Patterns, Process, and the Parable of the Coffeepot Incident: Arms Races Between Newts and Snakes from Landscapes to Molecules

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The mountains of the Oregon Coast Range are not high on the list of places most people would think of visiting to conduct fieldwork on snakes. Most mornings begin with fog so dense that you cannot see from your sleeping bag to Tenmile Creek, running only 15 meters away. Even within the shelter of a tent, there is a perpetual dampness to everything you own and the feeling that moss is beginning to colonize every surface. More often than not, the fog simply gives way to full-on rain and frequent toasts to the man who invented Gore-Tex. Snakes, and the herpetologists who study them, patiently await the few days when the sun burns through around midday, offering a rare opportunity to bask and warm their bodies. This environment is definitely one in which amphibians have the upper hand.

Many mornings are spent sipping too much coffee and waiting to find out whether fog or sun will win the morning battle and determine the day's activities. The sun dictates a frantic dash to the fields to collect garter snakes; the rain, the responsibility of notebooks and specimens to process (or maybe even a trip to town). The positive side of the rain is that it leaves plenty of time to think about all the fascinating things that one sees in the field, which inevitably leads to new questions and plans to test out possible explanations.

This was the world I inhabited while studying how patterns of natural selection might maintain the many different color patterns that are seen in a species of garter snake that is common throughout the Pacific Northwest. The fieldwork required capturing hundreds of snakes, recording their color patterns and their escape behaviors, and uniquely marking and releasing each one; then trying to recapture as many as I could over the next few years. More out of curiosity than design, I was also collecting and inspecting all other reptiles that I encountered, which pretty much meant one other species of garter snake and the occasional alligator lizard. (The Pacific Northwest is not known as a hotspot of reptile diversity).

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FIGURE 1 The author collecting garter snakes in the 1980s on a sunny day at Tenmile Creek.

One of the basic questions of interest to most ecologists is, "What does this creature eat?" The morphology and behavior of snakes make them ideal for answering such a question. Lacking limbs, snakes have no option but to swallow their prey whole. Because snakes are shaped inside and out like a long, thin toothpaste tube, recent meals can be coaxed out of the stomach by gently massaging the contents toward the mouth. After "barfing" the snake, the whole prey item can be identified and the snake sometimes will re-ingest its meal. Naturally, I wanted to know what the local garter snakes were eating, so part of my sunny-day routine was to barf the snakes that I encountered and to record the items in their diet.

The main species that I was studying, the northwestern garter snake, *Thamnophis ordinoides*, turned out to be fairly dull from the perspective of diet—earthworms and tiny slugs pretty much completed the picture. Barfing the common garter snake, *Thamnophis sirtalis*, on the other hand, was like unwrapping a present, never knowing for sure what would be inside. These big, red-headed snakes held rodents, giant slugs, nestling birds, earthworms, frogs, and several different kinds of salamanders of all shapes and sizes. One ambitious female snake had even swallowed a full-grown Pacific giant salamander, itself big enough to eat a small rodent. But the most impressive of all were rare cases of snakes that had eaten rough-skin newts, *Taricha granulosa*. I knew that those newts were deadly poisonous to most mammals, and I was shocked to find them in the bellies of these seemingly healthy snakes. Embarrassingly, I had forgotten, in my excitement, that I was not the first person to make this natural-history discovery. I, of all people, should have known that common garter snakes could survive encounters with poisonous newts.

FIGURE 2 This female garter snake is from the Coast Range of Oregon, where snakes were first observed eating toxic newts.

The discovery that newts were toxic began to unfold decades earlier, just over the mountain range from my field site at the college now known as Western Oregon University. In the early 1960s, an undergraduate student there, having recently completed his college football career, visited his biology professor to talk about doing some kind of research. Dr. Kenneth ("Doc") Walker leaned back in his chair, fussed with his pipe, and slowly told the student a local legend that he'd heard growing up on the coast of Oregon. Three hunters had been found dead at their campsite in the Coast Range, with no sign of struggle or injury. The only thing out of the ordinary about the scene was that a newt had been found boiled in their coffeepot. "Why don't you try to find out if those newts are poisonous or not?" Walker asked the student.

Within a few days, the student was given a tiny room in an old outbuilding, a single glass syringe, and a mortar and pestle for grinding newt skin. He quickly set about collecting a tank full of newts and trapping some field mice. The student soon found that tiny amounts of ground newt skin mixed with water and injected into mice killed them in only a few minutes. If the newt in the coffee pot truly had been responsible for the deaths of the hunters in the story, there must have been enough poison in a single newt to kill at least three grown men, if not more—tens of thousands times more than the amount of toxin needed

FIGURE 3 The rough-skin newt, *Taricha granulosa* (*top*), can be found in moist habitats throughout western North America. When physically threatened, newts assume the "unken" posture (*below*), exposing their bright orange bellies to advertise their toxicity to would-be predators.

to kill a mouse. Humans are many times larger than the natural predators of newts, the biggest of which is probably a raccoon or coyote. How could this degree of toxicity, far more than is needed to protect a newt from attack by predators, have evolved? What forces could have led to such a heavily armed prey?

I had heard the story of Doc Walker and the newt in the coffeepot untold times, because the student in the story was my father, Edmund D. ("Butch") Brodie, Jr. That story was his initiation into the research that he continues to this day. Over the next few years, he tested extracts of newt skin against all manner of potential predators, always finding the same result. Almost immeasurably small amounts of newt skin were lethal to every bird, mammal, or fish that might encounter a newt in the wild. The reason became clear when a group of chemists at Stanford University identified the main poison in newt eggs as

FIGURE 4 A garter snake (*Thamnophis sirtalis*) feeding on a poisonous newt from Oregon.

tetrodotoxin (or TTX), the same compound found in the skin and livers of some marine puffer fishes. In Japan and China, it had been known for thousands of years that eating puffer fish, or "fugu," could be deadly. In fact, it is this risk that makes fugu such a delicacy in Japan. Sushi prepared by licensed master chefs, including the highly toxic liver of fugu, is hotly sought after because of the chance for a "taste of death"—the tingling and numbness of the lips and mouth that accompanies properly prepared fugu. Unfortunately, this "taste" sometimes extends down the arms and into the chest, hampering breathing, and occasionally killing diners who ingest too much TTX.

My father had been unaware of another group studying the toxins of newts, and he was understandably crestfallen when his mentor came in waiving a copy of *Science* with a picture of a newt. As a young student on his first project, it seemed that his hard work had been scooped by the Stanford lab and published in the highest profile journal in the business. In retrospect, it was this identification of TTX as the poison in newts that became the cornerstone of his research career. Rather than having his work supplanted (for he was truly no chemist and probably never would have identified the toxin, anyway), my father came to realize that his interests were really in the "why" questions—the evolutionary problems of what forces and processes lead to such elaborate defenses. Even at this neophytic stage, his work had been aimed at determining what species of predators were susceptible to newt toxins.

