

Complex cocktails: the evolutionary novelty of venoms

Nicholas R. Casewell^{1,2}, Wolfgang Wüster¹, Freek J. Vonk³, Robert A. Harrison², and Bryan G. Fry⁴

¹ Molecular Ecology and Evolution Group, School of Biological Sciences, Bangor University, Bangor, LL57 2UW, UK

² Alistair Reid Venom Research Unit, Liverpool School of Tropical Medicine, Liverpool, L3 5QA, UK

³ Naturalis Biodiversity Center, Darwinweg 2, 2333CR, Leiden, The Netherlands

⁴ Venom Evolution Laboratory, School of Biological Sciences, University of Queensland, Qld 4072, Australia

Venoms have evolved on numerous occasions throughout the animal kingdom. These ‘biochemical weapon systems’ typically function to facilitate, or protect the producing animal from, predation. Most venomous animals remain unstudied despite venoms providing model systems for investigating predator–prey interactions, molecular evolution, functional convergence, and novel targets for pharmaceutical discovery. Through advances in ‘omic’ technologies, venom composition data have recently become available for several venomous lineages, revealing considerable complexity in the processes responsible for generating the genetic and functional diversity observed in many venoms. Here, we review these recent advances and highlight the ecological and evolutionary novelty of venom systems.

Venom in the animal kingdom

Venomous animals have been the subject of public fascination throughout human history, in large part due to the inherent danger associated with them, and the apparent incongruity between the small and often fragile-looking animal and the devastating damage it can inflict. Indeed, snakes, as the most widespread and most frequently lethal venomous animals encountered by humans, might have played a prominent part in the evolution of the primate brain and sensory systems [1]. Venoms offer interesting and often unique insights into several disparate biological fields, including pharmacology (drug discovery) [2], immunology (therapies for envenoming) [3,4], and structural biology (protein binding and interaction) [5]. Underpinning research in these areas is the ecology and evolution of the venomous animals and their venoms. Venom systems provide unparalleled models for investigating interactions between predators and prey, the influence of natural selection, and extreme cases of molecular evolution and protein neofunctionalization. Here, we review the evolutionary novelty of venoms and critically appraise their importance as models for investigating evolutionary processes in the animal kingdom.

Venom can be broadly defined as ‘a secretion, produced in a specialised gland in one animal and delivered to a target animal through the infliction of a wound “regardless

of how tiny it could be”, which contains molecules that disrupt normal physiological or biochemical processes so as to facilitate feeding or defense by the producing animal’ [6,7]. Venom serves multiple functions in the animal kingdom: most commonly as a foraging adaptation among trophically venomous taxa (e.g., most venomous mammals, snakes, some lizards, spiders, scorpions, centipedes, some insects, cephalopods, gastropods, and cnidarians), as a defensive adaptation in others (e.g., helodermatid lizards, most venomous fishes, echinoderms, lepidopteran larvae, and other insects), and potentially for intraspecific conflict (e.g., platypus). Predatory venom systems have also been proposed for extinct taxa, such as the theropod dinosaur *Sinornithosaurus* [8] and the pantolestid mammal *Bisonalveus browni* [9]. This taxonomic diversity highlights the importance of venom as an evolutionary innovation in that venomous animals are found across the animal kingdom (Figure 1). Consequently, a wide range of innovative structures have evolved to facilitate the delivery of venoms, including barbs, beaks, fangs or modified teeth, harpoons, nematocysts, pincers, proboscises, spines, sprays, spurs, and stingers [6,10,11].

Most animal venoms are highly complex cocktails of bioactive compounds. Venoms typically comprise a mixture of protein and peptides (commonly referred to as toxins), salts and organic components, such as amino acids and neurotransmitters [6,7,12–14]. The proteinaceous components are usually the most abundant. The composition and targeting of venom seemingly reflects its function, with defensive venoms, such as those from fishes or bees, being streamlined and highly conserved, with the primary action often being immediate, extreme localized pain [15–17]. By contrast, predatory venoms are more complex and often highly variable in composition and physiological effects [6]. This complexity creates the potential for variation in venom composition, which occurs at all levels in taxa where this has been researched. Such diversity can result in extreme variation in venom toxicity and mode of action between closely related taxa [18], populations of a single species [19,20], sex-related differences in siblings [21], and ontogenetic variations in the lifetime of an individual [22]. Venom diversity can also have severe consequences for the efficacy of human antivenoms that are designed to neutralize venom-induced pathology (Box 1). The causal factors underlying venom composition

Corresponding author: Casewell, N.R. (n.casewell@bangor.ac.uk).

Keywords: venom; evolution; selection; convergence; gene duplication; antivenom; drug discovery.

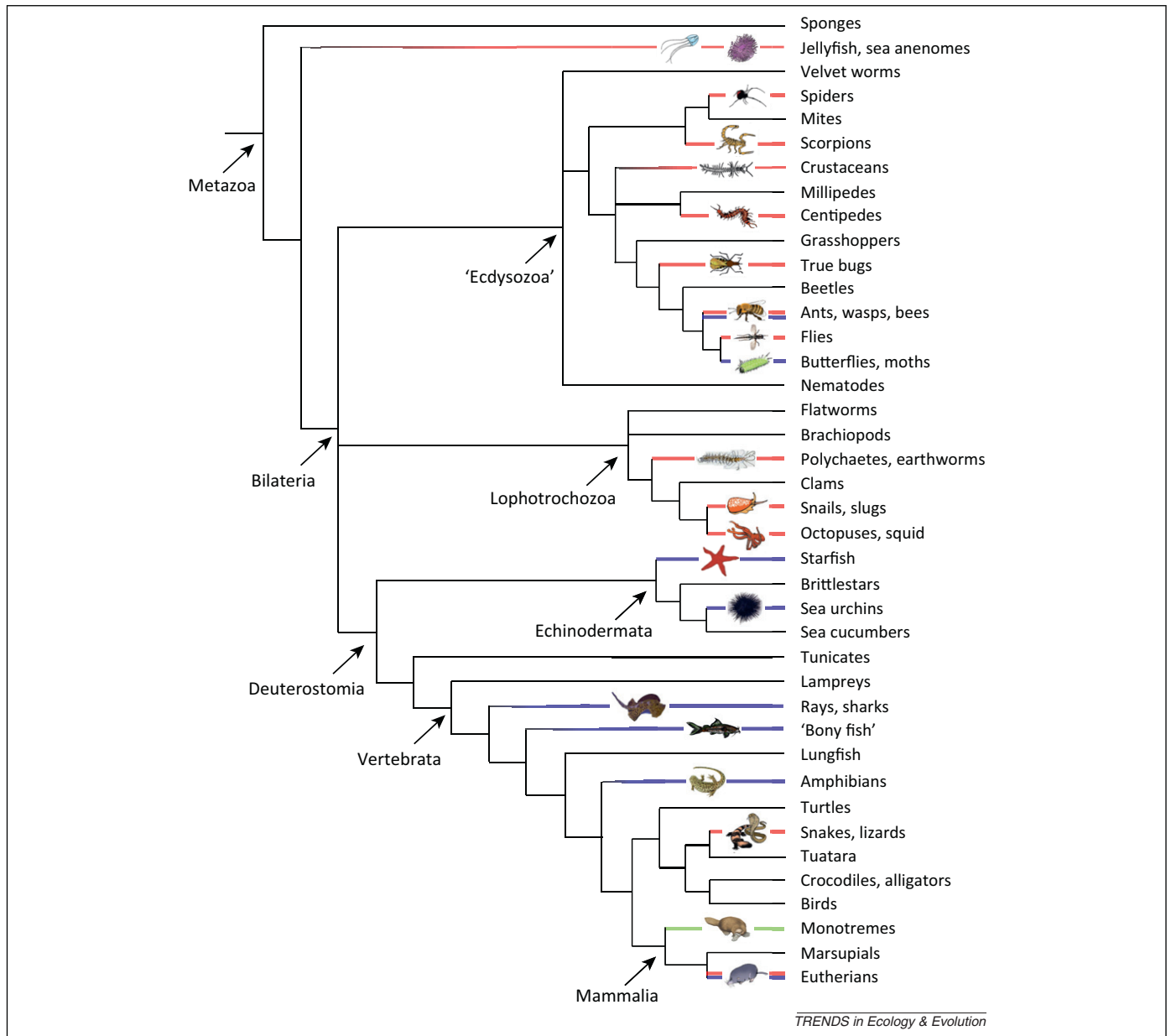


Figure 1. Schematic tree of venomous life in the animal kingdom. The tree demonstrates the evolutionary relation between animal lineages and highlights the frequency with which venom systems are found in the animal kingdom. Colored branches highlight major animal lineages that include members with venom systems. Red branches indicate a predatory role for venom, blue a defensive role, and green a role in intraspecific competition. The phylogeny is based on the tree of life presented in Pennisi [81]. Note that several animal lineages have been pruned from the tree to facilitate presentation.

and variation therein have been active fields of research in the recent literature.

