FEATURE ARTICLE

Of Newts and Neurotoxins: Coevolution in a Predator-Prey System Provides a Multifaceted Backdrop for Engaging Students

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ABSTRACT

Storytelling can stimulate learning by delivering scientific content within a narrative that increases comprehension and engagement. In this article, I describe the coevolutionary arms race between toxic newts and predatory garter snakes. This engaging story centers on the use of a deadly neurotoxin called tetrodotoxin (TTX) as an antipredator defense. Some species of newts contain TTX in their tissues, but resistance to TTX has developed through convergent evolution in garter snakes and other species. TTX resistance results from mutated voltage-gated sodium channels. These channels, whether TTX resistant or not, are found in all animals and are vital to the function of nervous and muscle tissues. Through reciprocal selection, coevolution has created phenotypic matching between toxic newts and TTX-resistant garter snakes across their range in the western United States. In other words, as newts became more poisonous, garter snakes became more resistant. These results and the scientific process behind them are discussed in detail. This story can be used by educators to provide a unifying and engaging backdrop as students learn multiple aspects of biology, such as protein structure, genetics, phylogenetics, electrical signaling, evolution, and the process of science.

Key Words: coevolution; tetrodotoxin; newt; garter snake; mutation; genetics; resistance; voltage-gated sodium channel; neurotoxin.

Introduction

Your thoughts race as you swallow the first bite. Is your mouth going numb or is your mind playing tricks on you? You know that numbness precedes paralysis and that paralysis precedes death. You wonder why you took this unnecessary risk.

You are eating fugu, a Japanese delicacy made from pufferfish. It is purportedly delicious but also potentially deadly (McCurry, 2016). As you savor the fugu’s texture and umami taste, you trust that the chef expertly prepared the dish without tainting the meat with the puffer’s poison, a paralyzing compound known as tetrodotoxin (TTX). This toxin is found in a diverse group of species, from bacteria to pufferfish to newts, and it disables the nervous and muscular systems of vertebrates, even at low doses. Some TTX-producing species, such as the rough-skinned newt, can contain enough toxin to kill 50 full grown humans (Yasumoto & Yotsuyamashita, 1996). Why would newts have such ridiculously high amounts of toxin if only a little will do?

Enter the humble garter snake. To them, newts are like fugu: a savory meal that could kill from TTX poisoning. But certain species of garter snakes eat with impunity, gobbling up entire newts and living to tell the tale. How do they achieve this remarkable feat, and does their resistance help explain why newts can have enough TTX to immobilize a potential predator 100,000 times its size? Is this some sort of coevolutionary arms race between predator and prey?

Storytelling such as this can be an effective pedagogical tool for increasing cognitive engagement in biology (Carroll, 2018). In this article, I explore one of the world’s most lethal neurotoxins, its mysterious origins, and a fascinating arms race between predator and prey. A dynamic evolutionary interaction between garter snakes and newts has produced remarkable adaptations that reveal important biological insights. This scientific tale can be utilized by educators to provide an engaging and authentic backdrop for teaching organismal biology, evolution, genetics, pathophysiology, phylogeny, and much more. As you read this article, consult Table 1 for a summary of topics and suggestions for how each can be used in the classroom.

The Mysterious Origin of Tetrodotoxin

Tetrodotoxin (TTX) gets its name from Tetraodontidae, the family that includes the various species of TTX-laden pufferfish. While humans have known since antiquity that pufferfish are toxic (Chau et al., 2011), the chemical was not isolated and purified as a crystal until 1950, first from pufferfish (Yokoo, 1950) and later from the newt genus Taricha (Brown & Mosher, 1963). It is now known
Table 1. A summary of topics related to tetrodotoxin and the coevolutionary arms race between newts and garter snakes, with suggestions for engaging students.