What had slipped my mind in the intervening years was the punch line to my father's first studies of newt toxicity. One of the most effective ways to sample adult newts is by pit trapping, wherein short, metal drift fences are erected on the border of a breeding pond with five-gallon buckets sunk to ground level along the drift fences. As newts approach the pond to breed, they follow the

fences until they fall into a bucket and are trapped. One season, while monitoring pit traps at a local pond, my mildly ophidiophobic father repeatedly encountered "big ugly" garter snakes in his buckets. Far from being stuck in the traps, the snakes would shoot up and out of the pits when approached, sprint past my father, and disappear into the brush. What the snakes were doing in the buckets, other than scaring approaching biologists, was not clear. Eventually, one of the snakes—the same species (*Thamnophis sirtalis*) that I was barfing some 50 miles and 25 years away—was caught in the act of eating a newt while in a bucket trap. My father immediately collected and tested some of those snakes, and discovered that they were unaffected by the newt toxin. He had found a resistant predator, one that could eat even a newt that contained enough poison to kill several humans.

With one puzzle solved, more arose. What ecological forces pushed snakes to eat poisonous prey? Was resistance an accidental by-product of garter-snake physiology, or did these snakes evolve resistance in direct response to eating poisonous newts? What mechanism could enable garter snakes to resist a poison that killed every other vertebrate predator? Were resistant snakes the evolutionary cause of the extreme toxin in the newts? These questions nagged my father long before I discovered for myself that *T. sirtalis* were eating poisonous newts at my field site. Now similar questions dogged my mind as I tried to understand the implications of what I had found in the belly of a snake.

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I was brusquely reminded of the story of the newts in the coffeepot and my father's subsequent explorations of *Taricha granulosa* toxicity when, the next time I was in town near a pay phone, I called the expert on salamander defenses to gauge the importance of my find. Although my father had never left behind his questions about *T. granulosa* and resistant garter snakes, circumstances had taken him out of the Pacific Northwest and had offered him other systems to study. He had investigated toxins in newts from eastern North America to China, but found nothing as dramatic as the *T. granulosa* he had studied as an undergraduate in Oregon. He discovered poisonous salamanders in Guatemala and snakes that could safely eat them, but he never found another predator that could survive an encounter with a *T. granulosa*.

Our subsequent conversations that summer began to hash out hypotheses that might explain our observations and designs for testing them. We came at the bigger problem from two very different perspectives, in terms of organisms and science. My father had always focused on the perspective of the defenses of amphibians and the ecological and behavioral factors that drove their evolution. I was studying snakes that were major predators of amphibians in the Pacific Northwest, and was training in a graduate program at the University of

Chicago that emphasized the role of genetics in determining evolutionary process. We were both curious about the potential for coevolution between predators and prey to drive the evolution of extreme traits, and so we designed an initial study to test whether coevolution might be taking place between newts and snakes.

The aspect that sets coevolution apart from other processes is reciprocity: natural selection imposed by one species—say, a predator—drives adaptation in a second species—say, a prey species. Evolutionary response by the prey species increases the level of defensive traits and, in turn, generates stronger selection on predators to better exploit the prey. If predators evolve increased adaptation to their prey, then the feedback loop of reciprocal selection and response is complete, and coevolution occurs. What is so dramatic about this process is that the forces of selection are themselves changing each generation, getting stronger and stronger and driving evolution faster and faster.

But natural selection is only one part of the adaptive process. The dogmatic mantra of evolution by natural selection is "variation, differential reproduction, heritability." Variation consists of the differences between individuals that present a substrate for selection. Differential reproduction means that some individuals leave more offspring than others. If those differences in reproduction are associated with differences in phenotypes, then natural selection occurs (some kinds make more babies than others). Heritability is the inheritance of those differences, so that the offspring resemble their parents; heritability is what transmits the variation in one generation to the next. Not all variation is heritable, however. If, and only if, the differences among individuals are heritable will selection result in evolutionary changes or in adaptation. Coevolution requires "genetic complementarity," or heritable variation, in both species for the traits that mediate an interaction.

This is where my father and I began our dissection of coevolution. If there is no heritable variation in snake resistance to TTX (as might be true, if all garter snakes possess the same TTX-insensitive physiology), then there could be no evolution by predators, and no coevolution between garter snakes and newts. To demonstrate the heritability of resistance, we would need to measure resistance in individual snakes and to compare differences among families. If individuals are more similar to family members than to nonrelatives, then that trait is heritable. Female garter snakes give birth to live young late in the summer. I collected pregnant female snakes in midsummer and shipped them back to my father's laboratory in Texas, and all we had to do was to measure each of several hundred babies and to compare the variation among families.

Measuring individual differences in resistance to TTX was the big trick. Traditionally, resistance and toxicity are evaluated at the level of populations or groups (if something is lethal, it is very hard to measure small differences in

FIGURE 5 E. D. Brodie, Jr. testing garter snakes for resistance in the lab. Neonate snakes are raced on a computer-controlled racetrack to determine their maximum sprint speed (*left*). Several days later, they are injected with a small dose of tetrodotoxin (TTX) and raced again. The percentage of normal performance they can achieve after exposure to TTX is taken as a measure of their resistance to the toxin.

its effect). A measure known as an LD50 (a lethal dose that kills 50% of the sample) is estimated by taking a group of subjects and repeatedly exposing each individual to a dose of toxin until an amount that kills half the group is found. We could obtain purified TTX, collected from the eggs of pufferfish and sold commercially, but the thought of killing hundreds of snakes to derive a single measure of resistance did not sit well with us. Besides, the LD50 approach would not work for us because we had to know the resistance level of every individual snake, not the resistance level of the group. My father knew, from testing newt extracts on various predators, that the first effects of TTX intoxication included a loss of muscle coordination. Even the resistant garter snakes he tested in the 1960s lost the ability to crawl and to right themselves when turned on their backs. Thinking back to these observations, he thought that measuring differences in the effect of TTX on locomotion might be the solution. We timed how fast a snake could crawl down a 2 m long racetrack lined with Astroturf and rigged with infrared sensors to automatically record the fastest speed of a snake. The next day, we injected each snake with a small amount of TTX, waited half an hour, and then timed how fast it could crawl down the racetrack once again. The difference in speed before and after injection with TTX gave us a measure of the TTX resistance of individual snakes. Snakes that crawled at the same speed after injection were judged to have 100% resistance to that

dose (that is, they could crawl 100% of their normal speed), whereas snakes that could only crawl half as fast as their baseline rate were judged to have 50% resistance to that dose.

My father perfected this assay over the summer, and waited for the snakes that I had sent from Oregon to give birth. In August, newborn babies appeared in the cages of females and were put through their paces on the racetrack. When I analyzed the data, I saw that we had clear evidence that TTX resistance varied substantially among litters of snakes. In fact, our numerical estimates indicated that almost all of the variation in resistance had a genetic basis. If natural selection favored increased resistance in snakes, there was ample heritability to produce an evolutionary response.

Interactions between natural enemies are often thought of as "arms races." Increases in the defenses of one species requires complementary increases in the offenses of the other. Natural selection drives this escalation–counterescalation process through evolutionary time without an obvious endpoint, as long as both species can continue to evolve and selection persists. Despite the popularity of the metaphor, there are good reasons to doubt that predators and prey become engaged in this kind of lockstep coevolution. The most persuasive counterargument is known as the "life–dinner principle." Simply put, in a race between predator and prey, the prey has more to lose (life) than the predator (a meal). This amounts to an asymmetry in selection that should lead to faster evolution of prey than predators, and so the prey might be expected to escape the arms race and to leave their predators behind. Many prey species seem to have evolved defenses against specific predators, though not vice versa. But what if the prey species are potentially deadly meals? Is the life–dinner principle suspended, with both parties risking the fatal consequences of a lost race?

