



## SYMPOSIUM

# Venom Resistance as a Model for Understanding the Molecular Basis of Complex Coevolutionary Adaptations

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## Synopsis

**Synopsis** Venom and venom resistance are molecular phenotypes widely considered to have diversified through coevolution between predators and prey. However, while evolutionary and functional studies on venom have been extensive, little is known about the molecular basis, variation, and complexity of venom resistance. We review known mechanisms of venom resistance and relate these mechanisms to their predicted impact on coevolutionary dynamics with venomous enemies. We then describe two conceptual approaches which can be used to examine venom/resistance systems. At the intraspecific level, tests of local adaptation in venom and resistance phenotypes can identify the functional mechanisms governing the outcomes of coevolution. At deeper evolutionary timescales, the combination of phylogenetically informed analyses of protein evolution coupled with studies of protein function promise to elucidate the mode and tempo of evolutionary change on potentially coevolving genes. We highlight case studies that use each approach to extend our knowledge of these systems as well as address larger questions about coevolutionary dynamics. We argue that resistance and venom are phenotypic traits which hold exceptional promise for investigating the mechanisms, dynamics, and outcomes of coevolution at the molecular level. Furthermore, extending the understanding of single gene-for-gene interactions to the whole resistance and venom phenotypes may provide a model system for examining the molecular and evolutionary dynamics of complex multi-gene interactions.

## Introduction

Coevolution between predators and prey is an important evolutionary force for the generation and maintenance of adaptive variation. Most studies of coevolving traits in nature have focused on accessible morphological variation (Benkman et al. 2003; Toju 2008). However, with recent advances in genomic and proteomic techniques, as well as an improving understanding of molecular function, we can now meaningfully examine coevolutionary dynamics at the molecular level (Hanifin et al. 2008; Nash et al. 2008; Zangerl et al. 2008; Jansa and Voss 2011;

Scanlan et al. 2011; Feldman et al. 2012). Among coevolving systems, the interactions between venomous and venom-resistant animals hold exceptional promise for investigating molecular coevolution. In this review, we outline how population-level, phylogenetic, and biochemical approaches can be applied to these systems to study the molecular and functional basis of complex phenotypic interactions.

Coevolution has been widely invoked to explain trait variation in venomous and venom-resistant animals, and is often discussed in terms of molecular “arms races” (Casewell et al. 2012b; Vonk et al. 2013). Studies of venom evolution have shown that

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molecular evolutionary processes such as positive selection, gene duplication, exon shuffling, and transcriptional splicing, among others play major roles in generating venom diversity (Fry et al. 2005; Doley et al. 2009; Casewell et al. 2012a, 2014; Vonk et al. 2013; Rokyta et al. 2015). In contrast, few studies have focused on the evolution of venom resistance, despite the fact that reciprocal evolution of weapons and defenses is required for an explanation based on an arms race or other coevolutionary dynamic to apply (Janzen 1980). The molecular basis of venom resistance has been investigated in only a few mammals and venomous snakes (Barchan et al. 1995; Sanchez and Rodriguez-Acosta 2008; Jansa and Voss 2011; Drabeck et al. 2015; Estevão-Costa et al. 2016), and rapid evolution and positive selection have been demonstrated for only two proteins that are targeted by venom toxins (e.g., Jansa and Voss 2011; Voss and Jansa 2012; Drabeck et al. 2015).

Diverse venoms span Animalia, with venom occurring in Cnidaria, Arthropoda, Annelida, Bryozoa, and Chordata (reviewed in Casewell et al. 2012b). While our review covers recent work focused on mammalian venom resistance due to its prevalence in the literature, studies of toxin resistance combined with other ecological and physiological data suggest that resistance is likely to have evolved in diverse groups of animals (Heatwole and Powell 1998; Heatwole et al. 1999; Voss and Jansa 2012). Thus, the mechanisms and approaches we discuss here have the potential to be applied across a diverse set of taxa beyond mammals. The diversity of venomous species and the broader network of species they interact with represent replicated opportunities to ask important questions about coevolution.

Here, we provide a brief review of what is known about the molecular basis of resistance to venoms, as well as outline research directions which would advance our understanding of coevolution between venom toxins and their targets and inhibitors. We first summarize the molecular mechanisms that underlie venom resistance, focusing on the mammalian systems in which resistance to venoms is best characterized. We then discuss conceptual approaches for studying the evolution of resistance within and between species. We focus on linking predictions from theory with empirical tests from specific systems, with an emphasis on how these approaches inform our understanding of coevolutionary interactions between venomous and resistant taxa. Finally, we describe gaps in our knowledge that should be addressed to fully pursue research on venom/resistance systems and suggest approaches to fill these gaps.

