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Chapter

CONE SNAIL BIOLOGY, BIOPROSPECTING AND CONSERVATION

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ABSTRACT

Cone snails are predatory marine gastropods that prey on worms, molluscs and fish. The venoms of these animals are true pharmacological treasures, and with the recent approval of the first cone snail venom-derived drug, the pressure on the resource is set to increase. While habitat loss and over-collecting for the shell trade are the major threat to cone snail diversity, every effort should be made to preserve this unique pharmacopoeia, including reducing to a minimum the number of specimens collected for scientific purposes. To this end, we show how recent improvements in sensitivity, miniaturisation of equipment, high throughput screens and novel technologies can help deliver valuable scientific and economic outcomes from small quantities of these limited samples.

INTRODUCTION

Natural products, such as those isolated from plants, animals and microbes, have been used to treat human diseases since the dawn of medicine (Harvey 2007). Nowadays, natural products or their derivatives still represent ~ 50 % of all approved drugs (Harvey 2008). This figure should not come as a surprise, since millions of years of evolution have often shaped these molecules into privileged structures that can be effective even via the oral route (Newman & Cragg 2009). While plant extracts remain the major source of novel bioactives, marine organisms have also been actively evaluated for a range of therapeutically useful properties, including anticancer and analgesic activities (Molinski *et al.* 2009).

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Our knowledge of marine biodiversity is likely underestimated, especially regarding deep water forms of life because access to the resource is very restricted, rendering the sampling effort difficult and expensive (Richardson & Poloczanska 2008). Yet, the remarkable hit rates of marine compounds in screening for drug leads have maintained the interest of pharmaceutical companies (Newman & Cragg 2004). Therefore, the major hurdle to the development of an active marine compound into a drug remains the procurement or manufacturing of large quantities in a sustainable way. Many of the marine organisms of interest are difficult to breed or grow *in vitro*, implying that the only source of the compound is from wild harvest. While it is quite challenging to evaluate the full extent of marine bioprospecting, concerns over the overexploitation of endemic species or small local populations have emerged (Hunt & Vincent 2006).

Recently, the approval of the first marine-derived drug for the treatment of intractable pain has further stimulated drug discovery programs from marine organisms (Miljanich 2004). This molecule, a short peptide called ω -MVIIA or Ziconotide and marketed as Prialt, was originally discovered in the venom of the magician cone snail, *Conus magus*, as a potent blocker of N-type calcium channels (Olivera *et al.* 1985). Several other “conopeptides” targeting ion channels, transporters and receptors are in various stages of clinical trials for the treatment of pain, but also myocardial infarction, epilepsy and neurodegenerative diseases (Lewis 2009, Lewis & Garcia 2003). As a result, cone snail venoms are regarded as pharmacological treasures (Wang & Chi 2004).

Cone snail venoms hold great promise for the discovery of new therapeutic leads, but unravelling their complex pharmacology and isolating minor individual active components from the thousands present in a single venom is challenging and typically requires significant amount of starting material. Historically, cone snails have been harvested from the wild, their venom gland dissected, the venom extracted from the venom duct, and the venom peptides tested in animals, isolated tissue preparations or a range of bioassays (Olivera *et al.* 1984). When more material is necessary to allow minor components to be characterised, further snails are collected, with tens to hundreds of cone snails sometimes necessary to obtain a single molecule of interest (Wang *et al.* 2009). This practise has been questioned as to whether it was sustainable and ethically acceptable (Chivian *et al.* 2003). Yet, a collective reply from researchers corrected that most world leading groups working with cone snail venoms are limited to 15-20 specimens per species per year (Duda *et al.* 2004). This number is dwarfed by the hundreds of thousands of snails harvested for the shell trade around the globe each year. Research efforts into the discovery of new drugs are certainly more valuable than ornamentation, with outcomes such as drugs that can directly benefit us. To allow access to the components from species that are small or more difficult to collect, most researchers are committed to further reduce the number of animals harvested from the wild using miniaturised approaches such as high throughput screening or next generation sequencing to accelerate discovery. In this chapter, we summarize the current knowledge on cone snail biology, and report on the recent improvements in various technologies that can produce high scientific and economic outcomes with minimal environmental impact.

