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Toxic Relationships and Arms-Race Coevolution Revisited

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Abstract

Toxin evolution in animals is one of the most fascinating and complex subjects of scientific inquiry today. Gaining an understanding of toxins poses a multifaceted challenge given the diverse modes of acquisition, evolutionary adaptations, and abiotic components that affect toxin phenotypes. Here, we highlight some of the main genetic and ecological factors that influence toxin evolution and discuss the role of antagonistic interactions and coevolutionary dynamics in shaping the direction and extent of toxicity and resistance in animals. We focus on toxic Pacific newts (family Salamandridae, genus *Taricha*) as a system to investigate and better evaluate the widely distributed toxin they possess, tetrodotoxin (TTX), and the hypothesized model of arms-race coevolution with snake predators that is used to explain phenotypic patterns of newt toxicity. Finally, we propose an alternative coevolutionary model that incorporates TTX-producing bacteria and draws from an elicitor–receptor concept to explain TTX evolution and ecology.

OVERVIEW OF ANIMAL TOXINS AND MECHANISMS LEADING TO TOXICITY

Toxins are ubiquitous throughout the tree of life. From bacteria to plants to marine and terrestrial animals (1), myriad toxins play crucial roles in the natural history and community ecology of species. The study of animal toxins remains a central topic of scientific inquiry, primarily because we often do not understand fully how animal toxins evolved, are produced, or are maintained. The immense diversity and ecological roles that toxins represent in ecosystems have required research from the cellular up to the community ecology level and have integrated evolutionary biology, biochemistry, pharmacology, physics, and analytical chemistry.

In this review, we define toxins as substances deployed by an animal to alter the physiology of a natural enemy. Toxins can be divided broadly into venoms or poisons. Although some have proposed a third category, toxungens (2), we focus on toxins only as venoms or poisons. The main differentiating factor between the two is the method of delivery into the target. Venoms are delivered by injecting the toxin(s) directly into the system, often into the bloodstream of the target. Poisons operate in a more passive manner, and must be ingested, inhaled, or absorbed through the skin to exert their effect. Poisons include a spectrum of substances, from a distasteful or noxious compound to potent neurotoxins. We must distinguish between the two categories to better understand differences in the evolutionary pathways of toxins, as well as the factors affecting their distribution and ecological impacts and any patterns of phenotypic variation.

From the start, it is important to realize that toxins and the associated toxicity of an animal are dose dependent. The extent of damage or lethality is affected strongly by the amount of toxin transferred to the recipient. Differences in dosage due to injection location, injection or ingestion volumes, and factors such as the mass of the poisoned target often determine whether a toxin exerts sublethal or fatal physiological effects. Clear examples come from the application of toxins as therapeutics. The bacterial toxin botulinum, produced by *Clostridium botulinum*, is used clinically at low concentrations and can lead to adverse paralytic effects when directed too close to motor endplate regions of muscles (3) and death at higher concentrations. There can also be extreme costs associated with toxin production. As a result, toxin-bearing species may have a threshold of toxin that they can produce or maintain. When toxins are metabolically expensive, it is also possible that they avoid delivering more toxin than is necessary. For example, some animals have evolved means to moderate the amount of toxin they use and adjust the dosage in proportion to their target's size. Malli et al. (4) found that the neotropical wandering spider (*Cupiennius salei*) adjusts venom injection volume based on the size of its prey. To further limit the energetic costs of venom production, spiders often do not envenomate their smallest prey. The authors found that in ~25% of the smallest prey (crickets), there was no detectable venom but instead mechanical damage from fang contact.

Although many mechanisms impact animal toxicity, ultimately toxin production can be separated into either endogenous or exogenous pathways. We first focus on these two categories to highlight how differences between them influence the ways we model, or even think about, toxin evolution and ecology. Broadly, we ask, how do animals obtain their toxicity, and how do modes of acquisition (endogenous or exogenous) impact toxicity and toxin composition? We highlight examples of endogenous producers that derive toxins via underlying genes or gene families and also present the pathways by which exogenous production can lead to toxicity. In both cases, we review known ecological and environmental factors that affect animal toxicity and how they govern toxin production. In many cases, an environmental change or ecological shift can drive an entire species to either completely develop or completely lose toxicity (5). Finally, we discuss predator–prey interactions and how coevolution can select for changes to toxins and potentially result in

a predator–prey arms race. We focus on the arms-race metaphor in detail because these types of interactions can produce extremely rapid and drastic changes in phenotypes and remarkable geographic variation among populations. Delineating differences in the means of production, understanding the impact of environmental conditions on toxicity, and evaluating coevolutionary dynamics will provide a better understanding of how we think about toxin evolution and ecology, help to establish links between changing environmental conditions and shifting toxin phenotypes, and offer perspective on the direction and rate of selection in toxin-bearing species.

MODES OF TOXIN ACQUISITION AND THE INFLUENCE OF ENVIRONMENTAL AND ECOLOGICAL FACTORS

Venoms are gene or gene family products that often are a cocktail of enzymatic and nonenzymatic proteins and peptides but can also include bioactive lipids, acids, and even viruses (6, 7). Venoms are produced endogenously. In many systems, venoms clearly evolved from duplicated genes, coopted for novel purposes, and remain under strong positive selection (8). Typically, venoms bind to specific channels in the cell membrane of the target organism. These proteins display high specificity and affinity toward channel binding sites due to selection for a fast-acting, efficient, and in many cases lethal chemical weapon (9). Although venoms are employed most commonly for predation and defense, venom composition varies greatly, and there are a wide range of functionalities (**Table 1**).

