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What *Hydra* can teach us about chemical ecology – how a simple, soft organism survives in a hostile aqueous environment

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ABSTRACT ***Hydra*** **and its fellow cnidarians - sea anemones, corals and jellyfish - are simple, mostly sessile animals that depend on bioactive chemicals for survival. In this review, we briefly describe what is known about the chemical armament of** ***Hydra*****, and detail future research directions where** ***Hydra*** **can help illuminate major questions in chemical ecology, pharmacology, developmental biology and evolution. Focusing on two groups of putative toxins from** ***Hydra*** **– phospholipase A2s and proteins containing ShK and zinc metalloprotease domains, we ask: how do different venom components act together during prey paralysis? How is a venom arsenal created and how does it evolve? How is the chemical arsenal delivered to its target? To what extent does a chemical and biotic coupling exist between an organism and its environment? We propose a model whereby in** ***Hydra*** **and other cnidarians, bioactive compounds are secreted both as localized point sources (nematocyte discharges) and across extensive body surfaces, likely combining to create complex "chemical landscapes". We speculate that these cnidarian-derived chemical landscapes may affect the surrounding community on scales from microns to, in the case of coral reefs, hundreds of kilometers.**

KEY WORDS: *chemical-ecology, Hydra, phospholipase A2, ShK domain*

Introduction

Back in the wild-and-wooly days of the old American west, the Colt revolver earned the nickname "the great equalizer", because it would allow any person –as weak as he or she might be – to survive in the dangerous and hostile environment of thugs, highwaymen and Clint Eastwood. Panning from the main street of the ghost town to a crevice under a nearby desert rock, the camera of the Western movie might catch a similar drama: a slow scorpion waiting in ambush to catch its faster, more alert and physically stronger prey, or the inverse – a jackal attacking a slow desert toad but immediately dropping it and retreating in convulsions. In both cases the "great equalizer" is chemical in nature – a venom or chemical defense system.

Cnidarians, and *Hydra* in particular, are one of the most extreme cases of "the great equalizer" – they are morphologically simple, soft bodied and sessile organisms surviving in an aquatic environment infested with potential predators, competitors and pathogenic organisms. Furthermore, most cnidarians are active predators, feeding on prey such as arthropods and fish that are fast, alert and well protected. For this reason, the chemical ecology of cnidarians, and especially their toxic chemical armament, has been intensely studied for several decades. With the sequencing of its genome (and that of another cnidarian, the sea anemone *Nematostella vectensis* (Chapman *et al.*, 2010, Putnam *et al.*, 2007) and the introduction of modern genetic techniques such as gene knock-down and transgenesis, *Hydra* is poised to become an important model organism in chemical ecology. In this review, we will briefly outline what is known about the toxic chemical armament of different *Hydra* species, focusing on the venom used to catch prey and defend against predators. More importantly, we aim to describe several ways in which *Hydra* can help understand how toxins and venoms evolve, how they are deployed and how they can affect marine ecosystems.

Abbreviations used in this paper: CRISP- cystein rich secretory protein; Hmp1- Hydra metalloproteinase 1; PLA2- phospholipase A2.

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What constitutes the chemical armament of *Hydra*?

Given the long history of *Hydra* as a research model, dating back to studies by Abraham Trembley (Trembley, 1744), it is surprising how little is known about the chemical arsenal of *Hydra*, especially in comparison to other cnidarians such as sea anemones (Moran *et al*., 2009a) (see also the special issue on cnidarian toxins and venoms in *Toxicon* 54, 2009). Initial studies of *Hydra* venom focused on describing the physiological effect of the venom on model organisms such as *Drosophila*, which simulate arthropod prey, and on isolated heart muscle. When injected into *Drosophila*, *Hydra* nematocyst venom causes a rapid spastic paralysis followed by a long phase of flaccidity (Weber *et al*., 1987, recently reviewed by Sher and Zlotkin, 2009). The latter, flaccid stage might help the *Hydra* manipulate the paralyzed prey, which may be as large as the *Hydra* itself, while engulfing it. The venom also has an inotropic effect, increasing the contraction force of vertebrate myocardium (Lesh-Laurie *et al*., 1989). The venom does not have strong tissue-degrading activity, suggesting that, unlike snake venoms (Fry and Wuster, 2004, Thomas and Pough, 1979), it does not initiate the digestion process.