CONE SNAIL BIOLOGY

Taxonomy

Conus is the type genus of the Conidae, which was validly established as a family by John Fleming in 1822 (Rockel *et al.* 1995). The Conidae together with the Turridae and Terebridae form the Superfamily Conoidea, also known as the Conacea or Toxoglossa (Rockel *et al.* 1995). As the largest genus of marine invertebrates, *Conus* arguably presents the most challenging taxonomy and nomenclature, with many new species described every year (Kohn 1980). Recently, a revised classification of the recent and fossil Conoidean gastropods has been published, based on radular morphology as well as other factors such as dietary habits and periostracum morphology (Tucker & Tenorio 2009).

Habitat

Generally, the genus *Conus* occurs throughout all tropical and subtropical oceans but is most diverse in the Indo-West Pacific region (Rockel *et al.* 1995). The few species found beyond the 40° N or S parallel are localized in South Africa, Southern Australia, Southern Japan and Mediterranean Sea. These marine snails are found mostly in coral reef areas, usually in shallow waters, under coral shelves, hiding in the sand, or under piles of rocks or rubble (Rockel *et al.* 1995). The density and diversity of cone snails decrease dramatically when the percentage of live corals is higher than 20 % (Kohn 1983). A maximum density of 40 individuals per square meter can be attained but they are usually much less abundant (Kohn 2001). There are also some cone snails that live among mangroves, and a reasonable number live offshore or in the deep waters up to 400 metres.

Reproductive Biology

Reproduction in cone snails has not been widely studied, but it appears that most have separate sexes and are fertilized internally (Rockel *et al.* 1995). Eggs are laid once a year and attached to substrate in capsules, with each capsule containing a varying number of eggs. Typically, egg masses are made of up to 25 egg capsules, and each capsule may contain up to 1,000 eggs. Therefore, each egg mass may contain about 25,000 eggs (Zehra & Perveen 1991). Two types of offspring or hatchlings have been described, the veliger (free-swimming larvae) and veliconcha (juvenile snails) stages. These early development stages are critical, and a large number will not survive beyond the first few days after hatching. The pelagic stage occurs between 1 and 50 days (Perron 1983).

In the life cycle of the genus *Conus*, larvae from the planktotrophic stage must feed on specific forms of plankton before settling and metamorphosing in the benthic environment. In a study of *C. pennaceus*, it was shown that over 99.93 % egg mortality occurs during this stage (Perron 1979). This critical larval stage has hampered most attempts to breed cone snails in captivity. The major hurdle is to find the correct food for each stage of development.

For instance, veliger larvae only feed on certain specific types of phytoplankton. After metamorphosis, the tiny cones shift to a diet that may be very different compared to adults or larvae. Indeed, piscivorous cones were shown to produce a “vermivorous-like” radula in animals with a shell length < 10 mm, implying that young cone snails may feed on worms rather than fishes (Nybakken 1990). One study has reported the successful breeding of *Conus* (*C. textile*), and the conditions found to be essential include: flowing sea water, presence of a biological film for metamorphosis and an appropriate food source (Perron 1980). The life span of a cone snail is estimated to be 10-20 years in the wild as well as in captivity, based on shell growth rate and marks. They can reach a maximum size of > 20 cm, but most species are < 8 cm and weight < 100 g (Rockel et al. 1995).

Behaviour

The adult cone snail hides under rocks or buries itself in sand during the day. Cone snails are usually solitary, but some species can be found in great numbers in particular areas, mainly due to their specialised habitats (i.e. a microhabitat) (Kohn 2001). At night, cone snails become active, leave their retreat and search for prey. Cone snails are highly specialised predators with some species feeding exclusively on worms (70%), molluscs (15%) or fishes (15%) (Rockel et al. 1995) (figure 1). Prey is detected using chemosensor organs such as the osphradium. The snail crawls towards it, extends its proboscis, and upon contact with any soft part of the body fires a barbed harpoon-like modified radula into the prey. The venom is injected through this hollowed tooth and envenomation proceeds rapidly, usually followed by paralysis. A few species will engulf the prey before stinging, including the deadly *C. geographus*.