Beyond predation and defense, species will deploy venoms in intraspecific competition. For example, toxic blenny fish (order Blenniiformes) experience intense intraspecific competition for territories and mates (10, 11), and competition among male blennies (*Meiacanthus grammistes*) may have selected for individuals with greater toxin concentrations with novel venom characteristics. Their venom plays a critical role in defense from predators, but males also use it in competitive intraspecific interactions (12). The venom is unique because its effects lead to hypotensive and proinflammatory responses that result in disorientation and poor coordination that make swimming difficult (13). As a consequence, envenomated competitors become easy prey and are dispatched from the competitive pool.

Other species use toxins to parasitize hosts. For example, the venom of parasitoid wasps (order Hymenoptera) is delivered via a precise, nonlethal sting to the brain of arthropod hosts, such as cockroaches (14). This paralyzes the host, disabling any escape response in the process. The wasp venoms can also contain symbiotic viruses or viruslike materials that assist with this process (15). The wasp will drag the zombified host to a nest and use the organism as a living food supply for their developing young. Eggs are oviposited into the living host body, allowing larvae to feed off the bloodstream. Of note, not all parasitoid wasp venom has a paralytic effect. Typically, ectoparasitoid wasp venom causes paralysis, whereas endoparasitoid wasp venom generally does not. Parasitoid wasps have evolved under strong selective pressures and often specialize on a limited suite of hosts (15). This precise degree of adaption to hosts could explain the extreme venom variation across the parasitoid wasps. In another example, tawny crazy ants, *Nylanderia fulva*, use their venom, which is not a polypeptide but rather formic acid, to neutralize and detoxify their own bodies from the venom of one of their major competitors, fire ants, *Solenopsis invicta* (16). These researchers found that *N. fulva* exhibits detoxification after conflict with various ant species but that this detoxification is greatest when the species interacts with *S. invicta*. They speculate that this is likely because the two species have coevolved in sympatry throughout South America.

Host diet can also influence venom production and composition. As such, interactions between genetic mechanisms and environmental conditions influence toxicity. In a study on saw-scaled vipers (genus *Echis*), the presence of arthropods and the proportion consumed correlated strongly

Table 1 Examples of toxic animals and influential factors affecting toxicity

Species	Affecting factor	Toxin(s) of interest	Impact	Reference
Saw-scaled vipers (*Echis* spp.)	Diet	Neurotoxins, cardiotoxins, hemotoxins, cytotoxins	Venom composition varied significantly in relation to the proportion of arthropod consumption in diet; presence of arthropods in diet was correlated to the level of toxicity.	Barlow et al. (17), Richards et al. (18)
Jellyfish (*Carukia barnesi*)	Diet	Tentacular venom	Ontogenetic diet shift caused an alteration in protein banding.	Underwood & Seymour (25)
Rattlesnakes (*Crotalus* spp.)	Diet	Concolor toxin, myotoxins	Ontogenetic diet shift caused no significant variation in toxicity between juvenile and adult snakes.	Mackessy et al. (33)
Some members of the genus *Vipera*	Diet	Presynaptic PLA2, neurotoxins (SPANs), vipoxin, vaspin, ammodytoxin	The presence of insects in the diet correlated to higher venom toxicity.	Starkov et al. (19)
Marbled sea snake (*Aipysurus eydouxii*)	Diet	3FTxs	The shift in dietary components produced a lack of need for prey capture, driving a deletion that led to a frame shift and a significant reduction in venom toxicity.	Li et al. (5)
Marine gastropod (*Conus* spp.)	Diet	Conotoxin	Introduction of a specialized diet caused a reduction in the expression of certain venom components.	Remigio & Duda (21)
Red-spotted newt (*Notophthalmus viridescens*)	Diet	TTX	Captive newts lost their toxicity after six years when provided with a toxin-free diet.	Yotsu-Yamashita et al. (75)
Elapid snakes (*Naja kaouthia*)	Diet	3FTxs	Ontogenetic diet shift caused a variation in venom toxicity and composition.	Modahl et al. (26)
Pit viper (*Calloselasma rhodostoma*)	Intraspecific variation (geographic)	Complex composition (96 distinct proteins)	Abiotic and biotic factors caused significant variation in venom toxicity in relation to geographic distribution.	Daltry et al. (31)
Mojave rattlesnakes (*Crotalus scutulatus*)	Intraspecific variation (climatic factors)	C-type lectin, myotoxins, phospholipase A_2, metalloproteinases, Mojave toxin	Directional selection led to the fixation of new venom phenotypes in snakes.	Strickland et al. (34)
Blennies (*Meiacanthus grammistes*)	Intraspecific variation (competition)	Opioid venoms	The increased selection pressure caused by intraspecific competition for territory among the species is thought to have led to the evolution of venom and fangs.	Harris & Jenner (12)

(*Continued*)

Table 1 (*Continued*)