Preliminary chromatographic analyses of *Hydra* venom revealed the occurrence of at least two differet protein toxic fractions, one causing spastic paralysis and hemolysis and the second causing long-lasting depressant paralysis (Klug *et al*., 1989b), however neither of these has been isolated and characterized to date (Sher and Zlotkin, 2009). Recent approaches making use of the ever-increasing amount of genetic data available have revealed the occurrence of transcripts with a high level of homology to at least eight groups of previously-described toxins (Sher *et al*., 2005b, Sher and Zlotkin, 2009). Two of these, actinoporins and ShK-domain containing proteins, have been shown to be associated with nematocysts (Hwang *et al*., 2007). Actinoporins are ~20 kDa cytolytic and lethal proteins common in sea anemones, which have been intensively studied to understand protein-membrane interactions (Anderluh and Macek, 2002, Edwards *et al*., 2002), whereas the ShK domain may block K^+ channels (Rangaraju *et al*., 2010 and see below). In addition, we have recently detected using mass spectrometry putative neurotoxic phospholipase A2 proteins in the nematocyst content, in agreement with biochemical analyses of the venom (Weber *et al*., 1987) (see below). Importantly, through millions of years of evolution, most venomous organisms have come to rely on complex chemical mixtures, rather than on one or two molecules, to catch their prey or defend themselves from predators. "Simple" venom, such as that produced by the honey bee, contains around 30 types of molecules (Peiren *et al*., 2005), whereas scorpion, spider or cone snail venom can contain hundreds and even potentially as much as one thousand different components (Escoubas *et al*., 2008). Thus, it is likely that additional families of toxins, potentially with novel functions or molecular folds, await discovery.

While our understanding of *Hydra* venom is still very patchy, the emergent picture is that of a venom cocktail different from that of many other venomous organisms studied to date. Ambush predators such as sea anemones, scorpions and cone snails need to immediately paralyze their prey, and this paralysis is caused

