

Toxicity in Cephalopods

Ira R. Cooke^{a,b,*}, Brooke Whitelaw^c, Mark Norman^d, Nikeisha Caruana^c and Jan M. Strugnelli^c

^aDepartment of Biochemistry and Genetics, La Trobe Institute for Molecular Sciences, La Trobe University, Melbourne, VIC, Australia

^bLife Sciences Computation Centre, Victorian Life Sciences Computation Initiative, Melbourne, VIC, Australia

^cDepartment of Ecology, Environment and Evolution, La Trobe University, Melbourne, VIC, Australia

^dSciences, Museum Victoria, Carlton, VIC, Australia

Abstract

Cephalopods are a morphologically diverse molluscan class that includes octopuses, cuttlefishes, squids, and nautilus. The behavior, morphology, and sometimes aposematic appearance of coleoid cephalopods (octopuses, cuttlefishes, and squids) are highly suggestive of the widespread use of toxins for predation and/or defense. Many cephalopods use a combination of their parrot-like beak and/or toothed radula to inject venomous saliva, thought to be produced in the posterior salivary gland, into prey through a bite wound or a hole drilled into the shell. However, relatively few toxins have been studied to date from only a narrow range of cephalopods. Active components that have been identified from cephalopod posterior salivary gland extracts (or saliva) include neurotoxins such as tetrodotoxin (also found in body tissues), tachykinins and cephalotoxins, biogenic amines such as serotonin and octopamine, and a diverse range of enzymes including serine proteases, phospholipase A2, hyaluronidases, and chitinases. Coleoid cephalopods represent excellent candidates for biodiscovery, being taxonomically distinct from heavily studied venom-producing organisms, and because their venoms appear to be complex mixtures of proteins and small molecules. Understanding the evolutionary history of toxicity in cephalopods remains a challenge, with many major taxa remaining unstudied and very little specific functional information available on most cephalopod toxins. The application of “omics” technologies to research on venoms and other toxic secretions (“venomics”) is an important and powerful way of characterizing entire suites of proteinaceous toxins from pure venom or gland extracts in cephalopods and is likely to yield future insights into the evolution of toxicity in this class.

Keywords

Cephalopoda; octopus; squid; venom; coleoid; tachykinin; cephalotoxin

Introduction

The molluscan class Cephalopoda contains the octopuses, squids, cuttlefishes, and nautilus. Although the greatest diversity of cephalopods is found in tropical shallow oceans, they occur in all marine habitats including the deep sea and polar regions. Cephalopods do not occur in freshwater environments. There are about 800 known species encompassing a large size range, from the giant squids (~14 m) to the pygmy squids (~2 cm). Most extant cephalopods do not possess an external shell, the exception to this being the nautilus (subclass Nautiloidea). The remaining cephalopods (subclass Coleoidea) possess an internal shell, which has been reduced or lost in many cases throughout the course of evolution and thus they are

*Email: I.Cooke@latrobe.edu.au

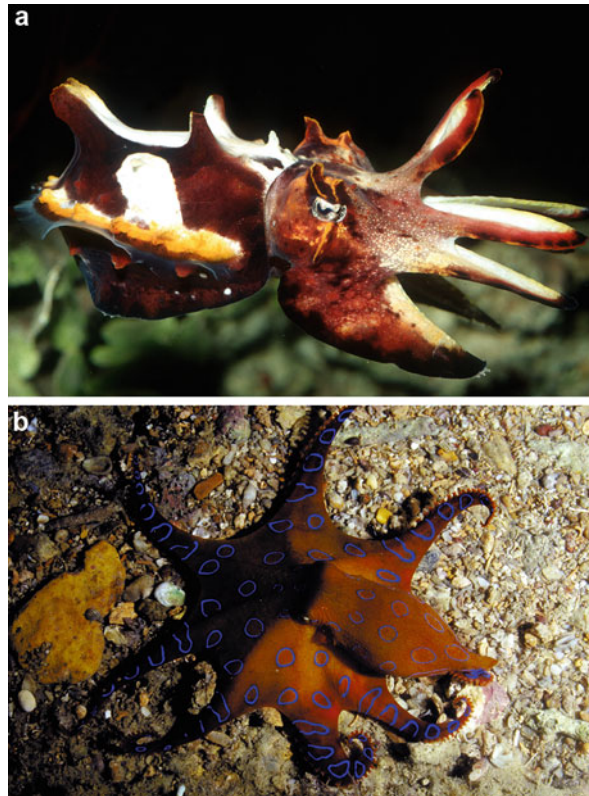


Fig. 1 Cephalopods with (possibly) aposematic coloration and displays. Photograph A is *Metasepia pfefferi* and B is *Hapalochlaena* sp. (Photograph A was taken by Roger Steene and B by Mark Norman)

vulnerable to predation. This lack of an external shell has likely driven the evolution of the remarkable ability of coleoids to change texture and color to blend in with their surroundings or, in some cases, to display warning colorations – potentially advertising their toxicity. In addition to the use of toxins in a defensive role, the behavior and feeding morphology of many coleoids is highly suggestive of the use of venom in subduing and/or externally digesting prey. The existence and use of toxins are so far unknown for nautilus, and this review will therefore focus exclusively on coleoid taxa.