Species	Affecting factor	Toxin(s) of interest	Impact	Reference
Common toad (*Bufo bufo*)	Intraspecific variation (competition)	Bufadienolides	Coexistence of greater numbers of intraspecific competitors drives higher production of toxins.	Bókony et al. (37)
California newt (*Taricha torosa*)	Intraspecific variation (geographic variation)	TTX	TTX concentration fluctuated, potentially due to environmental conditions; geographic variation was linked to coevolution.	Bucciarelli et al. (69), Hanifin et al. (76)
Cone snail (*Conus geographus*)	Interspecific variation (predation and defense)	Conotoxins	The original evolution of toxins as a defense mechanism has been repurposed to also serve predatory functions.	Dutertre et al. (42)
Hooded pitohui (*Pitohui dichrous*), variable pitohui (*Pitohui kirhocephalus*), rusty pitohui (*Pitohui ferrugineus*)	Sequestration from prey	Homobatrachotoxin	The toxin provides protection against predators and parasites.	Bartram & Boland (39)
Opossums (Didelphidae group)	Interspecific variation (venom resistance)	C-type lectin-like protein botrocetin	Venom resistance increased due to the high ratios of replacement to silent substitutions in the gene encoding von Willebrand factor (vWF).	Jansa & Voss (50)
Marine eels (*Gymnothorax hepaticus*, *Gymnothorax undulatus*)	030 (venom resistance)	3FTxs	Eels with a higher likelihood to be preyed upon by sea snakes displayed higher resistance to their toxins than eels who live in predator-free environments.	Heatwole & Poran (43)
Rhabdophis (family Colubridae)	Sequestration from prey	Steroid irritants	*Rhabdophis* snakes sequester and store toxins from their poisonous prey and later use it in defense and predation.	Yoshida et al. (22)
Poison-dart frogs (family Dendrobatidae)	Sequestration from prey	Lipophilic alkaloids	Poison-dart frogs have evolved the ability to sequester their poisons from their prey and use it in their defense mechanism.	Saporito et al. (66, 67)

Abbreviations: 3FTx, three-finger toxin; TTX, tetrodotoxin.

with toxicity levels among conspecifics (17, 18). A similar conclusion was reached from a study of the shield-headed *Pelias* subclade of the genus *Vipera*, where arthropod consumption also correlated with greater toxicity (19). Although there are observed differences in feeding preference within the group, species with the greatest preference for insects in their diet had the greatest toxicity. Brazilian yellow scorpions (*Tityus serrulatus*) also show modified venom profiles with changes to their diet. Pucca et al. (20) found that when scorpions were provided with a cockroach-based diet, venoms had a greater protein content. The study also highlighted correlations between post-starvation venom extraction time and venom toxicity, providing insight into the scarcity of dietary components in an environment that can affect venoms. Similarly, diet affects venom toxicity of

marine snails (genus *Conus*). When snail diets were specialized, researchers observed a reduced expression of critical components in their venom that consequently affected toxicity (21).

In some cases, animals actually sequester toxins from their prey. For example, *Rhabdophis* snakes (family Colubridae) do not possess venom glands and fangs but instead possess nucho-dorsal and nuchal glands where toxins are stored and expressed. Snakes in this group primarily sequester their toxins, bufadienolides, from their toad prey (family Bufonidae). Remarkably, a group of *Rhabdophis* snakes (*Rhabdophis nuchalis*) has shifted from anuran prey to earthworms, yet still maintains bufadienolides (22). The researchers determined that the group remained toxic by consuming fireflies (Lampyrinae). They further found that the bufadienolides in snakes that consumed fireflies were distinct from bufadienolides in *Rhabdophis* species that consumed toads. Poison-dart frogs (family Dendrobatidae) also rely on prey to maintain toxicity. Apparently, multiple lineages in the group have independently evolved the ability to sequester defensive alkaloids from their prey (23), and this is aided by autoresistance found across the clade (24).

Occasionally, shifts in diet or prey availability can lead to long-term physiological changes in toxin levels. In the marbled sea snake (*Aipysurus eydouxii*) and turtle-headed sea snake (*Emydocephalus annulatus*), loss of toxicity, loss of fangs, and degenerate venom glands are linked to a shift from fish predation to obligate egg consumption (5). The authors identified a deletion in the only gene responsible for expressing the three-finger toxin that translated to a loss of venom toxicity. Without positive selection acting on the genes coding the protein, venom functionality was lost. Ultimately, a unique evolutionary change in venom toxicity was linked to an ecological niche shift that rendered venom unnecessary.

As species progress through life stages, they are often subjected to differing abiotic and biotic conditions. Ontogenetic dietary shifts in animals are common phenomena often accompanied with significant physiological changes. For example, jellyfish (*Carukia barnesi*) shift from invertebrate prey when juveniles to vertebrate prey as adults (25). This transition is accompanied by alterations in venom protein–binding characteristics and physiological changes in tentacle structure and bell wart number, likely due to differing feeding tactics between the two life stages. A study focused on viperid snakes (*Bothrops insularis*) from São Paulo, Brazil, revealed similar patterns. Zelanis et al. (26) found that the toxicity level in adult snake venom was higher than in juvenile snakes, presumably due to changes in diet throughout development (26). Elapid snakes (*Naja kaouthia*) also undergo an ontogenetic diet shift (27). Although the researchers found that the abundance and diversity of three-finger toxins and many enzymes in venom did not differ between life-history stages, they did observe total phospholipase A_2 activity and isoform diversity between juveniles and adults. The authors speculate that the differences could have ecological consequences. Although these ontogenetic diet shifts often are associated with observable alterations on venom-related adaptations (28–32), that is not always the case. In rattlesnakes (*Crotalus oreganus*), the ontogenetic diet shift from small lizards in juvenile snakes to rodents and small mammals in mature snakes is not linked to significant changes in venom toxicity (33).

Other environmental factors can also impose changes on venom composition and toxicity. Abiotic and biotic factors such as the weather may impact prey availability, interactions with other species, and intraspecific competition, which can influence the expression of certain toxin phenotypes. Studies focused on pit vipers (*Calloselasma rhodostoma*) showed that the species displayed strong geographic variation in venom composition and that the variation was attributable to prey type and availability, as well as interactions with different species based on geographic location (31). In Mojave rattlesnakes (*Crotalus scutulatus*), climatic variables affected venom phenotypes (34). Specifically, coolest temperatures and annual precipitation strongly explained patterns of venom phenotypic variation. The researchers speculate that this may be correlated with prey distributions and/or underlying physiological adaptations.

