Background: Coevolutionary interactions between species have generated remarkable phenotypic diversity. These systems have facilitated asking mechanistic questions that have advanced our understanding of evolution and ecology[1]. Coevolution has led to unique molecular and cellular adaptations with the classic example in Evolution textbooks featuring the predator-prey interactions of toxic newts and toxin resistant garter snakes[2;3].

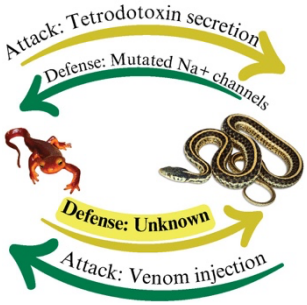


Figure 1: Current understanding of the newt-garter snake coevolutionary interaction.

The Rough-skinned Newt (*Taricha granulosa*) produces a toxin called tetrodotoxin (TTX) in its skin, which is a powerful neurotoxin that blocks voltage-gated sodium channels in nerve and muscle tissue, thereby inhibiting action potentials[4,5]. TTX is very potent and only a small amount is required to kill most species[6]. However, some Common Garter Snake (*Thamnophis sirtalis*) populations have evolved resistance through mutated sodium-gated ion channels which allows them to consume newts and survive[5]. The interaction between newts and garter snakes has led to a geographic mosaic of coevolution with differing levels of newt toxicity and garter snake resistance among populations. **Even though garter snake TTX resistance is a textbook example of coevolution, the story is incomplete because garter snakes are venomous and inject their own toxic cocktail into the newts, but it is unknown if newts have a defense against venom (Fig. 1).**

While rear-fanged snakes account for two-thirds of snake biodiversity, they have historically been overlooked due to their small size and low venom yield, thus little is known about their venom[7]. Garter snakes are rear-fanged snakes and secrete venom from their Duvernoy's gland which travels down grooves on their enlarged back teeth and enters prey, such as newts, as they chew[7]. Garter snakes produce large quantities of venom for rear-fanged snakes which is primarily composed of snake venom metalloproteinases (SVMPs) that damage tissue, cysteine-rich secretory proteins (CRISPs) which affect muscle contraction, and three-finger toxins (3FTxs) which have neurotoxic effects (Fig. 2)[8;9]. **Venom generates a strong selection pressure on prey (i.e., newts), which may select for the evolution of venom resistance (Fig. 1)[10].**

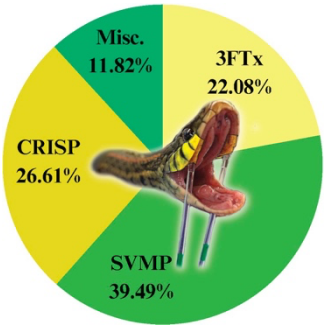


Figure 2: Based on my undergraduate thesis, garter snakes primarily express three toxin families in their Duvernoy's gland (yellow on the snake).

Four molecular mechanisms are known to facilitate venom resistance[10]. Venom inhibitors deactivate toxins before they reach their physiological target. Altered targets are mutated proteins that no longer allow toxins to bind, but maintain their original function (e.g., mutated sodium-ion channels in garter snakes that prevent TTX binding). Repurposed toxins occur when a mutated protein that was not targeted by the toxin initially is a better receptor to the toxin and prevents the physiological target from being attacked (e.g., scorpion venom preferentially binding to pleasure receptors rather than pain receptors in grasshopper mice)[11]. Acquired immunity occurs from repeated sub-lethal exposure to toxins.

Hypothesis: Newt populations will display differential resistance to garter snake envenomation due to at least one of the four molecular mechanisms.

Aim I: Establish the geographic mosaic of resistance of Rough-skinned Newts to Common Garter Snake venom: I will collect 20 newts and 10 garter snakes at the 28 previously studied locations including those that have recorded garter snake predation and populations with no recorded garter snake predation[5;12]. Garter snakes will be housed for six months in order to collect a substantial amount of venom to create pools for each location (Fig. 2). Before pooling, venoms will be used to identify intraspecific venom variation across locations using Reversed-phase High-Performance Liquid Chromatography (RP-HPLC)[13]. Venom resistance will be determined by injecting venom into the newts based on previous LD50 studies[13]. I will control for size, sex, and population to determine time until incapacitation as my measure of resistance. I predict it will take more venom and longer times to incapacitate newts injected with venom from locations where garter snakes prey upon them compared to newt populations that do not interact with garter snakes.