Coleoids are carnivorous, with many species, especially those that are benthic or demersal, specializing in hard-shelled prey such as bivalves, gastropods, crustaceans, and nautilus (Pilson and Taylor 1961; Chichery and Chichery 1988; Saunders et al. 1991). The feeding apparatus of all coleoids is a small parrot-like beak, which would appear to be unsuitable for dealing with hard-shelled prey by crushing. Although brute force dismemberment is possible for smaller prey, it seems likely that venoms are often employed to at least weaken the prey, if not to kill it. This is well documented among many octopods, whereby the hard shell of the prey is penetrated by drilling a small hole through which a venom is injected (Grisley et al. 1996; Mather and Nixon 1995; Pilson and Taylor 1961; Runham et al. 1997; Saunders et al. 1991; Wodinsky 1969). Close observation of prey capture by cuttlefishes (Chichery and Chichery 1988) suggests that they also inject toxins through a small wound inflicted in a soft joint area of their prey (crabs). Likewise, the tiny (7–12 mm) Japanese pygmy squid (*Idiosepius paradoxus*) is capable of paralyzing shrimp much larger than itself (20–30 mm) within 1 min of capture (Kasugai et al. 2004) and also uses salivary secretions for external digestion.

The brightly colored, or otherwise striking, appearance of some octopus and squid species suggests that they may use toxins for defense (based on the aposematic warning colorations of diverse vertebrates and invertebrates). In some cases, such as in the brightly colored flamboyant cuttlefish (*Metasepia pfefferi*, see

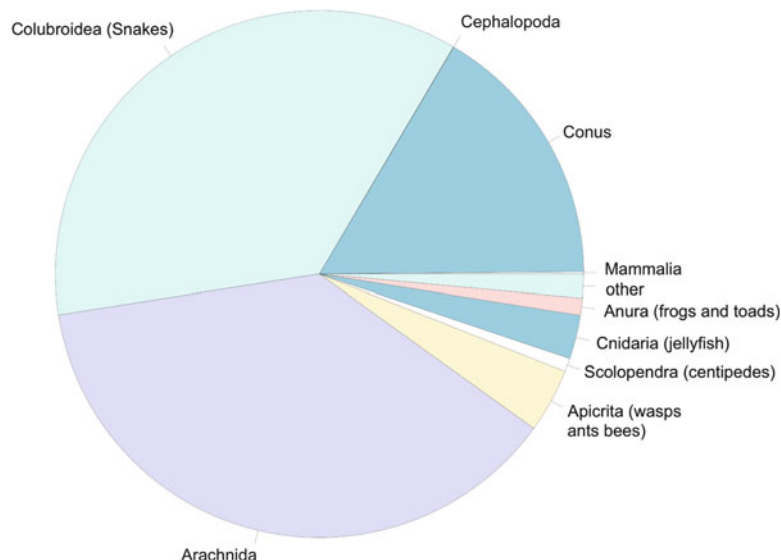


Fig. 2 Taxonomic distribution of 6,306 verified proteins in the UniProt toxin database. The database contains just a single cephalopod protein (SE-cephalotoxin). Note that at least one other cephalopod toxin (eledoisin) was erroneously missing from the UniProt toxin database at the time of writing

Fig. 1a), this striking coloration is coupled with diurnal activity in environments with high predator pressure (in contrast, many less colorful cephalopods are primarily nocturnal). The best-studied example of a cephalopod with a striking appearance is that of the blue-ringed octopuses (genus *Hapalochlaena*, see Fig. 1b), which flash iridescent blue markings when agitated (these cephalopods are also capable of excellent camouflage in order to stalk prey) (Norman 2000). *Hapalochlaena* species possess tetrodotoxin (TTX) in both the salivary glands and distributed throughout body tissues (Yotsu-Yamashita et al. 2007). The dramatic warning colorations and presence of the powerful neurotoxin throughout the body suggest more than a purely predatory role. Other examples include the striped pyjama squid (*Sepioloidea lineolata*) whose striking black-and-white striped color pattern suggests a warning to potential predators (Norman 2000). In addition, many coleoids that inhabit relatively shallow water environments produce ink that is released when attempting to evade a predator. Studies on the gastropod slugs known as sea hares (Aplysiomorpha), which also produce a form of ink, have discovered a complex cocktail of compounds in the ink that affect the behavior of predators such as lobsters (Derby 2007). Far less is known about the chemical composition and ecological role of cephalopod inks. Although some form of chemical defense in inks seems likely, this is yet to be demonstrated (Derby 2014; Wood et al. 2008).

These life history traits (hole drilling, aposematic coloration, lack of a hard protective shell, ink and slime production) suggest that cephalopods may possess a rich arsenal of toxic compounds in their venom and tissues. Nevertheless, the number of cephalopods for which any form of toxin research has been conducted remains low (fewer than 30 species), and the majority of these species appear in just four studies (Fry et al. 2009; Ruder et al. 2013; Ueda et al. 2008; Undheim et al. 2010). These four recent studies provide a glimpse into the diversity and potency of proteinaceous cephalopod toxins. Ueda et al. (2008) observed potent toxicity against crabs for posterior salivary gland (PSG) extracts of all three species of cuttlefish (*Sepia esculenta*, *Sepia lycidas*, *Sepia japonica*) studied and toxicity against both crabs and mice for PSG extracts of all three species of loliginid squids (Loliginidae) studied. This suggests that the use of potent venoms is widespread in cuttlefish and squids and that examination of a range of cephalopod taxa is likely to reveal different compounds (or variants) to account for varying target specificity. This is underscored by the studies by Ruder et al. (2013) and Fry et al. (2009) who provide a

list of nine putative toxin protein families coded for in the transcriptomes of cephalopod posterior salivary glands.