Intraspecific competition can also generate variation in the toxicity driven by competition over territory, mates, or hierarchy (35–37). Bókony et al. (37) found that in the common toad (*Bufo bufo*), coexistence with a greater number of intraspecific competitors led to increased toxin production. Specifically, tadpoles reared in greater densities had greater concentrations and a greater diversity of bufadienolides. Remarkably, they did not find that tadpoles increased toxin concentrations in response to predation risk, but they did with risk of desiccation. The authors concluded there is a cost to producing toxins that cannot be met if larvae need to invest rapidly in development to avoid pre-metamorphic death.

Competitive interactions between species also have a great influence on animal toxicity and can rapidly produce novel and drastic changes to phenotypes. One of the most remarkable examples of toxin evolution is found in pitohui birds (*Pitohui* spp.) (38–40). This group of birds possesses the toxin homobatrachotoxin, which is a member of the batrachotoxin group previously thought to occur only in dendrobatid frogs (genus *Phyllobates*). These are extremely efficacious toxins, and the birds appear to rely on them to protect their nests from predators and parasites, which they do by transferring the toxin from their skin and feathers to the surface of eggs and throughout the nest. Pitohuis—like poison-dart frogs—appear to rely on invertebrate prey (melyrid beetles that the birds consume) for their toxicity (38). Surprisingly, there are actually two genera of toxic birds: A species in the genus *Ifrita* was also found to possess batrachotoxin alkaloids and similarly employ it to deter nest predators (41).

Ecological interactions have also affected the toxin phenotypes of cone snails. *Conus geographus* actually employs two distinct types of venoms: one for predation and another for defense (42). The species is capable of switching rapidly between the two types of venoms and relies on stimuli in the proximal or distal regions of its body to determine which venom to use. Dutertre et al. (42) report that defensive stimuli elicit venoms high in paralytic compounds that target muscle receptors, whereas the toxins used in predation are prey specific. Selection likely favored specialization of predation and defense venoms to minimize costs associated with production.

In several systems, nontoxic prey have evolved resistance to their toxic predators and, in doing so, intensified the predator–prey relationship. Marine eels have developed high resistance to the venom of enemy sea snakes (43). In this system, sympatric eel species (*Gymnothorax hepaticus*, *Gymnothorax undulatus*) that are typical prey of venomous sea snakes (*Aipysurus laevis* and *Laticauda colubrina*) exhibit greater resistance to the snakes' venoms than eels that are not prey (*Heteroconger hassi*, *Gorgasia maculata*, *Anguilla rostrata*). Further, the local abundance of the venomous sea snakes appears to be positively correlated with resistance, such that locations with a greater likelihood of sea snake predation have a greater level of resistance to the snakes' venom. Broadly, it appears that *Gymnothorax* resistance evolved due to tight antagonistic interactions with *L. colubrina* that have led to a predator–prey coevolutionary dynamic (44). Similarly, researchers have determined that rock squirrels (*Spermophilus variegatus*) have evolved resistance to rattlesnake venom (*Crotalus atrox*, *Crotalus viridis*) (45). Specifically, *S. variegatus* has evolved an innate response to neutralize the venom by reducing metalloprotease and hemolytic activity, which they accomplish at a significantly greater rate compared to detoxification of venom from allopatric snakes. Grasshopper mice (*Onychomys torridus*) sympatric with toxic species of bark scorpion (*Centruroides exilicauda*) demonstrate extreme physiological resistance to the scorpions' potentially lethal venom (46). The researchers found a striking pattern of resistance consistent with a predator–prey selection hypothesis, such that populations of *O. torridus* sympatric with *C. exilicauda* have the greatest resistance, whereas allopatric populations have the lowest resistance. Ultimately, *O. torridus* have evolved a modified means of inactivating *C. exilicauda* venom by binding the toxin at the voltage-gated sodium channel, $Na_v1.8$. The $Na_v1.8$ has no effect on the mouse, and the result is a blocked pain

signal (47). Whether *C. exilicauda* has evolved a counterresponse is unclear, but the system offers an opportunity to understand selection imposed upon molecular and biochemical traits.

The evolution of prey toxicity can also force new ecological interactions, requiring predators to evolve counterresponses. A well-documented system involves honey badgers (*Mellivora capensis*) that feed primarily on venomous elapid snakes (family Elapidae). Honey badgers have evolved resistance to cobra venom by reducing its binding affinity to the molecular receptor (nAChR), the recognized target of the neurotoxic Indian cobra venom (48). Similarly, mongooses (family Herpestidae), which prey primarily on venomous snakes, have demonstrated poor binding affinity in their muscular receptor (AChR) to the active components in snakes' venoms, likely owing to mutations in their ligand-binding domain (49). Opossums (family Didelphidae) also consume a relatively high proportion of pit vipers (rattlesnakes and their allies, subfamily Crotalinae) and have evolved greater resistance to the viper's hemorrhagic venom (50). This adaptation is attributed to the high rate of replacement to silent substitutions in the genes encoding for a hemostatic blood protein typically targeted by the venom. Notably, these traits are largely considered adaptations selected for given their toxic and tightly linked predator–prey associations.

