick traces) of TsVIA. Fast inactivation of Na current evident in the control trace was blocked substantially by the presence of TsVIA.

of the peptide was obtained as described in Figs. S3 and S4. The active component is a 27-amino acid peptide with the following sequence: **C**AAFGS**FC**GLPGLVD**CC**SGR**C**FIV**C**LL (bold font indicates cysteine amino acids that form disulfide bonds). The

Table 1. Comparison of δ-conotoxin sequences from various cone snail species

Species type	Clade	*Conus* species	δ-Conotoxin (ref.)	Amino acid sequence
Worm-hunting				* * * ** *
	Tesseliconus	*C. tessulatus*	TsVIA	CAAFGSFCGL-PGLVDCCSGRCFIVCLL
		C. eburneus	ErVIA	CAGIGSFCGL-PGLVDCCSGRCFIVCLP
Fish-hunting				* * ** ** *
	Chelyconus	*C. purpurascens*	PVIA (33)	EACYAOGTFCGIKOGL--CCSEFCLPGVCFG#
		C. ermineus	EVIA (35)	EACYPOGTFCGIKOGL--CCSELCLPAVCVG#
	Pionoconus	*C. striatus*	SVIE (44)	DGCSSGGTFCGIHOGL--CCSEFCFLWCITFID
		C. magus	MVIA (34)	DGCYNAGTFCGIROGL--CCSEFCFLWCITFVDS#
		C. consors	CnVIA (34)	YECYSTGTFCGINGGL--CCSNLCLFFVCLTFS
		C. aurisiacus	AVIA (34)	DGCSNAGAFCGIHOGL--CCSEICIVWCT
Mollusc-hunting				*
	Cylinder	*C. textile*	TxVIA (31)	WCKQSGEMCNLLDQN--CCDGYCIVLVCT
		C. gloriamaris	GmVIA (45)	VKPCRKEGQLCDPIFQN--CCRGWNCVLFCV

The bold-font "C"s represent the cysteine amino acids that form the disulfide bonding framework of these delta conotoxin peptides. Asterisks represent conserved (non-Cys) amino acids within δ-conotoxins from fish-hunting cone snails. Underlining indicates conserved amino acids and conservative amino acid changes (similar biochemical properties) between worm hunters and fish hunters. All amino acid sequences were determined from peptides purified from venom, with the exceptions of MVIA and AVIA, which were determined by molecular cloning of the genes encoding these peptides. # indicates an amidated C terminus. O, hydroxyproline. Each dash is for alignment of conserved amino acids.

sequence is homologous to peptides in the δ-conotoxin branch of the O superfamily. Furthermore, the bioactivity observed on DRG neurons (Fig. 3) is consistent with that of a δ-conotoxin.

Evidence That the Excitatory Venom Peptide from *C. tessulatus* Is a δ-Conotoxin. The purified venom peptide was further characterized to test the hypothesis that it is a δ-conotoxin, which inhibits voltage-gated Na-channel inactivation. If the target Na channels were blocked, the effect of a δ-conotoxin should be masked in the calcium-imaging assay. As shown in Fig. 4 *A* and *B*, the effects of the purified peptide were abolished in the presence of tetrodotoxin (TTX), an Na-channel blocker. However, after unbound TTX and peptide were washed out of the bath, an amplified response to a depolarizing stimulus was observed, which persisted over the remaining time course of the experiment. This result is consistent with the peptide dissociating slowly relative to the known rapid dissociation kinetics of TTX from Na channels (29). The peptide was also tested on *Xenopus laevis* oocytes expressing mouse $Na_V1.6$, a voltage-gated sodium-channel subtype widely expressed in axons (Fig. 4*C*). The peptide dramatically inhibited channel inactivation, a hallmark of δ-conotoxins. Based on the amino acid sequence of the peptide, its observed biological activity on DRG neurons, and its activity on $Na_V1.6$, we conclude that the peptide is a δ-conotoxin. Consequently, we named this peptide δ-conotoxin TsVIA ("Ts" indicates that the peptide came from *C. tessulatus*; "VI" is a roman numeral designation of the cysteine arrangement; and "A" differentiates this peptide from other potential δ-conotoxins from *C. tessulatus*).