Selection pressures and venom evolution

Diet and venom evolution in snakes

Considering the primary function of most venoms is prey capture, natural selection on venom composition is a likely consequence. Its role in driving the evolution of venom composition has been studied most extensively in snakes, but has long remained contentious [23–25]. Although no evidence of adaptation in venom composition was found in some snakes [26], evidence from other studies suggests that venom composition is often adaptive. For example, patterns of venom variation in the Malayan pit viper (*Calloselasma rhodostoma*) were demonstrated to correlate significantly with variation in the diet of the species.

This was interpreted as reflecting natural selection for feeding on local prey [23], but the functional consequences of this variation remained unknown, leaving the question of its adaptive value unanswered [24,25].

The extreme lethality of snake venoms to laboratory model organisms has been interpreted as evidence against selection on venom composition [25]. The so-called 'overkill' hypothesis proposed that selection for venom potency is unlikely because the amount of venom injected into prey items is often greater than 100 times the lethal dose required [25]. However, this overlooks the fact that laboratory organisms might not reflect the response of natural prey to venom: specific resistance to snake venoms has evolved among both natural prey [27,28] and predators of snakes [29], presumably as a result of natural selection from snake predation, and can be extreme [27]. This

highlights the importance of testing the effect of venom on natural prey species rather than on convenient model organisms in artificial conditions [30,31].

In addition to venom-resistant natural prey, an additional evolutionary challenge for snakes is that venom synthesis appears to carry an appreciable metabolic cost, resulting in elevated metabolic rates after venom extraction [32], although the importance of this in the overall energy budget of the organism requires further research [33]. Moreover, at least some snakes display behavioral adaptations to optimize venom expenditure: in several rattlesnakes, the amount of venom injected has been found to correlate with the size of the prey, indicating a level of control over the use of this metabolically expensive resource [34]. The combined evidence of metabolic cost and behavioral adaptations to limit venom expenditure indicates that the energetic cost of venom might be an important constraint on its synthesis and use.

The combination of venom expense and resistance to venom among some prey leads to the expectation of intense natural selection for the optimization of venom to prey. Individual variation in venom composition has been shown to lead to differential venom effectiveness against different prey [35] and, thus, to potential differences in individual fitness, an essential precondition for natural selection. Accordingly, several studies of phylogenetically diverse snake lineages have detected increased prey-specific lethality to natural prey types, suggesting adaptive evolution [30,36,37]. Saw-scaled vipers (*Echis* spp.) also show evidence that venom economy, not kill speed, is the driving force behind this adaptation [30].

Evidence of adaptive evolution extends from overall toxicity to individual toxins, with prey-specific toxins, such as the bird-specific denmotoxin (GenBank: [DQ366293](#)) isolated from the mangrove snake (*Boiga dendrophila*), exhibiting increases in potency to specific prey types [38]. Finally, evidence that prey-specific venom toxicity undergoes ontogenetic change associated with dietary

shifts provides additional support for the adaptive hypothesis [22].

Diet and venom evolution in other animals

Although the relation between venom target and composition has been most comprehensively studied in snakes, other venomous organisms display many parallels suggestive of a more general evolutionary pattern. Venom synthesis has been shown to be metabolically costly in scorpions [39], and both these and spiders have evolved additional physiological and behavioral mechanisms to optimize energy expenditure associated with venom use, specifically venom metering and pre-venom. Venom metering in some spiders appears to be more sophisticated than in snakes, being dependent less on *a priori* decisions based on prey size than on the response of the prey: more venom is injected where the intensity and/or duration of prey movement is increased [40] or where dangerous prey are encountered [41]. Fascinatingly, scorpions have evolved a metabolically inexpensive, pain-inducing pre-venom [13], which appears to be utilized readily in defense, particularly in low threat encounters [42]. Once exhausted, or in high threat situations, scorpions inject the more energetically expensive, protein-rich main venom [42]. Thus, scorpions also appear to regulate venom expenditure during stinging.

Venom economy appears to be a major selective force in all taxa studied, and acts as a constraint that impedes the strategy of secreting larger quantities of a conserved venom to overcome prey resistance. Evidence for the alternative strategy of prey-specific venom has been observed in several other higher taxa. Among cone snails, associations between patterns of prey and toxin diversity [43–45], and the coevolution of specific toxin types and major prey classes [46], indicate a prominent role of natural selection for diet in venom evolution. Similarly, prey-specific venom lethality was also demonstrated in spiders with specialized diets [47]. In summary, most of the evidence points to

Box 1. Antivenom therapies: limitations and novel evolutionary approaches

Irrespective of ecological function, a wide range of venomous animals use their venoms in self-defense, and it is in this context that humans experience the effects of these secretions. Where human envenoming occurs frequently (e.g., venomous snakes account for 1.8 million envenomings, at least 94 000 deaths, and many thousands more suffering morbidity annually worldwide [82]) antivenoms, consisting of antibodies purified from the blood of horses or sheep hyperimmunized with venom, are produced to neutralize the injected venom. Although life saving, the efficacy of antivenom is typically restricted to the snake species whose venom was used in manufacture. Current venom-immunization protocols make no attempt to direct antibody specificities to the most pathogenic venom proteins and do not take into account the variant immunogenicity of different venom components. Consequently, conventional antivenoms contain numerous antibodies to weak or nontoxins that dilute the effectiveness of the toxin-specific antibodies, and often have low antibody levels to some pathogenic venom toxins. The result of this undirected production method is the need for large volumes of antivenom to effect cure (20–200 ml), which significantly increases the risk of patients suffering antivenom-induced adverse effects.

The application of new 'omic' techniques to elucidate the venom composition of medically important species presents a timely opportunity to develop antivenoms with better toxin specificity to improve clinical efficacy and safety [3,4]. Novel approaches are

now being applied to expand the therapeutic potential of antivenoms; for example, 'antivenomic' techniques [4] seek to identify venom toxins isolated from other medically important snakes (i.e., species not used in conventional antivenom manufacturing) that fail to bind to the antivenom, which are then used to supplement the venom immunization mixture. An alternative gene-based approach to developing toxin-specific antivenom has also been pioneered [83,84], which utilizes gene sequence data to distinguish venom toxins from nontoxins [85,86] and bioinformatic tools to identify regions (epitopes) common to related toxins (i.e., encoded by the same gene family) that are likely to induce high levels of antibody production (Figure 1). These epitopes are then linked to create an 'epitope-string', which, when used for immunization, stimulates the production of multiple, toxin-specific antibodies capable of neutralizing venom-induced pathology [3,87]. Applying this approach to the venoms of multiple species in a defined geographic area offers the potential to generate a single antivenom to neutralize venom pathology in all snakebite victims, irrespective of the biting species. This progression increasingly incorporates evolutionary analyses in the design of the epitope-string immunogens, including phylogenetics of polyspecies venom toxins to identify the presence and absence of gene homologs within each toxin family and the epitope sequences most conserved across the isoforms detected (Figure 1).