TIGHT ASSOCIATIONS BETWEEN PREDATOR AND PREY: COEVOLUTION OF TOXINS AND RESISTANCE

Coevolution is a central theory that biologists have used to explain patterns of phenotypic variation and specialization that arise from either mutualistic or antagonistic interactions between tightly associated species. Mutualistic coevolution promotes fitness gains for both species. Examples include specialization of plants and their pollinators (51–53) and ant–plant mutualisms (54, 55). Alternatively, antagonistic coevolution is the result of interactions between species that are not mutually beneficial and include predator–prey, plant–herbivore, and plant–pathogen coevolving systems. Ultimately, adaptive change in one species is to the detriment of the antagonist(s). Although antagonistic coevolution may manifest as a network of tightly linked and competing species, such as diffuse coevolution (or competitive coevolution), we concentrate on antagonistic coevolution in the narrow sense (i.e., trophic coevolution) that characterizes coevolving species in terms of an arms race (56, 57).

An arms race arises from antagonistic coevolutionary interactions between prey (or hosts) attacked by predators or natural enemies that leads to reduced fitness. Eventually, prey evolve a trait that diminishes predator pressure and ultimately increases their fitness. As a result, the trait and underlying allele(s) experience positive selection. This response will have consequences for predator fitness, and as such, the predator will counter the derived prey adaptation given its available genetic and physiological limitations. A counterresponse that increases fitness will be favored by natural selection, and the trait and associated allele(s) will eventually increase in frequency. This process is cyclic, and as the arms race escalates, the interacting species will remain in a lockstep process of coevolution such that predators and prey continuously exert antagonistic selection pressure on one another, with each new response negatively affecting the fitness of the other. The arms-race metaphor has provided a meaningful framework to understand and account for various coevolving systems (58–61), especially the evolution of toxins and counter-resistance (62, 63).

A FOCAL SYSTEM: NEWTS, SNAKES, AND TETRODOTOXIN EVOLUTION

As we have highlighted, chemical defenses are ubiquitous throughout the tree of life, and there are numerous examples of specialized toxins in terrestrial and marine taxa that are influenced by

ecological factors. However, the neurotoxin tetrodotoxin (TTX) is unique given its broad distribution across deeply divergent taxonomic groups. Currently, 5 classes of bacteria and 14 classes of eukaryotes are known to include descendants that bear TTX. As a group, amphibians have garnered extensive attention because four families of anurans (Rhacophoridae, Brachycephalidae, Dendrobatidae, and Bufonidae) and newts (family Salamandridae) are known to possess TTX. However, most published research has focused on Pacific newts (genus *Taricha*, henceforth "newts"), which are widely recognized as participants in an arms race with TTX-resistant garter snakes (genus *Thamnophis*; see 64). There are currently four recognized species in the genus *Taricha*, three of which are endemic to California and a fourth that ranges from California to southern Alaska. Newts and garter snakes are sympatric across most of *Taricha*'s range.

The prevailing hypothesis is that newts experienced antagonistic selection from garter snakes that gained resistance to TTX. In a compensatory response, newts have evolved ever-greater levels of TTX (65). Over time and various selection regimes, this has played out as a selection mosaic across the species' ranges, resulting in populations evolving geographically different TTX concentrations and resistance thresholds. Thus, newts appear to have evolved population-level differences in TTX concentrations because snake predators have evolved different resistance levels. The current pattern resembles a mosaic of hot and cold spots associated with relatively high or low TTX concentrations. Some populations of newts also seem to have no detectable TTX, or at least the levels are so low that the concentrations are below the limit of detection of the instrumentation.

Unlike other groups of toxin-bearing amphibians—for example, dendrobatid frogs—for which there is a reasonable understanding of toxin acquisition via diet and the downstream biosynthetic pathways (66, 67), there is no clear picture of how newts procure TTX, how they maintain it, and whether the trait is heritable. One possibility is that newts produce their TTX endogenously. The neurotoxin is a non-proteinaceous guanidine alkaloid and, therefore, presumably cannot be a direct product of a gene or gene family. As a result, newts and their associated TTX concentrations may not be driven by predator selection pressure if newts are not endogenous producers with genes producing TTX that experience selection. It is possible that TTX is produced through an endogenous biosynthetic pathway and that newts (and the myriad unrelated animal lineages possessing TTX) rely on precursor molecules to produce TTX, but to date there is no evidence that such a pathway exists in any taxonomic group known to have TTX. Instead, a substantial body of research indicates that many taxa are known to harbor TTX-producing bacteria (68). Thus, the alternative to endogenous production is that newts are hosts for TTX-producing symbionts.

Bucciarelli et al. (69) determined that newt TTX concentrations fluctuate within populations, which runs contrary to a strict model of antagonistic coevolution that argues the phenotype evolved in a step-wise process and is locked in an arms race. Using a nondestructive sampling method that requires only 2 mm of tissue from wild newts and data generated via a high-performance liquid chromatography with fluorescence detection system (70), we observed that the mean toxin concentrations of breeding populations cycled through a range of values that was as great as differences measured between populations separated by hundreds of kilometers (69). This could be due to extreme within-population variation, but we determined that individuals' toxin concentrations change through time by quantifying concentrations in adults from a large capture-mark-recapture population in Southern California (69). By measuring the same individuals across recapture events, we found that the TTX concentrations of some individuals increase and others decrease, and that this change was observable in adults captured year after year or within the same breeding season.

