Evolution of Resistance to Toxins in Prey

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Abstract

Venoms, as simple to complex mixtures of toxic components, are well understood to be used as trophic weapons by a range of predator species. Ecological predictions obviate the response of putative prey species against predator attacks, such as the development of biochemical defenses that allow prey species to evade predation, namely, resistance. Current hypothetical predictions indicate that venom toxicity and resistance form an antagonistic dyad that may be described as a coevolutionary chemical arms race. The development of resistance in prey populations is expected to drive the evolution of novel toxicities in predator populations and vice versa, given that predator-prey pairs are stably associated through evolutionary time. The utility of a chemical arms race model to describe

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toxicity-resistance systems as well as known information about natural resistance mechanisms derived against venomous predators are discussed across prey species of a wide range of venomous predators. The efficacy of resistance, mechanism(s) of resistance, phylogenetic breadth of resistance, and phylogeographic distribution of resistance are provided where information is available. For many predator groups, known prey resistance is not well described, and we discuss the cause(s) of such a gap in understanding, as well as future directions for resistance research and application of known resistance information for practical and theoretical purposes.

Keywords
Predator prey interactions • Resistance • Mechanism • Evolution • Chemical arms race

Introduction

Venoms are simple to complex mixtures of toxic components that are conveyed through specialized delivery systems to subdue prey (Mackessy 2002, 2010), and possibly to aid in predigestion of prey tissues (Pough and Groves 1983; Mackessy 1988). For prey species, on the defensive side of the predator/prey dyad, becoming a meal greatly decreases lifetime fitness, and predictably many forms of predator evasion have been documented. This essay discusses the nature of chemical defenses against predator venoms, often described as venom resistance, that have arisen in response to the selective pressure imposed by the chemical weapons of predators. For the purpose of this discussion, venom resistance is defined as the endogenous chemical/physiological capacity of a prey species to prevent or hinder the pathologic consequences of envenomation by a predator species. By this definition, in the absence of resistance mechanisms, venoms are pathological to prey species. This venom antagonism is in contrast to cases where a predator's venom has no bioactive effect on one or a group of potential prey species, but may be lethal to other species or groups of species (i.e., prey-specific venoms: Heyborne and Mackessy 2013; Mackessy and Saviola 2016; Pawlak et al. 2006, 2009). Venoms represent complex molecular weapons to defend against, and venom resistance is assumed to be conferred by venom-resistant molecules or mechanisms that are able to neutralize partially or fully the negative effects of a venom and its toxic constituents. Successful resistance should allow prey species to evade capture and digestion. There is evidence that in some cases, chemical neutralization of venomous components may not be sufficient to allow prey species to escape predator behaviors that enable prey capture, regardless of the effectiveness of venoms. However, behavioral responses that allow prey species to evade predators, or allow predators to successfully capture prey species, independent of the role of venom, will not be discussed.

This chapter focuses on known cases of prey resistance to predator venoms. Resistance in some groups, such as prey species of venomous snakes, is well described, but resistance in other groups, such as prey species of venomous insects,

is not well understood, and little information appears to be available even after extensive literature searches. Instances of resistance are discussed in relation to the venoms they are able to neutralize. Each section provides information regarding efficacy of resistance, mechanism(s) of resistance, phylogenetic breadth of resistance, phylogeographic distribution of resistance, as well as other relevant information about the nature of the predator/prey pairs in question. The discussion here centers on chemical arms races between venomous predators and resistant prey; that is, the focus remains only on animal/animal interactions, as there are no known cases of an animal venom used to subdue plant or prokaryote prey, or a plant that uses venom to dispatch prey species. Following the predator-specific sections is a concluding discussion of our current understanding of prey resistance to natural toxins, future directions for resistance research, and possible applications of resistance systems for practical and theoretical purposes.

Coevolution of Predator Venoms and Prey Resistance

When considering prey resistance, the underlying issue is whether a coevolutionary response to the selective pressure of predator venom exists within the system. Venoms, as derived trophic adaptations, are expected to experience selection pressure from mechanisms that allow prey species to evade predation. The appearance of resistance molecules in response to the derivation of new snake venom toxicities is expected to follow Dawkins and Krebs' (1979) model for an arms race between two taxa in an antagonistic coevolutionary relationship. A predator develops a chemical weapon (venom), which is used to subdue a prey species. As predators capitalize on susceptible individuals, the diversity of the prey population becomes limited to those individuals who are able to evade predation. These remaining individuals may persist because of phenomena like behavioral modifications, changes in microspatial distribution, or the appearance of a chemical mechanism that inhibits the toxic action of the predator's venom, namely, resistance. This resistance phenotype is expected to increase over time as the snake predator becomes increasingly incapable of incapacitating prey with the new resistance phenotype. Variations in predator and prey phenotypes are expected to follow each other through time in a frequency-dependent manner that creates new resistances to new toxicities and vice versa.