| Topic                                                                 | Learning concept                      | Student engagement                                                                 |
|-----------------------------------------------------------------------|---------------------------------------|------------------------------------------------------------------------------------|
| Molecular attributes of tetrodotoxin (TTX)                           | Organic chemistry                     | Interpret organic shorthand notation, identify functional groups, discuss biosynthesis of TTX (Chau et al., 2011). |
| Evolutionary origin of TTX                                           | Phylogeny and evolution                | Create and interpret a phylogenetic tree of organisms that produce TTX (which can be constructed from Chau et al., 2011). Is TTX production a homologous or analogous trait, or neither (Chau et al., 2011)? |
| Voltage-gated sodium (Na⁺) channels                                  | Electrophysiology; protein structure, binding, and inhibition | Discuss normal structure and function of Na⁺ channels. Explore the effect of TTX on functionality of Na⁺ channels. Compare the action of TTX to lidocaine, which acts as a noncompetitive antagonist for Na⁺ channels, as an example of protein-ligand mediated interactions (Sheets & Hanck, 2003; Yu & Catterall, 2003; Jost et al., 2008; Hannifin, 2010; Tikhonov & Zhorov, 2012). |
| Within a species there are multiple SCN genes, and expression of these genes creates different Na⁺ channels | Paralogous genes; differential gene expression | What are some potential advantages of gene duplication? Explain why Na⁺ channels in the brain are unaffected by TTX despite having no TTX-resistant mutations (answer: blood brain barrier) (Yu & Catterall, 2003; Zakon, 2013; McGlothlin et al., 2014; Brodie & Brodie, 2015). |
| Exogenous production of TTX                                          | Symbiosis; hologenome                 | What type of symbiotic relationships exist between TTX-producing bacteria and pufferfish and newts? How might the genes between newts and bacterial symbionts (collective referred to as the hologenome) be an example of coevolution (Chau et al., 2011; Vaelli et al., 2020)? |
| Mutations to the SCN genes create novel TTX-resistant Na⁺ channels | Mutation; protein function; transcription and translation | Why might a missense mutation that changes methionine to threonine inhibit binding of TTX to the Na⁺ channel? How are the properties of side chains relevant to protein-ligand binding? Is it possible for a mutation to result in no structural or function changes in a protein (Jost et al., 2008; Feldman et al., 2009; Feldman et al., 2010; Feldman et al., 2012)? |
| Snakes, newts, and pufferfish all have similar means of TTX resistance | Convergent evolution                   | Interpret a phylogenetic tree that demonstrates convergent evolution of TTX resistance. (For examples see Feldman et al., 2009; Hanifin & Gilly, 2015). |
| Phenotypic matching between garter snakes and toxic newts             | Coevolution                           | What does reciprocal selection mean, and how might this play out between toxic newts and predatory snakes (Hague et al., 2020; Reimche et al., 2020)? |
| Phenotypic mismatching between garter snakes and toxic newts          | Geographic mosaic theory of coevolution; gene flow, genetic drift | Why might phenotypic mismatching occur between toxic newts and predatory snakes? What other mechanisms contribute to genetic structure within a population (Hague et al., 2020; Reimche et al., 2020)? |

TTX occurs in a small number of species in at least seven animal phyla (Arthropoda, Chaetognatha, Chordata, Echinodermata, Mollusca, Nemertea, and Platyhelminthes) (Chau et al., 2011) and is used for defense against predators, or in the case of one type of flatworm, to subdue prey (Ritson-Williams et al., 2006). TTX also occurs in some species of dinoflagellates and bacteria (Chau et al., 2011).