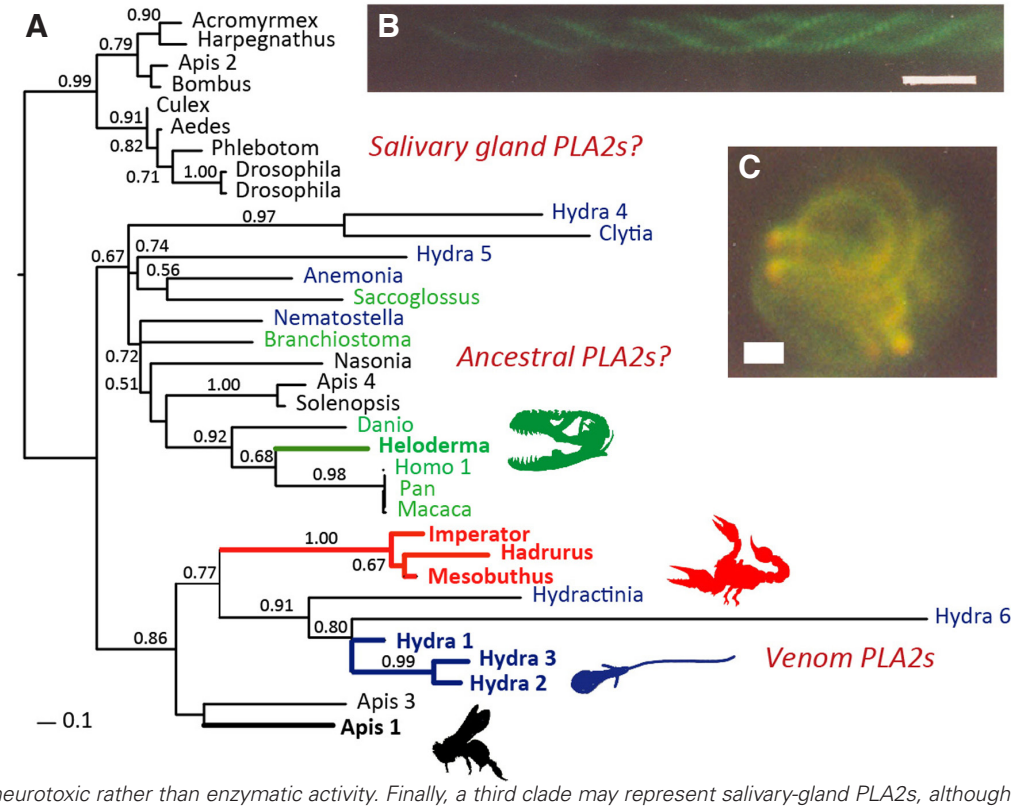

Fig. 1. Recruitment of type III phospholipase A2 proteins to animal venoms: a case of convergent evolution? (A) *A maximum likelihood phylogenetic tree is shown of the catalytic type III PLA2 domain from cnidarians, arthropods and deuterostomes (hemichordates, cephalochordates and vertebrates), with venom proteins highlighted by a thick branch, bold text and an icon of the producing organism. The organisms are color coded as follows: blue=cnidarians, red=arachnids, black=insects, green=deuterostomes. The tree was produced using PhyML, numbers above the branches denote aLRT confidence (Anisimova and Gascuel, 2006, Guindon and Gascuel, 2003). The sequences used to construct the tree can be divided into three major clades: The first contains PLA2s from a variety of organisms, and we propose that these represent ancestral PLA2s involved in arachidonic acid metabolism and through this pathway in multiple forms of endogenous signaling (Schaloske and Dennis, 2006). The second clade contains mainly known venom proteins, and we propose that these sequences may have undergone convergent evolution favoring a paralytic-neurotoxic rather than enzymatic activity. Finally, a third clade may represent salivary-gland PLA2s, although most of these sequences originate from sequenced genomes and their biological role is still unknown.* **(B,C)** *Immunohistochemical detection of a similar PLA2 on the tubules of undischarged* **(B)** *and discharged* **(C)** *nematocysts from the jellyfish* Rhopilema nomadica. *Bars: 2 μm in (B), 1 μm in (C). The images are from (Lotan* et al., *1995), reprinted by permission from Macmillan Publishers Ltd, Nature, copyright 1995.*

mainly by short (4-7kDa) peptide neurotoxins (Fry *et al.*, 2009, Moran *et al.*, 2009a, Morgenstern *et al.*, 2011, Olivera, 2002, Terlau *et al.*, 1996). Typically tens or even hundreds of such peptide neurotoxins are found in every venom cocktail, with different neurotoxins evolving to interfere with nerve and muscle activity at many different locations (e.g. pre- and post-synaptic ion channels, neurotransmitter receptors) (Fry *et al.*, 2009). From an ecological standpoint, *Hydra* belongs to this group of organisms, yet to date no genes or transcripts have been detected encoding short peptide neurotoxins (Sher *et al.*, 2005b, Sher and Zlotkin, 2009), and biochemical fractionation of the nematocyst venom supports the lack of low molecular weight (< 20 kDa) toxins (Klug *et al.*, 1989a).

How does *Hydra* paralyse its prey without using peptide neurotoxins?

Part of the neurotoxicity of the venom is likely due to the above-mentioned proteins containing ShK domains (Hwang *et al.*, 2007, Rangaraju *et al.*, 2010) as well as to neurotoxic PLA2s. Additional toxins, for example a large (>100 kDa) neurotoxin (which potentially also has cytolytic activity), are known to be found in the venom (Klug and Weber, 1991, Klug *et al.*, 1989a) but await identification and characterization. However, the total effect of the venom is likely more than a simple sum of the activities of its various molecular components, with venom components acting in synergy at various levels. At the molecular level, toxins can interact among themselves (or with non-toxic venom components) by allosterically modulating their binding to the relevant molecular target (Cohen *et al.*, 2006). At the cellular level, toxins can synergize by affecting complementary targets on the nerve or muscle fibers, for example through opening Na+ channels and blocking K+ channels which can lead to a "lightning-strike"-like tettanic paralysis (Terlau *et al.*, 1996). Finally, different components can interact at the system level, for example with tissue-disrupting components or components increasing vascular permeability enhancing the accessibility of neural tissue, or by affecting many critical systems in parallel (Gutierrez *et al.*, 2010, Wullschleger *et al.*, 2005).