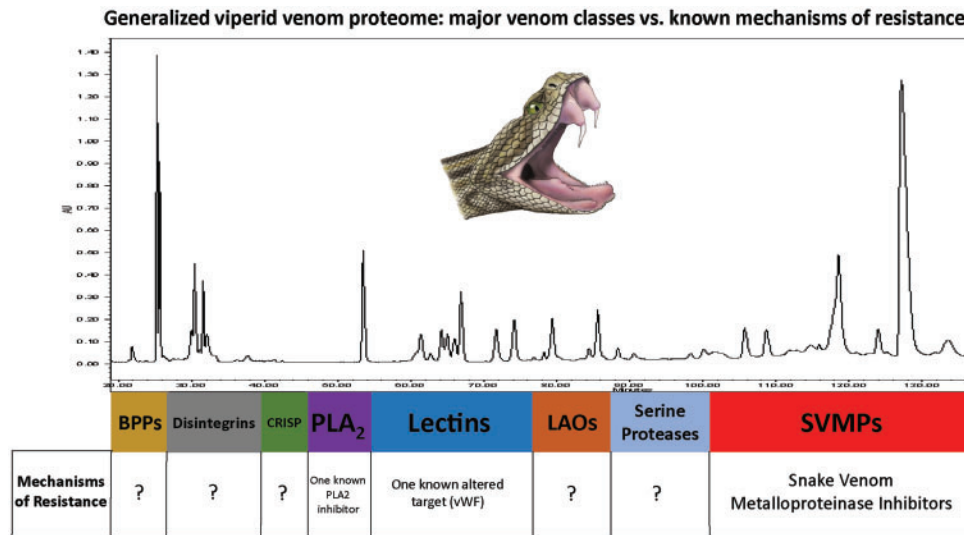
## Molecular mechanisms of resistance

Venom toxins target a wide array of biologically important molecules and disrupt numerous physiological functions. As such, understanding the molecular basis of venom resistance requires knowledge not only about how venom molecules exert their toxic effects but also about how resistant animals cope with these toxins at a molecular level. Though we understand some resistance mechanisms, our knowledge of the molecular basis of venom resistance lags well behind our understanding of venom composition and toxicity (Fig. 1). Consequently, an integrated picture of venom resistance and its role in the evolution of venom diversity and toxicity has yet to be realized. An important step in understanding how these traits might coevolve is to identify additional molecular mechanisms of resistance, because the molecular phenotypic interface between venomous and resistant species involves complex interactions between the venom components of one species, and the target or inhibitor proteins of the envenomated species. The interaction of these various molecules forms a complex phenotypic space where the outcome of envenomation is decided (Fig. 2).

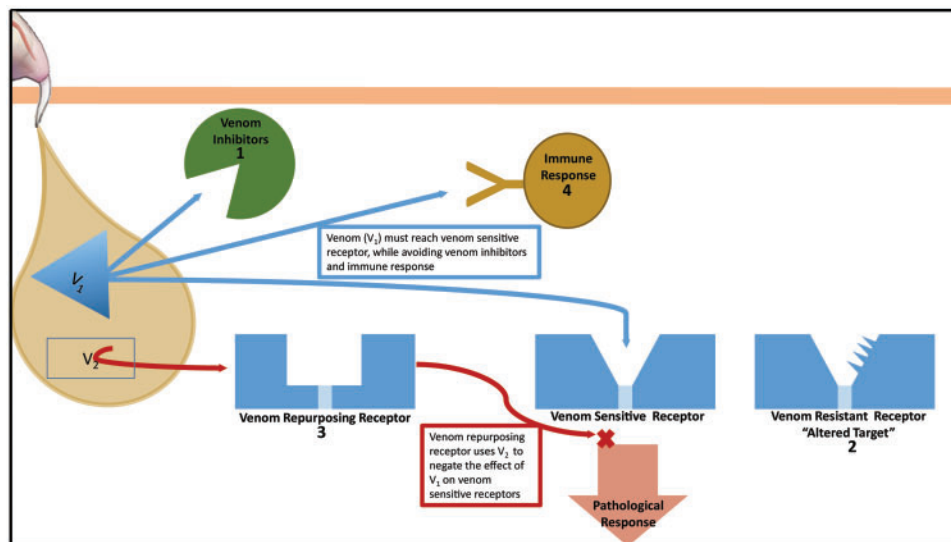
In this review, we characterize known mechanisms of venom resistance into four categories: (1) venom inhibitors, molecules that deactivate venom toxins before they reach their targets and benefit from a match to venom; (2) altered targets, proteins that have evolved to no longer bind venom toxins but that still retain their original physiological function and benefit from a mismatch to venom; (3) repurposed toxins, where venom toxins are used by the victim to short-circuit the physiological effect of the toxin and the resistant animal benefits from a match; and (4) acquired immunity, where resistance is acquired through repeated sub-lethal exposure to toxins.