TTX (Figure 1) is one of the most potent naturally occurring neurotoxins and is lethal to humans and most other vertebrates (Brodie et al., 2005; Feldman et al., 2010). Its toxicity results from its ability to inhibit transport proteins called voltage-gated Na⁺ (Na⁺) channels, which are found in the plasma membranes of muscle cells and neurons. Na⁺ channels are highly conserved among vertebrates and therefore remarkably similar in both structure and function (Brodie et al., 2005; Feldman et al., 2009; Hanifin & Gilly, 2015). TTX binds to the extracellular portion of the channel’s pore, which

![TTX Diagram](image-url)

**Figure 1.** Tetrodotoxin (C₁₃H₁₇N₃O₈) acts as a neurotoxin by inhibiting voltage-gated sodium channels. Its exact biosynthesis is unknown (Chau et al., 2011).
impedes the passage of Na⁺ and prevents the synthesis of electrical signals called action potentials (Tikhonov & Zhoro, 2012). Without action potentials, nerve and muscle cells cannot function, which can ultimately lead to paralysis and death (Brodie et al., 2005; Feldman et al., 2009). In humans, death typically occurs in four to eight hours, sometimes as quickly as 20 minutes, and there is no antidote (National Institute for Occupational Safety and Health, 2011).

How exactly TTX is produced is bit of a mystery, which adds the intrigue of this story. The fact that this toxin appears in such widely disparate taxa seems to rule out both homology and convergent evolution (Chau et al., 2011). If TTX production was a trait resulting from homology, it would have arisen long ago in a common ancestor of animals, algae, and bacteria, and thus the trait would have been handed down to a very large number of species, which is not the case. Also, TTX is such a unique and apparently difficult molecule to biosynthesize, it is unlikely that its creation would have independently evolved so many times in widely different animal taxa (Chau et al., 2011).

A more parsimonious hypothesis is that TTX is manufactured by a relatively small number of bacterial species and then bioaccumulates through food webs or is acquired directly via bacterial symbionts (Chau et al., 2011). Such is the case for the pufferfish. TTX-producing bacteria are known to live as symbionts with these fish. In fact, pufferfish that are raised in captivity and fed a controlled diet lose their toxicity over time, suggesting their inability to intrinsically produce the toxin (Noguchi et al., 2006).

Exogenous production of TTX by microbial symbionts, like that occurring in pufferfish, was suspected for all TTX-bearing animal taxa except certain species of toxic newts, most notably the rough-skinned newt (Taricha granulosa) (Hanifin, 2010). Endogenous production of TTX in rough-skinned newts was partially supported by the following observations. First, prior to 2020, researchers were unable to isolate and detect TTX-producing bacteria from toxic newts (Chau et al., 2011). Second, Taricha newts living in captivity increased TTX production over time despite being fed a diet known to decrease TTX production in other toxic animals. Thus, it was apparent that rough-skinned newts did not acquire their TTX through dietary means (Hanifin et al., 2002). Lastly, it appeared that TTX toxicity in newts is subject to evolutionary pressures, suggesting a genetic component related to its production (Brodie et al., 2005).

There are several counterarguments to these claims. First, it was possible that toxic newts did in fact harbor TTX-producing bacteria as symbionts and those bacteria had not yet been detected. It is estimated that only 1% of microbes are culturable (Chau et al., 2011). Thus, the inability to detect TTX-producing bacteria was not proof of their absence. Additionally, any genes acted upon by natural selection may be related to the newt’s uptake and storage of bacterially derived TTX, and not from its endogenous production (Hanifin & Gilly, 2015).

Evidence against endogenous production came from a novel isotopic feeding study. Taricha newts were administered four types of nutrients (acetate, arginine, citrulline, and glucose) constructed from radioisotopes of carbon (¹³C). These four nutrients were chosen because it was hypothesized that they could be used to create TTX in certain metabolic pathways. Results from the study demonstrated that newts used the ¹³C to make new metabolites, such as cholesterol derivatives and amino acid derivatives, but none of the ¹³C was found in newly produced TTX, suggesting that the newts’ metabolism was not responsible for its creation (Shimizu & Kobayashi, 1983).

The decades-long mystery of how rough-skinned newts acquired their toxicity was finally settled as I wrote this article. Researchers identified four genera of TTX-producing bacteria living on the skin of rough-skinned newts (Vaelli et al., 2020). Pseudomonas was one of those four and was especially important in characterizing the difference in microbiomes between the toxic and nontoxic newts included in the study. This research represents the first time that bacterial symbionts capable of producing TTX were identified in anything other than a marine animal species. Subsequently, it now appears that the evolution of toxicity in newts might involve the interplay of genes between the newt and its bacterial symbionts, something referred to as the hologenome (Vaelli et al., 2020).