Several expectations follow from this scenario of the development and maintenance of resistance in prey. First, predator/prey pairs are expected to associate with each other for stable periods of time. By definition, predators and prey should respond in sequential and reciprocal manners as the opposing partner develops a new offensive or defensive strategy to the other. Van Valen (1973) described this stable reciprocity in his postulation of the Red Queen hypothesis. Much as the Red Queen in Lewis Carroll's *Through the Looking Glass* tells Alice that to stay in one place she must keep running, Van Valen hypothesized that for either predator or prey to "stay in one place" (i.e., persist through evolutionary time), they must continue to evolve. By extension, if one of the predator/prey pair was unable to continue to respond to a newly derived trait in the other partner, they would soon become

extinct, assuming intense predation pressures on the susceptible prey phenotype. Extant predator/prey pairs should demonstrate some balance between the relative abundance of resistant and susceptible individuals, keeping in mind that this balance may be skewed toward one partner or the other at any given time point.

In addition to stable reciprocity, the timeline of coevolutionary relationships is expected to develop over longer rather than shorter timescales. When investigating the frequency and mechanism of resistance, it may be that the newly evolved resistance or toxicity is at such low abundance that detection of this functionality is nearly impossible. In the real time of academic research, the turnover of enough generations of predator or prey species to produce a new functionality may be too slow for any given researcher to describe in a lifetime. Additionally, whether novel toxicity or resistance are diversifying or are being selected against may depend on the historic length of predator/prey associations. Sunagar and Moran (2015) compared the rate of diversification of a variety of toxin groups against the relative age of a number of venomous species' lineages. These authors proposed a "two-speed" mode of venom evolution, where more recent lineages of venomous predators, such as cone snails and venomous snakes, show increased diversifying selection, and older lineages appear to be under increased levels of purifying selection. The authors proposed that diversifying selection for venomous predators would be associated with prey base or niche expansion; however, it is possible that diversifying selection may allow for maintenance of a stable relationship with current prey species and simply throw frequency-dependent selection of a chemical arms race into another round of novel toxicity and resistance development. In any case, younger or older lineages are not fixed in a selective regime and may experience a switch from purifying to diversifying selection and vice versa. Thus, it appears that the age of the lineage in question may increase the likelihood that resistance is a prominent feature of prey populations or that the toxicity of the predator may have an advantage over prey defenses (such as in Holding et al. 2016), again making resistance more difficult to detect.

It is cogent to note that while a chemical arms race scenario is presently a "best guess factor" as the driving force for biochemical diversification of venoms over evolutionary time, numerous cases of prey-specific toxicities and venom resistances are documented in the literature, which lends support to a coevolutionary relationship between toxicity and resistance. In support of the chemical arms race scenario, research into the relationships between venomous snakes and their resistant prey will serve as a test case. Current information about a diversity of resistant prey is prefaced by a discussion of theoretical and methodological approaches to evaluating the importance of coevolutionary processes in the development of resistance.

Resistance to Snake Venoms

Natural resistance to predator venoms is best described in prey species of venomous snakes, particularly mammals. The impetus for this wealth of knowledge comes from the attempt by snake venom researchers to elucidate the merits of the

hypothesis that diet has served as a major selective pressure shaping snake venom composition. Over the past several decades, researchers have demonstrated that venom composition may vary across geographic space and ontogenetically (see Mackessy (2010)) and has been purported to vary with diet (e.g., Gibbs and Mackessy 2009; Sanz et al. 2006). The more recent championing of diet as a major driver for venom compositional change is born out of an institutional debate over the origin of venom, i.e., whether venom is the product of neutral or selective processes over evolutionary time.

Near the end of the twentieth century, the issue of the origin of snake venoms as the product of neutral or selective processes became a major theoretical divide between venomous snake biologists. Scientists such as Dietrich Mebs (2001) and Mahmood Sasa (1999) argued that because snakes delivered venom in such large quantities, many times more than was sufficient to incapacitate prey, venom must not have arisen from selective processes and was "overkill." Considering the discrepancy between the minimum amount of venom required for prey capture and the actual amount delivered, they argued that venom components were too metabolically costly to be used in such large quantities. Additionally, they noted that the individual components of venom were so toxic across a variety of possible prey species that there did not appear to be a selection for specific toxicities. To these authors, venom arose out of neutral evolutionary processes that allowed for the sequestration and concentration of modified somatic molecules into what we observe today as the components of snake venom.