### Venom inhibitors

Many animal species have evolved at least partial resistance to venom in the form of circulating proteins that bind and inhibit venom proteins (Perez and Sanchez 1999; Perales et al. 2005; Biardi 2008). These blood-serum factors neutralize snake venom metalloproteinases (SVMPs) and phospholipases (Perez et al. 1979; Catanese and Kress 1993; Perales et al. 2005). They have been identified in at least 30 mammal species from six orders (Perez et al. 1978; Biardi 2008; Voss and Jansa 2012), and in a number of squamate reptiles that are either resistant to their



**Fig. 1** A representative HPLC venom chromatogram from a rattlesnake (*C. oreganus*) with elution regions roughly labeled according to the venom protein family based on peak identifications in Margres et al. (2014). Below the toxin names, the known mechanisms of resistance to these snake venom toxin classes are shown, highlighting that resistance is only known for less than half of the known toxin classes (Jansa and Voss 2011). BPP, Bradykinin potentiating peptides; PLA<sub>2</sub>, phospholipase 2s; LAO, L-amino oxidases.



**Fig. 2** Conceptual presentation of the molecular mechanisms of venom resistance (ordered 1–4 to correspond to order presented in text) in relation to the actions of venom, emphasizing the diverse ways in which resistance can be achieved and the evolutionary pressures encountered by all proteins at the molecular phenotypic interface of interactions between a venomous and resistant species. Pathological response induced by venoms (depicted by the red arrow) is interrupted through both direct and indirect mechanisms.

own venom or preyed on by venomous snakes (Perales et al. 2005).

Most of the known venom inhibitors function through direct interaction with venom proteins. For example, the  $\alpha\beta$  glycoproteins found in opossums, mongooses, and some rodents, and the inter- $\alpha$  trypsin inhibitor of ground squirrels irreversibly bind to a single venom protein to render it inactive

(Biardi 2008). A related mechanism exists in the European hedgehog (*Erinaceus europaeus*), where a  $\beta$ -macroglobulin draws venom proteases into close proximity with a string of amino acids that changes conformation to enclose the venom, acting as a molecular cage (de Wit and Weström 1987). Both of these mechanisms rely on inhibitors recognizing toxic molecules, where affinity of one protein for

another is a key part of the inhibitory process. Inhibition is also non-enzymatic, with inhibitors binding venom proteins in a 1:1 stoichiometry that inactivates both proteins (Perez and Sanchez 1999; Biardi et al. 2011).

Almost all of the known inhibitor proteins are associated with the Immunoglobulin (Ig) and Ficolin/Opsonin supergene family; for this reason, it has been suggested that these proteins are a derived part of the innate immune system (Perales et al. 2005; Sanchez and Rodriguez-Acosta 2008). As such, their evolution may differ from other classes of venom-resistant molecules described below. In particular, the serum inhibitors identified to date are all members of large, relatively old gene families, whereas the “altered targets” described below are typically members of small gene families (e.g., nAChR) or are encoded by single genes (e.g., vWF). Whereas positive selection may play an important role in the evolution of both toxin inhibitors and altered targets, the processes of gene duplication, gene turnover, and neofunctionalization may be the primary forces driving the evolution of circulating toxin inhibitors (for review see Taylor and Raes 2004).

### Altered targets

Resistant mammals can also cope with venom by evolving venom-targeted receptors that no longer bind damaging venom proteins, while retaining their essential physiological functions (Barchan et al. 1992; Jansa and Voss 2011; Drabeck et al. 2015). While therapeutic potential has focused attention on serum inhibitors, few studies have examined the role that modified venom targets play in resistance, and the implications of these adaptive changes on coevolutionary dynamics remain largely unexplored. Target alteration is typically mediated by a small number of point mutations that change the protein so that it no longer binds the toxin but still recognizes its endogenous ligand. To date, only three venom-targeted proteins have demonstrated resistance to their toxic ligands: the muscular nicotinic acetylcholine receptor (nAChR), the blood coagulation protein von Willebrand Factor (vWF), and the alpha-1-proteinase inhibitor.

The muscular nAChR mediates synaptic transmission from nerves to muscles, and is targeted by alpha-neurotoxins present in the venom of elapid and hydrophiid snakes (Neumann et al. 1989; Barchan et al. 1995). *In-vitro* and *in-vivo* research showed that the nAChR protein of hedgehogs, mongooses, and cobras—three taxa that survive

envenomation by neurotoxic snakes—shows strong binding resistance for alpha neurotoxins (Barchan et al. 1992, 1995; Takacs et al. 2004). Site-directed mutagenesis revealed that this loss of toxin binding ability is explained by amino acid substitutions at two sites on the molecule’s surface. A larger, comparative study subsequently showed that these same two amino acid sites have evolved independently under positive selection in four venom-resistant mammalian lineages: hedgehogs, mongooses, honey badgers, and pigs. This is the only known example of convergent adaptive evolution of a molecule involved in venom resistance; interestingly, the resistance seems to be mediated through two distinct biochemical mechanisms (Takacs et al. 2001, 2004). Cobras and mongooses have substitutions that confer resistance via glycosylation that leads to steric hindrance, whereas hedgehogs, honey badgers, and domestic pigs have substitutions that involve charge interference (Drabeck et al. 2015).

The second case of target evolution is the blood protein vWF, which mediates blood coagulation. The vWF protein is targeted by venom C-type-lectins (such as botrocetin), and has evolved under positive selection in a clade of didelphid opossums that prey upon venomous snakes and survive their bites (Jansa and Voss 2011). Although we do not yet know the functional significance of the observed amino acid changes in vWF in these taxa, eight sites are under strong positive selection in this lineage and seven of these are critical for botrocetin binding.