Are all of the toxins needed to capture the prey, or are there "core" toxins critical to venom activity? What is the role of non-paralyzing proteins (e.g. some types of hemolysins)? It is here that *Hydra* as a model organism has a unique power to help unravel the complexity of venom and dissect the role of specific venom components. This can be done through experimentally manipulating the venom composition by knocking down the expression of specific toxin genes (e.g. Chera *et al.*, 2006, Lohmann *et al.*, 1999 and others).

How did the chemical armament of *Hydra* evolve?

Venoms from vastly different organisms – cnidarians, arthropods, snails and snakes - have similar molecular components, yet it is highly unlikely that the last common ancestor of all metazoans was venomous (Fry *et al.*, 2009). How does a venomous organism "assemble its arsenal" (Fry and Wuster, 2004)? What are the basic molecular building blocks of venom, and where do they originate from? The current view of venom evolution suggests that toxins are often born when a gene encoding a protein involved in some aspect of the normal physiology of an organism is recruited and expressed in the venom gland (Fry *et al.*, 2009, Fry and Wuster, 2004). Such toxin genes are often replicated, for example by unequal crossover and recombination (Moran and Gurevitz, 2006, Moran *et al.*, 2009b, Moran *et al.*, 2008), to form multigene families. The various members of these genes can then either follow different evolutionary trajectories, often diversifying to form "combinatorial libraries" (Conticello *et al.*, 2001, Olivera, 2002, Sollod *et al.*, 2005), or conversely they can undergo "concerted evolution" which resists diversification and may facilitate high expression levels of important, functionally conserved, toxins (Moran *et al.*, 2008).

Type III secretory phospholipase A2s (PLA2s)

PLA2s are one of the first cnidarians venom proteins unequivocally shown to be injected by the nematocysts (Fig. 1 B,C; Lotan *et al.*, 1995; Lotan *et al.*, 1996), and are a good example of a protein scaffold that has been recruited multiple times into venom systems (Fry *et al.*, 2009). Using tandem mass spectrometry, we