Despite the high likelihood that cephalopods exhibit a diverse proteinaceous toxin arsenal, the number of well-characterized cephalopod toxins is extremely low. This is reflected in Fig. 2 which shows the number of proteins in the UniProt toxin database (verified toxins or components of venom) according to the taxonomic group from which the proteins were isolated. In this database, cephalopods, a taxonomic group of some 800 species, are represented by just one protein (SE-cephalotoxin), whereas a single well-studied genus of gastropods (*Conus*) accounts for 1,022 entries.

Although recent venomomics work provides strong evidence that cephalopod posterior salivary gland secretions are complex mixtures of many molecules (Cornet et al. 2014; Ruder et al. 2013), the effects of these compounds on potential prey (or predators) have not been established in most cases. In this review, we first focus on the most studied classes of molecules, including small molecules such as tetrodotoxin (TTX) and biogenic amines, as well as proteins such as cephalotoxin, chitinases, and peptidases. Later sections of the review summarize current knowledge on the evolution of toxicity in cephalopods and technological developments including the potential insights to be gained from venomomics studies.

Since cephalopods produce a range of potentially toxic substances (salivary venom, ink, body tissues), it is important to clarify our use of the terms venom, venomics, toxin, and toxic. In this review we use the term venom to refer to substances injected via a bite or drill hole (i.e., salivary secretions) but accept that the term “venomics” can sometimes be applied to studies of other toxic secretions. We use the term toxin to refer to specific molecules that confer toxicity and emphasize that toxins may be contained within various tissues in the cephalopod body or secreted as part of venoms or inks. All substances that contain toxins are referred to as toxic.

Significant Toxin Classes in Cephalopods

Tetrodotoxin

Tetrodotoxin (TTX) is an extremely potent low molecular weight neurotoxin. In cephalopods, TTX is known to be present in members of the genus *Hapalochlaena* (blue-ringed octopuses). Many studies have focused their attention on the genus *Hapalochlaena* due to the immediate threat it poses to humans. The first documented fatal envenomation from a blue-ringed octopus occurred in 1954 in Darwin, Northern Territory, Australia (Jacups and Currie 2008). Studies into the posterior salivary gland (PSG) of *H. maculosa* identified the fatal component as maculotoxin (Crone et al. 1976), which was later revised to TTX (Sheumack et al. 1978). A similar toxin to TTX was isolated from *H. maculosa* called hapalotoxin, distinguishable from TTX due to its higher polarity (Savage and Howden 1977).

Aside from cephalopods, TTX also occurs in a diverse range of other organisms including fishes, amphibians, arthropods, nemerteans, flatworms, arrow worms, echinoderms, and other molluscs. The origins of tetrodotoxin are not fully understood, but its sporadic appearance across unrelated taxa suggests that it is either ingested or produced by bacterial symbionts (Chau et al. 2011). Studies on puffer fish have shown that captive animals lose toxicity over time if fed a non-TTX containing diet (Noguchi et al. 2006), which suggests accumulation via ingestion. However, the newt, *Taricha granulosa*, is able to maintain and even increase TTX levels on a non-TTX containing diet (Hanifin et al. 2002), which suggests that it is produced by symbionts or even endogenously by the newts themselves. Studies on puffer fish (Yu et al. 2004; Wu et al. 2005) have identified TTX-producing bacteria from the tissues of TTX-bearing host species. TTX in blue-ringed octopuses is thought to be bacterial in origin, but it is not known whether these bacteria are housed within a particular organ, spread throughout the body, or ingested. Hwang et al. (1989) cultured 22 strains from the tissues of *H. maculosus*, [sic] of which 16 were

shown to produce TTX. TTX-producing strains were found in cultures made from the posterior salivary gland, tentacle, intestine, and ink sac and were identified as species of *Vibrio*, *Alteromonas*, *Pseudomonas*, and *Bacillus*.

Blue-ringed octopus are one group of only a small number of animals believed to use toxins for defense as well as to subdue prey. Evidence for a predatory role comes from the presence of TTX in the posterior salivary glands, which are the usual venom-producing organs in octopods, but its efficacy against typical prey has not been quantified (Williams 2010). Evidence for a defensive role comes from the characteristic iridescent blue warning rings that the animal displays when distressed, as well as from the relative distribution of TTX across tissues, eggs, and larvae. This intraorganismal distribution of TTX varies depending on species and geographic location (Hwang et al. 1989; Williams and Caldwell 2009). For example, Hwang et al. (1989) examined the relative concentrations of TTX in the posterior salivary glands versus other soft tissues, for two specimens collected at different locations. In one specimen (Cebu Island, Philippines), they found that the majority of total TTX was located in the soft tissues, with only around a quarter of total TTX being located in the PSG. In contrast, a specimen from Izu Peninsula (Japan) had concentrated almost all TTX in its posterior salivary gland. This suggests that the relative role (defense or predation) of TTX in blue-ringed octopus may vary between individuals or subpopulations. Further evidence for a defensive role for TTX comes from Yotsu-Yamashita et al. (2007), who examined six specimens from South Australia and found much higher levels of TTX in bulk body tissues (arms, cephalothorax, abdomen) than in the posterior salivary gland. Williams et al. (2011) found that while *H. maculosa* larvae contained TTX, it was not at a sufficient concentration to intoxicate or deter predators in controlled feeding experiments. Interestingly, they found that *H. maculosa* larvae were indeed distasteful to predators but speculate that this is due to some compound other than TTX, since TTX-spiked control items were not distasteful to representative fish or stomatopod predators.