Despite this breakthrough, a mystery still remains: how do bacteria make TTX? No genes or biosynthetic pathways have yet been identified (Chau et al., 2011). The enigma of TTX production remains to be solved, perhaps by one of your students!

**Vive la Résistance**

With an understanding of TTX and how it acts as a neurotoxin, I can now focus on the evolutionary arms race between toxic newts and the predatory garter snakes that stubbornly resist them. A handful of snake species have evolved resistance to TTX. How did they achieve this, and are these traits due to shared ancestry (homology) or convergent evolution (analogy)?

**Molecular Basis of TTX Resistance**

Students in introductory biology and physiology courses study electrical signaling and thus learn about Na⁺ channels. What they might not know is that all animals have these channels and the channels share genetic and structural similarities (Yu & Catterall, 2003). This is because Na⁺ channels are a homologous trait inherited from a common ancestor of animals that lived approximately 650 million years ago (Zakon, 2013). Na⁺ channels were critically important in the evolution of animals because of their central role in the development of the nervous system (Zakon, 2013).

Na⁺ channels are membrane proteins made of a single alpha subunit and one or more beta subunits (Yu & Catterall, 2003). The alpha subunit and its four domains are arguably the most important, as they contain the pore and gate that regulate diffusion of Na⁺ into cells, thereby initiating an action potential. Interestingly, TTX played an important role in how scientists came to understand the structure and function of Na⁺ channels (Yu & Catterall, 2003). As previously noted, TTX inhibits Na⁺ channels by interfering with the diffusion of Na⁺. Researchers used the ability of TTX to bind to these protein channels as a way of exploring the amino acid sequence of key structural segments (reviewed in Hanifin & Gilly, 2015).

For vertebrates, the genetic instructions for Na⁺ type 1 (hereafter Na₁, with decimals denoting subunits) channels reside in the SCN gene family, which codes for the alpha subunit of the channel protein complex. The number of genes in this family vary by taxa, from two in lamprey to ten in mammals (Zakon, 2013). Genes in the SCN family were created through multiple gene duplication events and now code for slightly different Na₁ channels (Zakon, 2013), with the different types often expressed in different tissues (Brodie & Brodie, 2015).

Genes created through gene duplication can diverge from one another over time and can take on new functions; such genes are described as paralogous. In mammals and reptiles, for example,
there are nine functional paralogous genes that code for nine types of voltage-gated sodium channels (as previously noted there are ten genes in mammals, but one of these, Na₉, has mutated and taken on a new function as a salt sensor) (Hiyama et al., 2002; Yu & Catterall, 2003). Students might be interested to know that in humans, mutations in these genes can result in such disorders as epilepsy (National Institutes of Health, 2020a), periodic paralysis, and muscle weakness (National Institutes of Health, 2020b).

Interestingly, some Na⁺ channels are naturally resistant to TTX or otherwise protected from it. In snakes (and similarly in humans), tests have demonstrated that channel subtypes Na⁺1.1, Na⁺1.2, and Na⁺1.3 are sensitive to TTX (Yu & Catterall, 2003). However, because they are expressed in the central nervous system, they are normally protected by the blood-brain barrier, which prevents TTX from entering that nervous tissue and impairing the channels (McGlothlin et al., 2014; Brodie & Brodie, 2015). Na⁺1.5 channels are expressed in heart muscle and are naturally resistant to TTX, as are the Na⁺1.8 and Na⁺1.9 channels of the peripheral nervous system (PNS). Lastly, the remaining three varieties of channels are susceptible to TTX: Na⁺1.4, found in skeletal muscle tissue, and Na⁺1.6 and Na⁺1.7 found in the PNS (Brodie & Brodie, 2015).