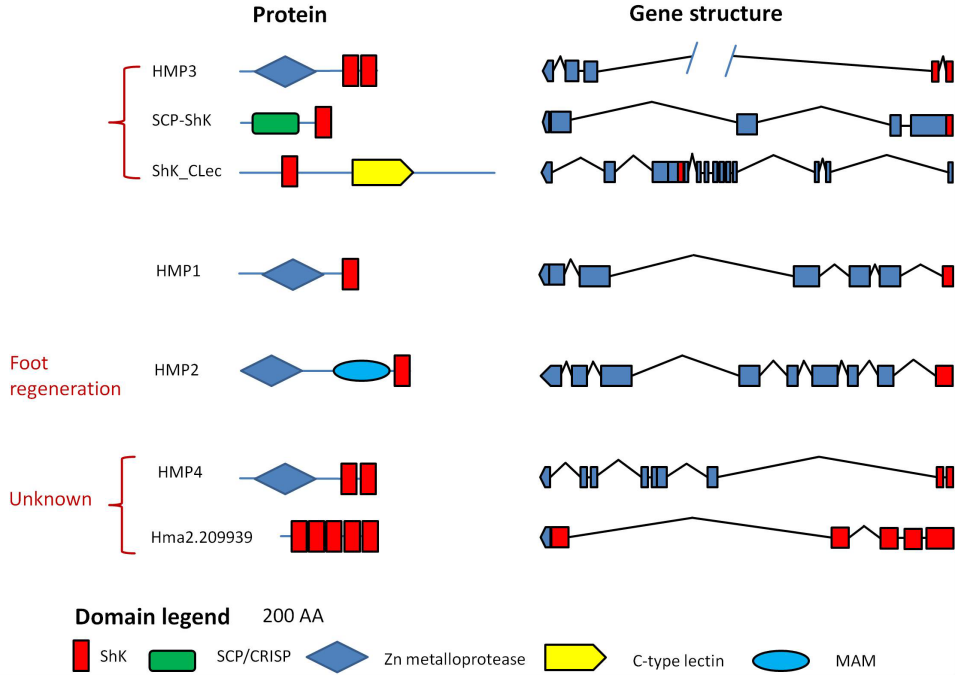

Fig. 2. ShK toxin-like domains: recurring features in venom-related and other proteins. *Schematic illustrations are shown of ShK-domain containing proteins and of their corresponding gene structures. The proteins are all drawn to the same scale, but the gene structures are not. The full length of the ShK-CLec protein is 1004 amino acids. The break in the gene model of HMP3 represents an unsequenced region of the genomic contig. Red exons are those encoding ShK domains. The gene model accession numbers in the* Hydra *genome are as follows: HMP3-Hma2.228615, SCP-ShK-Hma2.204028, ShK-CLec - Hma2.206417, HMP1 - Hma2.217198, HMP2-Hma2.214401, HMP4-Hma2.228188.*

have detected three different type III PLA2 proteins in extracts from *Hydra magnipapillata* nematocysts. These PLA2s are similar, on the one hand, to toxins found in bee, lizard and jellyfish venoms (Lotan *et al*., 1995, Lotan *et al*., 1996, Sher *et al*., 2005b), and on the other hand to mammalian PLA2s expressed in kidney, heart, liver and muscles (Valentin *et al*., 2000) (Fig 1A). In addition to the nematocyst PLA2s, the *Hydra* genome contains another three putative genes containing similar PLA2 domains, whose role is currently unclear (Fig 1A). Interestingly, the *Hydra* nematocyst PLA2s are more similar, in terms of their sequence, to venom PLA2s from scorpions and bees than the other PLA2 genes in the *Hydra* genome, or to type III PLA2 genes from other cnidarians such the hydrozoan *Clytia hemispaerica* and the sea anemones *Nematostella vectensis* and *Anemonia viridis* (Fig. 1A). Future studies are needed in order to determine whether the sequence similarity between venom PLA2s from different organisms represents convergent evolution of proteins originating from a similar scaffold towards a neurotoxic-paralytic activity rather than purely an enzymatic one.

Toxins with ShK-like domains

A second putative toxin, detected by Hwang and co-workers (Hwang *et al*., 2007) as a nematocyst-specific transcript, encodes a protein, which has a ShK-like domain (Fig. 2). ShK is a member of a group of peptide anemone toxins that block K^+ channels. When found in the context of a larger protein, the ShK domain may still bind and block K^+ channels, and as a non-venom, "endogenous" protein has been suggested to regulate the density of K^+ channels on the plasma membrane by trapping the channels in the endoplasmic reticulum (Rangaraju *et al*., 2010). The protein detected by Hwang and co-workers contains, in addition to a single ShK domain, also a second cysteine-rich domain (SCP or CRISP domain). A similar domain is found in many proteins potentially involved in pathogenesis, although its role is not clear. Additionally, more distant SCP/CRISP domains are found in several toxins from wasps, lizards, snakes and cone snails (Hwang *et al*., 2007, Sher *et al*., 2005b). In addition to this putative toxin, we have detected another three proteins in the nematocyst extract containing one or more ShK-like domains. Two of these proteins contain a zinc metalloprotease domain, and the third contains, in addition to a non-canonical ShK domain, also a C-type lectin domain (Fig. 2). Both these domains (Zn metalloproteases and C-type lectins) are common in snake venoms (Fry *et al*., 2009, Fry and Wuster, 2004). These proteins are good candidates for new families of cnidarian neurotoxins, and we are currently attempting to recombinantly express them and test whether they have the anticipated paralytic activity.