In the presence of a threat such as a potential predator, a different behaviour can be described. Firstly, the cone snail retreats into its shell, which represents an inviolable fortress for many predators. If the threat remains or intensifies, and while still hidden under its shell, the cone snail will extend its proboscis and wave it around, trying to sting the predator. Human accidents involving *Conus* stings are a consequence of this defensive behaviour (Olivera & Cruz 2001).

Among the 700 species of cone snails, only *Conus geographus* has been confirmed as responsible for several human deaths (Kohn 1958). It is estimated that more than 55% of stings from *C. geographus* may be fatal to humans, but such encounters are fortunately rare (Fegan & Andresen 1997). Over 30 human fatalities from *C. geographus* stings have been recorded in the medical literature (Alan J. Kohn, personal communication). Symptoms from a cone snail sting vary depending on the species, with extreme pain or a spreading numbness followed by paralysis often reported (McIntosh & Jones 2001). In general, the venoms of fish-hunting species are more lethal to vertebrates and most of the vermivorous and molluscivorous species are considered harmless to humans (Kohn *et al.* 1960). Cone snails are not aggressive by nature, and most accidents occur when a snail is picked up by divers and pressed against a wetsuit, or when collectors attempt to clean the shell of a live animal.



Figure 1. Live cone snails in their environment. Cone snails are marine predators that prey on worms, mollusks and fish. Upper panel shows two piscivorous species, *Conus geographus* (left) and *Conus striatus* (right). Middle section illustrates two molluscivorous species, namely *Conus marmoreus* (left) and *Conus textile* (right). Bottom panel shows two vermivorous species, *Conus coccineus* (left) and *Conus vitulinus* (right). Photos from Thierry Vulliet.

Venom Apparatus

Cone snails have evolved a specialised venom apparatus to subdue their prey. It is comprised of a venom gland, salivary glands (and accessory salivary glands), a radular sac, a pharynx, a proboscis and a radula (Marsh 1977) (figure 2). A cone snail's radula acts as a delivery system, and is hollowed and barbed to resemble a miniature harpoon up to 10 mm long (Kohn *et al.* 1972). These harpoons are produced and stored in a specialised organ, the radular sac, which is divided into two arms and connects to the pharynx (Marsh 1977). The short arm of the radular sac contains a limited number of mature, fully formed radula, while the long arm is the site of production, where radula in different stages of synthesis can be found. The total number of radula present in a radular sac is species-dependant, and seems to

vary according to the feeding habits (e.g. molluscivorous snails, which can inject venom multiple times into their prey, produce larger number of harpoons, see table 1). While the cone snail is hunting, a single radula is loaded from the short arm of the radular sac to the tip of the long and extendable proboscis (Salisbury *et al.* 2010). When the snail senses a prey it will extend its proboscis and fire the harpoon via a powerful muscular contraction, injecting a potent venom (Schulz *et al.* 2004). The average attack lasts only milliseconds and the prey is usually paralysed within a second.