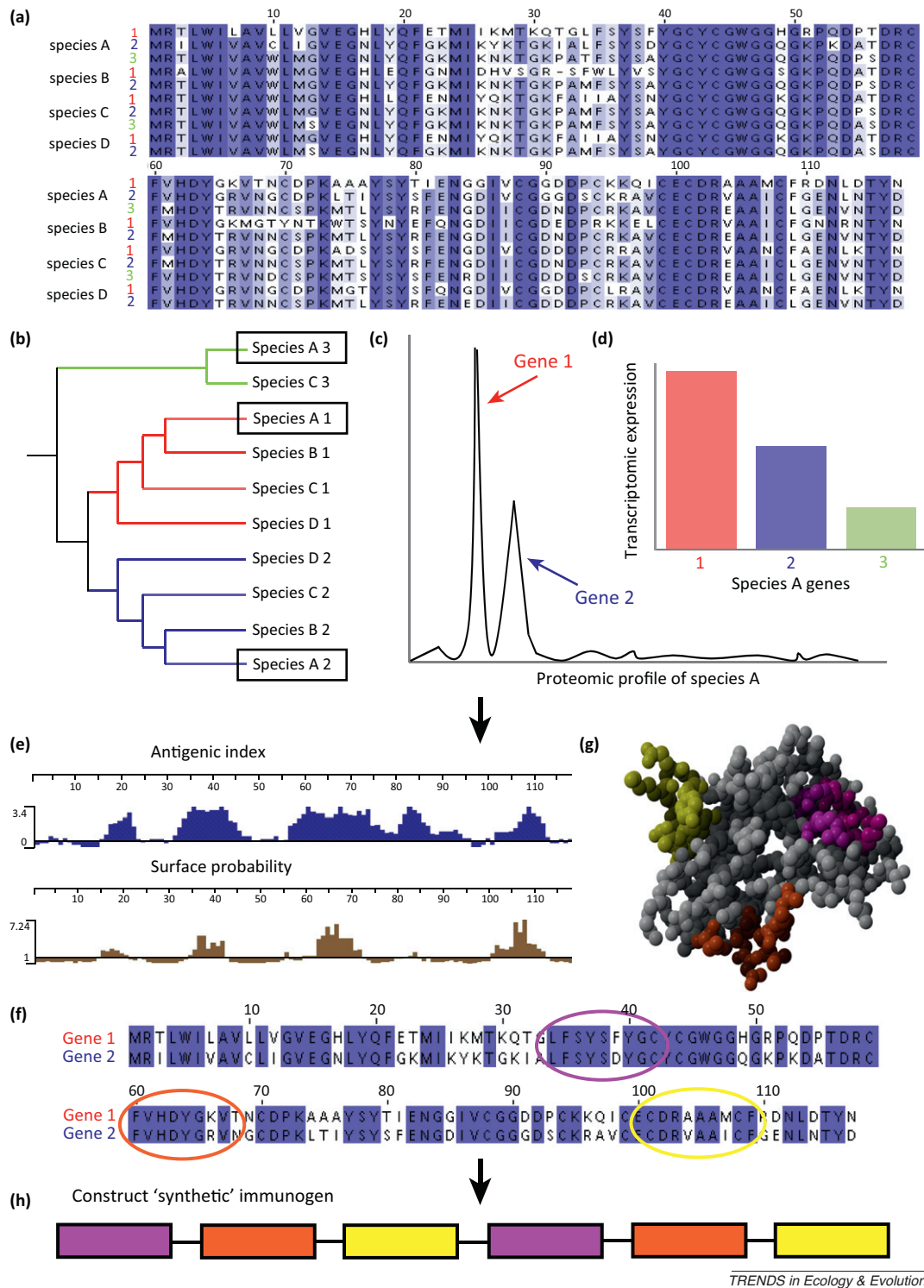


Figure 1. Schematic workflow to design 'epitope-string' immunogens for generating toxin-specific antivenom. Gene sequences of a group of pathologically important toxins expressed in venoms from related venomous species (in this case, four species A–D) are aligned (**a**). Phylogenetic analysis of the toxin family reveals the evolutionary history of the genes (**b**). Underpinned with venom gland transcriptomic data, proteomic analysis identifies the proteins expressed in venom of the snakes of interest (in this case species A) (**c**). Comparisons of venom gland transcriptome expression reveal the relative importance of the respective genes (**d**). In this case, gene 3 from species A is removed from downstream analysis because it exhibits low expression in the venom gland (**d**) and is not identified as secreted in venom (**c**). Selected venom toxin gene sequences are subjected to multiple bioinformatic tools to identify domains of the selected genes predicted to stimulate a high immune response (antigenic index) and that are surface exposed (surface probability) (**e**). Target 'epitopes' are selected from the sequence alignment based on these characteristics (**f**) and mapped to a template macromolecular structure to confirm that the epitopes are located on the surface of the protein (**g**). Selected epitopes are then linked together to prepare a single synthetic immunogen that is capable of generating multiple antibody specificities (**h**). This 'anti-toxin' serotherapy is therefore capable of targeting multiple distinct sites of the targeted venom toxin group [87]. The intent is to develop an antivenom comprising 'antitoxins' to each group of pathogenic venom proteins expressed in venoms of all the most medically important snakes in a defined geographic region [3].

wards natural selection for diet as the major driver of the evolution of venom composition across trophically venomous groups.

Other selective forces

The effects of selective pressures for functions other than foraging have not been studied in depth. Defense is a common secondary function of venom in many taxa in which foraging is its primary function, and is sometimes associated with defense-specific morphological and behavioral adaptations [48]. However, there is currently little evidence for defense-related selective pressures on venom composition in any taxon, and understanding of these pressures and their role in venom evolution remains poor.

In multiple snake lineages, parallel evolutionary shifts to undefended prey (e.g., eggs) or to constriction as the principal means of prey subjugation have been accompanied by atrophy of the venom apparatus and degeneration of toxin genes [7,49], suggesting that foraging is the principal selective force acting on venom and the retention of the venom apparatus.

Several hypotheses could explain the lack of evidence for the action of defensive selection pressures on venom. If defensively venomous animal–predator encounters occur infrequently, then selection is likely to be relaxed and venom components would not be expected to exhibit the same tempo of evolution identified in predatory venomous species. Alternatively, where predators are taxonomically and physiologically diverse, the evolution of specific anti-predator adaptations in venom would be difficult. This might account for the seeming incongruity between the frequent evolution of venom-associated defensive morphological structures in many taxa on the one hand, and the lack of obvious predator-specific defensive adaptations in venom composition in trophically venomous taxa or the conserved composition of venom in defensively venomous groups on the other hand. Additional studies of the role of venom in interactions between venomous animals and their predators are urgently needed to reveal the selective pressures required to maintain chemical defense systems in ecological communities.

Selection on venom and prey: evolutionary arms races

Although natural selection alone is unlikely to be responsible for generating variation in venom composition, the accumulated evidence suggests that natural selection for diet is often a potent driver of venom evolution in trophically venomous animals. This is potentiated by the dual factors of evolving resistance to venom in some prey and the metabolic expense of producing venom. The emerging picture is thus one of an evolutionary arms race [50], where evolving venom resistance in prey and the evolution of novel venom composition exert reciprocal selective pressures on each other, as encapsulated in the Red Queen hypothesis of Van Valen [51]. By contrast, in cases where venom is used for defense, the relatively meager evidence currently available suggests lower levels of adaptive evolution (consistent with rarity of use and/or predator diversity), but this remains a neglected area of research. These observations provide a fascinating basis to investigate the role and consequences of natural selection in response to

the frequency of interactions between predators and prey, and the multiple replicate origins of venom in animals provide an unparalleled opportunity to detect general evolutionary patterns.

Molecular evolution

Gene duplication

Venom systems provide unrivalled opportunities to investigate the interrelations between natural selection and the genetic and molecular processes responsible for generating the observed diversity and, hence, variation, in toxin composition and action. Many venom toxins are thought to evolve via the ‘birth and death’ process of gene evolution [52], by which a gene encoding a normal ‘physiological’ body protein, usually one involved in key regulatory processes or bioactivity, is duplicated and a duplicate copy selectively expressed in the venom gland [53,54] (Figure 2a,b). These ancestral physiological proteins appear to be expressed in a variety of different tissue types and exhibit diverse ancestral activities [7,55]. Once a particular gene has been recruited into the venom gland, additional gene duplication often occurs, coupled with protein neo- and/or subfunctionalization, typically resulting in large multilocus gene families that encode toxins exhibiting a variety of functional activities and potencies [53,54,56–58]. Until recently, this recruitment process of toxins into the venom gland had been assumed to be a rare, one-way process. However, recent phylogenetic analyses of toxin gene homologs expressed in other tissues provide evidence that toxins can be ‘reverse recruited’ from the venom gland for a role in physiological tissues (Figure 2c,d), whereas other toxin types appear to be coexpressed in the venom gland and other tissues [59]. These findings provide a framework to investigate the distinction between ‘toxins’ and ‘non-toxins’, which is currently poorly known. In addition to tracing the evolution of new protein functions within gene families, elucidating the mechanisms that control the location and extent of toxin gene family expression will provide a fascinating basis for understanding the evolutionary dynamics of proteins produced for internal (the physiology of the animal) and external (venom) functions.