This premise suggested that dangerous prey might impose selection on the abilities of their predators. Upon careful consideration, however, it is very hard to see how either fatal poisons or resistance to them can be selectively favored. The selective advantage to toxic prey results from their ability to repel a predator. Killing the predator has no inherent advantage. Moreover, if the prey dies or is injured in the act of poisoning the predator, then there is no advantage to the individuals with more poison. On the other side of the interaction, if prey are so toxic that all predators die after attacking one, then there is no chance for more-resistant predators to gain an incremental advantage. Despite the apparent advantage of lethal toxins and resistance to them, selection can favor their coevolution only if individuals with more toxin or more resistance gain an advantage.

To discover whether there was a solution to this paradox, we had to look more closely at the behavioral interaction between predators and prey. In a test arena, we allowed one-on-one interactions between snakes that had been

scored for resistance and newts from a locality with high toxicity. Snakes usually attack newts at the middle of the body, sometimes using their own body to pin the prey, then they carefully walk their jaws forward to the head of the newt to begin swallowing. Moving the jaws from side to side and slowly working over the head and shoulders of the newt, it may take a snake up to two hours to fully swallow its prey. What surprised us in this sequence was that, once a snake had begun the swallowing phase, it did not always complete the action. In many cases, a snake would work to swallow a newt for up to an hour, then would eventually stop, open its jaws, and spit out the newt. The amazing result was that every newt, even those that had had their heads down the gullet of a snake for almost an hour, walked away with no apparent injury.

What determined whether a newt lived or died? The most resistant snakes were the only ones that successfully swallowed newts (indicating an individual advantage to increased resistance). We were not able to measure the toxicity of individual newts at the time, but it is likely that newts that were more toxic also had an advantage. The reason some snakes gave up on swallowing newts is unknown, but probably results from direct intoxication. When attacked, newts assumed a curled defensive posture and visibly secreted toxin out of their pores around the bite, but they did little else to resist physically. Snakes that eventually rejected newts usually exhibited impaired mobility for several hours after the interaction, indicating that they had absorbed a substantial amount of toxin even without swallowing the newts.

So reciprocal selection could operate through such a behavioral mechanism, but what evidence indicates that coevolution has taken place over the long term? We needed a way to compare the toxicity and resistance in current newts and snakes to that of their ancestors to find out if the trait levels had increased. Evolutionary biologists have a time machine of sorts in the form of phylogenies and trait reconstruction. Phylogenetic trees reflect the relationships between present-day species and populations based on how similar they are in genetic and phenotypic traits. By mapping the distribution of traits onto such a tree and making some assumptions about how evolution occurs, it is easy to see what character state the presumed ancestor of a species or group probably had. In our case, we scored the TTX resistance of as many garter snakes and their nearest relatives (called natricine snakes) as we could test, along with some more distant relatives. The picture was unequivocal. The only snakes with elevated TTX resistance were the *Thamnophis sirtalis* from Oregon, which we knew preyed upon newts. All other natricines had only a tenth the resistance to TTX, clearly demonstrating that our resistant population had evolved increased resistance compared with its nearest relatives. When we compared natricines to other snakes, such as rattlesnakes, racers, and rubber boas, we found that the whole group of natricines had slightly higher resistance, suggesting there was

something unique in the physiology of natricines that might have predisposed them to engaging in the arms race.

Taricha granulosa, too, had dramatically higher levels of TTX than other newts. All of the newt species that have been sampled to date have some detectable amount of TTX in their skin, including the closest relatives to the genus *Taricha* in North America, the three species of the genus *Notophthalmus*, and a number of European and Asian salamanders. The amount of TTX in *T. granulosa* from Oregon is as much as 100 times greater than that in any other species. One of the biggest remaining mysteries about TTX is its source. We still do not know if newts synthesize their own TTX, sequester it through diet, or perhaps obtain it from a symbiotic bacterium. The fact that TTX is found in so many kinds of organisms (ranging from bacteria to flatworms, mollusks, and fish) suggests that there is some exogenous source. However, our attempts to detect a dietary or bacterial origin for TTX in newts all suggest that newts make their TTX themselves. This gap in our knowledge is still one of the most critical impediments to our understanding of how the coevolutionary dynamic plays out.

Common garter snakes are found throughout most of North America, but *Taricha* species occur only in the far west, from the Sierra and Cascade mountains to the coast. This geographic disconnect offered us the chance look for the signature of coevolution within these species. The hypothesis was simple: where garter snakes lived with newts (sympatry), they should have coevolved resistance to newt toxin. Where they occurred outside the range of newts (allopatry), snakes would have no selective pressure to evolve resistance and therefore should be sensitive to the effects of the toxin.

By now, I had begun a "real job" as a faculty member. My father and I had been working together on the system for only a few years, and both of us thought we could tie up some loose ends with this comparison before moving on to other projects of our own. The fieldwork appealed to me, and somehow we came to the arrangement that I would collect samples throughout western North America, while my father stayed in the lab and ran all of the resistance assays. (Luckily for me, it is critical to minimize the variation in the assay scores by always having the same person run the behavioral tests.) To generate the kind of statistical power we needed to compare what we expected to be minor differences, we needed to sample roughly 8 to 10 localities each of snakes sympatric with newts and those outside the range of the poisonous prey. I spent most of the next several summers camping out of a pickup and collecting pregnant garter snakes to ship back to the lab. Meanwhile, my father played midwife to several hundred pregnant female snakes, and then raced the literally thousands of babies down a racetrack to score their resistance. Research is always an uneven combination of adventure and tedium.

What we discovered from this survey was that populations of garter snakes did indeed differ in their resistance to TTX, but not in the simple pattern that we had predicted. Snakes that had come from localities without newts were uniformly nonresistant. This result supported the obvious expectation that resistance evolves only in the presence of poisonous prey. Snakes that were sympatric with newts, however, ranged 1,000-fold in their resistance to TTX from one place to another. We could not afford to buy enough TTX to impact the performance of some of these snakes, while others were no more resistant than a garter snake from the Midwest, where no newts of the genus *Taricha* had probably ever lived. Each of these localities had abundant newts, so why did coevolution seem to occur in some places and not in others?

Rather than wrapping up a side project, these new findings completely overturned our understanding of the newt–snake interaction and our picture of coevolutionary process in general. How could we explain the lack of resistance in so many populations of snakes that co-occurred with *Taricha* if an arms race between predator and prey were really taking place?