Utilization of a toxin necessarily requires an organism to also contain these highly detrimental proteins or compounds. As a result, the organism requires a degree of resistance to these toxic elements (Flachsenberger and Kerr 1985). The tolerance of one *Hapalochlaena* species (most likely *H. maculosa* based on the collection site) was examined by Flachsenberger and Kerr (1985), who found no negative effects when the specimen was injected with high doses of purified TTX or salivary extract. This suggests that any predatory or defensive function of *Hapalochlaena* venom is not directed at others of the same genus. In contrast, the male platypus utilizes its venom almost exclusively for intraspecies competition (Wong et al. 2012).

Cephalotoxin

The term cephalotoxin was first used by Ghiretti (1959) to describe a protein purified from the posterior salivary gland of *Sepia officinalis*. The defining characteristic of this protein was its ability to induce complete paralysis in crabs, a phenomenon that had previously been observed for whole octopus saliva as early as 1897 (Krause 1897). Later work by Ghiretti (1960), McDonald and Cottrell (1972), Songdahl and Shapiro (1974), and Cariello and Zanetti (1977) isolated protein fractions with similar neurotoxic effects from the common octopus (*Octopus vulgaris*), the curled octopus (*Eledone cirrhosa*), and the giant Pacific octopus (*Octopus dofleini*, now *Enteroctopus dofleini*). Although all of the proteins in these early works have been referred to as cephalotoxin, no sequencing or other detailed molecular characterization was performed, and it is not clear that they were homologues. Indeed, Songdahl and Shapiro (1974) noted that the molecular weight of their protein (23 kDa) was very different from the protein studied by McDonald and Cottrell (30–70 kDa) and suggested that cephalotoxins may be a diverse group.

Much more recently, Ueda et al. (2008) performed the first direct protein sequencing on a compound with the neurotoxic effects characteristic of cephalotoxin. This protein, purified from the posterior salivary glands of the golden cuttlefish (*Sepia esculenta*), was named SE-cephalotoxin and is one of

only a small number of cephalopod toxins that have been purified and sequenced. At 1,052 amino acids, it is exceptionally large for a venom protein. Currently, the Tox-Prot database of over 6,000 proteins contains just 14 longer sequences and has a median length of 79 amino acids (<http://www.uniprot.org/program/Toxins>). Other features of the protein sequence are the presence of a signal peptide, pro-peptide, and multiple cysteine-rich regions, all of which are commonly observed in other venom proteins (Ueda et al. 2008). It is likely that SE-cephalotoxin is indeed a homologue of at least some of the proteins previously called cephalotoxin. It is highly glycosylated, which is consistent with observations made by Cariello and Zanetti (1977) on cephalotoxins isolated from the *O. vulgaris*.

The evolutionary origin of cephalotoxin remains unclear as very few homologues have been sequenced. SE-cephalotoxin homologous sequences have been found in *Sepia officinalis* (Cornet et al. 2014), Hawaiian bobtail squid (*Euprymna scolopes*) (Collins et al. 2012), the coral *Acropora millepora* (Ramos-Silva et al. 2013), and most recently the Australian ghost shark (*Callorhinchus milii*) (Venkatesh et al. 2014). In addition, Ruder et al. (2013) sequenced cephalotoxin homologues in the broadclub cuttlefish (*Sepia latimanus*), the pharaoh cuttlefish (*Sepia pharaonis*), and the southern reef squid (*Sepioteuthis australis*) but did not publish the assembled sequences or present a phylogenetic analysis for cephalotoxin. The existence of homologues in the coral and ghost shark genomes indicates an ancient (pre-molluscan) origin for the protein, and functional work (Collins et al. 2012) indicates that within *Euprymna scolopes*, the protein plays an important role in the organism's immune system that is possibly unrelated to its use as a venom.

Tachykinins

Tachykinins comprise a large family of highly conserved neurotransmitters found in vertebrates and invertebrates including cephalopods. They participate in both the peripheral and central nervous systems via afferent and efferent pathways, being involved in smooth muscle contraction, peripheral sensing, and neurogenic inflammation (Khawaja and Rogers 1996). Tachykinin receptor proteins (TKRP) mediate the effects of tachykinins via specific binding between each tachykinin and its associated receptor.

The first peptide toxin to be isolated and sequenced from cephalopod venom was the tachykinin eledoisin (Erspamer and Anastasi 1962), from the musky and curled octopuses (*Eledone moschata* and *Eledone cirrhosa*, respectively). This followed the discovery of substance P, a mammalian tachykinin in 1931 (von Euler and Gaddum 1931), and it was soon realized that these molecules were structurally and pharmacologically similar, being part of a large family widespread across many taxa (Khawaja and Rogers 1996). Eledoisin is present in the salivary glands of octopods from the genus *Eledone* (Anastasi and Erspamer 1963; Erspamer and Anastasi 1962). It induces hypotension and contraction of gut muscles in vertebrate (dog and guinea pig) assays (Anastasi and Erspamer 1962) but its effects on invertebrates have not been assayed.

