Evolution of Resistance to Toxins in Pre

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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 Pre 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

extinct, assuming intense predation pressures on the susceptible prey phenotype.

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

often used and synthesized to confirm coevolution (Futuyma and Slatkin 1983). In the case of resistance/toxicity systems, the demonstration of resistance through

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 *N a*)

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 *fl*oridana), 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, *O*) 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

Resistance to Snake Venoms in the Opossums (Famil 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/Toxicit 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 diversifi

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

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

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 pro-

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 hypo

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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