Later work established that toxin concentrations could be induced to increase (71), and this was established by tracking the toxin concentrations of captive adults. Our research indicated that unlike other TTX-bearing animals, such as pufferfish (72), newts maintain TTX in captivity. The

observed increase of toxin concentrations (measured as total milligrams of TTX per individual; see 73) occurred within the first few days of captivity, and the extent of change (1.5- to 3.0-fold increase) certainly would have ecological consequences, especially regarding predator–prey relationships. Others have found similar patterns (74), although in one newt species (*Notophthalmus viridescens*) it appears to eventually diminish to a nondetectable level (the decline required six years; see 75). In addition, larval siblings reared in the laboratory or in the wild had drastically different TTX concentrations through development (71), an unexpected result if TTX is only genetically based with a high degree of heritability. Surprisingly, larvae in the wild had lower TTX concentrations and greater body condition (a ratio of mass to length), whereas larvae in the laboratory had greater TTX concentrations and lower body condition. The overarching conclusions from this body of work are that individual- and population-level chemical defenses are not static and appear to depend on environmental conditions.

Ultimately, the TTX phenotype is not locked at a population-level mean. There is a great deal of phenotypic variation within populations, and there is also a considerable degree of variation between adjacent populations that is not logically explained by the arms-race or geographic mosaic theories. A mean population toxin value is often used to quantify the toxicity of a population, but given that the trait interacts with the environment (i.e., it is plastic), the mean may not be a particularly useful way to represent toxin concentrations. In the context of an arms-race model, the mean does not adequately describe what a predator can be expected to experience. If the trait is inducible, then predators must often deal with a gamut of toxin concentrations, in which case the minimum, maximum, range (maximum minus minimum), and duration that individuals spend at a given toxin level arguably better represent the target phenotype. The TTX patterns spatially depicted in **Figure 1** highlight the TTX concentrations we have measured from 2,396 adults from 57 breeding populations across California. The data represent values from all four currently recognized *Taricha* species sampled over eight years and span a large part of their distribution in California. Although these data collapse time, thereby removing the critically relevant temporal fluctuations within and across individuals, they summarize the extreme range of phenotypic variation observable in many populations.

Several research groups have determined that predators have genetically determined levels of resistance to TTX and that resistance varies between snake populations, presumably as a result of differing selection regimes imposed by toxic newts (see figure 3 in Reference 76). Hanifin et al. (76) assessed resistance based on reduced functionality (escape speed) when snakes were orally administered TTX. They determined population-level mean resistance values based on the amount of TTX needed to elicit a 50% reduction in escape speed. This work shows that some snake populations have very low resistance to TTX (0.01 mg oral dose TTX reduced escape speed by 50%), whereas other populations have extreme resistance (~58.49 mg oral dose TTX). The researchers also determined the 85–15% range of escape speed reduction within each snake population. That range serves as a threshold of resistance, such that any newt populations within a specific threshold were considered to be matched with the snake predator resistance. One major result of this work showed that snake resistance–newt toxicity phenotypes were mismatched in several regions. However, it appeared that snakes always had greater resistance in these populations rather than newts having greater toxicity, suggesting that snakes largely outpaced newts in the arms race.

One striking result of Hanifin et al.'s (76) substantial work is that their data indicate a great amount of variation in toxin concentrations within populations relative to measured resistance in snake predator populations (76, figure 3). This is surprising under a strict coevolutionary arms race interpretation, but less so if there is no clear role of genetics with the newt TTX phenotype. The constraints on sodium channel functionality, such as voltage-gated sodium channel morphology,