This neutral view was quickly challenged by research showing that the notion of overkill was unlikely. Hayes et al. (2002) demonstrated that venomous snakes had control over the amount of venom released in striking a prey item. The amount of venom delivered was more than absolutely necessary to subdue prey items, but control over venom delivery indicated that there was a functional role for allowing large volumes to be expressed in snakebite envenomation. Saviola et al. (2013) demonstrated that, at least in venomous snakes from the family Viperidae (vipers, pit vipers, and other solenoglyph venomous snakes), a large bolus of venom was required in order to deliver a particular molecule in high enough concentration to allow the snakes to recover their envenomated prey item. Viperid snakes often use a sit-and-wait ambush strategy and strike prey as they cross the snake's path; prey that has fled the ambush site and succumbed to the effects of the venom is then recovered, often at some distance to the ambush site. The process of prey relocation may be challenging because prey may escape in any direction in three-dimensional space, and thus a relocator molecule is needed to track the envenomated prey item effectively. At this point, an arms race hypothesis was explored to explain the evolution of the complex phenotype of snake venom and associated delivery systems.

A number of prey species groups show resistance to snake venoms, and a wide variety of evidence helps to corroborate a chemical arms race scenario. Each species group will be treated separately, and data has been compiled on the prevalence and mechanism of resistance. Any study attempting to uncover coevolutionary relationships between species pairs faces the challenge of using extant and historical evidence to infer reciprocity across evolutionary time. A number of approaches are

often used and synthesized to confirm coevolution (Futuyma and Slatkin 1983). In the case of resistance/toxicity systems, the demonstration of resistance through standard toxicity assays is required. Anecdotal evidence for prey ability to avoid predation may not be explained by chemical resistance; resistance must be confirmed through direct challenges with physiologically and biologically relevant doses of venom. As novel phenotypes should appear in a single individual or small population of individuals and radiate out in the direction of gene flow, locality of both predator and prey must be taken into account. A record of the geographic distribution of populations with resistance or susceptibility may further allow for spatial correlation with the range of the venomous partner species. Thus, a biogeographic account of current resistance may be constructed. Longitudinal documentation of the biogeography of a particular resistance mechanism may offer some insight into the rate of change in the dynamics of resistance and toxicity for a given species pair. To date, it does not appear that this type of long view has been established for any system involving snakes, and even if one could be constructed, if reciprocal responses occur over evolutionary time, this may preclude any detection of active flux in the relationship between toxicity and resistance within the lifetime of a given researcher.

Following initial screening for resistance, mechanistic descriptions are often elucidated that demonstrate the direct ability of prey physiologies to negate the pathologic effects of venoms. As mentioned earlier, prey species are challenged by the (often) complex phenotype of predator venom, and their responses may range from a wholesale attempt to neutralize the diversity of toxins in a venom to mechanisms that attack a limited number of toxins. Finally, some attempt must be made to connect species pairs in evolutionary time and demonstrate stepwise evolutionary change. This correlation through time is the most difficult line of evidence to obtain, as current technologies limit these types of studies to phylogenetic comparisons between predator and prey species complexes (Filipiak et al. 2016; Page 2002; Suchan and Alvarez 2015). Correlation between the divergence of predator and prey clades would seem to indicate reciprocal evolutionary divergence; however, correlational analyses are limited in their ability to confirm causality between the coevolution of toxicity and resistance and speciation or divergence in predator and prey taxa. It is also possible that some common biotic or abiotic pressure, unrelated to potential coevolutionary scenarios, caused cladogenesis in both predator and prey species, and resistance is secondarily derived.

Resistance to Snake α-Neurotoxins

A resistance mechanism that has been confirmed across a diversity of mammalian predators and prey is the ability to tolerate snake α-neurotoxins, acetylcholine receptor (AChR) agonists. Ovadia and Kochva (1977) demonstrated that mongoose sera challenged with venoms from snakes in the family Elapidae (cobras, kraits, and other opisthoglyphous snakes) was able to neutralize the effects of the venom.

Later research uncovered that this resistance to elapid venoms is directed against α-neurotoxins that make up a significant portion of the total venom protein. Barchan et al. (1992) sequenced the mongoose AChR and detected a number of non-synonymous mutations in the ligand binding site of the AChR. Hypothesized structures for these mutations indicate a confirmation change in the ligand binding site that prevents α-neurotoxins from binding while still allowing acetylcholine (ACh) to bind its receptor. Later work (Asher et al. 1998) further demonstrated that the mongoose's resistant AChR prevented α-neurotoxins from binding while still allowing ACh to bind with higher affinity than non-resistant type AChR found in rats. This elevated binding affinity indicated that mongoose AChR was able to prevent complete binding of α-neurotoxins while allowing ACh to bind with little steric or concentration-dependent competitive hindrance from α-neurotoxins that had inundated synaptic junctions. A slight conformational change was sufficient to create near complete resistance to α-neurotoxins.