Positive selection at the molecular level

The evolution of venom toxin families via the ‘birth and death’ model is often accompanied by strong evidence of accelerated evolution and positive selection [53]. In particular, positive selection appears to be near-universal among studied trophically venomous taxa, including snakes [54,57,60,61], scorpions [56,62], spiders [63], and cone snails [58,64]. The A-superfamily of conotoxin genes isolated from venomous cone snails contains some of the most rapidly evolving protein-coding genes identified in metazoans to date [58]. In addition to exceptional nonsynonymous substitution rates following gene duplications, the gene turnover of A-conotoxins was found to be greatly accelerated, with duplication events occurring at two to three times the rate identified in all other multilocus gene families [58]. Multiple studies have demonstrated that positive selection acts predominately on amino acid residues that are surface-exposed on the protein macromolecular

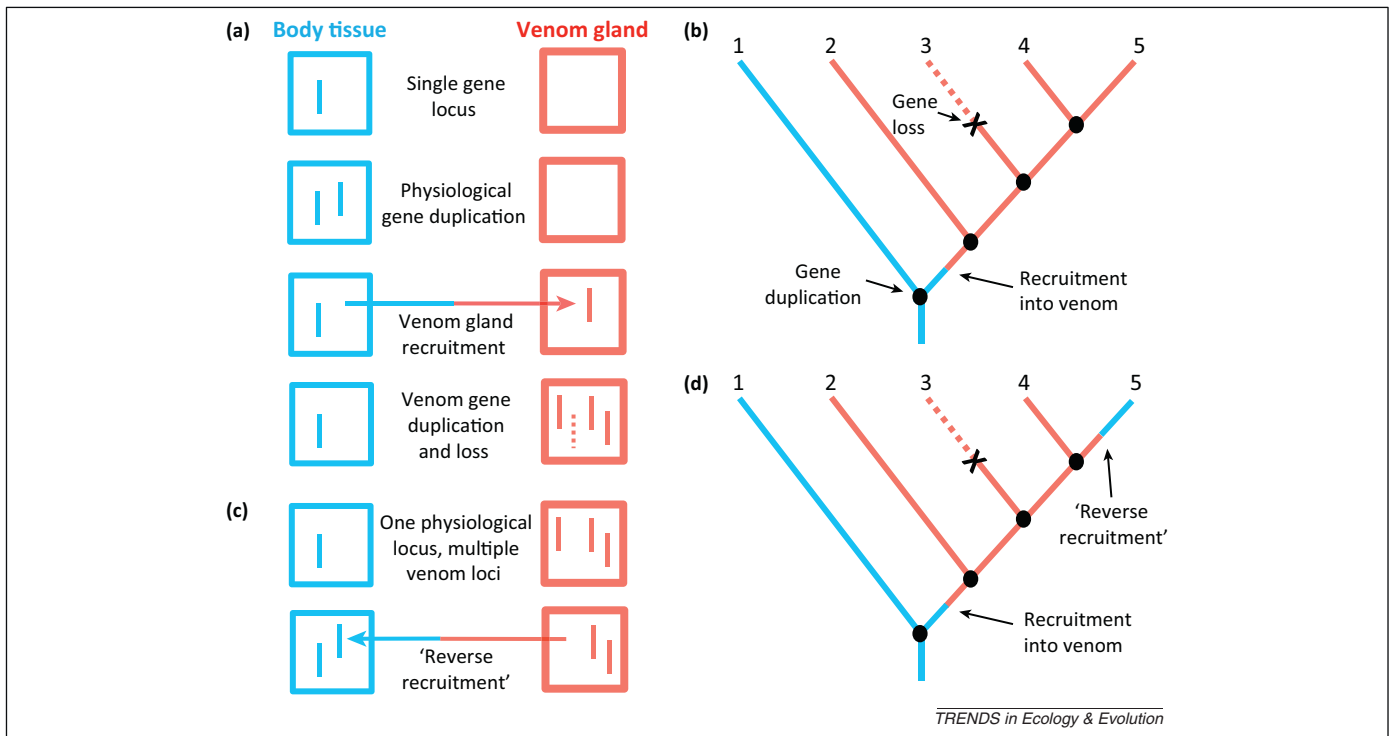


Figure 2. The recruitment and evolution of toxin families under the ‘birth and death’ model [52]. Schematic (a) and gene tree (b) of toxin recruitment into the venom gland and subsequent evolution. A physiologically expressed gene (blue vertical bar) is duplicated and the location of expression of the duplicate is transferred to the venom gland (red vertical bar). Subsequently, additional gene duplication may occur, resulting in multiple venom-expressed genes, whereas some copies degenerate into pseudogenes (broken vertical bar). The cladogram (b) depicts the processes described in the schematic (a) in an evolutionary manner, by demonstrating the evolutionary history of the gene family, including changes in the sites of gene expression (indicated by blue and red branch colors). In this case, gene duplications are indicated by black circles and gene loss events (degeneration into pseudogenes) by a cross. Some toxin genes also appear capable of ‘reverse recruitment’, whereby a venom-expressed gene (red vertical bar) ultimately becomes expressed back in physiological tissues (blue vertical bar) [59]. A schematic (c) and gene tree (d) of the ‘reverse recruitment’ process outline how changes in the sites of expression of some venom toxins, alongside gene duplication events, can result in complex evolutionary histories.

structure [57,61,65]. By retaining a largely stable structural core, the modification of surface-exposed residues is thought to facilitate neofunctionalization of the toxin by modification of protein-target interactions (e.g., by increasing affinity to existing targets or facilitating targeting of new receptors). Gene duplication, positive selection, and protein neofunctionalization therefore appear to work in unison to provide the evolutionary novelty that allows adaptation of venoms to different prey [64], as well as overcoming prey defenses against venom [27,28].

Venom genomics

Large multilocus toxin gene families appear to provide a selective advantage to trophically venomous organisms. In most cases, a large number of these paralogous genes are found retained and expressed in venom. It has been postulated that the retention of related toxin isoforms might provide a selective advantage over the ‘optimization’ of a single gene product, particularly where synergistic bioactivities can be predicted [57] or perhaps where different prey are targeted. However, a key postulation of the ‘birth and death’ model [52] is that some duplicate genes are not successful and ultimately degenerate into pseudogenes. However, the surprising paucity of genomic information from venomous animals [the only genomes sequenced to date are those of the platypus [66], honeybee [67] and three species of predatory wasp (*Nasonia* spp.) [68]] has prevented the testing of this key prediction. Although

inferring gene loss from transcriptomic data is theoretically feasible through analyses such as gene tree reconciliation [69], reverse recruitments into other transcriptomic tissues [59] and the technical challenges of reconciliation [70] make quantifying the success of duplicate genes problematic in the absence of greater genomic resources. However, the completion of several ongoing venomous genome sequencing projects, in conjunction with multiorgan transcriptomic data, is likely to provide exciting insights into the dynamic evolutionary history of complex multilocus gene families.