Thus, to resist succumbing to the effects of TTX, both newts and garter snakes require mutations in the Na⁺1.4, Na⁺1.6, and Na⁺1.7 channels, with the Nav1.4 channel being especially critical for its role in controlling muscle movement and breathing.

**Common Solutions**

Resistance to TTX has developed repeatedly in multiple animal taxa, among both predators and prey. It is important to recognize that prey, such as newts and pufferfish, also require resistance to TTX because their Na⁺ channels are just as susceptible as those in snakes, humans, or any other vertebrate. What is remarkable is that TTX resistance has independently evolved multiple times through convergent evolution, and animals such as newts, snakes, and pufferfish have all arrived at a common solution.

That solution involves mutated SCN genes that produce amino acid substitutions and structural changes in Na⁺ channels (Figure 2). These changes reduce the ability of TTX to bind to Na⁺ channels by physically blocking TTX or disrupting normal electrostatic interactions with it, such as hydrogen bonds (Feldman et al., 2012). Approximately 80 amino acids compose the region of the outer (extracellular) pore where TTX binds to Na⁺ channels (Tikhonov & Zhorov, 2012) and it takes as little as a single amino acid substitution in this region to substantially reduce the binding affinity of TTX (Feldman et al., 2009). For example, compared to the ancestral condition, the Sierra garter snake (Thamnophis sirtalis) has an amino acid substitution in domain III of the Na⁺1.4 channel that replaced a methionine and its hydrophobic side chain with a hydrophilic threonine (Feldman et al., 2009). This very same mutation developed in toxic pufferfish and rough-skinned newts through convergent evolution. Studies of this particular amino acid substitution found that it increased resistance to TTX by a factor of 15 (Jost et al., 2008).

While I just highlighted the effects of just a single mutation, species with TTX resistance often have several mutations in the outer pore of Na⁺ channels. For example, rough-skinned newts can have three missense mutations (resulting in three amino acid substitutions) in the outer pore region of the Na⁺1.6 channel. Each mutation independently contributes to TTX resistance, and the additive effect of all three is extreme resistance (Vaelli et al., 2020). Similarly, TTX resistance increases in garter snakes with an increasing number of mutations (Feldman et al., 2009; Feldman et al., 2010). Overall, it is remarkable that taxonomically diverse species have independently evolved such similar solutions to resisting TTX through modification of the outer pore of Na⁺ channels (Figure 3). In some cases, snakes, newts, and pufferfish have convergently evolved the same amino acid substitutions in their Nav1.4 channels.

**Figure 2.** The outer pore of Na⁺1.4 channel is shown in two models. In the middle of each is tetrodotoxin. The models also indicate how mutations have produced multiple amino acid substitutions among several species. Shown at the bottom of this figure is the amino acid sequence for the four Na⁺1.4 domains, with amino acids known to affect TTX binding shown in bold and mutations found in some snake species indicated with triangles. Figure taken from Feldman et al., 2012.

**Figure 3.** Convergent evolution of TTX resistance. (A) The four domains of Na⁺1.6 are shown, with amino acid substitutions that confer TTX resistance shown in lighter shading. (B) A phylogeny for select species along with amino acid sequence alignments of the four Na⁺1.6 domains. Included are the rough-skinned newt (Taricha granulosa) and the common garter snake (Thamnophis sirtalis). Lighter font indicates TTX-resistant species and lighter shading denotes substitutions that provide TTX resistance. Figure taken from Vaelli et al., 2020, which is licensed under a Creative Commons Attribution license.
Coevolution

Many students are likely familiar with the concept of predator-prey arms races due to watching nature documentaries. What makes this case study interesting to students is that newts and garter snakes are potentially common “backyard” species, especially for those living along the western coast of the United States. Thus, the newt/snake arms race may be a more accessible and regionally relevant example of coevolution than what is typically presented in documentaries.