In addition to mongooses, similar conformational changes in acetylcholine receptors have been documented in the Chinese cobra (*Naja atra*), the Javelin sand boa (*Eryx jaculus*), the dice snake (*Natrix tessellata*), and also in the European hedgehog (*Erinaceus europaeus*) (Barchan et al. 1992; Neumann et al. 1989). Resistance in *N. atra* is most likely protection against auto-envenomation; however, it is possible that this resistance may allow evasion from cannibalism or predation by other sympatric elapid snakes. The example of *E. europaeus* provides an additional mammalian example of resistance to α-neurotoxins, but perhaps the most intriguing example of resistance is the case of the three non-venomous snakes. Considering the ongoing debate among snake venom toxinologists about the ultimate origin of snake venom proteins and the delivery apparatus (e.g., Fry et al. 2012), the appearance of α-neurotoxin resistance across more basal snake taxa begs the question of whether resistance is intrinsic to snake physiology or has appeared independently several times throughout the radiation of the snakes. In any case, a better understanding of the molecular origin of snake resistance to snake venoms could indicate a coevolutionary predator-prey situation if the hypothesis that resistant, non-venomous snakes were once or are currently preyed upon by venomous snakes is supported.

Resistance in Woodrats (Genus *Neotoma*)

As a follow-up study to anecdotal evidence of resistance in Southern Plains woodrats (*Neotoma micropus*), Perez et al. (1978) challenged woodrats with venom from the western diamondback rattlesnake (*Crotalus atrox*), showing that these rodents had greatly elevated tolerance to the venom compared to a laboratory mouse control. Perez et al. (1979) further showed that this resistance mechanism was able to significantly decrease the hemorrhagic effects of *C. atrox* venom for *N. micropus*. De Wit (1982) screened a second *Neotoma* species, the eastern woodrat (*Neotoma floridana*), with the venom from Osage copperhead (*Agkistrodon contortrix phaeogaster*) and detected a similar resistance to hemorrhagic toxins. It appeared

that venom resistance was shared across the genus. Using electron microscopy, Huang and Perez (1982) further showed that *N. micropus* suffered little hemorrhage or muscle damage following envenomation. Some mitochondrial and myofibril damage were detected, but it appeared that resistance also prevented myotoxic pathologies, especially in comparison to laboratory mouse controls. A candidate antihemorrhagic resistance molecule was purified and partially described by Garcia and Perez (1984). This single, non-enzymatic resistance molecule was able to bind and neutralize *C. atrox* toxins. Binding was shown to be non-polyvalent, and the authors concluded that this candidate molecule was not an immunoglobulin. Unfortunately, it does not appear that further descriptive work has been completed on this resistance molecule, and no biogeographic or further phylogenetic information is available regarding the distribution and prevalence of this resistance mechanism in *Neotoma*.

Resistance of Ground Squirrels (Genus, *Otospermophilus*) to Snake Venom Metalloproteases

Another well-described example of snake venom resistance are endogenous snake venom metalloprotease inhibitors (SVMPIs), best documented in a number of squirrel species in the genus *Otospermophilus* (formerly *Spermophilus*). Biardi and Coss (2011) showed that rock squirrel (*Otospermophilus variegatus*) serum was able to neutralize the pathological effects of venom from two species of rattlesnake, the western diamondback rattlesnake (*Crotalus atrox*) and prairie rattlesnake (*Crotalus viridis viridis*), which were sympatric to assayed squirrel populations. Challenges with venom from an allopatric species of rattlesnake, the northern Pacific rattlesnake (*Crotalus oreganus oreganus*), were not successfully neutralized. Interestingly, the venom used in these experiments was commercially purchased; however, even without a confirmation of matching locality between predator and prey samples tested, there still appeared to be an inhibitory effect against individuals from a sympatric predator species. In the same year, another team (Biardi et al. 2011) published a description of an SVMPI isolated from *O. beecheyi* serum. This molecule was able to prevent tissue damage and hemorrhage normally expected from envenomation by the sympatric *C. o. oreganus*. Further, resistance was positively correlated with the proximity of rattlesnake population to resistant *O. beecheyi*; that is, resistance was ineffective against distant populations of *C. o. oreganus*, indicating that resistance is geographically localized and requires predation (or at least offensive) pressure from the colocalized rattlesnake population to select for resistance. The authors recognized that while other mammals do not have similar SVMPIs that serve as resistance molecules, there appears to be convergence of defenses against hemorrhagic toxins, a hallmark of many viperid snake venoms. Future work in mammalian resistance to viperid venoms will confirm or reject convergence to defenses against hemorrhagic toxin classes of snake predators.

Resistance to Snake Venoms in the Opossums (Family Didelphidae)

A final group of prey items with described resistance to venomous snake predators are the opossums (Mammalia: Didelphidae). Jansa and Voss (2011) reported an increased number of non-synonymous changes in gene sequences of a hemostatic protein, von Willebrand factor (vWF), in opossums known to exploit venomous snakes as prey items. These researchers found that these non-synonymous changes are associated with binding sites for C-type lectin-like proteins found in some viperid snake venoms; changes to these regions were inferred to decrease binding affinity with these toxins. These data do not indicate that opossums preyed upon by venomous snakes have similar resistance, but later work (Voss 2013) found that a number of opossum species could be confirmed as venomous snake prey and that their relationships to known, resistant species of opossums make it plausible that they would also likely show changes to vWF. However, beyond these types of phylogenetic correlations, evidence for resistance against venom challenges is not available, and physiological data would be required to verify that resistance to C-type lectin-like proteins is sufficient to allow for evasion from predation by venomous snakes.