Other mechanisms of gene evolution

It is important to note that the recruitment of genes for expression in the venom gland is not exclusively reliant on gene duplication. Some identified toxin genes are simply modified, alternatively spliced, or generated through alterations in the structure of domains (Box 2). The ecological role of venom is likely relevant to the mode of toxin evolution utilized. For example, many of the most pathogenic toxin families in trophically venomous taxa are multilocus in nature [54,56–58,61,71]. However, the evolutionary processes governing toxin evolution in defensively venomous taxa have received less attention than have their predatory counterparts, but might provide novel insights into modes of molecular evolution under different selective regimes. The success of holistic approaches to study predatory venoms, utilizing combinations of

Box 2. Mechanisms of toxin evolution

Although gene duplication appears to be a key mechanism that facilitates toxin family evolution and neofunctionalization in many venomous taxa [53,54,56–58] (Figure 1a), it is not a prerequisite for toxin recruitment. In several cases, venom genes have been found to be homologous to gene loci that are physiologically expressed in nonvenomous taxa and therefore appear to have been ‘hijacked’ (and presumably subsequently modified) for a role in venom [55,88] (Figure 1b). Although largely unstudied in venomous animals to date, alternative splicing has also been proposed as a mechanism capable of generating novel toxins [89,90]; evidence from the gene encoding acetylcholinesterase (*AChE*) of the elapid snake *Bungarus fasciatus* (GenBank: [AF045238](#)) supports this hypothesis, where a single gene is alternatively spliced to produce venom and physiological forms [89] (Figure 1c). Domains present in toxin genes can also be important in the generation of novel toxins. For example, two types of sarafotoxin isolated from *Atractaspis* snake venom comprise tandem domains that have been repeated multiple times [91], whereas genes found in helodermatid lizards exhibit evidence of domain duplications giving rise to both single, multidomain product toxins (helofensins) and multiple, single and/or multidomain product toxins (natriuretic peptides) [92] (Figure 1d). Finally, the loss of toxin domains has also been proposed as a mechanism that appears to facilitate adaptive evolution and neofunctionalization of toxins, as found in the metalloproteinases of viperid snakes [57] (Figure 1e).

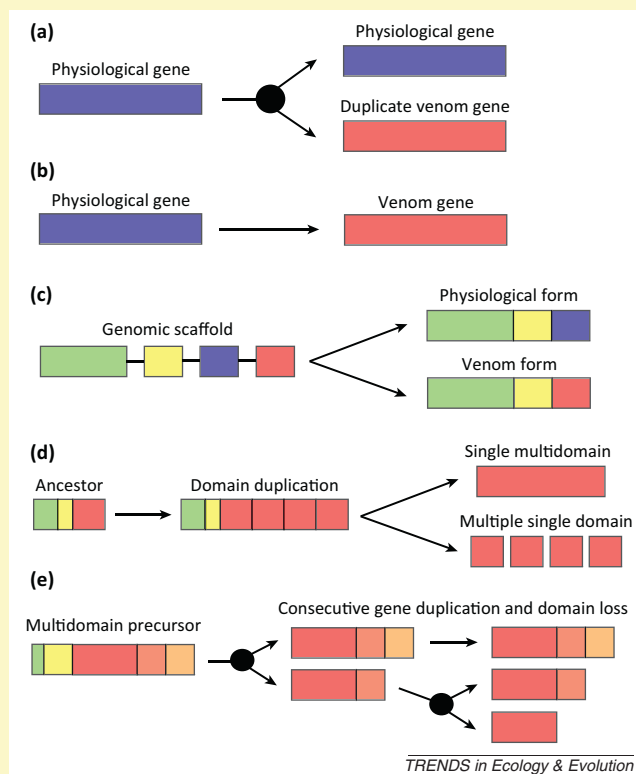


Figure 1. A schematic of the mechanisms that underlie toxin evolution. (a) Gene duplication of a physiological gene and subsequent evolution of a duplicate gene into a venom toxin, (b) modification of a physiological gene into a venom gene, (c) alternative splicing of exons resulting in physiological and venom toxins encoded by the same gene, (d) duplication of an ancestral domain resulting in a multidomain precursor that encodes either single, multidomain products or multiple, single domain products, and (e) consecutive loss of domains from duplicate multidomain precursor genes produces multiple related venom toxins. Circles signify gene duplication events.

molecular, proteomic, morphological, and functional data [71–73], could also be readily applied to defensive venoms to elucidate the respective evolutionary history of a plethora of important, yet largely overlooked, venomous

lineages. Such analyses will be vital to evaluate the importance of natural selection and gene duplication in the context of protein evolution.

Convergent evolution

Venoms are some of the most complex biochemical secretions found in the animal kingdom. Despite this complexity, recent studies have revealed a remarkable degree of convergence in the physiological targeting of the components and the basic molecular building blocks utilized in toxin construction. Targets of venom action include most major physiological pathways and tissue types accessible by the bloodstream; these characteristics have resulted in interest from the pharmaceutical community for the development of novel therapeutics and diagnostics from venom (Box 3). Interestingly, convergent targeting across taxa is a common occurrence and particularly evident in the hemostatic and neurological systems, where venom components recruited independently into different venomous lineages act on the same molecular targets (Figure 3) [6]. Toxins disrupting hemostasis by inhibiting or triggering many of the multiple steps of the coagulation cascade, thereby causing hemorrhage, have evolved convergently in several lineages (Figure 3a). Disruption of neurotransmission, both presynaptically (sodium, potassium, and calcium channels) and postsynaptically (muscarinic and nicotinic receptors) has also evolved convergently on multiple occasions (Figure 3b).

The most intriguing type of convergence concerns the molecules selected for use as toxin scaffolds. Recent studies have revealed that, of the plethora of available building blocks, 14 protein types have been convergently recruited by two or more venomous lineages [6,66,73,74]. In some cases, the same protein type has been recruited on multiple

Box 3. Pharmaceutical development of venom toxins

The past two decades have seen a surge in projects exploiting the extraordinary biological diversity and potency of venom components to develop novel drugs and diagnostics for human diseases, or as probes to study cells, receptors, or physiological pathways [2,93,94]. Encouraged by the substantial medicinal and fiscal success of the Bristol-Myers Squibb angiotensin-converting enzyme (ACE) inhibitor, captopril [95], many other pharmaceutical companies have invested in venom-based drug discovery programs [2]. The majority of the currently approved products were developed from snake venom proteins with distinct cardiovascular specificities, particularly thrombin, fibrinogen, and integrin receptors [2,96]. The rapid advances in proteomics, genomics, and transcriptomics have since resulted in affordable, high-throughput technology platforms [14,97–99] enabling efficient drug discovery mining of venom toxins from species, which unlike snakes, produce venom in small quantities. For example, the toxin repertoires of spiders and cone snails are estimated to contain more than 10 million compounds available for bioprospecting [12,14]. This is important, because the small venom pool studied to date, often with particular focus on certain toxin types through selective assaying, represents an infinitesimally small representation of the true diversity available. Drug-bioprospecting activity will likely continue to rise as largely unstudied venomous animal lineages are exploited for novel lead compounds. A thorough understanding of the evolutionary and ecological biology relating to different venomous animal lineages is critical to the success of such directed programs, by informing and guiding the optimal selection of biological targets for the development of future pharmaceuticals and therapeutics.