At roughly this time, John Thompson, an evolutionary ecologist who studies plant–insect interactions, was wrestling with similar problems in his own empirical system. Thompson's plant, a delicate wildflower called woodland star (the genus *Lithophragma*), is pollinated by a small moth in the genus *Greya* that lays its eggs on the *Lithophragma* flower. In most areas, *Greya* moths are the only pollinators of the flower, and the system seems to have evolved along a mutualistic trajectory. *Greya* moths pollinate the plant while laying their eggs, and the moth larvae later feed on the developing seeds. Individuals of both species yield a fitness benefit from the interaction. However, Thompson noticed that, in some localities, the *Lithophragma* plants had lower fitness when *Greya* moths laid eggs in the flowers: the relationship had turned parasitic. The difference seemed to be the presence of a small fly that also pollinated *Lithophragma*. On the hillsides and in the river valleys where these flies occurred, *Lithophragma* did not require *Greya* to fertilize its seeds, so the presence of *Greya* larvae had a net negative effect on the *Lithophragma* plants. These observations led Thompson to think about coevolution on a finer scale. Instead of viewing the world of species interactions as an even landscape that is the same everywhere the two species lived together, he began to imagine a crazy quilt of interactions that might differ from one spot to the next, depending on the details of the local ecology.

These emergent ideas became known as the Geographic Mosaic Theory of Coevolution (GMT). The stripped-down version of the GMT starts with the realization that, although species and populations are the units that evolve over time, the interactions that drive coevolution occur between individuals and therefore take place on a local scale. The consequences of those interactions

then depend on a multitude of factors that range from the physical (geology, climate) to the biotic (other competitors, predators, parasites) and might differ at a very fine grain. The primary prediction of the GMT is that, over the geographic range of an interaction, there will be hotspots where coevolution between species is intense and coldspots where coevolution is weak or nonexistent. From that mosaic, many other corollaries emerge, but the presence of hotspots and coldspots of coevolution is the foundation of the perspective.

When we looked at a map of TTX resistance in garter snakes, hotspots and coldspots were exactly what appeared. The entire range of allopatry with newts appeared to be a giant coldspot (just as predicted), as did much of the zone of sympatry. In fact, only two hotspots of resistance were obvious, one in Oregon and the other in the Bay Area of California. Snakes in both of those regions were 100 to 1,000 times as resistant as those from other regions. Visualizations of the landscape of resistance revealed that, away from these hotspots, resistance gradually decreased as would heat from a source. This pattern, too, was consistent with the GMT and could reflect individuals from hotspots and coldspots migrating and interbreeding to generate intermediate resistance phenotypes. In some localities, dramatic levels of genetic variation were apparent, again suggesting that alleles determining different resistance phenotypes were moving through the landscape. In one California population, families of snakes differed so much in resistance that we could not test them all with the same amount of TTX. Extremes of resistance in this locality matched both the lowest and the highest resistance of neighboring populations.

The existence of two resistance hotspots does not necessarily mean that the strength or history of coevolution varies across the range of newts and snakes. Resistance could have arisen only once in garter snakes and been distributed in its current pattern through reinvasion of the west after the most recent glaciation events. If this alternative hypothesis were valid, then the snakes from the Oregon and California hotspots should be each other's nearest relatives. To test this alternative, we needed a picture of evolutionary relationships among western *Thamnophis sirtalis*. The generation of such phylogeographic trees is fairly straightforward for those trained in molecular-genetic techniques and phylogenetic reconstruction. Unfortunately, neither my father nor I could claim those tools in our toolkits. We turned to Fred Janzen, a friend of mine from graduate school, to help us out. Janzen and his lab members routinely sample reptile populations and use PCR (the polymerase chain reaction technique) and gene sequencing to reveal genetic differences among groups in regions of the mitochondrial genome. These regions are preferable for reconstructing phylogeographic relationships because differences accumulate quickly and randomly; more differences indicate more distant relationships. Shared derived features that differ from other groups indicate close relationships.

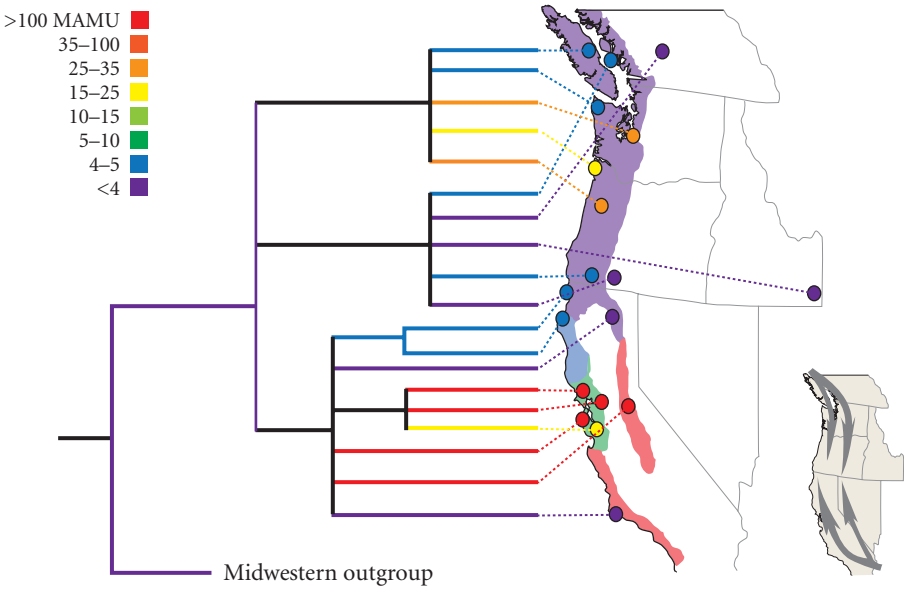

FIGURE 6 The evolutionary relationships of western *Thamnophis sirtalis* populations show multiple origins of TTX resistance. The phylogeny at left is based on relationships determined from mtDNA sequences and indicates at least three related groups of western *T. sirtalis*. The Northwest Coastal clade at the top includes resistant snakes in Oregon and Washington. The Intermountain clade is closely related but does not include any resistant populations. At the bottom is the California clade, which includes resistant populations. The phylogeny supports a biogeographic pattern (*lower right*) in which California populations stem from a southern ancestor and the Northwest Coastal and Intermountain groups come from a northern ancestor, both of which are expected to be nonresistant to TTX. The colors refer to TTX resistance levels measured in Mass Adjusted Mouse Units.

Genetic analyses suggested that the group of populations now present throughout California came from reinvasions from a southern refuge population, while the lineages along the coast and mountains of the Northwest claimed an ancestor to the north, probably from a refuge in the Haida-Gwaii Islands off the coast of British Columbia. Resistance to newt toxin had arisen at least once in each of these major modern lineages. History could not explain our distribution of hotspots.

If an arms race were truly driving the repeated evolution of TTX resistance, then we would expect the resistance levels of the garter snakes to closely match toxicity levels of the local newts. If this were true, then many populations of newts must lack toxicity. To test this prediction, we needed a way to quantify

the TTX levels in individual newts. Once again, the traditional approach of toxicologists to developing an LD50 was too coarse to accommodate our questions. Japanese scientists had been investigating TTX levels, mostly in pufferfish, using HPLC (high pressure liquid chromatography) to precisely measure quantities of toxin in a given sample. HPLC is a notoriously finicky methodology that requires very specific reaction conditions, an obsession with technology, great persistence, and a bit of voodoo. The reaction to measure TTX is one of the fussiest, and no one in North America had ever successfully developed a working HPLC setup for this task. Uncowed by our technical ignorance, a graduate student in my father's lab decided to give it a go. Charles Hanifin made connections with the Japanese lab that developed the HPLC methodology for TTX and arranged for a six-week stay in Japan to learn from the masters. He returned with great confidence and cobbled together a working HPLC unit from spare parts and a few supplies. New HPLC units cost more than our combined labs netted in total research funding in a year. The first attempts to quantify TTX in our lab were complete flops. The pump on the HPLC unit was not precise enough, the glass columns cracked under too much pressure, seals on fittings blew, everything seemed to go to pieces. Hanifin kept at his tinkering until he finally had the machine producing repeatable runs of standards, then replicated the sample runs he had performed in Japan. Who knows what he did during the many all-nighters to make the machine work. (I still suspect that he made some deals with minor deities.)