More recent studies on *O. vulgaris* have revealed considerable additional detail about the role of tachykinins in octopods. Kanda et al. (2003) isolated two tachykinins from the posterior salivary glands of *O. vulgaris* (Oct-Tk-I, Oct-TK-II), and subsequently, Kanda et al. (2007) detected a total of seven tachykinin-related peptides in *O. vulgaris* brain tissue. The first octopod tachykinin receptor (Oct-TKRPR) was identified by Kanda et al. (2007) from heart tissue of *O. vulgaris*, and it was found that this receptor is not responsive to tachykinins expressed in salivary glands (Oct-Tk-I), but that it is responsive to tachykinin-related peptides expressed in brain tissue (Oct-TKRP's I–IV). This suggests that production of tachykinins in saliva is likely to be specifically related to venom production rather than participating in the octopus' endogenous neurologic pathways.

Tachykinins and tachykinin-related peptides are usually small (approx 10–150 amino acids) and are often produced by cleavage of a larger precursor protein. It has been noted that vertebrate tachykinins often possess a motif of the form FXGLM, whereas in invertebrates it is FXGXR. A study by Ikeda

et al. (1999) showed that the C-terminal residue within this motif (M for vertebrates, R for invertebrates) was crucial to the potency of neuromuscular effects for a tachykinin from an echiuroid worm and one of mammalian origin. In particular, they assayed invertebrate and vertebrate tachykinins against invertebrate and vertebrate assays and found that substitution of the C-terminal residue (R to M for invert tachykinin and M to R for vertebrate) could induce a switch in assay specificity from invertebrate to vertebrate and vice versa. Interestingly, tachykinins from the *O. vulgaris* posterior salivary gland and Eledoisin (found in posterior salivary glands of *Eledone*) all possess the vertebrate motif (FXGLM) despite the fact that their dominant prey items are invertebrates. Ruder et al. (2013) assayed the relative activity of three octopus tachykinins (two from *O. vulgaris* and one from *Octopus kaurna*), all of which had an FX[SG]LM motif using vertebrate and invertebrate assays. They found that all peptides elicited a response from both assays and that their relative potency was the same on invertebrate and vertebrate tissues. These results and others (e.g., Poels et al 2009) point to a deficiency in our current understanding of the differences between vertebrate and invertebrate tachykinins and their receptors, and that vertebrate vs invertebrate tachykinin specificity cannot simply be induced by analysis of the tachykinin motif amino acid sequence alone.

Chitinases, Peptidases, and Other Degradative Enzymes

Many venomous animals include degradative enzymes as components of venom and which may be classed as toxins due to their ability to cause cell or tissue damage or even neurotoxic effects (Kini 2003). These often target specific structurally important molecules whose degradation may have anticoagulant effects, leading to increased tissue permeability, cell lysis, or hemorrhage (Gutierrez and Rucavado 2000; Kini 2003; Kang et al. 2011; Wong and Belov 2012). This in turn can enhance the spread of other venom components and/or hasten the immobilization or death of the victim. It is likely that cephalopod venoms include molecules to perform these functions, but little is known about them. Transcript sequences from several major venom enzyme classes have been identified in cephalopod posterior salivary gland extracts (Fry et al. 2009; Ruder et al. 2013) including hyaluronidase (*H. maculosa*, *O. cyanea*), serine peptidases (probably ubiquitous), and phospholipase A2 (*Loliolus*, *Sepia*, *Sepiotheuthis*). In addition, whole PSG extracts of four Antarctic octopus species (*Adelieledone polymorpha*, *Megaleledone setebos*, *Pareledone aequipapillae*, and *Pareledone turqueti*) have been assayed for alkaline phosphatase, acetylcholinesterase, phospholipase A2, hemolytic, and proteolytic activities (Undheim et al. 2010) with three species showing some activity in all assays. That these degradative enzymes are important components of cephalopod venoms is suggested by the fact that they have undergone diversification (in the case of serine proteases) within the cephalopod lineage (Fry et al. 2009) and have acquired adaptations to cold temperatures within Antarctic species (Undheim et al. 2010).

Chitinases and peptidases appear to be a ubiquitous component of cephalopod posterior salivary gland extracts, being found in all species for which transcriptomic sequencing has been performed (Fry et al. 2009; Ruder et al. 2013; Cornet et al. 2014), as well as being identified in numerous other species via bioassays (Grisley and Boyle 1987; Undheim et al. 2010; Grisley 1993) or by purification and direct sequencing (Ogino et al. 2014). As components of venom, they are likely to cause considerable damage to prey, breaking down muscle or connective tissue and/or assisting with cephalopod hole boring into the exoskeletons of crustaceans. A key role for chitinases, peptidases, and other digestive enzymes in cephalopod venom is likely to be external digestion. The cephalopod body plan includes a very small mouth and a narrow esophagus that passes through the doughnut-shaped brain, thereby placing strong constraints on the size of food particles that can be ingested. External digestion is particularly well documented for octopus, where the injection of salivary secretions greatly increases the ease with which crustacean prey can be dismembered (Grisley and Boyle 1987; Nixon 1984). By devising a specific bioassay for detachment of crab muscle from carapace, Grisley and Boyle (1987) were able to attribute this activity to a proteolytic enzyme contained in milked salivary extracts from *Eledone cirrhosa*.