The coevolutionary arms race between garter snakes and toxic newts is well established (Brodie & Brodie, 1990) and is defined by iterations of adaptation and counteradaptation (Janzen, 1980; Brodie et al., 2005; Hague et al., 2020; Reimche et al., 2020). Newts evolved the capacity to use TTX as an antipredator defense, whereas predatory garter snakes evolved resistance to TTX in a process of reciprocal selection (Hague et al., 2020). Presumably, newts that could fortify their bodies with TTX had a selective advantage due to reduced predation. In response, snakes with TTX resistance had a selective advantage because they survived when preying on toxic newts and got a tasty meal out of it. Consequently, in some areas newts have extremely high TTX levels (enough to kill 50 people) and garter snakes have extreme TTX resistance.

Analyzing geographic variations of phenotypes is presently the leading method used by ecologists to validate the coevolutionary link between populations of rough-skinned newts and garter snakes (Figure 4). These ecologists have investigated such questions as “Does TTX resistance in snake populations increase in areas where newts are more toxic?”, and “When toxic newts are absent, do snake populations have low TTX resistance?” For the common garter snake (Thamnophis sirtalis) and the rough-skinned newt (T. granulosa) in particular, the coevolutionary arms race is well documented. Biologists have discovered that substantial intraspecific variation exists for both TTX levels in T. granulosa and TTX resistance in T. sirtalis, and these two traits strongly covary across the US West Coast (Hague et al., 2020). When newts produce high levels of TTX, garter snakes tend to have high resistance, and the opposite is also true (Hague et al., 2020; Reimche et al., 2020). Similar geographic patterns of phenotypic matching have also been

![Figure 4. Evolutionary ecologists map trait variation at the population level to find evidence of coevolution: (A) TTX levels in prey (Taricha newts), (B) TTX resistance in predator (Sierra garter snake), and (C) phenotypic mismatch between newts and snakes. Warm colors denote higher levels of TTX, TTX resistance, and phenotypic matching, respectively. Figure taken with permission from Reimche et al., 2020; copyright 2020 British Ecological Society.]

Figure 4.

Some argue that it is more important for students to learn the scientific process than scientific facts (National Science Board, 2008). This case study in coevolution provides many opportunities for educators to discuss, and perhaps demonstrate, the biotechnology and experimental methods used by researchers (Table 2). For example, genetic analysis was critical for elucidating the mechanism and convergent evolution of TTX resistance. Educators can replicate this process for their students by extracting and amplifying DNA (preferably from nonpoisonous sources and without harming any vertebrates), and perhaps even sequencing the DNA. Similarly, students can use publicly available bioinformatics databases like BLAST to compare DNA or protein sequences for Na, channels among different species.

While such DNA analysis may seem relatively straightforward from a conceptual standpoint, students might be perplexed about other parts of the research presented here. For example, how did...
biologists determine that “an amino acid substitution in domain III of the Na\_1.4 channel … increases resistance to TTX by a factor of 15”? Genetic analysis can determine if and where amino acid substitutions occurred, but how did the researchers determine the effect of a mutation on the phenotype (in this case, TTX resistance)? Answering that question can be enlightening for students. In short, scientists quantified TTX resistance by measuring changes in membrane potential, first in normal cells and then in cells having the mutated (TTX-resistant) form of Na\_ channels, such as those found in some garter snakes. Na\_ channels normally promote changes in membrane potential by allowing the diffusion of Na\(^{+}\) into the cell. As Na\(^{+}\) enters the cell, membrane potential becomes more positive and this helps create action potentials. In species susceptible to TTX, such has humans, the toxin binds to Na\_ channels and this reduces or stops the influx of Na\(^{+}\), which results in little or no change in membrane potential. This is harmful to the organism because without that change, action potentials cannot be created. Scientists can use the patch clamp technique (Neher & Sakman, 1992) to measure changes in membrane potential (or lack thereof) in a single cell following exposure to TTX. Thus, they can quantify the impact that TTX has on the functionality of Na\_ channels. A mutated Na\_ channel from garter snakes that is resistant to TTX would demonstrate normal changes in membrane potentials despite the presence of TTX.