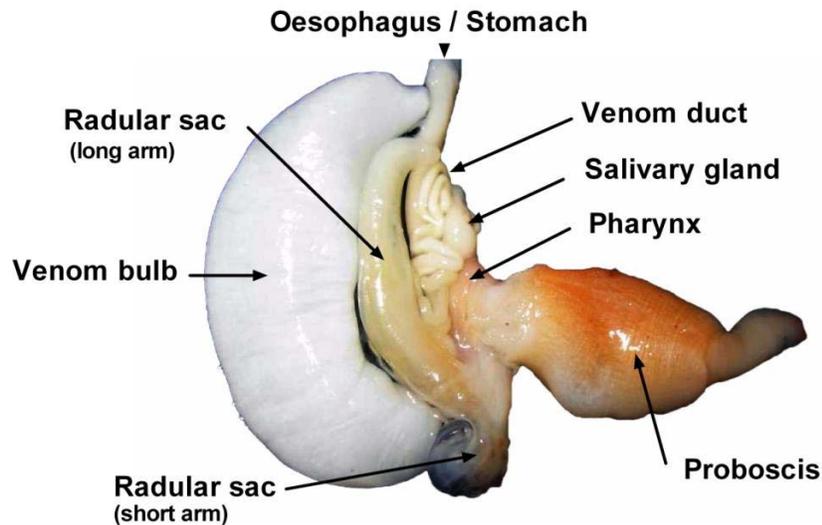


Figure 2. The venom apparatus of cone snails. This panel illustrates the dissected venom apparatus of *Conus striatus*.

Table 1. Number of radulas stored in the radular sac per species.

Species	Diet	Short arm	Long arm	Total
<i>C. miles</i>	V	1	18	19
<i>C. striatus</i>	P	6	34	40
<i>C. geographus</i>	P	23	27	50
<i>C. marmoreus</i>	M	13	61	74
<i>C. textile</i>	M	29	46	75

V, vermivorous; P, piscivorous; M, molluscivorous.

Conotoxins and various enzymes are produced in a long, convoluted duct connected to the pharynx (distal end) next to the radular sac, and is linked to a muscular bulb at the other extremity (proximal end). Some toxins are only expressed in particular regions of the venom duct, as demonstrated using molecular biology and mass spectrometry methods (Garrett *et al.* 2005, Marshall *et al.* 2002). It is interesting to note here that comparison of the venom contents extracted from the duct and the milked venom (ejected from the radula) from the same animal revealed significant differences (Biass *et al.* 2009, Jakubowski *et al.* 2005). Indeed, while a number of compounds were common to both venoms, up to 50 % of the masses detected in the injected venom were unique. Therefore, other organs such as the

salivary glands may participate in the elaboration of the injected venom, as demonstrated for *Conus pulicarius* (Biggs *et al.* 2008).

Venom Pharmacology

The venom of cone snails has evolved to rapidly subdue their prey. As a result, the biological effects seen following the injection of venom into the prey are often reminiscent of muscle paralysis and complete nervous system shut down. If the venom is fractionated and each fraction injected into the brain of a mouse, then a multitude of pharmacological actions are observed (Olivera *et al.* 1990). Indeed, the venom of cone snails is made up of over a thousand small disulfide rich peptides, as well as numerous proteins, amino acids and small molecules (Davis *et al.* 2009). Each of these conotoxins potentially targets an ion channels or other key membrane receptors with exquisite potency and selectivity; the challenge is to find its target (Dutertre & Lewis 2010). An impressive number of pharmacological targets for conotoxins have already been identified, but more are likely to be revealed in the future, ever expanding the use and applications of these peptides (see table 2).

Table 2. Pharmacology of Conus peptides.

Class	Mode of action	example
ω -conotoxin	Ca _v 2.2 inhibitor	MVIIA
μ -conotoxin	Na _v inhibitor	SIIIA
μ O-conotoxin	Na _v 1.8 inhibitor	MrVIB
δ -conotoxin	Na _v enhancer	EVIA
κ -conotoxin	K _v inhibitor	PVIIA
χ -conopeptide	NET inhibitor	Xen2174
α -conotoxin	nAChR inhibitor	Vc1.1
σ -conotoxin	5HT ₃ R antagonist	GVIIIA
ρ -conopeptide	α_1 -adrenoceptor inhibitor	TIA
Conantokin	NMDAR antagonist	conantokin-G
Conopressin	Vasopressin agonist	conopressin-G
Contulakin	neurotensinR agonist	contulakin-G

BIO-PROSPECTING

Bioprospecting is broadly defined as the exploration of biodiversity for commercial and/or scientific purposes to generate valuable genetic and biochemical resources (Arico & Salpin 2005). Clearly, the primary goal of such activity is often to find novel molecules to cure human diseases. Plants, microbes and marine invertebrates are currently the main focus of academic and industrial discovery programs (Harvey 2008). Indeed, more than half of our modern medicines are or derived from natural chemical compounds provided by nature (Harvey 2007). Among the most famous natural compounds is the antibiotic penicillin which was derived from a type of mould. More recently, the cancer drug taxol isolated from the bark of the yew tree has found important uses in the management of cancer.