Correlational Evidence for Resistance/Toxicity Coevolution in Venomous Snakes

The extent of information regarding resistance to snake venoms varies depending on the species group of interest and may include as little as an initial confirmation of resistance to a full description of the resistance mechanism. In relatively few cases, functional information can be paired with evolutionary analyses to test the underlying assumptions of a chemical arms race. Barlow et al. (2009) investigated a potential coevolutionary relationship between venom specificity toward scorpion prey in four species groups of the genus *Echis* (saw-scaled vipers). They used a Bayesian inference method to plot a phylogeny of these four groups and compared the relative amounts of scorpion versus rodent prey found in the stomach contents of museum specimens, as well as toxicity assays (LD_{50}) toward scorpions (*Scorpio maurus*), to species relationships. Venoms of species groups with the highest amounts of scorpions in their diet were the most toxic against scorpion prey, while the *E. coloratus* group, rodent specialists, showed the lowest toxicity. Relative abundance of a particular type of prey scaled with the relative toxicity of the venom; for example, the *E. ocellatus* group had an intermediate amount of dietary scorpions and showed an intermediate toxicity toward live scorpion prey. The implication of this increased toxicity toward preferred prey group was that *Echis* venom has undergone selection favoring increased toxicity toward a preferred prey type. While Barlow et al. (2009) did not test for scorpion resistance, the demonstration of prey specificity that follows the best resolution of *Echis* phylogenetic relationships indicated a positive selective pressure for enhanced toxicity, perhaps

driven by prior prey resistance mechanisms. For example, a common ancestor to *Echis* may have retained toxicity toward scorpions, while sympatric Rodentia developed resistance, to the point that only *Echis* phenotypes that could shift to non-rodent prey were able to persist. Secondary diversification of the venom toxins may have restored high toxicity toward rodent prey, favoring a shift in those lineages to specializing on rodents. The availability of non-scorpion taxa, preference toward these taxa (how often they attempt to predate), and the relative resistance or susceptibility of these taxa would be needed to corroborate reciprocal selectivity of venom and resistance.

In the case of opossums, antihemorrhagic toxicity has been correlated with phylogenetic comparisons of predator and prey species. Voss and Jansa (2012) compared South American opossums and vipers, revealing that species of opossums that were too large as adults to be ingested by vipers showed no resistance to venom. Nonresistance in larger prey taxa was interpreted as the result of non-predation that venomous snakes had no behavioral inclination to attempt predating these overly large meals and thus no selective pressure to develop resistance was present. Verifying the assumption of reciprocity between predator and prey, resistance may arise or be maintained only in prey lineages that are likely targets of venomous snake predators.

Natural Resistance in Prey of Other Venomous Taxa

Presently, little information is available regarding the appearance or mechanisms of resistance in prey species of cone snails, insects, helodermatid lizards, cnidarians, centipedes, shrews, scorpions, arachnids, and anemones. The sporadic and sometimes tangential evidence that exists for resistance against a number of these venomous predators will now be discussed. Literature searches for documented cases of resistance in prey species of insects were unproductive, but protective immune reactions in non-prey species may indicate a set of mechanisms that provides resistance for prey. Metz et al. (2006) described the ability of mast cells in inbred laboratory mice to confer protection against hypothermia and death associated with envenomation by the European honeybee (*Apis mellifera*). Palm and Medzhitov (2013) later demonstrated that whole honeybee venom and the isolated pore-forming toxin, melittin, was able to induce inflammatory pathways in in vitro and in vivo experiments. The honeybee does not use its venom for prey capture; however, it may be that resistance to venoms of bee relatives in the order Hymenoptera, such as predatory wasps, rely on the escalation of similar immune and allergic responses to evade predation.

Immune responses conferring resistance to envenomation have been documented for some arachnids. Schenone et al. (1970) induced resistance to challenge doses of venom in laboratory rabbits through repeated sublethal doses of venom from the Chilean recluse spider (*Loxosceles laeta*). A ramping of immune response to venom dosing was detected by observing the increasing presence of antibodies in rabbit serum across the dosing period. Similarly, Njau et al. (1986) induced resistance to

paralysis in laboratory rabbits through repeated sublethal infestations of red-legged ticks (*Rhipicephalus evertsi evertsi*). Later, Reck et al. (2009) used serum from tick-infested cattle to confer protection again the anti-hemostatic properties of tick saliva in in vitro and in vivo assays. While defenses to parasitism by tick species do not fit with a definition of prey resistance to venom, the apparent excitation of the immune system in cattle speaks to a convergent mechanism by which arachnid venoms may be neutralized. As arachnid toxins are quite diverse, hypothesizing a general convergent mechanism may be too simplistic, but it stands to reason that in the absence of other candidate resistance mechanisms to explore, immune responses to arachnid venoms are plausibly productive.