We investigated whether species in the same clade as *C. tessulatus* have related δ-conotoxins. From *C. eburneus*, a species closely related to *C. tessulatus*, we purified a venom component that exhibited the same biological activity as native δ-conotoxin TsVIA (Fig. S5). The observed mass and amino acid sequence of the native *C. eburneus* δ-conotoxin matched the predicted mass and amino acid sequence obtained from molecular cloning (Table 1). Thus, *C. tessulatus* and *C. eburneus* venoms contain related δ-conotoxins, with striking sequence similarity to the δ-conotoxins from the venoms of fish-hunting *Conus* species.

Physiological Role of δ-Conotoxins. The δ-conotoxins from *C. tessulatus* and *C. eburneus*, δ-conotoxin TsVIA and δ-conotoxin ErVIA, belong to a widely distributed family of *Conus* peptides that span several lineages of cone snails. The first δ-conotoxin discovered was δ-conotoxin TxVIA (also known as the "King Kong" peptide) from the mollusc-hunting species *C. textile* (30, 31). Four fish-hunting cone snail δ-conotoxins were subsequently characterized from venom (Table 1): δ-conotoxins NgVIA, *C. nigropuctatus*; δ-PVIA, *C. purpurascens*; δ-SVIE, *C. striatus*; and δ-EVIA, *C. ermineus* (32–35).

The role of δ-conotoxins in fish hunting is critical in piscivorous cone snail species that belong to the *Pionoconus* or *Chelyconus* lineages; δ-conotoxins are a major component of the so-called lightning-strike cabal (8, 36). This is a key physiological strategy for the capture of fish by the much slower-moving cone snails that causes an extreme hyperexcitability of the fish nervous system, resulting in an almost instant tetanic paralysis, as though the fish had been hit with a Taser. The basis of this pharmacological activity is the combination of δ-conotoxins that inhibit Na-channel inactivation and κ-conotoxins that inhibit K-channel activation in axons at the injection site. Normally, Na-channel inactivation and K-channel activation repolarize the membrane to terminate an action potential. Consequently, inhibiting both Na-channel inactivation and K-channel activation blocks membrane repolarization following an action potential, resulting in massive depolarization, generating both retrograde and anterograde action potentials. In effect, the nervous system is subjected to an electrical storm that originates from axons near the venom-injection site. Thus, the evolution of appropriate δ-conotoxins and κ-conotoxins was postulated to be key to the evolutionary shift from worm hunting to fish hunting in cone snails (9, 36).

Different lineages of fish-hunting cone snails have structurally unrelated κ-conotoxin peptides, with each lineage having recruited κ-conotoxins from different gene superfamilies, including the M superfamily (*Phasmoconus* clade) (37, 38), O superfamily (*Chelyconus* clade) (8), and conkunitzin superfamily (*Pionoconus* clade) (39). In contrast, when all of the relevant fish-hunting lineages are compared, δ-conotoxins are found to be highly conserved; all belong to the O superfamily, and share strong sequence similarity. We demonstrate a striking sequence similarity with δ-conotoxins TsVIA and ErVIA from *C. tessulatus* and *C. eburneus* but not with the δ-conotoxins from molluscivorous *Conus* species (36, 40). The sequences of δ-conotoxins from two mollusc-hunting species, six fish-hunting species, and the two species from the *Tesseliconus* clade, *C. eburneus and C. tessulatus* (Table 1), demonstrate the remarkable sequence similarity of the δ-conotoxins from worm-hunting and fish-hunting species and the greater sequence divergence of δ-conotoxins from mollusc-hunting species.

The striking sequence similarity between the δ-conotoxins of worm-hunting *Tesseliconus* species and fish hunters is consistent with these having all evolved from a homologous ancestral δ-conotoxin. Consistent with this hypothesis, these peptides all potently inhibit inactivation of vertebrate Na_V channels. In contrast, δ-TxVIA and δ-GmVIA have no known effects on the inactivation of vertebrate Na_V channels; presumably, these peptides were under selection to target molluscan Na channels.