Venoms are now subject to more intense investigation. While a number of traditional medications derived from the venom of snakes, spiders and frogs have shown promise over the years (Harvey 2002), the relative infancy of venom peptide bioprospecting is mainly due to the difficulty in obtaining the material, and to the nature of the active components of venoms, which comprise mainly small proteins and peptides. Indeed, proteinaceous molecules have long been regarded as poor drug candidates, due to their poor bioavailability and short half-life in biological fluids such as blood and serum. Peptides typically have greater selectivity and affinity compared to small molecules, providing better efficacy and safety, and potentially fewer side effects. In addition, novel chemical strategies have greatly improved the stability and oral-bioavailability of peptides (Clark *et al.* 2005, Clark *et al.* 2010, Muttenthaler *et al.* 2010). As a result, we are now observing a surge of interest in peptide-based drugs from pharmaceutical companies.

Conservative estimates suggest each venomous animal may contain between 50 and 200 active components in their venom (Escoubas *et al.* 2006), although recent reports on cone snail venoms indicated numbers at least an order of magnitude higher (Biass *et al.* 2009, Davis *et al.* 2009). There are more than 40,000 species of spiders and 10,000 species of venomous marine snails, indicating that natural libraries of millions of bioactive compounds can be generously produced by these animals. It is estimated that for the cone snails alone, less than 2% of the biodiversity has been uncovered, and less than 1% has been pharmacologically characterised (Kaas *et al.* 2008). With so many potential compounds and organisms to choose from, how should scientists proceed with their bioprospecting efforts? It mostly depends on the target of interest, the equipment and assays available to each laboratory. Naturally, the largest, the most abundant, and medically important species were initially studied. Early work aimed at understanding the lethality of *Conus geographus*, the only species of cone snail responsible for human deaths (Olivera *et al.* 1999). Other large species such as *C. textile*, *C. striatus*, and *C. marmoreus* have also been intensively investigated. Yet, small, rare or inoffensive species may be just as interesting and promising as the common or larger species. Unfortunately, many of the rarer species are also of great interest to collectors, and scientists rarely get a chance to study them. Collectors are only interested in the shell, and discard the animal inside, while scientists often break the shell to access the tissues of interest. A better communication and cooperation between scientists and collectors may actually be rewarding to both parties.

From a recent study on all conotoxin sequences deposited in the online database Conoserver, it appears that toxins extracted from species of the Atlantic regions or South African region and/or belonging to clades IX, X and XV are underrepresented, whereas others have not yet been studied at all (e.g., clade IV) (Kaas *et al.* 2008). Therefore, research efforts targeted at these species are likely to uncover novel molecules of interest. Understandably, deep water species, as well as endemic species to remote places have largely eluded bioprospecting efforts. Novel techniques of collecting, in close association with local populations, are likely to provide interesting specimens from this untapped biodiversity (Serony *et al.* 2009).

CONSERVATION

Scientific investigations have so far mostly focused on common species of cone snails, and therefore the impact of collecting for scientific purposes has remained limited. By far the most serious threat to cone snail diversity is the destruction of their habitat, which in most cases consists of fragile reef ecosystems (Rockel et al. 1995). Coral reefs around the world are threatened by pollution, destructive fishing practices, coastal developments and mass tourism, not to mention acidification and warming of oceans due to climate change. The collection of large number of shells for the ornamentation trade may also contribute to the decline of some species. However, it should also be mentioned here that none of the 700 species of cone snails is listed on CITES (Convention on International Trade in Endangered Species). Nonetheless, concerns about overharvesting some cone snail species for research purposes have emerged, with estimates of “hundreds of thousands” sacrificed annually (Chivian et al. 2003). While there is no statistical study on the extent of cone snail harvesting, these numbers were largely overestimated (Duda et al. 2004). Nowadays, the number of specimens collected for research in most laboratories ranges from tens to even one single animal per species annually. Indeed, thanks to continuous improvements in sensitivity and miniaturisation of analytical methods and high throughput screening assays, small samples from a unique specimen can nowadays yield large amount of data (see figure 3). A brief overview of some of the strategies that can reduce the number wild-collected animals for research is given below.