Finally, another altered target apparently exists in the Virginia opossum (*Didelphis virginiana*). The alpha-1-proteinase inhibitor ( $\alpha$ 1-antitrypsin) serves as an important inhibitor of endogenous proteases in the Virginia Opossum, but it is uniquely not deactivated by crotaline snake venoms, suggesting that it too has acquired functionally important amino acid changes (Catanese and Kress 1993). However, the evolution of this molecule has not been examined in any detail.

### Repurposed toxins

A novel mechanism of resistance has been demonstrated in species of grasshopper mice (*Onychomys* sp.) that regularly attack and eat bark scorpions (*Centruroides* sp.) and sustain their stings (Rowe and Rowe 2006, 2008). Scorpion stings are extremely painful to most mammals, but grasshopper mice show a reduced pain response compared to laboratory mice (Rowe and Rowe 2008). This pain resistance works by binding a scorpion toxin to a previously untargeted pain receptor, which induces



analgesia, blocking the effects of other pain-inducing venom components (Rowe et al. 2013). While one bark scorpion venom protein, CvIV4, induces pain by activating the sodium channel Nav1.7, it does not directly interact with Nav1.8, a downstream sodium channel which is an essential part of the pain signaling pathway (Rowe et al. 2013). However, rather than evolving a change to the direct target (Nav1.7), grasshopper mice have been shown to have amino acid changes on Nav1.8. These changes on Nav1.8 bind to another venom toxin, which induces numbness, negating painful effects of CvIV4, without altering its direct target, Nav1.7 (Rowe et al. 2013).

Similar to altered venom targets, this molecular adaptation seems to be associated largely with a small number of amino acid changes. Comparative work along with mutagenesis revealed that although a particular amino acid site determines most of the sensitivity to venom (resistance via analgesia) in grasshopper mice, this same residue is present across a diverse array of mammals, all with diverse natural histories that are not necessarily associated with venom exposure (Rowe et al. 2013). Thus, scorpion venom inhibition in grasshopper mice is likely an exaptation which perhaps has predisposed this species to exploit a toxic prey item (Rowe and Rowe 2006, 2008; Rowe et al. 2013).

### Acquired immunity

Laboratory mice (*Mus musculus*) have been used to determine baseline toxicity of snake venoms for decades, but recent work suggests that mice may mount an immune response via mast cell (MC) activation when injected with venom from various snake species. Metz et al. (2006) found that MCs released carboxypeptidase A (CPA) that protected against systematic consequences of venom injections. Furthermore, MC-deficient mice have increased susceptibility to certain venoms (Schneider et al. 2007; Akahoshi et al. 2011; Marichal et al. 2013). Mouse IgE, FcεRI-expressing effector cells and MCs are all involved in acquired immunity to venom and can result in increased survival to lethal doses of venom (Starkl et al. 2016). However, this effect varies between strains of mice, suggesting that potential for acquired resistance via type 2 immunity is a trait that may be inherited, and as such subject to natural selection. These studies represent a mechanism for mitigation of venom morbidity and mortality previously undescribed for any species, including those for which venom resistance is well documented.

Though little is known about acquired immunity as a mechanism of venom resistance in free-living mammals, it may play a substantial role for both predators and prey that survive bites from venomous snakes. Additionally, ophiophagous mammals are known to eat the whole snake, including the venom glands (Almeida-Santos et al. 2000; Begg et al. 2003). This suggests that venom proteins come into contact with mucus membranes before digestive enzymes are able to degrade them, thus providing an additional route of sub-lethal exposure which may subsequently serve to bolster immunity to venoms. Whether via mucosal or subdural (injected) exposure, this mechanism may serve to supplement general innate mechanisms of resistance described above (Mowat and Weiner 1999; Ogra et al. 2001).

### Coevolution of resistance and venom at different evolutionary timescales

Coevolution operates and can be studied at different evolutionary timescales (Thompson 2005). At short timescales, coevolution plays out among geographically structured populations that are connected by gene flow, creating a selection mosaic across the landscape (Thompson 2005). This has been termed the Geographic Mosaic Theory of Coevolution, and its predictions have been supported in a variety of predator–prey systems (Hanifin et al. 2008; Nash et al. 2008; Toju 2008), where hot-spots of reciprocal selection and cold-spots without it exist for all pairs of enemy species. Studies have documented population-level variation in venom resistance (Poran et al. 1987; Biardi 2008; Rowe and Rowe 2008; Biardi and Coss 2011; Holding et al. 2016; Pomento et al. 2016), but the possibility that this variation reflects geographically variable coevolutionary selection pressures requires parallel assessments of variation in both the venom and venom-resistance phenotypes to determine whether local venom variability selects for variable resistance, and vice versa (Janzen 1980; Gomulkiewicz et al. 2007).