Other than immune-based resistance to arachnid venoms, research into the application of arachnid toxins as insecticides has revealed another possibly fruitful avenue of study regarding prey resistance to arachnid venoms: the prevention of toxin binding to nervous cell receptors by structural interference. Bende et al. (2014) identified two residues in a particular region of American cockroach (*Periplaneta americana*) voltage-gated sodium channels that conferred resistance against β-Diguetoxin-DC1a from the desert bush spider (*Diguetia canities*). These researchers were attempting to discover novel targets for insecticide development and in the process uncovered the mechanism whereby some insects may avoid envenomation by desert bush spiders. Differential toxicity to prey nervous tissue has been identified for other spider predators. For example, Liu et al. (2016) documented the ability of *Araneus ventricosus* venom to block cockroach, but not mouse, voltage-gated sodium channels, suggesting the binding mechanism causes lethal effects in insects while inactive toward vertebrates. In both cases, the experiments were motivated by the development of insecticides that are insect-specific; however, these lines of inquiry reveal possible candidate resistant prey species.

Another group with preliminary evidence for resistance in prey is the sea anemones (phylum, Cnidaria; class, Anthozoa). Some species of this group capitalize on prey species that are powerful enough to escape the grasp of an anemone, such as teleost fishes, or have durable defenses to infiltrate, such as mollusks, which necessitate the use of venom for prey capture (Frazão et al. 2012). While direct evidence of the development of resistance in putative prey species is not available, there are a number of studies that indicate two mechanisms that confer resistance to mutualistic anemone fishes (genera *Amphiprion* and *Premnas*) and crustaceans (representatives from several genera; Mebs 2009). First, mutualistic partners may develop or acquire a mucus coat that neutralizes defensive compounds on the surface of the anemone, or else allow the partner to associate closely with the anemone without eliciting the firing of venom-delivering stinging cells, nematocysts (Frazão et al. 2012). A second line of defense in mutualistic partners of sea anemones are internal defenses that allow the partners to neutralize venom toxins, should the nematocysts fire. Mucus coat defenses appear to be the main defense for mutualistic crustaceans (Mebs 2009), and mutualistic anemone fish appear to use combinations of both strategies. Mebs (1994) tested three mutualist *Amphiprion* anemone fish species against the venom of four sea anemone species, finding limited endogenous resistance in cohabitating fish species. In some cases, the mutualist fish was not

resistant to the venom of its own host anemone. Together, these trends indicated that the development of a protective mucus coat was the main defense against host venom for anemone fish and that resistance may or may not be necessary for successful mutualistic relationships. A survey by Nedosyko et al. (2014) of the number of associations between all 26 species of mutualist anemone fishes and all ten species of host anemones indicated that anemones with the least and most toxic venoms were inhabited by the fewest numbers of mutualist species. Intermediate toxicity was associated with the greatest diversity of mutualist species, and these authors concluded that there must be a trade-off in the amount of protection versus the amount of risk for potential mutualist species. For putative prey species of anemones, differential toxicity across anemones may reflect a variegated landscape of selective pressures that could lead to the development or refinement of resistance mechanisms. However, no evidence of resistance in prey species is currently available. One mechanism of resistance that may be of interest for future investigation is changes in the architecture of ion channels of sea anemone prey species. Gasparini et al. (2004) compared the previously documented ability of scorpion and sea anemone venoms to block voltage-gated potassium channels, indicating convergence on the same toxic mechanism, i.e., binding a specific portion of the pore complex to prevent the passage of current through these channels. Thus, candidate resistance mechanisms to sea anemone venoms may arise as the result of non-synonymous changes to exposed surfaces of ion channels that reduce the ability of toxins to bind and block physiological currents. This kind of change has given rise to the tetrodotoxin resistance seen in red-sided garter snakes (*Thamnophis sirtalis*), allowing the predator to capitalize on otherwise deadly prey (Feldman et al. 2012; McGlothlin et al. 2014).

Finally, resistance to scorpion venoms has been documented, but further investigations of the mechanisms or biogeography of resistance have yet to appear in the literature. Israeli-Zindel et al. (1973) derived LD_{50} values for venom of the yellow scorpion (*Leiurus quinquestriatus*) toward seven species of beetles and a strain of laboratory mouse. They found a wide range of susceptibility and resistance and demonstrated that several beetle species tested had several orders of magnitude greater tolerance to the venom than the laboratory mouse. When the hemolymph of the most resistant beetle was analyzed 24 h following envenomation, detectable venom concentration had dropped to 40% of the original level. A further assay testing the specificity of resistance revealed that an enzyme-deactivating mechanism confers resistance to this beetle species. However, beyond this early study, few have tested the ability of plausible prey species to defend against scorpion envenomation, and most studies focus on species that are unlikely prey of scorpions, such as rodent predators of scorpion (Rowe and Rowe 2008). As in other non-snake predators mentioned above, there is evidence from tests in model organisms that immune responses may be likely resistance mechanisms for some prey items (see Akahoshi et al. 2011; Kamon and Shulov 1965), but it remains to be seen whether these are mechanisms present in scorpion prey species. Collectively, the literature presents a range of possible resistance mechanisms to venomous predators, and future research may confirm the presence of resistance in prey species.