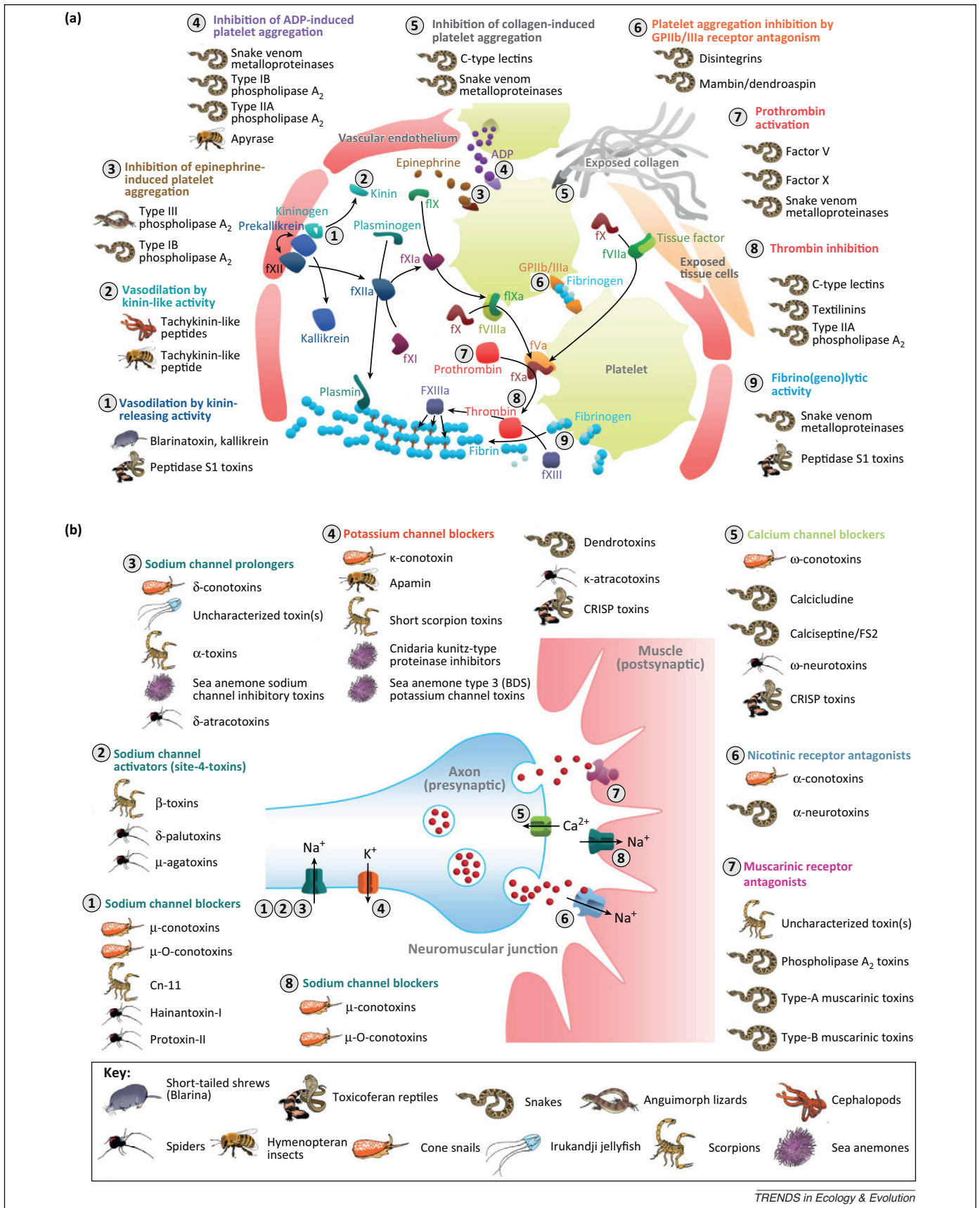


Figure 3. Convergence of toxin action in the animal kingdom. **(a)** Sites of convergent hemotoxic toxin activity are displayed and are represented by numbers (1–9). Each number represents a different physiological target that is targeted convergently by different toxins present in different venomous lineages or in the same venomous lineage. Toxin names and animal lineages acting on each target are listed below each numbered legend, with pictures of the venomous lineages relating to the key at the bottom of the figure. **(b)** Sites of convergent neurotoxic toxin activity are displayed and are represented by numbers (1–7). Adapted, with permission, from [6].

occasions into the same venomous clade, such as the four times that phospholipase A₂ appears to have been recruited into squamate reptiles [75]. Notably, phospholipase A₂ has also been recruited into the venoms of cephalopods, cnidarians, insects, and scorpions [6]. Similarly, kunitz-type toxins have been independently recruited into the venoms of cnidarians, cone snails, insects, scorpions, reptiles, and twice in spiders [6]. A consistent feature of such protein types is stabilization of the molecular scaffold through extensive cysteine crosslinking [55]. This characteristic appears to facilitate modification of nonstructural residues, often resulting in extensive protein neofunctionalization. Observations of toxin convergence occurring throughout the animal kingdom therefore provide a model system to investigate the structural, functional, and regulatory characteristics that make certain protein families amenable for a 'toxic' role in venom and the evolutionary changes involved in turning a physiological protein into a toxin.

Concluding remarks and future directions

Despite much research, we remain ignorant of many facets of the natural history of venoms and the interactions between that natural history and the modes of evolution of these chemical arsenals. The multiple parallel origins of both defensive and trophic venoms provide an ideal model system for investigating the evolutionary dichotomy between venomous predators and their prey and between venomous prey and their predators. Understanding the frequency and ecological importance of interactions between these animals and the role of venoms therein is required to illuminate the diversity and intensity of selective pressures acting upon them. The implications extend far beyond the evolution of venom itself, for instance through the evolution of mimicry and its role in diversification. Some of the most well-known cases of mimicry involve a venomous animal acting as the model for Batesian mimics [76]. In snakes, the evolution of venom and consequent avoidance of associated patterns by predators might have favored the diversification of nonvenomous snakes, by shielding these otherwise vulnerable animals through the protective umbrella of mimicry [77]. However, several venomous animals also use mimicry themselves, ranging from Batesian to Müllerian, and even aggressive mimicry [76,78]. These observations stress the importance of assessing interactions between venomous predators and/or prey in the context of an ecological community, and highlight the potential, but hitherto understudied, role of venom in the structuring of ecological communities.

Many facets of the molecular evolution of venom and its underlying mechanisms also remain insufficiently understood. The inherent evolutionary complexity observed in many toxin families should be viewed as an asset for evolutionary biologists, because the genetic and functional diversity of such proteins make them ideal systems for testing the models postulated to underlie gene evolution and adaptive change in organisms [79]. Importantly, these often extreme examples of gene evolution could also be applied as robust models to test the boundaries of evolutionary bioinformatic software.

The role of gene duplication is thought to be crucial for organismal evolution by facilitating the evolution of new protein functions [79]. However, gene duplication can also contribute to gene dosage effects (where protein dosage is increased by the duplication of protein-encoding genes) [80], which might be particularly relevant for the production of highly potent venom. Although the generation of genetic toxin diversity is well described in predatory venomous animals [54,56–58,60,61,64], the importance of gene dosing remains completely overlooked, despite this being one mechanism that could be responsible for overcoming prey resistance or differences in prey physiology. Assessing the influence of gene dosing (and the discovery of other genetics mechanisms relevant to toxin evolution) is currently limited by the paucity of genomic information available for venomous animals. Future genomic and transcriptomic characterizations of venomous taxa have the potential to elucidate the molecular mechanisms operating on venom toxin gene evolution and, importantly, the elements that control their regulation and expression. Several fundamental questions relating to the production, maintenance, and evolution of venom thus remain, yet advances in 'omic' technologies offer great potential for elucidating the fascinating mechanisms responsible for generating some of the most complex and potent biochemical secretions found in the animal kingdom.

Acknowledgments

We thank two anonymous reviewers for their valuable advice relating to the content of the manuscript. We apologize to the authors of several relevant papers that could not be included in the review owing to space restrictions. We acknowledge support from the Natural Environment Research Council, UK (N.R.C., NE/J018678/1; W.W., NER/SA/2006/14086), the Wellcome Trust, UK (R.A.H., 072426) and the Australian Research Council and the University of Queensland (B.G.F.). We thank Camila Renjifo (Liverpool School of Tropical Medicine, UK) for providing assistance with the creation of Figure 1 in Box 1.

References

- 1 Isbell, L.A. (2006) Snakes as agents of evolutionary change in primate brains. *J. Hum. Evol.* 51, 1–35
- 2 King, G.F. (2011) Venoms as a platform for human drugs: translating toxins into therapeutics. *Expert Opin. Biol. Ther.* 11, 1469–1484
- 3 Harrison, R.A. *et al.* (2011) Research strategies to improve snakebite treatment: challenges and progress. *J. Proteomics* 74, 1768–1780
- 4 Williams, D.J. *et al.* (2011) Ending the drought: new strategies for improving the flow of affordable, effective antivenoms in Asia and Africa. *J. Proteomics* 74, 1735–1767
- 5 Dutertre, S. and Lewis, R.J. (2010) Use of venom peptides to probe ion channel structure and function. *J. Biol. Chem.* 285, 13315–13320
- 6 Fry, B.G. *et al.* (2009) The toxicogenomic multiverse: convergent recruitment of proteins into animal venoms. *Annu. Rev. Genomics Hum. Genet.* 10, 483–511
- 7 Fry, B.G. *et al.* (2012) The structural and functional diversification of the Toxicofera reptile venom system. *Toxicon* 60, 434–448
- 8 Gong, E. *et al.* (2010) The birdlike raptor *Sinornithosaurus* was venomous. *Proc. Natl. Acad. Sci. U.S.A.* 107, 766–768
- 9 Fox, R.C. and Scott, C.S. (2005) First evidence of a venom delivery apparatus in extinct mammals. *Nature* 435, 1091–1093
- 10 Smith, W.L. and Wheeler, W.C. (2006) Venom evolution widespread in fishes: a phylogenetic road map for the bioprospecting of piscine venoms. *J. Hered.* 97, 206–217
- 11 Vonk, F.J. *et al.* (2008) Evolutionary origin and development of snake fangs. *Nature* 454, 630–633
- 12 Olivera, B.M. (2002) 'Conus' venom peptides: reflections from the biology of clades and species. *Annu. Rev. Ecol. Syst.* 33, 25–47