At longer evolutionary timescales, phylogenetic analyses allow us to investigate the long-term dynamics of coevolution. Integrating phylogenetic predictions with molecular biology techniques for the expression and *in-vitro* testing of mutations and ancestral protein states permits assessment of the evolution of adaptive function. Thus, we can empirically test hypotheses about the mode and tempo of adaptive coevolution. Below we review the evolutionary and coevolutionary insights that can be gained through comparisons made at both timescales for species of venomous and resistant animals.

### Short timescales—population-level variation

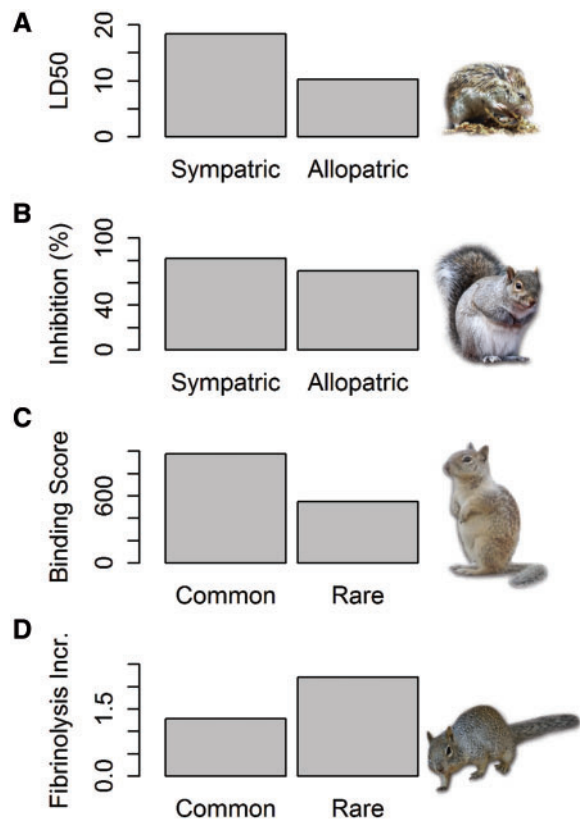
Population-level variation in venom composition is common (Chippaux et al. 1991; Daltry et al. 1996; Alape-Girón et al. 2008; Gibbs and Chiucchi 2011; Rokyta et al. 2015) but only a few studies have quantified intra-specific variation in levels of venom resistance (Poran et al. 1987; Poran and Coss 1990; Biardi et al. 2006; Biardi 2008; Rowe and Rowe 2008; Biardi and Coss 2011; Pomento et al. 2016). These few studies demonstrate a general pattern where the frequency of encounters with venomous enemies plays an important role in maintaining resistance (Fig. 3). For example, in the southern grasshopper mouse, one population that is sympatric with the Arizona bark scorpion was more resistant to the venom of this scorpion than a different population living without scorpions (Rowe and Rowe 2008). Similarly, squirrels seem to be more resistant when sympatric with rattlesnake predators. In the California ground squirrel (*Otospermophilus beecheyi*), LD<sub>50</sub> and overall serum-to-venom binding scores are higher, and serum-based inhibition of metalloproteinase and hemolytic activity are more effective in areas with many snakes, compared to sites where snakes are absent or rare (Poran et al. 1987; Biardi et al. 2000, 2006; Biardi 2008). The closely related rock squirrel (*O. variegatus*) shows a similar pattern in its ability to limit adverse effects of venom fibrinolytic activity (Biardi and Coss 2011). Finally, serum from the eastern gray squirrel (*Sciurus carolinensis*) is more effective at inhibiting timber rattlesnake (*Crotalus horridus*) metalloproteinase activity in a population where the snakes occur, than in a population where the snakes are absent (Pomento et al. 2016).

These population-level patterns suggest a significant role for local selection from venomous enemies in maintaining high resistance in the face of potential costs; however, the role of coevolution in these systems is less certain. For example, the encounter rate with scorpions may select for grasshopper mouse resistance, with no reciprocal evolutionary response in the scorpion population. Coevolution requires an evolutionarily response in the venomous species to variation in some aspect of the resistance phenotype, and vice versa (Janzen 1980). Analysis of selection gradients and reciprocal selection (Lande and Arnold 1983; Brodie and Ridenhour 2003), tests for trait correlations among populations in each species (Hanifin et al. 2008), and analysis of whether trait variation reflects local adaptation to variation in the other species (Blanquart et al. 2013) can provide such support.