Explanations of a Limited Literature on Natural Resistance

In general, it appears that natural resistance to predator toxins should appear, yet available information is limited. Reaffirming the likelihood that predation pressures, particularly the trophic adaptation of venom, should drive coevolutionary development of resistance, several explanations for a lack of information on resistance emerge. First, a dearth of reported resistance may result from variable and insufficient research effort: the simplest explanation would be that little or no effort has been made to screen candidate resistant prey. Even in the most well-described resistance systems, resistance to venomous snakes, mammalian resistance dominates the literature, despite abundant natural history accounts of venomous snakes consuming nonmammalian prey (but see Mackessy and Saviola (2016)). Second, while some effort may have been made to investigate predator/prey interactions, the documentation of local specificity in some of the prey resistance systems discussed suggests that analyses may not detect resistance because of mismatches between the localities of predator and prey that are tested. The maintenance of resistance in a population of prey species may be dependent on the presence of a particular venom profile that in turn is delimited by the overlapping ranges of local populations of predator and prey. Thus, assaying for resistance using a venom from outside of assayed individuals' local area may lead to the false conclusion that resistance is not present in a prey species or population. Third, beyond mismatching of predator/prey populations, small sample sizes also may allow resistant prey to be overlooked. Under a Red Queen dynamic, the frequency of resistance is expected to cycle through periodic minima. Low-frequency resistance phenotypes would be increasingly harder to detect by random sampling. All in all, future investigations in these least described predator/prey systems and continuing investigations in known resistance systems must consider that limitations in research design and effort may not capture the evolutionary processes driving reciprocal flux between resistance and toxicity.

Another explanation for limited information on prey resistance is the possibility that these predators do not exert enough predation pressure to cause selection for prey resistance. Simply, prey resistance may not exist, despite the logic of coevolution under a chemical arms race hypothesis, because venomous species are not significant predators. If predators move from specialist to more generalist diets over time, selection of novel toxicities may be favored, and therefore reciprocal resistance may not appear. Initial development of toxicity against a limited number of prey species may allow predators to capitalize later on a wider range of related prey species with similar physiologies. With a wider prey base, predators would be able to take advantage of other food sources in the event that resistance does appear in some prey individuals. Therefore, if selective pressure from venomous predators is negligible, and the appearance of resistance alleles in a population only happens as a result of random mutation, the fixation of prey resistance in the population is unlikely, because these rare resistance alleles risk early extinction due to their low abundance. Finally, over time, overcoming the toxic action of venom by prey may prove insurmountable, and our present-day analysis would detect venom toxicity to a

variety of locally available prey, but no or extremely small numbers of resistance mechanisms in prey. The present discussion only considers chemical resistance to predators' venoms, but other strategies may evolve in response to the selective pressure of venom toxicity. Behavioral modifications, and/or reproductive strategies that allow further generations of prey to persist in an area, may subvert the predation pressures of venomous animals and bypass chemically based coevolutionary processes. For example, in one of the better described toxicity/resistance dyads (between Pacific rattlesnakes and ground squirrels), several behaviors that prevent predation are documented. Certain populations of squirrels are known to tail flag to signal their awareness of a nearby predator, resulting in the retreat of the approaching rattlesnake (Putman and Clark 2014); others bombard approaching rattlesnakes with substrate to motivate predator retreat (Goldthwaite et al. 1990), and some rub themselves against shed skins of local rattlesnakes to mask individual scent and evade chemosensory detection (Clucas et al. 2008). While these populations may also have chemical defenses against predator venoms, behavioral modifications that disrupt predatory episodes exist as well, demonstrating that other prey species may not require physiological resistance mechanisms if behavioral modifications are sufficient to elude detection and/or envenomation.