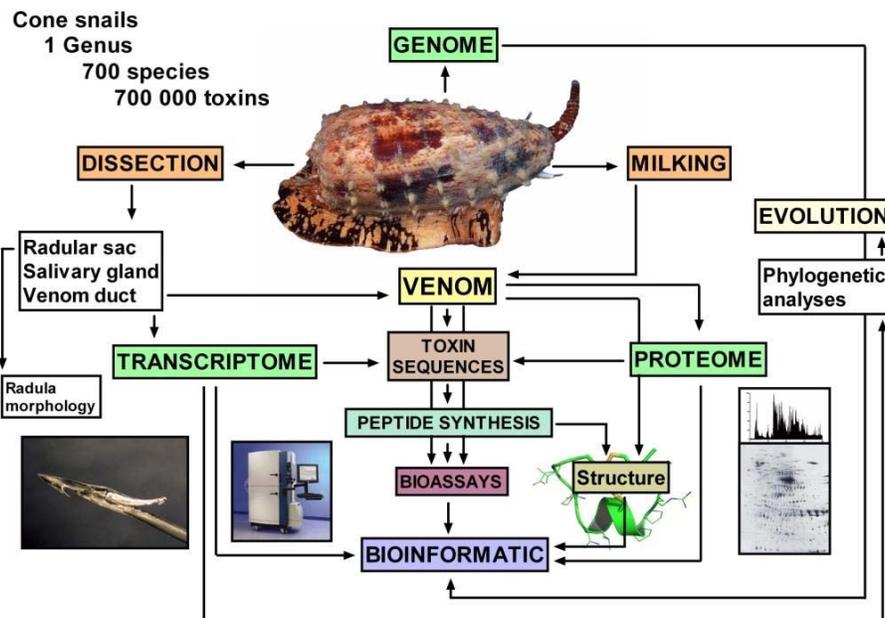


Figure 3. Current strategies to maximize scientific outcomes from a unique specimen. Here we illustrate how a single specimen of cone snail can generate a large amount of data, including genomic, transcriptomic and proteomic data.

High Throughput Screening (HTS) Assays

A range of HTS platforms are already available (such as fluorescence resonance energy transfer or homogeneous time resolved fluorescence), permitting the screening of thousands of samples per day. The miniaturization of HTS assays is an important objective for the pharmaceutical industry as well as for fundamental science (Houston & Banks 1997). Miniaturisation is one way to access and evaluate a greater diversity of compounds from very limited samples such as natural extracts. One critical step is the reduction of the volume in which the assay is performed so that the concentration of the active compounds reaches levels high enough to produce a detectable response. Currently, typical assay volumes in 96 well plates are in the range of 50–500 μl with a medium throughput potential of about 20,000 assays per day. Assay volumes can be reduced to 10 μl using 384 well plates, increasing the throughput to 50,000 assays per day. The development of even higher throughput in 1536 well format should allow assay volumes to be reduced to less than 5 μl , potentially opening further novel discovery opportunities.

Mass Spectrometry

Mass spectrometry has become the method of choice to study the complexity of venoms (Escoubas 2006). In particular, soft ionisation technologies such as matrix-assisted laser desorption-ionization (MALDI) and electrospray (ESI) are heavily utilised to unravel the composition of these proteinaceous mixtures (Davis *et al.* 2009, Escoubas *et al.* 2008). While venoms can be studied as a whole sample (mass fingerprinting, profiling), they are usually pre-fractionated for better resolution, diminution of the ion suppression effect and higher coverage. A liquid chromatography step can be carried out off-line (MALDI) or online (ESI), each method providing high quality yet complementary data sets (Biass *et al.* 2009). Recent improvements in sensitivity (dynamic range) and accuracy of mass spectrometers are allowing high throughput analysis from minute samples (Nilsson *et al.* 2010). For instance, using a combined Orbitrap-ETD with a targeted chemical derivatization strategy, the full sequences of 31 peptide toxins could be obtained from just 7% of the crude venom of a single *Conus textile* (Ueberheide *et al.* 2009).