In all of these cases, the ShK domains are encoded by a separate exon, and at least 20 additional exons encoding this motif are found in the *Hydra* genome (Fig 2). These ShK-domain encoding exons may provide a "venom recruitment shortcut", as secreted proteins into which these exons are integrated will, most likely, bind K^+ channels found on excitable tissue – the main target of neurotoxins. A similar mechanism, where a K^+ channel toxin "leads" a PLA2 to nerve terminals, focusing the PLA2 enzymatic membrane-deforming activity on excitable tissue, has been suggested to underlie the potency of the snake toxin -bungarotoxin (Montecucco and Rossetto, 2000, Rowan, 2001).

Hydra metalloproteinase 1 (HMP1)

Finally, one of the ShK-domain-containing proteins we have detected in *Hydra* nematocyst extracts, *Hydra* Metalloproteinase 1 (HMP1), has previously been shown to be involved in head development (Yan *et al*., 1995; Yan *et al*., 2000), also see in this issue (Sarras, 2012). Future work is needed to both test whether HMP1 exhibits some form of toxic activity and to reconcile our observation of this protein in nematocyst extracts with its immunohistochemical localization to the extracellular matrix and endodermal cells in previous studies (Yan *et al*., 2000, Yan *et al*., 1995). However, if indeed HMP1 is a *bona-fide* venom protein, it may represent an early stage in the recruitment of proteins to venom, where the same protein fulfills both the ancestral, developmental, role and a new role in prey paralysis.

How does *Hydra* deliver toxins to its target?

Like all cnidarians, *Hydra* have evolved in their stinging cells, an amazing apparatus named nematocyst, to deliver their toxic chemistry into the target organism. These "high-tech cellular weaponry" systems (Tardent, 1995) are in essence sub-cellular nano-syringes, capable of explosively punching into the prey cuticle at accelerations of up to one million g (Holstein and Tardent, 1984, Nuchter *et al*., 2006), see in this issue the review by Beckmann and Ozbek (2012). *Hydra* produce four nematocyte types, each of which is postulated to have a different biological role: large stenoteles and smaller desmonemes are organized in batteries and are involved in prey capture; holotrichous isorhizae are distributed all over the *Hydra*'s body, and are used for defence of the *Hydra* against predators, and atrichous isorhizae are not used at all against target organisms; instead, these adherent nematocytes are used for locomotion (Ewer, 1947, reviewed in Sher and Zlotkin, 2009). Previous studies have suggested that two of these nematocyst types, stenoteles and holotrichous isorhizae, contain hemolytic proteins (Klug *et al*., 1989a), and recently it has been shown that each of these nematocysts contains a different actinoporin-like toxin (Hwang *et al*., 2007). Thus, it is likely that different nematocysts contain different toxins, and that *Hydra* in fact may have two venoms rather than one – one for catching prey and one for predator deterrence, each delivered by different nematocytes. *Hydra* now provide an excellent platform with which to study the adaptation of venom to specific ecological roles, although efficient methods must first be developed to separate nematocysts into different types to enable functional analyses of the venom (e.g. Wiebring *et al*., 2010).

The fascinating details on how nematocysts are built, how they function and how they are integrated into the nerve network of cnidarians are reviewed elsewhere (Tardent, 1995; Kass-Simon and Scappaticci 2002; David *et al.,* 2008; Beckmann and Ozbek, 2012). As the developmental processes controlling the assembly of these complex secretory structures are slowly coming to light, it will be interesting to determine when and how the toxins are incorporated into the nematocyst (when and how the "gun is loaded"). Additionally, toxins (like any other protein) have a limited "shelf life", yet nematocysts can be stored intact in the cnidarian tissue and ready to fire, for extensive periods of time, presumably without the venom losing significant activity. Does *Hydra* possess specific

molecular mechanisms to retain the activity of the "loaded" venom? Are there quality control mechanisms monitoring the venom to make sure its components are appropriately folded? Answers to such questions may, beyond their scientific value, also have important biotechnological applications.

Can the chemical armament of a cnidarian affect the environment at different scales?