- 13 Inceoglu, B. *et al.* (2003) One scorpion, two venoms: prevenom of *Parabuthus transvaalicus* acts as an alternative type of venom with distinct mechanism of action. *Proc. Natl. Acad. Sci. U.S.A.* 100, 922–927
- 14 Escoubas, P. and King, G.F. (2009) Venomics as a drug discovery platform. *Expert Rev. Proteomics* 6, 221–224
- 15 Church, J.E. and Hodgson, W.C. (2002) The pharmacological activity of fish venoms. *Toxicon* 40, 1083–1093
- 16 Peiren, N. *et al.* (2005) The protein composition of honeybee venom reconsidered by a proteomic approach. *Biochim. Biophys. Acta Proteins Proteomics* 1752, 1–5
- 17 de Graaf, D.C. *et al.* (2009) Bee, wasp and ant venomics pave the way for a component-resolved diagnosis of sting allergy. *J. Proteomics* 72, 145–154
- 18 Mackessy, S.P. (2010) Evolutionary trends in venom composition in the western rattlesnake (*Crotalus viridis* sensu lato). Toxicity vs. tenderizers. *Toxicon* 55, 1463–1474
- 19 Glenn, J.L. and Straight, R. (1978) Mojave rattlesnake (*Crotalus scutulatus scutulatus*) venom: variation in toxicity with geographic origin. *Toxicon* 16, 81–84
- 20 Calvete, J.J. *et al.* (2010) Snake venomics of the Central American rattlesnake *Crotalus simus* and the South American *Crotalus durissus* complex points to neurotoxicity as an adaptive paedomorphic trend along *Crotalus* dispersal in South America. *J. Proteome Res.* 9, 528–544
- 21 Menezes, M.P. *et al.* (2010) Sex-based individual variation of snake venom proteome among eighteen *Bothrops jararaca* siblings. *Toxicon* 47, 304–312
- 22 Andrade, D.V. and Abe, A.S. (1999) Relationship of venom ontogeny and diet in *Bothrops*. *Herpetologica* 55, 200–204
- 23 Daltry, J.C. *et al.* (1996) Diet and snake venom evolution. *Nature* 379, 537–540
- 24 Sasa, M. (1999) Diet and snake venom evolution: can local selection alone explain intraspecific venom variation? *Toxicon* 37, 249–252
- 25 Mebs, D. (2001) Toxicity in animals. Trends in evolution? *Toxicon* 39, 87–96
- 26 Williams, V. *et al.* (1988) Variation in venom properties from isolated populations of tiger snakes (*Notechis ater niger*, *N. scutulatus*) in South Australia. *Toxicon* 26, 1067–1075
- 27 Heatwole, H. and Poran, N.S. (1995) Resistances of sympatric and allopatric eels to sea snake venoms. *Copeia* 1995, 136–147
- 28 Biardi, J.E. and Coss, R.G. (2011) Rock squirrel (*Spermophilus variegatus*) blood sera affects proteolytic and hemolytic activities of rattlesnake venoms. *Toxicon* 57, 323–331
- 29 Jansa, S.A. and Voss, R.S. (2011) Adaptive evolution of the venom-targeted vWF protein in opossums that eat pitvipers. *PLoS ONE* 6, e20997
- 30 Barlow, A. *et al.* (2009) Coevolution of diet and prey-specific venom activity supports the role of selection in snake venom evolution. *Proc. R. Soc. B* 276, 2443–2449
- 31 Richards, D.P. *et al.* (2012) Venom lethality and diet: differential responses of natural prey and model organisms to the venom of the saw-scaled vipers (*Echis*). *Toxicon* 59, 110–116
- 32 McCue, M.D. (2006) Cost of producing venom in three North American pitviper species. *Copeia* 2006, 818–825
- 33 Pintor, A.F.V. *et al.* (2010) Costs of venom production in the common death adder (*Acanthophis antarcticus*). *Toxicon* 56, 1035–1042
- 34 Hayes, W.K. (1995) Venom metering by juvenile prairie rattlesnakes, *Crotalus v. viridis*: effects of prey size and experience. *Anim. Behav.* 50, 33–40
- 35 Galán, J.A. *et al.* (2004) Neutralization of venoms from two Southern Pacific Rattlesnakes (*Crotalus helleri*) with commercial antivenoms and endothermic animal sera. *Toxicon* 43, 791–799
- 36 Jorge da Silva, N., Jr and Aird, S.D. (2001) Prey specificity, comparative lethality and compositional differences of coral snake venoms. *Comp. Biochem. Physiol.* 128, 425–456
- 37 Gibbs, H.L. and Mackessy, S.P. (2009) Functional basis of a molecular adaptation: prey-specific toxic effects of venom from *Sistrurus* rattlesnakes. *Toxicon* 53, 672–679
- 38 Pawlak, J. *et al.* (2006) Denmotoxin, a three-finger toxin from the colubrid snake *Boiga dendrophila* (Mangrove Catsnake) with bird-specific activity. *J. Biol. Chem.* 281, 29030–29041
- 39 Nisani, Z. *et al.* (2007) Cost of venom regeneration in *Parabuthus transvaalicus* (Arachnida: Buthidae). *Comp. Biochem. Physiol. A* 147, 509–513
- 40 Malli, H. *et al.* (1999) Effects of size, motility and paralysation time of prey on the quantity of venom injected by the hunting spider *Cupiennius salei*. *J. Exp. Biol.* 202, 2083–2089
- 41 Kuhn-Nentwig, L. *et al.* (2004) Biochemistry, toxicology and ecology of the venom of the spider *Cupiennius salei* (Ctenidae). *Toxicon* 43, 543–553
- 42 Nisani, Z. and Hayes, W.K. (2011) Defensive stinging by *Parabuthus transvaalicus* scorpions: risk assessment and venom metering. *Anim. Behav.* 81, 627–633
- 43 Remigio, E.A. *et al.* (2008) Evolution of ecological specialization and venom of a predatory marine gastropod. *Mol. Ecol.* 17, 1156–1162
- 44 Duda, T.F., Jr *et al.* (2009) Geographic variation in venom allelic composition and diets of the widespread predatory marine gastropod *Conus ebraeus*. *PLoS ONE* 4, e6245
- 45 Elliger, C.A. *et al.* (2010) Diversity of conotoxin types from *Conus californicus* reflects a diversity of prey types and a novel evolutionary history. *Toxicon* 57, 311–322
- 46 Duda, T.F., Jr and Palumbi, S.R. (2004) Gene expression and feeding ecology: evolution of piscivory in the venomous gastropod genus *Conus*. *Proc. Biol. Sci.* 271, 1165–1174
- 47 Pekár, S. *et al.* (2008) Dietary and prey-capture adaptations by which *Zodarion germanicum*, an ant-eating spider (Araneae: Zodariidae), specialises on the Formicidae. *Naturwissenschaften* 95, 233–239
- 48 Wüster, W. and Thorpe, R.S. (1992) Dentitional phenomena in cobras revisited: fang structure and spitting in the Asiatic species of *Naja* (Serpentes: Elapidae). *Herpetologica* 48, 424–434
- 49 Li, M. *et al.* (2005) Eggs-only diet: its implications for the toxin profile changes and ecology of the marbled sea snake (*Aipysurus eydouxii*). *J. Mol. Evol.* 60, 81–89
- 50 Dawkins, R. and Krebs, J.R. (1979) Arms races between and within species. *Proc. R. Soc. Lond. B: Biol. Sci.* 205, 489–511
- 51 Van Valen, L. (1973) A new evolutionary law. *Evol. Theor.* 1, 1–30
- 52 Nei, M. *et al.* (1997) Evolution by the birth-and-death process in multigene families of the vertebrate immune system. *Proc. Natl. Acad. Sci. U.S.A.* 94, 7799–7806
- 53 Kordiš, D. and Gubenšek, F. (2000) Adaptive evolution of animal toxin multigene families. *Gene* 261, 43–52
- 54 Fry, B.G. *et al.* (2003) Molecular evolution and phylogeny of elapid snake venom three-finger toxins. *J. Mol. Evol.* 57, 110–129
- 55 Fry, B.G. (2005) From genome to ‘venome’: molecular origin and evolution of the snake venom proteome inferred from phylogenetic analysis of toxin sequences and related body proteins. *Genome Res.* 15, 403–420
- 56 Weinberger, H. *et al.* (2010) Positions under positive selection – key for selectivity and potency of scorpion α -toxins. *Mol. Biol. Evol.* 27, 1025–1034
- 57 Casewell, N.R. *et al.* (2011) Domain loss facilitates accelerated evolution and neofunctionalization of duplicate snake venom metalloproteinase toxin genes. *Mol. Biol. Evol.* 28, 2637–2649
- 58 Chang, D. and Duda, T.F., Jr (2012) Extensive and continuous duplication facilitates rapid evolution and diversification of gene families. *Mol. Biol. Evol.* 29, 2019–2029
- 59 Casewell, N.R. *et al.* (2012) Dynamic evolution of venom proteins in squamate reptiles. *Nat. Commun.* 3, 1066
- 60 Nakashima, K-I. *et al.* (1993) Accelerated evolution of *Trimeresurus flavoviridis* venom gland phospholipase A2 isozymes. *Proc. Natl. Acad. Sci. U.S.A.* 90, 5964–5968
- 61 Juárez, P. *et al.* (2008) Evolution of snake venom disintegrins by positive Darwinian selection. *Mol. Biol. Evol.* 25, 2391–2407
- 62 Zhu, S.Y. *et al.* (2004) Adaptive evolution of scorpion sodium channel toxins. *J. Mol. Evol.* 58, 145–153
- 63 Binford, G.J. *et al.* (2009) Molecular evolution, functional variation, and proposed nomenclature of the gene family that includes sphingomyelinase d in sicariid spider venoms. *Mol. Biol. Evol.* 26, 547–566
- 64 Duda, T.F., Jr and Palumbi, S.R. (1999) Molecular genetics of ecological diversification: duplication and rapid evolution of toxin genes of the venomous gastropod *Conus*. *Proc. Natl. Acad. Sci. U.S.A.* 96, 6820–6823
- 65 Gao, B. *et al.* (2011) Molecular divergence of two orthologous scorpion toxins affecting potassium channels. *Comp. Biochem. Physiol. A* 159, 313–321