To compare newts and snakes at each locality, we would have to collect samples of newt skin from almost 30 localities for which we had estimates of predator resistance. Hanifin followed a tip from his dermatologist father and discovered that a 5-mm skin punch used by doctors to take biopsies from humans yielded us a consistent sample that did not require sacrificing the donor. To see if we were on the right track, we tested TTX levels in skin samples that we had collected over the years and stored in a freezer. Comparing five localities of newts and snakes in the northern part of their range showed an almost perfect match. Where snakes had no resistance, newts had no detectable TTX; where resistance levels were elevated, so was newt toxicity. However, this pattern was based on only a tiny part of the phenotypic and geographic range of the interaction.

Mismatched abilities of interacting species are a major prediction of the Genetic Mosaic Theory of Coevolution. If mosaics of hotspots and coldspots exist, then the movement of genes and individuals from one locality to another should result in some places wherein predator traits and prey traits are out of step. Defining and recognizing a mismatch is a deceptively difficult proposition. A mismatch between individuals indicates that one player dispatches the other without harm or cost. At the population level, where it really matters for

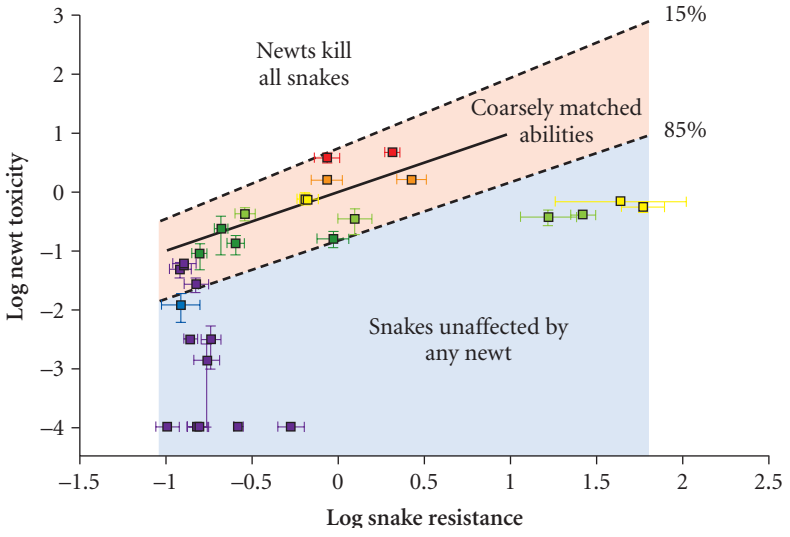

FIGURE 7 The phenotypic mismatch between newt toxicity and snake resistance. Each locality appears on this plot as a point with the range of resistance or toxicity shown by bars around that point. The zone in orange outlines all of the combinations of toxicity and resistance that could result in impairment to the predator and a risk of death to the prey. Above that zone are combinations wherein newts are so toxic they would kill any snake that ingested them; below that zone are combinations wherein any snake could eat a newt without any effect on its performance. Colors of the symbols refer to newt toxicity as in Figure 7.

coevolution, one player might interact with any of the possible players from the interacting population. Thus, a snake might attack the most toxic or the least toxic newt in a population. We reasoned that, if the abilities of one species were so far ahead of the other that they could handle an interaction with no fitness consequences, this would constitute a mismatch. Moreover, the mismatch would have to hold for every possible pair of individuals at a given locality. If newts were "ahead," then even the least toxic newt would have to be able to fully immobilize the most resistant of local garter snakes. Similarly, for the snakes to be ahead, the least resistant snakes would have to be able to swallow the most toxic newt in the vicinity without any ill effects.

The evaluation of a mismatch, then, requires comparing distributions within the context of a functional relationship. Just having lots of toxin does not necessarily make a newt well defended if, for example, the snakes it encounters are very resistant to the toxin. Bit by bit, we had amassed the pieces of this functional puzzle: how does the toxicity of a skin sample relate to total newt toxicity,

how does snake resistance to an injected dose of TTX compare with an ingested dose, and what are the relationships with size and sex, both for the snakes and for their prey? All of these elements allowed us to generate a reasonably accurate picture of the outcome of an interaction between a newt of given toxicity and a snake of given resistance. Three phenotypic spaces are clear: above a certain level of toxicity, a snake is likely to die after attacking a newt. Below a different level, newts do not have sufficient toxin to deter or impair a snake. In between, a zone of possible matching occurs wherein many different outcomes could result. Because selection requires differences in individual fitness, only the middle zone could be considered a phenotypic space in which reciprocal selection and coevolution could occur.

When we mapped the newt-toxicity data against snake-resistance data, our simple story of enemies with matched abilities fell apart, as did our picture of hotspots. Roughly half of the localities we sampled were so mismatched in predator and prey abilities that no current selection is expected to occur. Although snake resistance and newt toxicity shared similar geographic patterns of elevation, suggesting matched abilities throughout the ranges, functional comparisons revealed a very different picture. In fact, some of the places that we had labeled as hotspots based on snake phenotypes alone (such as the Bay Area of California) now turned out to be true coevolutionary coldspots. The geographic pattern of trait exaggeration is a poor indicator of the pattern of potential for reciprocal selection.

The one-sided nature of the interaction was truly shocking. While we might have expected some mismatches, we never could have guessed that one species, the snakes, would always come out ahead in the arms race. For all of the variation in traits, environment, and a geographic range that spanned most of the western coast of North America, we did not see a single case in which the prey were too toxic for the local snakes to handle. Within this group of mismatched populations, two groups of localities were apparent. On one end of the spectrum, both predators and prey had elevated traits. In fact, those localities included the most resistant snakes yet found—resistant enough to safely ingest so much TTX that it would kill hundreds of humans—as well as some of the more toxic newts. At the other end of the distribution were localities in which both species had values of resistance or toxicity consistent with the ancestral trait predicted for the lineage. For these populations, there was no evidence that phenotypic escalation had ever occurred in either species.

The picture that emerges of the arms race between newts and snakes looks like this: in some places (especially in Northern California and British Columbia), both predators and prey exhibit low levels of defenses and appear to never have engaged in an arms race. In these cases, snake resistance is low and at ancestral levels, but is still strong enough to enable them to eat any local newt.