### Case study

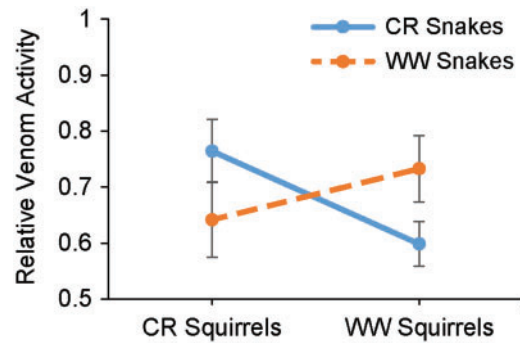
To provide an example of using measures of local adaptation in venom and resistance to study coevolution, we describe our recent work on venomous rattlesnakes and their resistant squirrel prey (Holding et al. 2016). Local adaptation was measured using reciprocal crosses of venom from northern pacific rattlesnakes (*C. oreganus*) and blood serum from California ground squirrels collected among 12 populations where these species interact. The SVMP activity of venoms was measured twice, once on the venom alone, and again after incubation with squirrel serum containing inhibitors. This allowed venom inhibition to be scored as a measure of the snake–squirrel interaction in sympatric and allopatric combinations of venom and serum. The fully reciprocal cross of all 12 populations showed that the snakes possessed venom that is locally adapted to overcoming inhibition of metalloproteinase activity by squirrel serum factors, while the sign and magnitude of local adaptation varied across the sites in a way that is partly predicted by environmental variation. Whereas all rattlesnakes maintained some venom metalloproteinase activity following treatment with ground squirrel serum, populations of snakes retained more SVMP activity with local than with foreign ground squirrels (Fig. 4).

This work provides evidence that venom resistance does not just vary as a function of location, but that different venom and resistance phenotypes can show complex interactions in their effect on the outcome of envenomation. The predator-genotype  $\times$  prey-genotype ( $G \times G$ ) interactions necessary for local adaptation, and  $G \times G \times$  environment interactions characteristic of geographic selection mosaics (Gomulkiewicz et al. 2007), seem to exist for venom and resistance in this system. Furthermore, the snake was the locally adapted species in this interaction, suggesting that the prey are not always ahead in coevolutionary interactions involving venom and resistance as suggested by the general Life-Dinner Principle (Dawkins and Krebs 1979). Finally, the existence of local adaptation merits a reappraisal of the idea that escalatory arms races generate much of the variation in venom and resistance, as such dynamics are not predicted to lead to local adaptation. Instead, the outcome of envenomation in this system appears to be determined by how effectively inhibitor X binds to venom protein Y. In the context of Fig. 2, the venomous species is hypothesized to benefit from a match to target and a mismatch to inhibitors, antibodies, and repurposed venom receptors, while the opposite is true for



**Fig. 3** Various measures of venom resistance from the four prey species where resistance has been analyzed for variation at the population level, where populations were assayed that vary in terms of presence/absence or density of specific venomous predators. Each species shows a pattern of local adaptation where there is higher venom resistance in locations where selection from a specific venomous predator is expected to be stronger due to greater abundance of snake predators (A) southern grasshopper mice (*O. torridus*) require higher lethal doses of bark scorpion (*C. exilicauda*) venom when sympatric with the scorpions (Rowe and Rowe 2008); (B) eastern gray squirrels (*S. carolinensis*) are more effective at inhibiting SVMPs when sympatric with timber rattlesnakes (*C. horridus*; Pomento et al. 2016) (C) California ground squirrels (*O. beecheyi*) have higher serum to venom binding scores when in areas where northern Pacific rattlesnakes (*C. oregonus*) are more dense (Biardi 2008); and (D) the serum of rock squirrels (*O. variegatus*) limits the increase in venom fibrinolytic activities observed when venom and serum are mixed when taken from areas where western diamondback rattlesnakes (*C. atrox*) are more common (Biardi and Coss 2011).

the resistant species. These molecular matching mechanisms, which pit venom phenotypes against resistance phenotypes in a binding-avoidance-binding-seeking fashion (Dybdahl et al. 2014; Cagliani et al. 2016) are distinctly different in action from a quantity-based escalatory arms race. The discovery of a role for phenotype matching (as in phenotype matching versus phenotype differences: Ridenhour



**Fig. 4** A pattern of rattlesnake local adaptation to avoiding metalloproteinase inhibitors in mammal serum from a reciprocal crossing study of two California populations of northern Pacific rattlesnakes (*C. o. oregonus*) when paired with sympatric and allopatric California ground squirrel (*O. beecheyi*) serum. Shown is the percentage of venom metalloproteinase activity maintained after incubation with inhibitory serum relative to venom-only trials (mean  $\pm$  SE). The venom of 10 snakes and 10 squirrels from each site was used, with snake venom and squirrel serum were randomly paired. Animals were collected from Wind Wolves Preserve (WW) and Chimineas Ranch Ecological Reserve (CR).

and Nuismer 2007) in coevolving venom and venom resistance proteins does not exclude a parallel role for arms race dynamics, which could govern the speed of enzymatic action or the overall concentration of each protein (Holding et al. 2016). Future work to characterize the role alternative coevolutionary dynamics in shaping the overall complex venom phenotype will be valuable.