So far, we have discussed *Hydra* venom assuming it is delivered by the nematocysts as a "point source", locally injected into the target organism. However, several studies suggest that nematocysts-derived compounds act as "feeding hormones", initiating the feeding response (Burnett *et al.*, 1963), and inhibiting the discharge of additional nematocysts once the *Hydra* is satiated (Ruch and Cook, 1984). Since *Hydra* are often found in nature as dense patches (Elliott *et al.*, 1997), such signalling may also occur between individuals, facilitating group responses, for example to a dense patch of zooplankton prey. Furthermore, if these venom components can be sensed by *Hydra*'s prey, these could act as "predator-released kairomones", causing the prey to change behaviour and potentially avoid predation (Blaustein *et al.*, 2010, Fainzilber *et al.*, 1994).

From a wider perspective, the chemical arsenal of *Hydra* is much wider and more complex than the nematocyst-derived venom. *Hydra* produce a multitude of potentially bioactive compounds, including hemolysins likely involved in digestion (Sher *et al.*, 2008, Sher *et al.*, 2005a, Zhang *et al.*, 2003), other potential pore-forming proteins involved in development and immunity (Amimoto *et al.*, 2006, Miller *et al.*, 2007, Sher and Zlotkin, 2009), neuropeptides and signaling peptides (Bosch and Fujisawa, 2001, Bottger *et al.*, 2006, Takahashi *et al.*, 1997), see in this issue (Fujisawa and Kayakawa, 2012; Pierobon, 2012) and several antimicrobial peptides (Augustin *et al.*, 2009, Fraune *et al.*, 2010). These proteins and peptides are produced by different body regions or cells, and several are secreted either into the gastrovascular cavity (Sher *et al.*, 2008), into the developing oocyte or onto the ectodermal surface (Fraune *et al.*, 2010). Importantly, when a signal is secreted from one location and diffuses or is transported to another, gradients are formed, and these gradients can convey information, for example during development and morphogenesis (Bosch and Fujisawa, 2001). Such gradients can mediate localized interactions between *Hydra* and its associated microbiota, for example by limiting bacterial load or modulating the composition of the microbial population in specific body regions (Fraune *et al.*, 2010, Fraune and Bosch, 2007). We therefore propose that bioactive chemicals secreted both as "point sources" (e.g. discharged nematocysts) and across larger epithelial regions form concentration gradients and combine to create complex "chemical landscapes" (Fig. 3). These chemical landscapes form the micron-scale environments in which *Hydra* and its associated microbiota interact.

Finally, how far does the "chemical sphere of influence" of *Hydra* – and other cnidarians – extend? To what extent are the microenvironments of *Hydra* coupled to the ecosystem at larger scales? The usefulness of tiny *Hydra* to study such large-scale questions may seem questionable at first; however, as predators in aquatic environments, the potential of *Hydra* to affect prey densities and zooplankton population structure has already been suggested (Elliott *et al.*, 1997, Link and Keen, 1995). Furthermore, *Hydra* harbour specific microbial communities, which differ between species and likely also differ from that of the surrounding water (Fraune and Bosch, 2007). As benthic animals, *Hydra* can therefore serve as "microbial refuges", and when they detach and become planktonic (Elliott *et al.*, 1997, Reisa, 1973), their associated microbiota may inoculate new regions of the water body, mediating bacterial dispersion (Grossart *et al.*, 2010). Finally, other cnidarians such as soft corals have been shown to release sufficient bioactive compounds into the water to reach concentrations which potentially affect surrounding microbes or the settlement of larvae of competing species (Krug, 2006). Through these three mechanisms – selective predation, microbial coupling and direct chemical influence -, *Hydra* can potentially have far reaching ef-