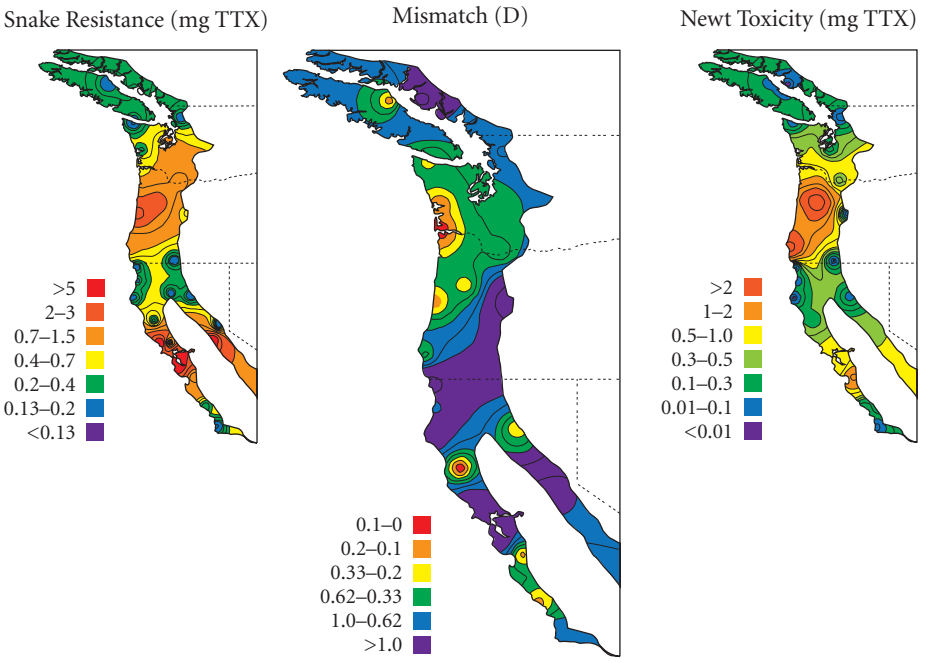

FIGURE 8 Hotspots of TTX, TTX resistance, and reciprocal selection in the arms race between newts and snakes. The upper maps show distributions of resistance in snakes (*left*) and toxicity of newts (*right*) where both occur. The general pattern of trait exaggeration is the same in both taxa, suggesting that coevolution has led to the elevation of toxicity and resistance. The map of trait matching (*middle*) shows a different pattern. The mismatch index, D, measures the degree of functional mismatch between newt and snake traits. Where traits are grossly mismatched, reciprocal selection does not occur. These regions are shown in colder colors (*green, blue,* and *purple*). Hotspots of coevolution are expected to occur where newts and snakes have closely matched abilities, shown in warmer colors on this map.

Newt toxicity is apparently never severe enough to deter snake predators, and so reciprocal selection does not occur. In a few localities, something has caused newt toxicity to increase to a level that enables newts to exert selection on snake resistance, and these populations enter into an arms race. A number of localities show the signature of this arms race through coarse matching, and coevolution seems to push them along a line of escalating predator and prey abilities to ever-increasing phenotypic extremes. Then, in at least two lineages of snakes in California, resistance reaches a level that allows predators to escape the arms race altogether. Resistance is so extreme that the prey no longer represent a

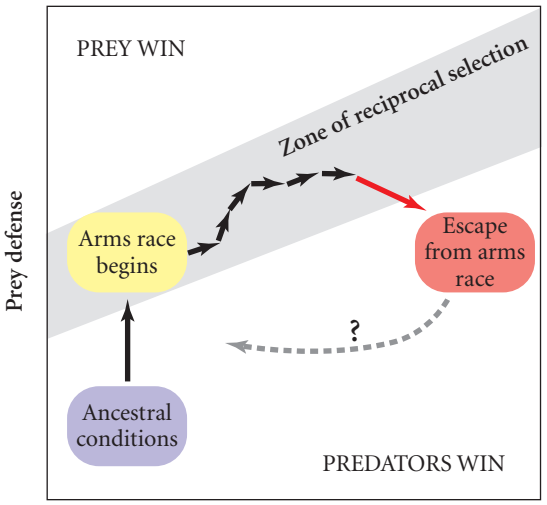

FIGURE 9 The general dynamic of arms race coevolution observed between newts and snakes. Localities begin at lower left, with snakes slightly ahead in the race. Some force causes newt toxicity to increase until the locality resides in the gray zone where traits are closely enough matched to generate reciprocal selection. At this point, arms race dynamics can ensue, and localities wander through this zone of coevolutionary selection, following a pattern of escalation and counter escalation. At some point well into the arms race, some populations appear to experience a mutation with large phenotypic effect that jumps populations out of the zone of reciprocal selection and into a zone of escape (red circle). What happens next is unclear, but one possibility is that costs of adaptation cause the decrease in toxicity and resistance until populations once again enter the zone of reciprocal selection and arms race dynamics resume.

selective pressure. The toxicity of the prey remains high in these areas, but it did not keep pace with resistance, and now selection does not favor increases in toxin levels. The future fate of these localities in which predators have the advantage is unknown. One possibility is that the costs of maintaining toxicity and resistance will eventually cause those traits to decrease until the populations re-enter the phenotypic space where reciprocal selection can once again restart the arms race.

Most of the details of this dynamic are still mysterious to us. What force causes the initial escalation of newt toxicity? Is it selection from another predator? Do exogenous sources of toxicity play a role in causing sudden changes in toxin levels? Why do prey never seem to get ahead in the arms race? Is there some physiological limit to how much toxin can be produced or held by a

newt? What allows the TTX resistance of snakes to increase so dramatically that reciprocal selection is precluded? The answer to the last question may lie in the physiological and molecular mechanisms of TTX resistance in snakes.

Throughout our studies of the newt–snake arms race, my father and I have been curious about the physiological mechanism that enables snakes to resist the effects of TTX, but we did little to push that direction. Evolutionary biologists often focus on the "why" questions regarding the process of evolution, without concentrating too much on the reductionist details of how the beasts we study work mechanistically. This bias can sometimes leave us blind to the adaptive steps that must occur in order for evolutionary change to take place. In our case, we knew that garter snakes were resistant to a potent neurotoxin, but we did not know how they accomplished this feat at the physiological level. Our ignorance of mechanisms did not prevent us from exploring coevolutionary dynamics and patterns of evolutionary convergence, but we had little idea how much more we would come to understand when the mechanism was revealed.

At first, the range of possible mechanisms of resistance was daunting. Many organisms neutralize poisons by breaking them down in the digestive system with special enzymes, and others have compounds in their circulatory systems that bind to toxins and render them inactive. Still others change basic elements of their own physiology so that the active sites where toxins bind are not recognized by the molecule. Our earliest experiments used simple approaches to try to narrow the scope. TTX mixed with the blood of resistant snakes was still lethal to mice, so we reasoned that the toxin was not inactivated in the circulatory system. Similarly, delivering TTX into a snake intraperitoneally bypasses the digestive system, but snakes were just as resistant to injected toxin as they were to ingested toxin, indicating that the toxin was not broken down during digestion. So we were left looking for some other aspect of physiology, but what? And in which tissues?

We were lucky to be studying a toxin that was so well understood at the physiological level. TTX has been used for decades in neurophysiological studies because its action is simple and well known. In all animals, information is transmitted along nerves and muscles via electrical impulses. These impulses are propagated as action potentials that are generated by moving charged ions across cell membranes, thereby creating differences in electrical charge. As the inner and outer charges change, electricity moves along the membrane. In vertebrates, the voltage-gated sodium channels are among the most critical components of this machinery. These channels selectively move positively charged sodium ions across membranes and into cells and then back out again on a scale of milliseconds. In concert with a similar potassium pump, these channels generate rapid changes in membrane potential that drive information along nerves and muscles. This mechanism is incredibly highly conserved among all

vertebrates, so that not only is the sodium–potassium pump the same, but the very molecular structure of the proteins that form the ion channels is nearly identical from fish to humans.