Amines

Amines are low molecular weight organic compounds that are ubiquitously found in biological systems. Many amines are neurotransmitters, including epinephrine, norepinephrine, dopamine, serotonin, and histamine. They are frequently encountered as components of invertebrate venoms, and, while they are often responsible for producing an acute pain response (Welsh 1964), they are not often the cause of more serious effects such as paralysis or death (Welsh 1964).

Early work on cephalopod venoms identified a substance with the ability to induce smooth muscle contractions in mammals from posterior salivary gland extracts of *O. vulgaris*. This substance was then named enteramine, but in 1952 (Erspamer 1952) it was found to be identical to serotonin, a substance that had been studied independently by another group of researchers working on mammalian blood (Whitaker-Azmitia 1999). In addition to identifying enteramine/serotonin, Erspamer (1952) also discovered octopamine from the posterior salivary gland extracts of *O. vulgaris*. The effects of octopamine in invertebrates and in vertebrates have been extensively studied (David and Coulon 1985). It acts as a neurotransmitter, neurohormone, and neuromodulator with effects on both the central and peripheral nervous systems (David and Coulon 1985). It has been shown that both octopamine and serotonin elicit a neurophysiological response from crustaceans (Livingstone et al. 1980), but because these molecules are so ubiquitous in biological systems, it is unclear whether their presence in posterior salivary glands indicates an active role in octopus venom itself.

Taxonomic Coverage and the Evolution of Toxicity in Cephalopods

The evolutionary history of toxicity in cephalopods remains relatively unknown, and in order to better understand it, two major challenges need to be overcome. The first is poor taxonomic coverage. Several taxonomic groups have received very little (e.g., cirrate octopuses [Cirrata], vampire squid [Vampyroteuthidae]) or no (e.g., argonauts, blanket octopus [Argonautoidae], bottletail squids [Sepiadariidae]) investigation. Also, relatively few species of some speciose groups have been investigated. For example, around six species from the family Sepiidae (cuttlefishes) have been investigated to date, yet the family is known to contain around 100 species. Similarly there are over 300 species known from the super family Octopodoidea, Strugnell et al. (2013), but only ~7 species have been investigated at the present time. Only a single species (*Todarodes pacificus*) from the order Oegopsida has been studied, yet at least 250 species are known to belong to the order. Therefore, we cannot be certain that the toxins identified to be present within those species studied are “representative” of the broader taxonomic group.

The second challenge is that almost no cephalopod-specific functional information exists regarding the molecular components of venoms. This means that although we may be able to identify protein variants that are specific to cephalopods, or perhaps to a particular cephalopod clade, we are limited in our ability to draw links with feeding behaviors or life history traits that would explain these evolutionary events. In the absence of cephalopod-specific functional information (e.g., studies on the specific effects of pure venom fractions), the best that can be achieved is to infer function based on sequence similarity with venom components from well-studied species such as cone snails, snakes, and arachnids. This is problematic because some families of venom proteins (e.g., PLA2, Kini 2003) include many variants that have evolved diverse and highly specific functions that cannot readily be determined from the amino acid sequence alone. Furthermore, since all venomomics studies on cephalopods to date have used posterior salivary gland extracts as a proxy for venom, it is sometimes difficult to rule out the possibility that a putative venom component is in fact merely part of the salivary gland apparatus and not present in the actual saliva produced.

Several recent studies have made progress toward addressing these challenges and have provided the first significant insights into toxin evolution in cephalopods. A study by Ueda et al. (2008) assayed posterior salivary gland extracts from three cuttlefish species (*Sepia*), three loliginid squid species (*Loligo*, *Sepioteuthis*), and one oegopsid squid species (*Todarodes pacificus*) for lethal activity against mice and crabs (*Potamon dehaani*). They found that extracts from all cuttlefish species were most potent against crabs but had no activity against mice, whereas all the loliginid squids exhibited some potency against both mice and crabs, and the oegopsid squid had no lethal activity in either assay. This finding suggests that *Sepia* and loliginid squids possess specialized toxins to suit their preferred prey, with loliginids consuming a greater proportion of vertebrates (fish) than *Sepia* whose main diet is crustaceans (Hanlon and Messenger 1996). The complete lack of lethal activity from PSG extracts of *Todarodes pacificus* is also interesting because this species is from a different taxonomic group (order Oegopsida), contained within the Decapodiformes, from the six toxic species studied (which belong to the order Myopsida and family Sepiidae). Another recent study that used assays on whole PSG extracts to demonstrate toxin evolution was that of Undheim et al. (2010). They provide evidence that some Antarctic octopods have evolved cold-adapted enzymes as part of their venom. In particular, they showed that alkaline phosphatase activity was cold adapted (higher activity at 0 °C than at 37 °C) in all species and proteolytic activity was also cold adapted in three of four species tested.