Interestingly, scientists used genetically engineered frog cells to conduct these tests (Jost et al., 2008). Rat skeletal muscle was the source of cDNA for creating the normal, TTX-susceptible Na\_1.4 channel. Using a process called site-directed mutagenesis, the rat cDNA was mutated to introduce the necessary amino acid substitution to create a TTX-resistant form of the channel, like that found in some garter snakes. These two versions of the gene were then injected into different *Xenopus* oocytes in the form of synthetic RNA transcripts (called cRNA). There, the cRNA was expressed to create either the normal or mutated version of Na\_1.4 channels (Jost et al., 2008).

A similar process using genetically engineered *Xenopus* oocytes was used to determine TTX resistance in rough-skinned newts, with a couple notable differences. First, the DNA was sourced from the mouse *Mus musculus* instead of rats. And second, site-directed mutagenesis introduced three amino acid substitutions to match those found in rough-skinned newts (Vaelli et al., 2020).

TTX resistance in garter snakes can also be determined using a whole-organism bioassay. This technique involves intraperitoneal injections of TTX to determine any negative impact on locomotion. Snakes that move more slowly exhibit TTX susceptibility, whereas those that are resistant are unaffected (Brodie et al., 2005; Feldman et al., 2009). Through analysis of both techniques mentioned here (whole-organism bioassays and bioengineered cellular testing), educators can facilitate discussions with students about why scientists might choose to answer the same research question by looking at different scales of biological organization. Students might also consider the pros and cons of each method.

Making connections between the scientific facts and the scientific process can be enlightening for students. Whether using liquid chromatography and mass spectrometry to quantify TTX levels or a DNA sequencer to find mutations in SCN genes, students can benefit when connections are made between course content and real-world world applications (Brown et al., 2009).

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### Table 2. Summary of the scientific processes used to understand TTX resistance and toxicity, and the coevolution of both traits. Discussing these processes within the context of newt and garter snake coevolution can help students better understand the process of science.

| Process of science | Details |
|--------------------|---------|
| Radioisotopic feeding studies | Use of carbon radioisotopes to determine if newts synthesized TTX (Shimizu & Kobayashi, 1983). |
| Quantifying TTX levels | Determining TTX levels in newts using high-performance liquid chromatography, mass spectrometry, (Brodie et al., 2005; Vaelli et al., 2020), and competitive inhibition enzymatic immunoassay with TTX-specific antibodies (Hague et al., 2020; Reimche et al., 2020). |
| Comparing DNA and protein sequences in SCN genes and Na\_ channels, respectively | DNA extraction, polymerase chain reaction, DNA sequencing, and alignment of DNA and computer-translated amino acid sequences (e.g., Feldman et al., 2010; Hanifin & Gilly, 2015; Vaelli et al., 2020). |
| Determining toxic effect of TTX (molecular scale) | Measuring changes in membrane potential in genetically engineered cells expressing either normal or resistant version of Na\_ channels, following exposure to TTX (Geffeney et al., 2005; Jost et al., 2008). |
| Determining toxic effect of TTX (organismal scale) | Whole-organism bioassay involves injections of TTX to determine effect on speed of locomotion in garter snakes (Brodie et al., 2005; Feldman et al., 2009). |
| Phylogenetic analysis of Na\_ protein sequences | Amino acid sequences are compared among various types of animals to assess convergent evolution of TTX resistance (Feldman et al., 2009; Hanifin & Gilly, 2015; Hague et al., 2017). |
| Determining population structure and geographically analyzing the distribution of traits among prey and predators | Sample populations for traits of interest (genetic variation, TTX resistance, etc.), map the distribution of these traits for both prey and predators, and use models and statistical analysis to test for covariance (Brodie et al., 2002; Hague et al., 2016; Hague et al., 2020; Reimche et al., 2020). |
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