#### Longer time-scales—phylogenetic and functional reconstruction

Understanding the evolution of the molecules involved in venom resistance can also benefit from the application of phylogenetic approaches at deeper evolutionary timescales. For example, the identification of multiple instances of convergent acquisition of a venom-resistant nAChR molecule was only revealed through a comparative phylogenetic study that included species of resistant and non-resistant mammals (Drabek et al. 2015). Similarly, powerful phylogeny-based tests of positive selection (Yang et al. 2005; Yang 2007) have revealed instances of positive selection on particular amino acid sites in particular venom-resistant lineages (Jansa and Voss 2011; Drabek et al. 2015). Robust phylogenies also provide the essential framework for reconstructing ancestral character states, including ancestral protein sequences (Pauling and Zuckerkandl 1963). Integrating these types of phylogenetic approaches with laboratory studies of ancestral protein function

has been coined the “functional synthesis” (Dean and Thornton 2007), and provides much promise for understanding the evolution of protein function across diverse systems (for review see Hartley et al. 2006; Hoekstra et al. 2006; Dean and Thornton 2007; Harms and Thornton 2013)

While the phylogenetic approaches mentioned above have laid the groundwork in this field of research, the promise of the functional synthesis has yet to be applied to understanding the evolution of proteins involved in venom resistance. Golding and Dean (1998) suggest the ideal candidates for this empirical functional approach are those in which there is a clear and measurable physiological shift, strong evidence for selection, and robust phylogenetic histories. With the growing number of identified instances of venom resistance in mammals (reviewed in Voss and Jansa 2012), case studies of adaptive evolution of venom-resistant molecules (Jansa and Voss 2011; Drabeck et al. 2015), the explosive growth of phylogenetic knowledge for mammals, and the development of heterologous expression systems for these proteins, we are now poised to apply the functional synthetic research program to understanding how resistance evolves (Supplementary Table 1). Although much of this research is in its infancy, below we highlight a case approach which shows how the functional synthesis could be applied to understanding the evolution of molecules involved in venom toxicity and resistance.

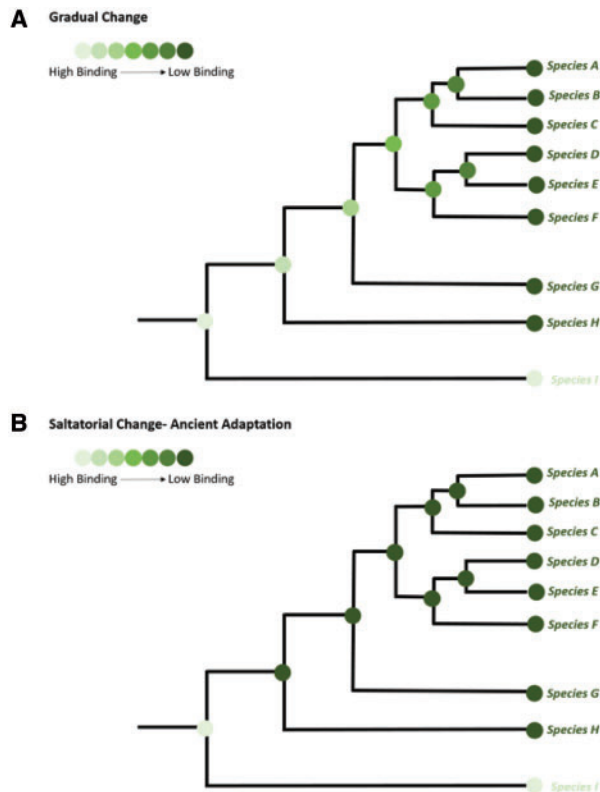
#### Case study

Using an existing system as an example, we will expand on one case outlined in Supplementary Table 1 for which the functional synthetic approach is tractable. Specifically, molecular models suggest that vWF can no longer bind botrocetin, a venom C-type lectin (CTL), in resistant opossums (Jansa and Voss 2011), and recent physiological assays provide strong evidence that opossum vWF in fact does not respond to very high doses of botrocetin (D. H. Drabeck, unpublished data). Of the nine species of didelphid marsupials in the clade Didelphini, seven are either known to eat venomous snakes and/or be resistant to vWF-binding venom CTLs. Though members of this clade show accelerated adaptive evolution at the CTL binding site (vWF A1), sequence variability at these sites is present between species, suggesting that there may be functional variability in vWF resistance across Didelphini (Jansa and Voss 2011). A robust species-level phylogeny for New World opossums makes it possible to employ powerful modern phylogenetic tools to infer ancestral protein sequences of vWF for all members of this

clade. Biochemical assays that quantify binding affinities of opossum vWF for venom CTLs can provide functional data for each amino acid site of this protein, including its ancestral states, across the clade on which it has evolved. These data can illuminate the molecular and functional tempo and mode of evolution of adaptive traits. For example, Fig. 5 illustrates two competing hypotheses for the evolution of a resistant target to a venom toxin. In the first hypothesis evolution of resistance is gradual, and mutations appear progressively (and perhaps convergently) along the lineages leading to the resistant phenotype. In the alternative scenario, evolution is saltational—the mutations responsible for resistance all arise at the base of the resistant clade, and subsequent mutations (if any) have no effect on the resistance phenotype. Importantly, the only way to distinguish between these two hypotheses is through functional laboratory studies of reconstructed ancestral proteins. If resistance evolved suddenly at the base of the clade, then ancestral proteins will not vary in their binding ability and will have similar binding ability as the modern proteins, all of which exhibit the resistant phenotype. Alternatively, if acquisition of resistance is a gradual process, then ancestral proteins should vary in their ability to bind the toxin protein.