Recently, two studies (Fry et al. 2009; Ruder et al. 2013) have specifically attempted to gain insights into toxin evolution in cephalopods by applying high-throughput transcriptome sequencing to posterior salivary gland extracts across ten cephalopod species from major octopod and decapod groups. These offer the first molecular phylogenetic insights into toxin evolution in these taxa, in addition to providing a broad overview of the protein composition of cephalopod venoms. Of particular interest is the finding by Fry et al. (2009) (later expanded upon by Ruder et al. (2013)) that a large number of S1 peptidases exist in the venoms of all ten species studied. At least for *H. maculosa*, *O. kaurana*, and *S. latimanus*, these molecules are collectively distinct from non-cephalopod taxa while being present throughout the cephalopod clade (Fry et al. 2009). Fry et al. (2009) argue that at least four successive gene duplication events have occurred prior to the divergence of the decapodiform and octopodiform lineages (revised to six events by Ruder et al. 2013) and that this suggests an ancient origin of the posterior salivary gland organ in cephalopods and, by extension, its use in venom production. In addition, Ruder et al. (2013) applied site-specific selection analyses to serine proteases and pacifastins. They found that although most sites were under negative selection, 26 serine protease sites and three pacifastin sites were under positive selection, with the majority of positively selected serine protease sites likely to be at the surface of the folded protein. Since changes at the protein surface are most likely to affect receptor binding and/or enzymatic function, positive selection at these sites agrees with a model for toxin evolution in which genes are duplicated and then one of the copies takes on a role as a toxin (Wong and Belov 2012). Given that venom proteins are injected into a foreign victim, they are likely be subject to some positive selection in order to optimize efficiency against novel receptors or substrates.

New Technologies and Shifting Emphasis

Over the past 50 years, technologies for separating, assaying, and characterizing venom extracts and toxin molecules have become more sensitive and are providing increasing quantities of data. Constrained by older technologies, early studies tended to focus on a small number of abundant species (available commercially), used large volumes of starting material, and attempted to isolate a single molecule for detailed study. For example, Erspamer and Anastasi (1962) used salivary glands from 10,000 *Eledone* individuals in order to characterize the tachykinin peptide, eladoisin, and 30,000 *O. vulgaris* individuals

were used by Ersparmer and Asero (1953) to isolate enteramine. Such large quantities of material are astonishing by the standards of most modern studies where less than ten individual animals is the norm (e.g. Ruder et al. 2013; Ueda et al. 2008; Undheim et al. 2010). This trend toward the use of fewer animals is undoubtedly a positive one as it enables study of less abundant species, is significantly cheaper, and is more ethically sound.

Despite using reduced sample volumes, modern studies are able to generate vastly more data than ever due to the adoption of modern “omics” technologies. These allow whole genomes and transcriptomes to be sequenced via next-generation nucleic acid sequencing technology and for whole proteome surveys to be conducted using mass spectrometry. Collectively termed “venomics” (Escoubas and King 2009), these techniques allow data to be obtained on whole suites of molecules (e.g., whole animal or whole tissue), at low cost, from a small (milligram to gram) quantity of sample. Techniques related to venomics (indicated by arrows l, m, n, and o in Fig. 3) have so far seen very little application to cephalopod venom research. Notable exceptions are the studies by Fry et al. (2009) and Ruder et al. (2013), which performed transcriptome sequencing on posterior salivary gland (PSG) extracts of several species, and the study of Cornet et al. (2014), which performed both transcriptomic and proteomic analyses on PSG of *S. officinalis*. These studies identified numerous transcripts with homology to venom proteins observed in other taxa (snakes, arachnids, and cone snails) including CAP (CRiSP/Allergen/PR-1) proteins, carboxypeptidases, chitinases, hyaluronidases, pacifastins, phospholipase A2 proteins, SE-cephalotoxin, and serine proteases (Table 1).

Notable missing lines of inquiry from Fig. 3 include those corresponding to direct protein analysis, for example, by mass spectrometry-based proteomics, and particularly analysis of venom or pure toxic fractions. These are important because venom proteins are often posttranslationally modified (Buczek et al. 2005) and because transcripts may be expressed in venom gland tissue without necessarily being