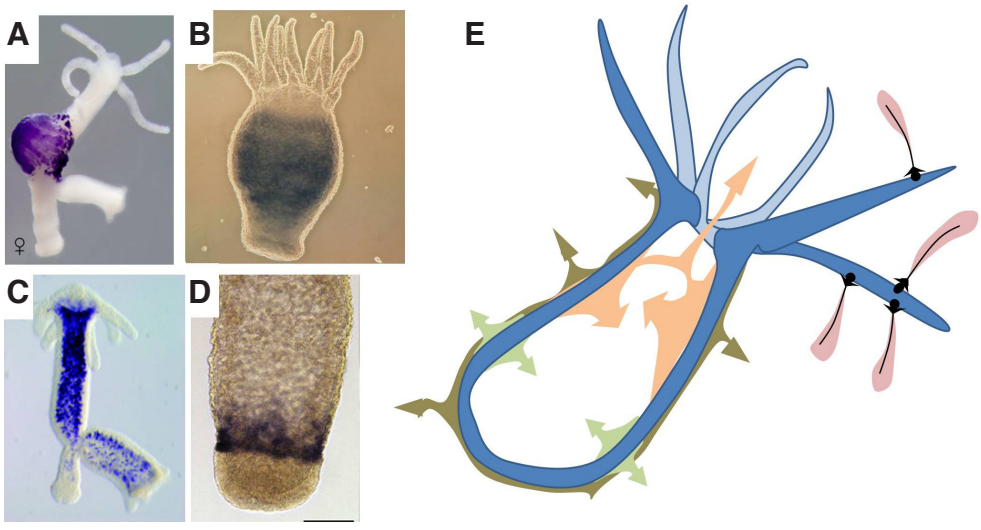

Fig. 3. Schematic representation of the "chemical landscape" produced by secreted bioactive proteins in Hydra. (A-D) *Examples of genes encoding antimicrobial and cytolytic peptides and proteins expressed in different regions of* Hydra. **(A)** Periculin 1A, *an antimicrobial peptide which modifies the microbial community, is expressed in the female germline of adult* Hydra vulgaris, *as well as in developing embryos (not shown).* **(B)** Hydralysins *expressed in the endodermal digestive cells of* Hydra viridissima, *encode paralytic and cytolytic pore-forming proteins that are secreted upon feeding into the gastrovascular cavity.* **(C)** Hy-MAC *encodes a protein containing a MAC-PF pore-forming domain, it is expressed in gland cells throughout the body of* Hydra magnipapillata. **(D)** *Anklet also contains a MAC-PF domain,* Anklet *is expressed at the border of the peduncle and the basal disk in* PalmatoHydra robusta, *and is involved in basal disk formation and maintenance.* **(E)** *Multiple bioactive compounds, secreted both as point sources (nematocysts) and across large body regions, combine to form a chemical landscape in which* Hydra*-microbe interactions occur. A is from (Fraune* et al.*, 2010), B is from (Sher* et al.*, 2008), C is from (Miller* et al.*, 2007) and D is from (Amimoto* et al.*, 2006) reproduced with permission from the Proc. Natl. Acad. Sci. USA, FASEB Journal, Genome Biology and Elsevier, respectively.*

fects on their surrounding ecosystems.

Cnidarians are dominant predators (and prey) in many aquatic ecosystems, and are famous for forming the largest biogenic structures on Earth – coral reefs – which are currently threatened (Pandolfi *et al.*, 2005). Characterizing the chemical landscape of cnidarians and how it affects freshwater and marine ecosystems will require an interdisciplinary effort by ecologists, molecular and developmental biologists, biochemists, physicists, mathematicians and oceanographers or limnologists. As an experimentally tractable cnidarian, *Hydra* is uniquely poised to answer many of the questions on how these sessile, simple invertebrates survive in and affect their hostile aqueous environment.

Acknowledgments

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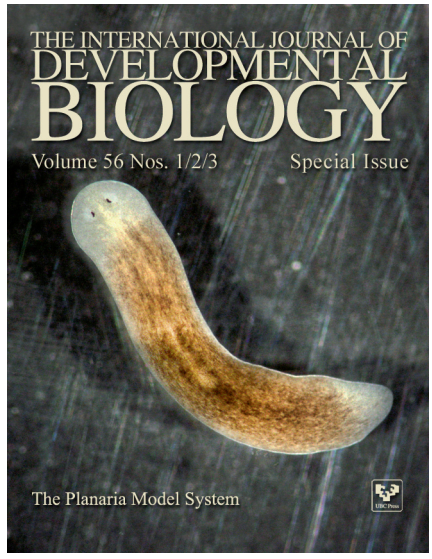

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