TTX acts as a cork to voltage-gated sodium channels. The toxin binds with remarkably high affinity to sodium channels in skeletal muscle and nerves and plugs the pore so that sodium ions cannot enter or leave the cell. Whole subdisciplines of neurophysiology and structural biochemistry have been devoted to trying to identify the structure of voltage-gated sodium channels, but, as membrane-bound proteins, they are difficult to visualize. Sodium channels are a family of proteins encoded by 6 to 10 different genes, each of which is expressed in different tissues. One major effort currently underway is to examine how easily different toxins bind, and thereby to reveal the protein charges and structures. This is where TTX comes in. Over several decades, researchers have experimentally changed sodium channels a few amino acids at a time and then have asked whether the binding of toxins has changed as a result. In the process, we have learned where in the channel TTX is most likely to bind, thereby illuminating the basic protein structure of sodium channels that are locked inside of a membrane.

This perspective on the action of TTX led us to suspect that garter snakes might have evolved resistance to the toxin by changing some aspect of the sodium channels in their nerves and muscles. This mechanism would be extraordinary, because the genetic sequence that codes for a given sodium-channel protein is almost exactly the same across all vertebrates, yet it seemed like the most logical place for us to start. Unfortunately, neither my father nor I knew anything about neurophysiological techniques. For many years, we just shelved the notion of looking at mechanisms of resistance in favor of taking our research in other directions.

Then, along came a neurobiology graduate student with an interest in evolutionary biology. Shana Geffeney had training in biophysics, but unlike many in her subdiscipline, she was also keen to explore the processes and pressures that drive evolutionary changes in neurophysiology. Her first strategy was to ask if our resistant and nonresistant snakes differed in skeletal-muscle sensitivity to TTX. Using a simple textbook technique in neurobiology, she removed skeletal muscle from snakes, pinned it out in a saline bath, and measured the propagation of action potentials along the muscle fiber. Then she added a concentration of TTX to the saline solution and tested the muscles again. Geffeney discovered that populations of snakes differed dramatically in how their muscles responded to TTX. For populations of snakes outside the range of newts, low concentrations of TTX totally shut down action potentials so that no electrical current at all was moving along the muscles. For populations we classified as mildly resistant to TTX based on whole-animal performance, it took 10 to100

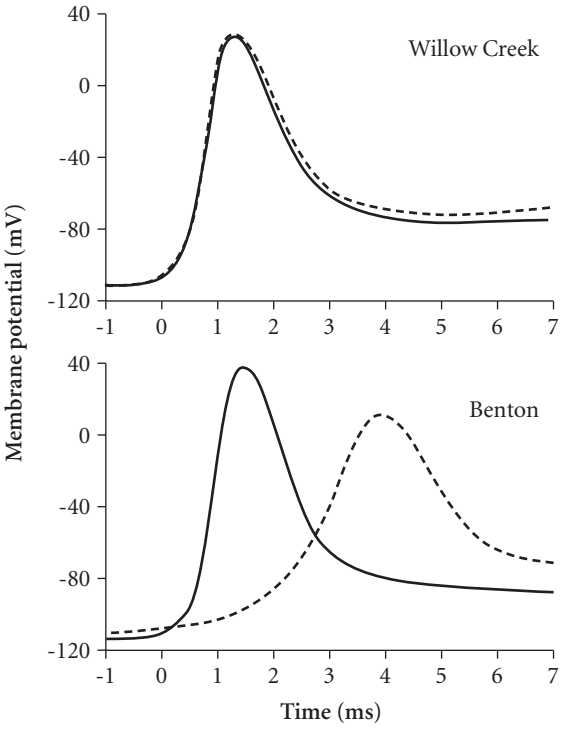

FIGURE 10 Action potential traces from an experimental test of skeletal muscle resistance to TTX. Solid curves show the membrane voltage of a muscle held in saline; dashed lines show the same curve after application of a dose of TTX. The Benton population of snakes is highly resistant, but still has its action potentials dramatically slowed. The Willow Creek population is one of the most resistant ever tested. Its action potentials are unaffected by TTX. The test concentration of TTX would completely block any action potential in a human or mouse muscle.

times as much TTX to even slow down the transmission of electricity. For the most resistant population tested, she was never able to affect action potentials, even with 1,000 times the concentration that completely arrests a mouse or human channel.

Geffeney's results nailed down the physiological mechanism of resistance. Because the action of TTX is so specific, it was clear to us that the differences in how muscles from different localities responded to toxin were due to differences in how TTX binds at the sodium channel. Moreover, we saw differences between individuals that were not expected. Most aspects of neurophysiology are normally viewed as species-specific characters—that is, they were thought to be the same among all individuals in a population. If that were true, it would

be difficult to imagine how selection at the individual level could generate the observed differences in physiology among populations. Our data demonstrated the same kind of physiological variation among individuals within populations that explained differences in resistance among populations of snakes. Ironically, some biophysics researchers did not think Geffeney's work was exciting enough to publish because it used an old-fashioned technique. Imagine our satisfaction when, because of its importance in understanding coevolutionary process and the diversification of neurophysiology, it was eventually published in *Science*.

Bolstered by the confidence that sodium channels in skeletal muscle were involved, we were inspired to delve a bit deeper and to try to understand exactly what changes in sodium channels might confer resistance to TTX in snakes. Whatever the specifics, they would have to explain a range of resistance that varied as much as 1,000-fold and that evolved no less than twice in a single species. Using genetic tools developed for sequencing the human sodium channel from skeletal muscle, Geffeney attempted to amplify a region of the sodium channel from each of the four populations of snakes that she had previously tested for physiology. What we found was a clear pattern of sequence differences in one region of the gene that indicated that different amino acids were substituted in each population. In a nonresistant population of snakes, the amino acid sequence was identical to that seen in humans or mice—once again demonstrating the incredible conservatism across vertebrate lineages, even at the molecular level. Resistant snakes, on the other hand, had between one and four substitutions, depending on the population. Some of those changes were common to all lineages, but others were unique to a single population.

Although these amino acid substitutions correlate with population differences, it was not clear that they were, in fact, the molecular changes that were responsible for resistance evolution in snakes. After all, we only examined a portion of a single gene. Snakes are expected to have six to eight total sodium channels, and any one of them might influence whole-animal resistance. More to the point, the amino acid changes that we noted between nonresistant and resistant populations might have no bearing on how TTX binds to a channel and therefore be irrelevant to the adaptation in which we were interested.