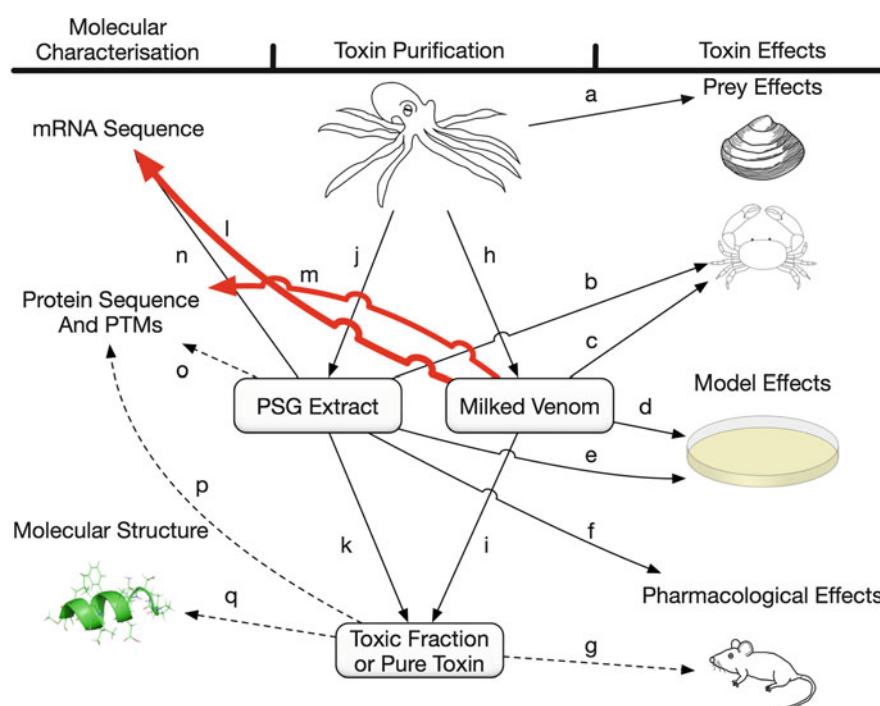


Fig. 3 Summary of research on proteinaceous cephalopod toxins to date. Toxin purification is shown in along the *center line*, with methods for measuring toxin effects shown on the *right*, and for molecular characterization on the *left*. Notable missing lines of inquiry are shown in *thick red* (no studies) or as *dashed lines* (very few studies). Full details describing each labeled arrow are given in Table 1 with example studies

Table 1 Methodologies and lines of inquiry pursued in cephalopod toxin research. Labels correspond to arrows in Fig. 3

Label	Description	Examples
a	Behavioral and morphological studies demonstrating venom use by cephalopods	Fiorito and Gherardi 1999; Kasugai et al. 2004
b, c	Injection of salivary extract or milked venom into representative prey species (as an assay)	Ghiretti 1960; Songdahl and Shapiro 1974
d, e	Model assays on milked venom or posterior salivary gland extract	Erspamer 1948; Grisley and Boyle 1987; Key et al. 2002
f, g	Investigation of potential medicinal effects of whole venom or a pure toxin isolated from venom	Karthigayan et al. 2007
h	Extraction of pure saliva via milking or dissection	Ballering et al. 1972; Ghiretti 1960
j	Dissection of posterior salivary glands and homogenization and/or extraction with solvent	Erspamer 1948; Cariello and Zanetti 1977
k, i	Isolation of pure active fraction via bioassay-guided fractionation	Anastasi and Erspamer 1962; Cariello and Zanetti 1977; Ghiretti 1959
l, m	mRNA sequencing (transcriptomics) or proteomics (via mass spectrometry) on pure cephalopod saliva	No cephalopod studies. See Corrêa-Netto et al. 2011 for an example in other taxa
n	Transcriptomic sequencing on posterior salivary gland extracts	Fry et al. 2009; Ruder et al. 2013
o	Proteomics on posterior salivary gland extracts	Cornet et al. 2014
p	Direct sequencing of pure toxic peptides or proteins	Anastasi and Erspamer 1963; Ueda et al. 2008
q	Secondary or tertiary structure determination	Grace et al. 2003

translated to protein and/or secreted into the mature venom. Posttranslational modifications can include modifications to the amino acid sequence (e.g., cleavage of pro-peptides, signal peptides, etc.) and site-specific modifications (Buczek et al. 2005; Kapono et al. 2013).

An additional issue identified by Fig. 3 is that no venomics studies have been performed on pure cephalopod saliva (as opposed to posterior salivary gland extracts). Obtaining pure saliva is clearly a technical challenge, but one that a number of studies in the past have overcome through milking (Grisley and Boyle 1987) or careful dissection of the venom ducts (Ghiretti 1960). Studies on pure saliva are important because they allow unambiguous identification of venom products without the presence of proteins related to other functions of the posterior salivary gland. Nevertheless, future studies to tackle this issue will need to employ a combined transcriptomic/proteomic strategy since protein production (and hence related mRNA) occurs in the gland and not in the saliva/venom itself. Thus, proteomics of saliva/venom combined with a database of transcript sequences obtained from a variety of body tissues including posterior salivary gland is required.

While venomics studies can identify the protein sequences of many potential toxins simultaneously, their ability to make inferences about the biological significance of these molecules is heavily reliant on homology with sequences where detailed functional studies, or bioassays, have previously been conducted. One of the few recent studies to pursue such detailed work is that of Ueda et al. (2008), who used between three and 18 specimens per species to study posterior salivary gland toxins across three cuttlefish and four squid species. This study observed toxic activity in six of seven species and was able to fully isolate and sequence one toxic protein (SE-cephalotoxin) from *Sepia esculenta*. Unfortunately, toxic proteins from squids in this study were not amenable to separation on the basis of salt concentration; however, it is clear that those proteins were potent against both mice and crabs (whereas cuttlefish venom was effective only against crabs) and would be interesting targets for future purification and sequencing efforts.

Conclusion and Future Directions

Research into the nature of toxicity in cephalopods dates back over 100 years with detailed biochemical studies dating from the 1950s. Despite this long history, our understanding of cephalopod venoms, and the toxins they contain, remains extremely rudimentary. In particular, very few examples exist where a particular molecule has been isolated as a pure (or near pure) fraction, assayed to determine its physiological effects, and then characterized to determine its structure or amino acid sequence. Notable exceptions are the tachykinin eledoisin, SE-cephalotoxin, and TTX. Toxins of octopuses, squids, and cuttlefishes show a very high potential for biodiscovery. The application of “venomics” technologies to toxin research is emerging as an important and powerful way of characterizing entire suites of proteinaceous toxins from pure venom or gland extracts in cephalopods.

Cross-References

- ▶ [A Critique of the Toxicoforan Hypothesis](#)
- ▶ [Mutation, Duplication, and More in the Evolution of Venomous Animals and Their Toxins](#)
- ▶ [Systematics of Cephalopods](#)

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