Transcriptomics

The Human Genome Project, with the unprecedented throughput requirement for DNA sequencing, has fuelled the development of novel technologies that can benefit many different research projects, including those dealing with non-model organisms such as cone snails (Metzker 2010). The so-called “next-generation sequencing” technologies, as opposed to the traditional Sanger sequencing, have delivered on the promise of sequencing DNA at unprecedented speed, thereby enabling impressive scientific achievements and novel biological applications (Morozova *et al.* 2009). While several different platforms are available, the 454-pyrosequencing technology (Roche) provides the longest reads (300–350 bp on average), ensuring a more accurate assembly of contigs in the absence of a reference genome (Droege & Hill 2008, Margulies *et al.* 2005). Next generation sequencing

technologies are not limited to genome projects, and are particularly relevant to venom-based discovery projects. Transcriptome analysis of venom gland transcripts have the potential to reveal all toxin sequences in one single experiment (Hu *et al.* 2011). Unfortunately, the high prevalence of post-translational modifications in cone snails venom peptides preclude a direct discovery process from transcriptomic data alone, and proteomic data integration is required.

Sustainable Venom Production

Dissection and tissue extraction have been the methods of choice for cone snail venom collection for decades. The procedure consists of collecting tens or hundreds of cone snails, snap freezing them and extracting the venom gland. This simple procedure is still highly successful, leading to the discovery of a number of interesting compounds from cone snail venoms, including the marketed pain killer (Ziconotide) (Olivera 2006). However, venom can be obtained from live cone snails in a similar way that snakes and spiders are "milked" for their venom to manufacture the life-saving anti-venom and to provide the scientific community with "natural libraries of compounds" (Hopkins *et al.* 1995). While this is a tedious procedure and only amenable to certain species, it provides many advantages. Milked venoms are free of cellular debris and contain few traces of unprocessed toxins and degradation products when compared to dissected venoms. In addition, milked venoms have a much higher solubility compared to dissected venoms. The milked venom from piscivorous cone snails is highly soluble as opposed to the thick paste obtained after venom duct dissection. This unique property of milked venom appears well-suited to biological screening assays and should facilitate the discovery of novel pharmacological tools and drug leads (Bingham *et al.* 2009). Some conotoxins are abundantly expressed in the milked venom, and in some cases, direct HPLC purification may provide sufficient material for structural studies. Furthermore, venom composition can be compared between individuals using mass spectrometry, and can be followed over time to reveal dramatic and unexpected intraspecimen variations (Dutertre *et al.* 2010). Finally, unique and original molecules are found only in the milked venoms. Indeed, other organs may participate in the elaboration of the injected venom, such as the salivary glands or the radular sac (Biggs *et al.* 2008, Marshall *et al.* 2002). The specific molecules produced in these organs are obviously absent in the venom obtained from the dissection of the venom duct only. Further work is clearly needed to discover how to milk more cone snail species and to better understand the specific role of each organ in venom production. Given milking is a sustainable collection method that requires only small number of individual per species, and it avoids sacrificing the animals, these efforts may be well rewarded.

CONCLUSIONS AND PERSPECTIVES

In the near future, marine bioprospecting efforts will likely focus not only on natural extracts from ocean plants, animals, and microbes, but also on the genetic information stored in the genomes and transcriptomes of these organisms. Thanks to the Human Genome Project, the expertise and technology assembled now benefits sequencing of non-human

genomes. The genome and venom gland transcriptome of one species of *Conus* has been published already (Hu et al. 2011) and more can be expected in the future. Coupled to high throughput mass spectrometry and miniaturised bioassays, these integrated approaches will likely increase the rate of discovery from this valuable resource.

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