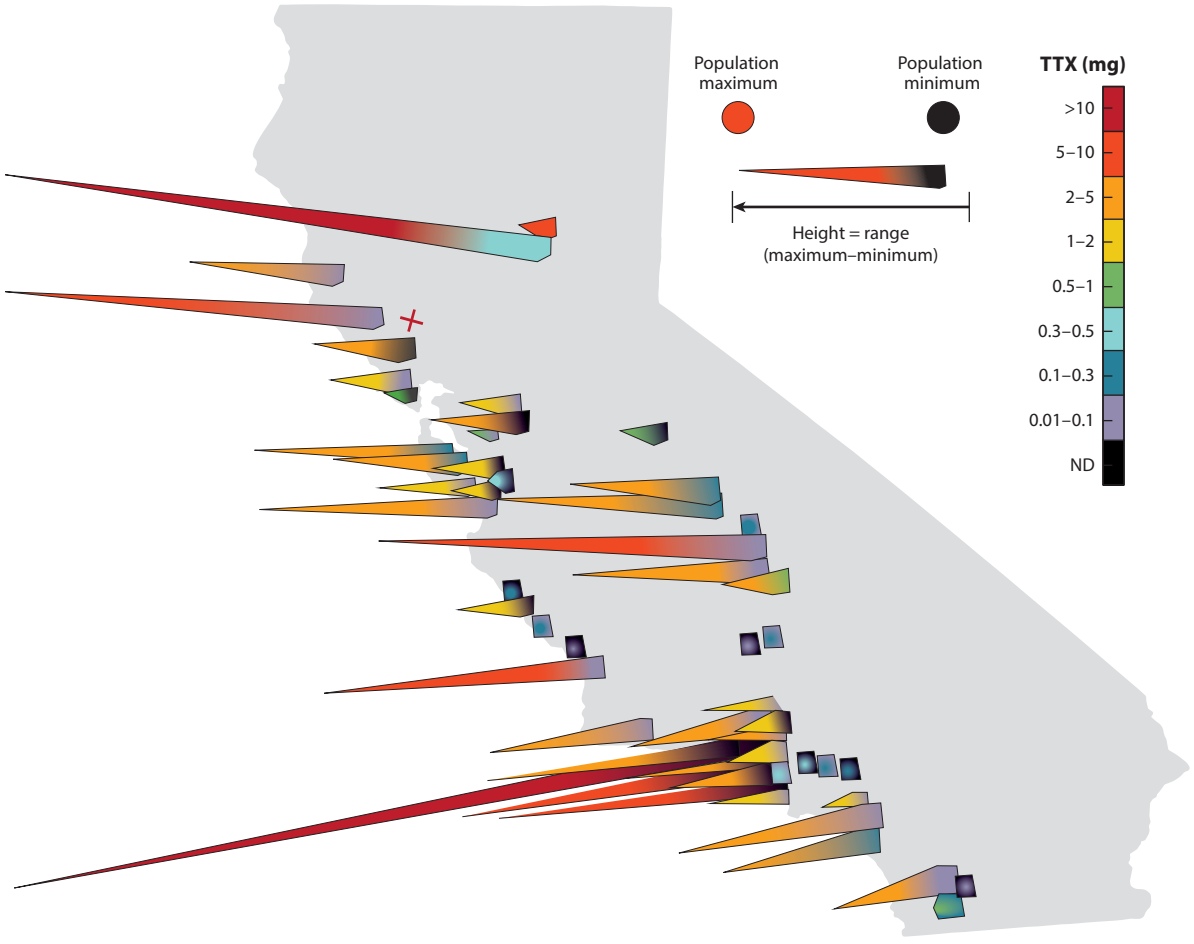

Figure 1

Max TTX (estimated total mg TTX per individual) concentrations from adult *Taricha* newts ($n = 2{,}396$) at 57 breeding populations across California (USA). Peak height represents the mathematical range (maximum minus minimum) of TTX concentrations from adults in a population. The colors at the tip and base of each peak represent the population maximum and minimum values. One population with a maximum of 31.33 mg (range = 31.27) was excluded from the graph, but its location is marked (x). Abbreviations: ND, nondetectable; TTX, tetrodotoxin.

may be a factor that limits resistance variation, whereas TTX appears to be less constrained, likely because the phenotype is not controlled by genes or genes alone.

Collectively, these results challenge how we think about the evolution of TTX, the role of predators, and the hypothesis of an arms race between newts and snakes. One of the most interesting questions that remains is, what is driving temporal variation and the large degree of phenotypic variation in TTX expression? An answer has emerged recently.

THE ROLE OF BACTERIA IN NEWT TTX PRODUCTION

TTX production in newts appears to involve bacterial symbionts. Recently, Vaelli et al. (77) determined that four genera of bacteria (*Pseudomonas*, *Aeromonas*, *Shewanella*, and *Sphingopyxis*) found

on the skin of the newt *Taricha granulosa* could produce TTX. The researchers isolated and cultured bacteria and then screened the cultivation media for TTX. They identified 11 bacterial strains from the 4 genera that could produce detectable quantities of TTX using a liquid chromatography with tandem mass spectrometry system. Comparisons of the bacterial communities between newt populations with detectable and nondetectable TTX concentrations showed stark differences in the relative abundance of bacterial organizational taxonomic units (OTUs). In particular, the researchers observed a much greater relative abundance of *Pseudomonas* OTUs in newts with TTX compared to newts with no detectable TTX. This genus is known to produce TTX, as are *Aeromonas* and *Shewanella*, but all previous research identified these TTX-producing bacteria only from marine organisms, including pufferfish, octopus, and shellfish (78). Contrary to previous work that found bacteria from newts likely had no role in TTX production (79), the results of Vaelli et al. (77) indicate that TTX production for terrestrial organisms may also result from the associations between bacteria and their hosts.

The next logical question is how newts as hosts influence their symbionts and the larger role that bacteria play in the interactions between predator(s) and toxic prey, a question that can actually be extended much more broadly to numerous TTX-bearing animals, both marine and terrestrial. In general, this is a challenging question to answer because we know so little about the evolution and chemical ecology of the TTX phenotype, especially for amphibians. However, one important insight from Vaelli et al. (77) is that *T. granulosa* newts have evolved modified voltage-gated sodium channels that provide extreme resistance to TTX. Specifically, three mutations in $Na_v1.6$ at DI, DIII, and DV were identified and determined to confer extreme auto-resistance, collectively providing newts with resistance to ~1,132 mg of TTX [estimated half-maximal inhibitory concentration (IC_{50}) = 3,551 uM +/− 469]. Having only the DI mutation provides resistance to ~243 mg of TTX (IC_{50} = 763 uM +/− 284), only the DIII mutation ~0.6 mg of TTX (IC_{50} = 2.43 uM +/− 0.23), and only the DV mutation ~1.5 mg of TTX (IC_{50} = 4.73 uM +/− 0.42). The three mutations were found together in a population of newts known to have high toxin concentrations but also in a population with no detectable TTX [a population isolated in Idaho (USA) and considered nontoxic because all individuals from this site appear to possess no measurable amount of TTX]. These results suggest that auto-resistance may be shared across *T. granulosa* populations and perhaps across the genus, although this remains to be established. One major implication of this result is that at least one species of newt appears to have the capacity to maintain TTX at quantities that rival the greatest resistance measured in any snake population across the arms-race mosaic (the largest resistance value based on a performance of 50% function was 58.49 mg; at 15% function the greatest resistance was 723.2 mg). If other newt populations possess similar mutations that confer comparable levels of auto-resistance, then newts would broadly possess the necessary molecular foundation to protect themselves from copious amounts of TTX being produced by symbionts. One important question remains: What limits the maximum TTX concentrations that an individual or population can reach? This may be a factor largely influenced by the newt microbiome and interactions with the environment, rather than what was presumed to be the result of the newt genotype and its evolved ability to produce TTX.