Behavioral Observations of *C. tessulatus*. *C. tessulatus* specimens were kept in tanks with various worm and fish species with the hope of observing a three-way interaction between the cone snail predator attacking its worm prey and interacting with a potential competitor, teleost fish. The snails attacked and devoured certain worms (as shown in Fig. 1), particularly nereids, which they clearly preferred to the other worms present in the aquarium (e.g., terebellid polychaetes). However, we also observed *C. tessulatus* attacking fish, which the snail clearly regarded as potential prey. In one instance, the snail extended its proboscis, and the tip of the proboscis explored the gill areas of the fish. The snail may have released venom into the gill areas, because the fish later exhibited muscle spasms and was engulfed by the rostrum (false mouth) of the snail. On two other occasions, the snail attempted to inject venom into the fish, but on both occasions failed to pierce the skin and a cloud of venom was released instead, after which the fish appeared to be unaffected (Fig. 5). A snail was observed attempting to swallow a weakened fish; *C. tessulatus* will opportunistically eat dead fish (which Indo-Pacific fish-hunting *Conus* species do not touch). Movies S1 and S2 illustrate both the worm-hunting behavior and fish-hunting behavior of *C. tessulatus*.

We conclude from these observations that *C. tessulatus* is primarily a worm hunter, and certain types of worms such as nereid polychaetes are its primary food resource. However, this species appears to have a range of secondary prey, including terebellid polychaetes and fish. Thus, a primarily vermivorous predator opportunistically preys on fish, even though it does not have the specializations of the most common piscivorous *Conus* species that make them much more efficient fish hunters (such as a harpoon-shaped tooth for tethering envenomated fish).

Reconstruction of the Shift in Prey. We have presented evidence that supports a specific hypothesis for how the transition from worm hunting to fish hunting occurred in cone snails. We previously suggested (36) that as an ancestral lineage of vermivorous cone snails attacked their worm prey, fish would try to steal the prey and that vermivorous *Conus* may have evolved δ-conotoxins to deter fish competitors. The basis for this hypothesis was that in diverse lineages of fish-hunting cone snails, the same gene superfamily had been recruited for the δ-conotoxins used to capture fish. To effectively immobilize a fish, a δ-conotoxin has to be coupled to a K-channel blocker. In contrast to the structurally conserved δ-conotoxins, different lineages of fish-hunting snails have recruited different K-channel blockers from diverse conotoxin gene superfamilies. Within a particular lineage (or clade) of fish-hunting cone snails, peptides that block K channels are clearly homologous. However, the peptides that block K channels from different fish-hunting clades have no sequence similarity. This suggested that δ-conotoxins were already in place before fish hunting evolved, and the subsequent evolution of κ-conotoxins led to various double-toxin combinations that rapidly immobilize fish.

The molecular evolutionary smoking guns consistent with this hypothesis are the δ-conotoxins found in *C. tessulatus* and *C. eburneus* venoms. These *Conus* species are in a worm-hunting clade closely related to fish-hunting and mollusc-hunting *Conus* lineages. Worm hunting likely originated in ancestral conoideans in the Late Cretaceous; the data suggest that during the Miocene or earlier, a peptide very similar to δ-conotoxin TsVIA evolved in an ancestral worm-hunting cone snail that ultimately gave rise to fish-hunting lineages (e.g., *Chelyconus* and *Pionoconus*). The striking sequence similarities between δ-conotoxins found in fish-hunting cone snails (Table 1) and the δ-conotoxins characterized from the primarily worm-hunting *Tesseliconus* predict the sequence features of the ancestral δ-conotoxin in the last common ancestor of the three clades (*Pionoconus*, *Chelyconus*, and *Tesseliconus*) (Table 1). This common ancestor was "preadapted," having evolved a potent δ-conotoxin targeted to vertebrate Na channels. The subsequent recruitment of two types of K channel-blocking toxins, conkunitzins (for the *Pionoconus* clade) (39) and κ-conotoxin (for the *Chelyconus* clade) (8), presumably provided the trigger for the shift from worms to fish as primary prey in the two piscivorous lineages.

Cumulatively, the phylogenetic position of *Tesseliconus* relative to the fish-hunting clades, the biotic interactions of *C. tessulatus* with worms and fish, the similarity in sequences of the worm-hunting and fish-hunting δ-conotoxins, and the biological activity of δ-TsVIA and δ-ErVIA on vertebrate Na channels