To nail down whether we had really identified the molecular basis of TTX resistance, we needed to isolate the sodium channels and test their response to TTX in a novel background. To do this, Geffeney engineered new sodium channels and expressed them in egg cells from the African clawed frog (*Xenopus*). These new channels, called chimaeras, were based on human sodium-channel genes, which had only the amino acid substitutions that we thought might be important. For example, the Warrenton, Oregon, population of snakes had a single substitution of interest: valine was substituted for isoleucine at position 1561 in the protein. Geffeney took a human DNA sequence and switched only

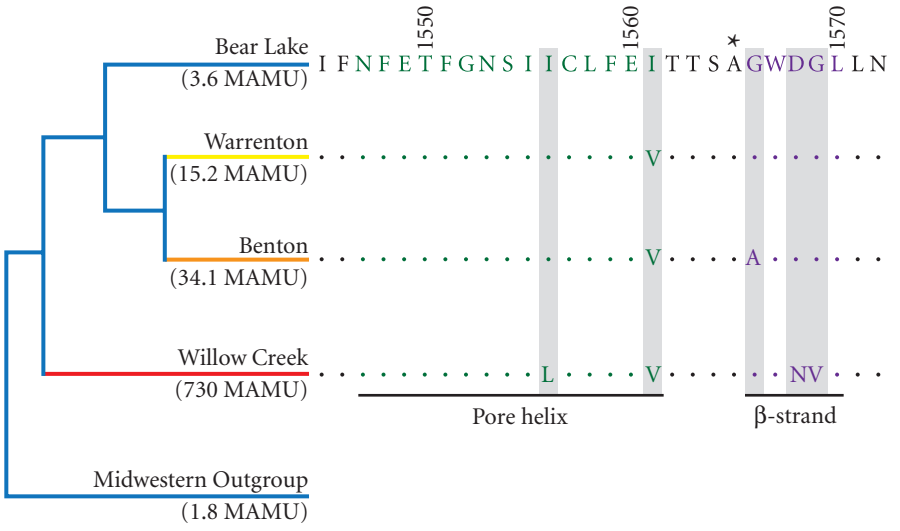

FIGURE 11 The amino acid sequence that confers resistance to TTX in garter snakes. The sequence shown is from Domain IV of the skeletal muscle sodium channel, $Na_V1.4$. The sequence from the Bear Lake population does not confer resistance to TTX, and is the same amino acid sequence found in most vertebrates, including humans. The other populations include snakes from Oregon populations (Warrenton and Benton) with high resistance and snakes from a California population (Willow Creek) with extremely high resistance. Resistance levels are shown in Mass Adjusted Mouse Units (MAMUs). The phylogeny on the left is taken from a larger study that clearly shows Willow Creek belongs to a different lineage than the Oregon populations and represents a novel origin of TTX resistance.

the codon that altered the isoleucine at position 1561 to generate a "Warrenton chimaera." Otherwise, the gene was like any other TTX-susceptible human sodium channel. She then introduced this chimaera into the developing *Xenopus* egg cell, so that the protein would be expressed in an entirely novel environment. No other aspects of snake physiology could influence the protein's function in this context. She repeated the process for each population and tested whether electrical current across these new chimaeric channels was blocked by TTX.

The differences in current blockage by TTX among the engineered channels matched our population differences in whole-animal resistance almost perfectly. Not only could we identify a gene responsible for population differences in resistance, but we now knew the specific changes to the protein that

conferred differences in resistance among our snake populations. A 1,000-fold difference in resistance could be explained by four or fewer amino acid substitutions. The most common substitution was the isoleucine-to-valine switch at position 1561 in the protein. In one fortuitous accident, Geffeney left this change out of some sodium channels and learned that this substitution alone had only a two-fold effect on resistance. The less common substitutions must be responsible for the majority of extreme TTX resistance in some populations. These results do not rule out other important changes in other genes, but they clearly establish these mutations in the skeletal muscle sodium channel, $Na_V1.4$, as a major source of adaptive differences across garter snake populations.

Our exploration of the mechanisms of TTX resistance in snakes has given us unexpected tools to address fundamental questions about convergent evolution and predator–prey arms races. Comparing the molecular changes in sodium channel function across the evolutionary history of *Thamnophis sirtalis*, we see evidence both of repeated answers and unique answers to evolutionary challenges. The most common amino acid change appears to have evolved independently in both the California and Pacific Northwest lineages of garter snakes. Other substitutions are unique to specific populations of snakes, and they seem to convey different levels of resistance. All of these changes occur in one very limited region, known as the S5-6 linker region of Domain IV, of a single sodium channel gene. This point alone indicates strong constraints in the evolution of sodium channels: there must be very few parts of the protein that can be changed to reduce TTX binding without serious detrimental effects on sodium channel function.

The molecular basis of resistance is likely at the root of why some populations of snakes have escaped the arms race with toxic newts. As Geffeney learned with her chimaeric proteins, it only takes one or a few amino acid substitutions to have dramatic impacts on TTX binding. As new mutations arise, they may have such huge effects on the magnitude of TTX resistance that a snake population is shot suddenly out of the zone of coevolutionary selection. These adaptive jumps are so massive that only one or two steps are needed for snakes to escape the arms race.

Decades later, the answer to Doc Walker's question of whether a newt in a coffeepot might have killed those hunters in the Oregon Coast Range is a clear yes. Not only do we now know that newts of the genus *Taricha* in general are poisonous, but we also know that the newt of legend would have come from the area with the highest toxicity levels that we have ever measured (and more than enough to kill three adult humans). The site of the Coffeepot Incident is, in fact, one of two that we can point to as coevolutionary hotspots in the arms race

between snakes and newts. In some ways, it could be said that it is really garter snakes that killed the legendary hunters.

But the answered questions are not even half of the story. The project that started for us as an intellectual detour that we thought might be finished with a few quick experiments has grown to be our major research effort. Instead of wrapping up a line of research, each hard-won finding has opened a door into an immense new area of questions and techniques. With the help of outstanding collaborators, my father and I have been able to add tools from chemical ecology, phylogenetics, neurophysiology, and molecular genetics to our research toolbox. Each of these has opened our eyes and minds to the importance of integrating different levels of inquiry when addressing evolutionary problems.

Ultimately, however, our work still garners its fundamental inspiration from discoveries made in the field. Just as the initial impetus for our studies of predator–prey arms races came from unexpected observations of natural predation, our current directions are driven from surprising findings from field studies of our own and others. For many years, we were convinced that the arms race between newts and snakes involved only *Thamnophis sirtalis* as a predator, even though some six or seven other species of garter snakes might be expected to feed on newts in Western North America. Anecdotal accounts of newt predation by two other species, *T. atratus* and *T. couchii*, started to reach us from colleagues working in localities that we had not had a chance to visit. When we pursued these leads, sure enough, we found these species of snakes were feeding on newts and had evolved dramatically strong TTX resistance. We now have an exceptional tool—at least three species and five independent origins within garter snakes—to explore repeated evolution of resistance.

It is really no surprise that we are far less likely to run out of questions about evolution than we are to run out of time to pursue them. After all, the cycle of fieldwork leading to hypotheses, and tests of hypotheses to new questions, was the foundation of modern studies of evolution. Darwin and Wallace each constructed their theories of natural selection on the firmament of many years of field trips and natural-history observations from all corners of the world. The rest of us have spent the ensuing century and a half trying to understand the nuances of the process they outlined. For us, just as for Darwin and Wallace, it is the knowledge of organisms in the natural world that reveal the paradoxes that demand explanation. You can never predict when something nasty barfed out of a snake will lead you down a path of discovery with an endless array of detours to explore.

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