- 66 Warren, W.C. *et al.* (2008) Genome analysis of the platypus reveals unique signatures of evolution. *Nature* 453, 175–183
- 67 Weinstock, G.M. *et al.* (2006) Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature* 443, 931–949
- 68 Werren, J.H. *et al.* (2010) Functional and evolutionary insights from the genomes of three parasitoid *Nasonia* species. *Science* 327, 343–348
- 69 Maddison, W.P. (1997) Gene trees in species trees. *Syst. Biol.* 46, 523–536
- 70 Casewell, N.R. *et al.* (2011) Gene tree parsimony of multilocus snake venom protein families reveals species tree conflict as a result of multiple parallel gene loss. *Mol. Biol. Evol.* 28, 1157–1172
- 71 Fry, B.G. *et al.* (2008) Evolution of an arsenal: structural and functional diversification of the venom system in the advanced snakes (Caenophidia). *Mol. Cell. Proteomics* 7, 215–246
- 72 Fry, B.G. *et al.* (2006) Early evolution of the venom system in lizards and snakes. *Nature* 439, 584–588
- 73 Yang, S. *et al.* (2012) Chemical punch packed in venoms makes centipedes excellent predators. *Mol. Cell. Proteomics* 11, 640–650
- 74 Whittington, C.M. *et al.* (2008) Defensins and the convergent evolution of platypus and reptile venom genes. *Genome Res.* 18, 986–994
- 75 Fry, B.G. *et al.* (2012) Novel transcripts in the maxillary glands of advanced snakes. *Toxicon* 59, 696–708
- 76 Pough, F.H. (1988) Mimicry of vertebrates: are the rules different? *Am. Nat.* 131, S67–S102
- 77 Greene, H.W. and McDiarmid, R.W. (2005) Wallace and Savage: heroes, theories and venomous snake mimicry. In *Ecology and Evolution in the Tropics. A Herpetological Perspective* (Donnelly, M.A. *et al.*, eds), pp. 190–208, Chicago University Press
- 78 Alexandrou, M.A. *et al.* (2011) Competition and phylogeny determine community structure in Müllerian co-mimics. *Nature* 469, 84–88
- 79 Innan, H. and Kondrashov, F. (2010) The evolution of gene duplications: classifying and distinguishing between models. *Nat. Rev. Genet.* 11, 97–108
- 80 Kondrashov, F.A. *et al.* (2002) Selection in the evolution of gene duplications. *Genome Biol.* 3, research0008.1–0008.9
- 81 Pennisi, E. (2003) Drafting a tree. *Science* 300, 1694
- 82 Kasturiratne, A. *et al.* (2008) The global burden of snakebite: a literature analysis and modelling based on regional estimates of envenoming and deaths. *PLoS Med.* 5, e218
- 83 Harrison, R.A. *et al.* (2000) Antibody from mice immunized with DNA encoding the carboxyl-disintegrin and cysteine-rich domain (JD9) of the haemorrhagic metalloprotease, Jararhagin, inhibits the main lethal component of viper venom. *Clin. Exp. Immunol.* 121, 358–363
- 84 Harrison, R.A. (2004) Development of venom toxin-specific antibodies by DNA immunisation: rationale and strategies to improve therapy of viper envenoming. *Vaccine* 22, 1648–1655
- 85 Wagstaff, S.C. and Harrison, R.A. (2006) Venom gland EST analysis of the saw-scaled viper, *Echis ocellatus*, reveals novel $\alpha_9\beta_1$ integrin-binding motifs in venom metalloproteinases and a new group of putative toxins, renin-like aspartic proteases. *Gene* 377, 21–32
- 86 Casewell, N.R. *et al.* (2009) Comparative venom gland transcriptome surveys of the saw-scaled vipers (Viperidae: *Echis*) reveal substantial intra-family gene diversity and novel venom transcripts. *BMC Genomics* 10, 564
- 87 Wagstaff, S.C. *et al.* (2006) Bioinformatics and multiepitope DNA immunization to design rational snake antivenom. *PLoS Med.* 3, e184
- 88 Wong, E.S. *et al.* (2012) A limited role for gene duplications in the evolution of platypus venom. *Mol. Biol. Evol.* 29, 167–177
- 89 Cousin, X. *et al.* (1998) Identification of a novel type of alternatively spliced exon from the acetylcholinesterase gene of *Bungarus fasciatus*. *J. Biol. Chem.* 273, 9812–9820
- 90 Zhijian, C. *et al.* (2006) Genetic mechanisms of scorpion venom peptide diversification. *Toxicon* 47, 348–355
- 91 Ducancel, F. (2005) Endothelin-like peptides. *Cell. Mol. Life Sci.* 62, 2828–2839
- 92 Fry, B.G. *et al.* (2010) Novel venom proteins produced by differential domain-expression strategies in Beaded lizards and Gila monsters (genus *Heloderma*). *Mol. Biol. Evol.* 27, 395–407
- 93 Bohlen, C.J. *et al.* (2011) A heteromeric Texas coral snake toxin targets acid-sensing ion channels to produce pain. *Nature* 479, 410–414
- 94 Diochot, S. *et al.* (2012) Black mamba venom peptides target acid-sensing ion channels to abolish pain. *Nature* 490, 552–555
- 95 Cushman, D.W. and Ondetti, M.A. (1991) History of the design of captopril and related inhibitors of angiotensin converting enzyme. *Hypertension* 17, 589–592
- 96 Koh, C.Y. and Kini, R.M. (2011) From snake venom toxins to therapeutics – cardiovascular examples. *Toxicon* 59, 497–506
- 97 Olivera, B.M. *et al.* (1995) Combinatorial peptide libraries in drug design: lessons from venomous cone snails. *Trends Biotechnol.* 13, 422–426
- 98 Liang, S. (2008) Proteome and peptidome profiling of spider venoms. *Expert Rev. Proteomics* 5, 731–746
- 99 Vetter, I. *et al.* (2011) Venomics: a new paradigm for natural products-based drug discovery. *Amino Acids* 40, 15–28