Aim II: Determine the resistance mechanism responsible for the biological response to venom in newt populations: To determine the mechanism of venom resistance, I will use genome sequencing and molecular assays (Fig. 3). To test for the presence of venom inhibitors, a blood assay will be performed to determine if there is significant variation in venom toxin activity. I will use published methods to measure SVMP, CRISP, and 3FTx activity and screen for inhibition within newt blood samples[14]. To test for the presence of altered targets and repurposed toxins, whole genome sequencing will be utilized and compared to identify mutations associated with sodium-gated ion channels and nicotinic acetylcholine receptors (nAChR)[11;15;16]. Sodium-gated ion channel mutants are known for resistance adaptation in other species specifically with repurposed toxins[11]. nAChR are recorded to have specific amino acid mutations that cause toxins to preferentially bind to them preventing negative venom effects[15;16]. I will identify structural variants, search for point mutations in regulatory genes, and identify mutations within the amino acid sequence. If I find mutations in either protein family, I will confirm the venom resistance mechanism via biological assay[11;15;16]. If no mutations are found, I will expand my search to other proteins in the genome and follow the same process. Finally, to test for the presence of acquired immunity, I will determine if mast cell carboxypeptidase (Mc-cpa) enzymes are in higher concentration in resistant newts. Mc-cpas are an essential protective enzyme and their presence reduces the morbidity and mortality associated with venom[17]. An increase in Mc-cpa expression within newts exposed to snake venom indicates acquired immunity.

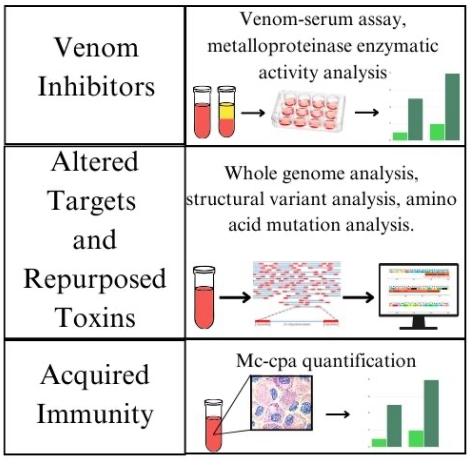


Figure 3: Methods schematic to identify venom resistance mechanism.

Intellectual Merits: The newt-garter snake system is used to teach biology students evolutionary concepts including coevolution. My research project would complete the story of this coevolutionary relationship by investigating resistance from the newt's perspective. My Ph.D. research will expand on the topics of predator-prey interaction, rear-fanged snake venom analysis, and the garter snake-newt system. The methodology I will use to analyze multiple molecular mechanisms will provide a road map for other resistance studies wanting to investigate all four mechanisms of molecular resistance.

Broader Impacts: I will teach, train, and mentor at least five undergraduates during my Ph.D. who will assist me in field sampling and molecular work, learn how to use bioinformatic pipelines, and practice coding. I will seek out women in STEM and other minorities as my mentees to provide the dedicated mentorship I received during my undergraduate. With their help, I will create learning modules with slides, interactive activities, and conceptual checkpoints for students to learn about coevolution from the perspective of the newt-garter snake system that expands on textbook content (Fig. 1). I will work through these modules on my YouTube channel to provide resources for K-12 classes and make the modules available freely to the public. Additionally, I will create the modules in the same format as the textbooks' online learning platforms, Pearson Plus and Macmillian Achieve, so they can be easily disseminated by the publishers and implemented in classrooms. The modules will highlight graduate and undergraduate research and contribute to student learning outcomes in evolution.

References: [1] Thompson and Pagel. *Ency. of Life Sciences*, 2001. [2] Herron and Freeman. *Evolutionary Analysis*. Pearson, 2014. [3] Emlen and Zimmer. *Evolution: Making Sense of Life*. Macmillan Learning, 2019. [4] Huey et al. *Science*, 2002. [5] Geffeney et al. *Science*, 2002. [6] Abal et al. *Toxins*, 2017. [7] Hofmann et al. *Toxins*, 2021. [8] Gutiérrez et al. *Toxins*, 2016. [9] Yamazaki et al. *Toxicon*, 2004. [10] Holding et al. *Integrative and Comparative Bio.*, 2016. [11] Rowe et al. *Science*, 2013. [12] Hanifin et al. *PLoS Biology*, 2008. [13] Margres et al. *MBE*, 2017. [14] Biardi et al. *Toxicon*, 2011. [15] Barchan et al. *PNAS*, 1992. [16] Drabeck et al. *Toxicon*, 2015. [17] Metz et al. *Science*, 2006.