Conclusion and Future Directions

The diversity and efficacy of prey resistance appears to be shaped by the selective pressure of predator toxicity as predicted by chemical arms race hypotheses. However, the fact that only a handful of well-described resistance systems exist in the literature demonstrates the need for further investigations into the diversity and extent of prey resistance. Future directions in the study of natural resistance to venoms must include screens for resistant prey species, using in vitro or in vivo assays to identify the capacity of prey species to avoid the normally pathological consequences of envenomation. Development of a well-supported alternative to LD_{50} determinations is crucial to reduce the number of native prey animals needed to demonstrate resistance and increase throughput, but at present there is no sufficient model to replace whole animal toxicity tests, particularly for unknown systems. Special attention should be paid to the interaction of local populations of predators and prey versus the effects of predator venoms on nonlocal populations of (possible) prey. Further, the prevalence of resistance mechanisms that appear specific to local predators indicates that the development and propagation of resistance genotypes could be modeled to predict or detect the appearance of new resistance mechanisms or to track the spread of resistance mechanisms through prey populations across large landscapes that connect multiple populations. The detection of local resistance also may indicate that current information about the relative abundance of resistance in a given prey species is underestimated; multiple pairwise comparisons between local predator and prey populations would be required across a significant portion of their sympatric range to document resistance or susceptibility unequivocally. Understanding that evolutionary processes are adequate but not necessarily ideal,

reciprocal stepwise modifications to either toxicity or resistance mechanisms are expected to be the norm in coevolutionary systems, rather than wholesale changes to composition. The recent use of genome/transcriptome/proteome comparisons (i.e., Cardoso et al. 2010; Gibbs et al. 2009) could shed light on underlying trends in molecular evolution: how often do resistance genotypes change, how often do novel genotypes appear, and what resistance mechanisms are likely to experience the strongest selection?

Beyond research opportunities focusing on the evolutionary history and development of prey resistance, a better understanding of resistance mechanisms may provide a source for future biomedical innovation. Currently, clinical treatment, both medical and veterinary, of envenomation by venomous species commonly relies on the use of antivenom therapeutics and complementary treatment regimens to combat systemic pathologies such as hypofibrinogenemia, thrombocytopenia, myotoxicity, neurotoxicity, and many other symptoms (Chippaux and Goyffon 1998; Diaz 2004; Rhoads 2007). The incidence of envenomation by spiders, scorpions, and snakes are of particular concern considering their common occurrence, dramatic impacts to global health, and significant financial impacts to health systems. In an attempt to improve treatment, the World Health Organization (WHO 2007) deemed envenomation by snakes and scorpions to be a neglected public health issue and has suggested strategies to develop better antivenom therapeutics. While improvement of existing antivenom therapeutics promises to increase the efficacy of envenomation treatment, the addition of venom resistance molecules to treatment protocols may further improve clinical outcomes. Resistance molecule therapeutics are not intended to replace antivenom therapies, but instead work synergistically with existing treatment protocols to combat venom toxicities. As proof of concept, two classes of anti-snake venom compounds derived from resistant prey species have been cited as promising candidates for drug discovery. Thwin et al. (2010) provide a summative review of a number of these molecules, including a group of phospholipase A_2 inhibitors (PLIs) derived from venomous snake blood sera (Viperidae, Elapidae). The biological roles of these molecules is to prevent complications from auto-envenomation or envenomation by other sympatric (intra- and interspecific) venomous snakes. Hypothetically, clinicians could administer the appropriate antivenom to combat broad spectrum effects of envenomation and additionally employ a derived PLI in cases where patients present with envenomations from snakes with PLA_2-rich venoms. Treatment schedules that incorporate such molecules could be better tailored to individual patient needs to improve the efficacy of medical intervention and patient health outcomes.

In addition to PLIs, another promising class of resistance molecules for drug development are snake venom metalloprotease inhibitors (SVMPIs). As mentioned earlier, SVMPIs have been isolated from a wide range of mammalian prey species of snake predators. Especially in the Americas, SVMPIs promise an excellent addition to combat the hematologic pathologies experienced in a large number of snakebite envenomations (owing to a higher proportion of venomous taxa with snake venom metalloprotease-rich venoms; Mackessy 2010). Metalloproteases have been described as “gateway toxins” (Biardi et al. 2011) because they break down

structural elements within tissues, potentially increasing the rate that other toxic components of the venom may infiltrate and access the bloodstream. Biardi et al. (2011) postulated that the therapeutic use of an SVMPI would limit access of venom components by destroying the ability of the venom to spread from the envenomation site. The biochemical functions of metalloproteases (hemorrhage, tissue destruction) would be blocked, and spread of venom would be attenuated, and the hope is that this temporary neutralization of one part of the venom and subsequent sequestration of other toxins would allow antivenom therapeutics time to propagate to and neutralize the locally envenomated tissue. In short, resistance molecules such as PLIs and SVMPIs are expected to shorten treatment regimens by increasing immediate efficacy of antivenom therapeutics.

In conclusion, our understanding of the prevalence and mechanisms of prey resistance to natural toxins remains limited to a small number of predator/prey systems. However, the prediction that prey species in tightly coupled predator/prey relationships should develop reciprocal chemical arms against predator toxins motivates a continued effort to discover and describe resistance. Future studies should focus on assessing not only the mechanistic nature of resistance but also the demography of resistance in natural populations of prey. Dedication to interdisciplinary approaches that couple molecular and ecological information will exponentially increase what we understand of the interactions between venomous predators and their resistant prey.

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