Applying these same methods to the evolution of interacting snake venom proteins, such as botrocetin, would allow us to examine the functional evolution of a potentially co-evolving molecule. Work like this would provide the first example of a hypothesized coevolutionary interaction in which both interacting partners have been functionally characterized at the molecular level, and would serve to test assumptions about how evolutionary and ecological mechanisms shape functional changes in ostensibly coevolving proteins (Gomulkiewicz et al. 2007; Ridenhour and Nuismer 2007). In particular, the simplest but most important assumption to test is whether there is evidence of reciprocal evolutionary change in molecular function in a natural system, which has long been an assumption of many canonical studies of coevolution, but has yet to be demonstrated (Bull and Molineux 1992; Brockhurst et al. 2003; Mizoguchi et al. 2003; Jessup et al. 2004; Hanifin et al. 2008; Jansa and Voss 2011; Scanlan et al. 2011). Venom resistance is a system which is plentiful with opportunities such as this for evolutionary biochemists, as there are several other putative protein–protein coevolutionary interactions (Supplementary Table 1), and likely many more to come as this field develops.





**Fig. 5** Alternative hypotheses for the evolution of a venom-resistant protein. Under the gradual-change hypothesis (A), venom resistance is acquired gradually during the evolution of the venom-resistant clade. Under the saltatorial-change hypothesis (B), venom resistance (measured as ability to bind a venom toxin) is acquired once at the base of the venom-resistant clade. Under both scenarios, extant taxa have equivalently low binding affinities to the venom toxin (indicated with dark green dots at the tips of the tree). The only way to distinguish between the two hypotheses is to assess binding affinity for reconstructed ancestral proteins, which will vary in their binding affinity under hypothesis (B), but not hypothesis (A).

## Future directions

### Integrated phenotypes and the evolving resistome

The “resistome” can be considered an “integrated phenotype” in the same sense that the term has been applied to a wide variety of morphological traits (Murren 2012). Specifically, an integrated phenotype is defined as a set of functionally related traits that interact with each other in a way that affects their overall function (Murren 2012). The “resistome” then is the collection of molecules that confer venom resistance through the mechanisms outlined above. Methodologically, the use of “antivenomics” (Calvete et al. 2011) to isolate individual molecular components of resistance and then use them in *in-vitro* tests of function involving single

versus multiple components allows us to address how these parts of the “resistome” might interact to confer organismal resistance. Are these interactions functionally synergistic or simply additive (Yeh et al. 2006)? Can information on functional interactions between proteins be used to organize individual molecular components of resistance into functionally defined modules (Yeh et al. 2006)? Do components of resistance show negative tradeoffs with other fitness-related traits in prey that could limit the evolution of overall resistance? Direct estimates of function are difficult to conduct for components of morphological traits, and so we see a special role for resistance (and venom) for assessing phenotypic integration in complex phenotypes from a functional perspective.

This “antivenomics” approach is a specific example of the general approach of using high-throughput sequencing, modern bioinformatics, and comparative analysis to identify the molecular basis of adaptive traits. Such an approach can also provide a guide for researchers to identify venom targets and inhibitors that are currently undetected. Subsequent comparative studies of these newly identified molecules holds enormous potential for understanding the molecular evolutionary processes involved in generating and maintaining variation in venoms as well as in venom resistance. The application of these and other integrated methods requires robust species and gene phylogenies, which are becoming increasingly common. Additionally, for both venom and resistance proteins which are members of large protein families and purported to be rife with gene duplication and neofunctionalization, it is vital to understand complete gene histories to apply methods such as ancestral reconstruction of phenotypes and tests of positive selection. Though only a few mechanisms resistance are known and even fewer examined in detail, applying these methods will doubtless illuminate the way forward in this field.

## Summary

The hypothesis that venom and resistance are co-evolved traits has been invoked because of the matched nature of these traits, yet evidence for reciprocal selection leading to evolutionary change in both venom *and* resistance traits has yet to be convincingly demonstrated. Here, we suggest several approaches which aim to provide strong evidence for reciprocal molecular coevolution in the context of venom and venom resistance evolution. Population level approaches which utilize reciprocal pairwise testing, analyses of selection gradients, and

tests for trait correlations, can identify sources and strength of selection, potential costs and trade-offs, and examine statistical support for correlated adaptive variation. Evolutionary biochemical approaches allows the examination of the ancestral functional changes of proteins involved in venom and resistance, elucidating the mode and tempo of (co)adaptive change. Expanding the examination of single venom-target interactions to understanding the full complexity of venom resistance is necessary to examine this trait as an integrated molecular phenotype, and is now feasible with modern high-throughput approaches. Given these insights, we stress the value of understanding venom resistance as a coevolving adaptive trait, and its potential for providing insights as to how coevolution occurs at a molecular level.

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## Supplementary data

Supplementary Data available at *ICB* online.

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