In general, this recent work indicates that the TTX phenotype has been oversimplified and misinterpreted. The current model of antagonistic coevolution between snakes and newts is inadequate and does not account for the role of bacterial symbionts that appear to produce TTX. The arms-race model assumes that the TTX phenotype is heritable and responsive to predation pressure that leads to reciprocal genetic change and that newt TTX changes at evolutionary and not ecological timescales. Because these assumptions are no longer fully supported, we must reevaluate the processes that have led to the current phenotypic patterns observed across the species' ranges. This requires a new model that considers the role of symbionts, genes, and the environment.

A NEW MODEL TO EXPLAIN TTX EVOLUTION AND ECOLOGY

Antagonistic interactions between coevolving species can be modeled in at least two ways. The first is what would be expected under an arms-race scenario. In its most basic framework, it conceptualizes prey as toxin producers and predators as toxin resistors. Prey produce toxins that impede or poison predators, and predators possess the trait(s) necessary to lessen or diminish the effects of the prey toxins. This type of antagonistic coevolution has been termed a toxin–detoxifier interaction (80). This model broadly informs our current understanding of TTX evolution and ecology.

However, based on the recent evidence reviewed here, we propose an alternative model to explain TTX evolution and ecology that is adapted from the plant–enemy/host–pathogen literature and theoretical framework (80). Numerous studies demonstrate how plant–enemy and host–pathogen systems involve interactions that lead to induced systemic defensive responses when plants or hosts sense their antagonist(s). The induced response is elicited by molecular and cellular processes: Enemies or pathogens produce substances or signals that bind to host/plant cellular receptors and initiate the response. This model is referred to as an elicitor–receptor interaction (80). Kniskern & Rausher (80) termed this type of coevolution "information coevolution" and proposed that selection favors receptors in plants/hosts that recognize enemies and reciprocal responses in enemies that evade detection by modifying elicitor molecules. As such, coevolution would proceed in general through sensing, with selection on receptors and elicitor molecules.

An updated model to explain TTX ecology and evolution in newts must incorporate a role for bacterial symbionts and integrate ideas from information coevolution. Given that we know that TTX concentrations of newts can be induced (71), there is the potential for elicitor–receptor interactions that mediate this induced response. This could be due to molecular signals from the environment to the host that initiate a physiological response, which is then sensed by symbionts, resulting in modified TTX production. Early work demonstrated that larval newts detect waterborne TTX that elicits antipredator behavior (81, 82). In these experiments, the source of TTX was nearby adults, and the larval response was to flee and seek shelter because adults can be cannibalistic. TTX as a chemical cue also appears to impact breeding site fidelity of adult newts (69). Males with low TTX concentrations abandon breeding pools if competing males in the pool have higher TTX concentrations. Anecdotally, we have observed adult males in pools snout to snout in a sort of standoff, presumably assessing one another, until one flees. In the *Taricha* system, we have also found that the macroinvertebrate community alters its foraging behavior in the presence of TTX (83, 84). More broadly, other taxa rely on TTX as a chemical cue, including pufferfish, in which it serves as a sexual attractant (85), and snails that cue in on it for feeding (86). Incorporating symbiotic relationships and information coevolution into the newt TTX system requires that newts also have sensors to gauge their TTX concentrations. In other symbiotic relationships, there is evidence that hosts have the physiological capacity to sense microbial products (87). If this physiological capacity applies to newts, then they may be able to gauge their individual toxin concentrations and coerce symbionts to up-/downregulate production. Signals that induce a response could be predators, mates, competitors, or changes in the abiotic environment. It is also possible that newts do not control production, or have a limited role, and that the bacteria simply respond to conditions of the environment (including their host) to optimize their fitness. This may include the broader physical environment or conditions within or on the skin of newts, including intra- and interspecific bacterial interactions.

Research over the coming years will need to determine how bacteria produce TTX and how production is regulated, including the environmental cues that initiate, influence, and guide production. A crucial next step will be to understand how TTX is transported within hosts. To date, little is known about the ways TTX is shuttled from symbiont to host, although TTX-binding

proteins initially discovered in pufferfish are a potential candidate (88, 89). Of course, there is also much to understand regarding the host–symbiont–environment interaction (here, we specifically use environment to capture a variety of signals that may be in the environment, *vide supra*). It will be important to determine what molecules may serve as elicitors and what the receptors are, how they differ between populations, and what selection pressures have led to any observable differences. These questions extend beyond the newt coevolution system and pertain to any TTX-bearing species. Answers to these questions in any TTX-bearing group will provide meaningful insight into the evolutionary patterns that the arms-race model alone fails to address. Eventually, broad-scale phylogenetic analyses will help shed light on the nature of the TTX phenotype and how it has come to evolve.

CONCLUSION

We close on a broader thought. There are thousands of toxin-producing species and just as many systems that are influenced by the various factors we have discussed, including endogenous or exogenous modes of acquisition, and a plethora of ecological factors that influence toxicity, from diet to ontogeny to intra- and interspecific interactions to phylogeny. One exciting aspect of toxins research is that it is collaborative and requires a combination of natural history, ecology, evolutionary biology, analytical chemistry, and natural products synthesis. Through these collaborations, we can learn a great deal about the evolution and ecology of chemical defense phenotypes. Our focus on one coevolutionary system shows us that we can come to understand a few ways that toxins can affect ecology, and that toxin evolution is much more complex than hypothesized previously. Through collaboration and interdisciplinary research, there is much more we can learn about toxin phenotypes of many other species and the evolution of their roles that extends far beyond toxins purely as a means of defense.

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