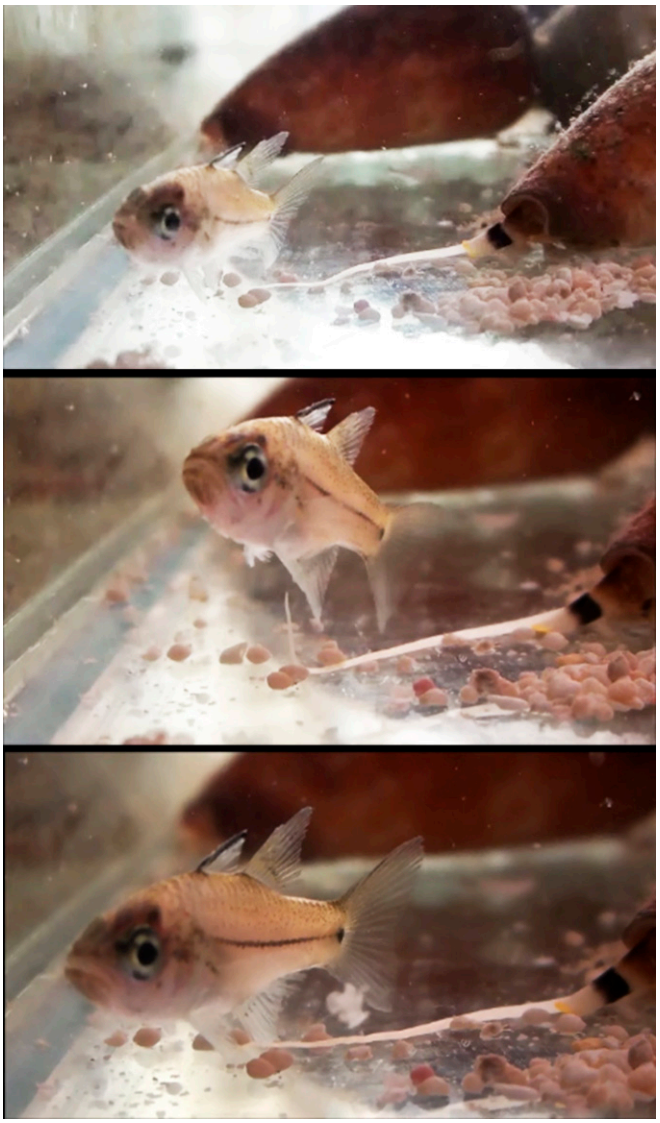

Fig. 5. *C. tessulatus* attempting to envenomate a fish. The snail has extended its proboscis; shortly after the *Middle* frame, the tip of the proboscis touched a fin of the fish, and the snail expelled a cloud of venom near the fin, which is visible (*Bottom*) floating above the extended proboscis and near the lower edge of the tail fin.

suggest how a worm-hunting ancestral species evolved into fish-hunting descendants. Although *C. tessulatus* is not highly specialized for fish prey, it will attempt to envenomate fish, but it is less effective than true piscivorous species. This raises the possibility that the ancestral species had a lifestyle much like that of *C. tessulatus*, namely vermivorous, with a spectrum of secondary prey that could be envenomated if the primary prey were scarce. We suggest that *Tesseliconus* may be a relict clade, relatively unchanged from the last common ancestor of *Tesseliconus*, *Chelyconus*, and *Pionoconus* (although the possibility that *Tesseliconus* evolved from a fish-hunting ancestor that secondarily reverted to a worm-hunting lifestyle cannot be rigorously eliminated). However, if *C. tessulatus* and the *Tesseliconus* clade indeed closely resemble the ancestral vermivores that gave rise to fish-hunting *Conus* clades, the ancestral δ-conotoxin may not only have been defensive but could also have been used for gratuitous fish hunting.

Materials and Methods

Additional details are described in *SI Materials and Methods*. *C. tessulatus* venom was fractionated as described previously (41). Aliquots of pooled and individual venom fractions were assayed for activity on dissociated DRG neurons by calcium imaging. The active component of pooled fractions 40–46 was purified by following the activity in calcium-imaging assays, as shown in Fig. 3. The calcium-imaging methods have been described in detail previously (25–28). The amino acid sequence of δ-conotoxin TsVIA (shown in Table 1) was determined by a combination of Edman degradation, MS/MS sequencing, and molecular cloning, as described previously (41) and in *SI Materials and Methods*. The amino acid sequence of δ-conotoxin ErVIA (shown in Table 1) was identified by molecular cloning as previously described (42) and by MS/MS sequencing, as described in *SI Materials and Methods*. Use of *X. laevis* frogs, which provided oocytes for this study, and use of C57BL6 mice followed protocols approved by the University of Utah Institutional Animal Care and Use Committee that conform to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (43). Preparations of cRNA and the injection of cRNA into oocytes were done as previously described (29). Electrophysiology methods were also previously described (29). Detailed methods for phylogenetic analysis are provided in *SI Materials and Methods*.

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