Feeding Ecology and Tetrodotoxin Resistance in Eastern *Thamnophis sirtalis*

Leleña Ann Avila
Sacramento, California

M.A., Indiana University, Bloomington, 2006
B.S., University of California, Berkeley, 1996

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Abstract

Why do predators consume toxic prey and evolve potentially costly resistance to prey toxin? Ecological and behavioral factors may explain why the benefit of consuming toxic prey might outweigh the cost of experiencing the toxin and result in resistance evolution. Toxic prey may be an important resource temporally, spatially, or for different classes of predators, and may be mediated by the predator's condition (e.g., hungry predators or those with high energetic requirements). Evolved prey recognition behaviors may constrain predators to consume, or may facilitate inclusion of toxic prey into the predator's diet. Through experience, predators may also learn to prefer certain prey. In an eastern population of garter snake (*Thamnophis sirtalis*), I measured resistance to tetrodotoxin (TTX) to determine if the trait had evolved from the ancestral condition as observed in western populations. I examined the ecological context in which *Th. sirtalis* preys upon sympatric *Notophthalmus viridescens* (red-spotted newt), which are chemically defended by TTX. I also explored behavioral hypotheses for why toxic newts are consumed.

At Mountain Lake Biological Station (MLBS) in Virginia, *Th. sirtalis* exhibit moderate resistance to TTX (7.66 mass adjusted mouse units (MAMU), range = 1.5 - 72.7 MAMU) compared to other populations of garter snakes, but do not display the expected molecular variation in a gene known to confer resistance in other snakes. On average, *Th. sirtalis* are predicted to experience minimal effects of TTX after consuming local newts, but yearling and juvenile snakes may be more susceptible to the amount of toxin found in sympatric efts. *Notophthalmus viridescens* made up 3.3% of the observed

diet of *Th. sirtalis* at MLBS and newts were consumed only by females. A combination of congenital responsiveness toward, and learned preference for *N. viridescens* contributes to the inclusion of newts in the diet of those *Th. sirtalis* with sufficient resistance to consume them. The availability of *N. viridescens* during critical periods for *Th. sirtalis*, such as during gestation or prior to overwintering, may provide the ecological context that drives evolved consumption of toxic newts and may also be a means of selecting for TTX resistant snakes in this population.

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Dedication

This dissertation is dedicated to my family. It is through their infinite love and support that I am able to pursue and achieve my ambitions. As the first member to obtain a doctorate, I hope to inspire others in my family to set goals and expectations for themselves, and to know that with hard work and perseverance they, too, can realize their dreams, whatever they may be.

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Chapter 1:

Introduction to Dissertation:

Feeding Ecology and Tetrodotoxin Resistance in Eastern *Thamnophis sirtalis*

Introduction

A substantial portion of any community may be composed of chemically defended plants or prey (Kingsbury 1964; Whiteman et al. 1990; Schall and Ressel 1991; Glendinning 2007) so it is likely that predators and herbivores will encounter them. When predators encounter toxic prey, they may either avoid these prey or the predator may evolve traits that allow them to resist the toxin (Brodie and Brodie 1999a). Avoidance may be expected because toxin resistance can be energetically expensive or have persistent physiological trade-offs making it a costly option (Després et al. 2007; Kliot and Ghanim 2012). On the other hand, evolved avoidance of toxic prey may result in sacrificing a food resource, the cost of which may be great for a hungry predator (Abrams 1986; Brodie and Brodie 1999a). On the surface, the costs associated with evolved resistance may seem greater than those associated with avoidance especially for opportunistic feeders that may have several prey options. However, some predators do evolve potentially costly traits, such as resistance, to cope with prey toxins (Després et al. 2007; Glendinning 2007). Why would a predator consume toxic prey and evolve costly resistance when more innocuous resources are available?

There may be several reasons why a predator would evolve costly resistance, rather than avoidance, of toxic prey. Evolving resistance may allow predators to exploit a common resource, whether it is seasonally or generally common (Thompson 1994; Hileman et al. 1995). A predator's condition (e.g., hunger), due to reduced prey availability or competition, may drive predators to consume an underexploited and chemically defended resource (Sexton et al. 1966; Gelperin 1968; Hileman et al. 1995;

Barnett et al. 2007; Barnett et al. 2012). Predators may be genetically constrained to respond positively to some prey types (Arnold 1981c) or young predators may learn to prefer prey (Burghardt and Hess 1966; Fuchs and Burghardt 1971; Punzo 2002; Darmaillacq et al. 2014) before defensive chemicals reach toxic levels. For some predators, the consumed toxin may provide a benefit, such as protection from its own predators (Nishida 2002; Hutchinson et al. 2007; Mori et al. 2012).

Toxic prey may be an important resource during a particular time of the year (Thompson 1994) or when other food is scarce. For example, the black-eared mouse, *Peromyscus melanotis,* is insensitive to the cardiac glycosides and pyrrolizidine alkaloids found in lipid-rich overwintering monarch butterflies (Glendinning and Brower 1990) and are, therefore, able to exploit this resource when other nutrient-rich foods are scarce. These mice feed almost exclusively on monarchs during the winter months and produce significantly more offspring compared to other sympatric mouse species that cannot exploit this resource (Glendinning and Brower 1990). Consuming toxic prey allows *P. melanotis* to consume an abundant resource during a time of year when other high quality prey are scarce, which results in a fitness benefit in the form of increased offspring.

Hungry predators may be more likely to choose toxic prey regardless of season (Sexton et al. 1966; Gelperin 1968; Hileman et al. 1995; Barnett et al. 2007; Barnett et al. 2012). For example, hungry diving beetle larvae (*Dytiscus verticalis*) will attack tails of the toxic newt, *Notophthalmus viridescens,* more often than satiated beetles and continued contact results in learned aversion of the toxic newts, until the beetles become hungry again (Hileman et al. 1995). Evolving mechanisms such as resistance to withstand

these toxins allows predators to exploit resources that other taxa cannot. The ability to exploit these resources may provide fitness benefits that result in selection favoring resistant phenotypes.

Behavioral or genetic constraints can also bias predators to interact with toxic prey. Predators may be born with positive responsiveness to prey types, which allows the predator to recognize species or classes of animals as potential food (Burghardt 1970a; Burghardt and Abeshahe 1971; Arnold 1981c; Cooper et al. 2000; Aubret et al. 2006; Cooper 2007; Cooper and Secor 2007). Recognition of toxic or otherwise dangerous prey may also be correlated with recognition of an important non-toxic resource through genetic correlation (Arnold 1981a; b; c). The predator may, therefore, be unable to avoid the toxic prey without also avoiding the non-toxic prey. Such a correlated response has been observed in coastal populations of *Thamnophis elegans* where recognition of frequently consumed slug prey is correlated with hazardous leeches resulting in snakes attacking leech odors despite their hazardous nature (Arnold 1981c). Correlated responses have also been observed in *Th. melanogaster*. *Thamnophis melanogaster* are unable to distinguish prey odors of carrion eating and blood-sucking leeches that are capable of killing the snake, and the snake will attack and attempt to ingest both prey types when presented (Drummond and Garcia 1995).

Predators may also learn to prefer prey experienced early in life. This phenomenon has been demonstrated in a variety of predators (Burghardt and Hess 1966; Fuchs and Burghardt 1971; Punzo 2002; Darmaillacq et al. 2004). For example, food naïve cuttlefish (*Sepia officinalis*), that innately prefer shrimp, learned to prefer crabs

after a single food exposure (Darmaillacq et al. 2004). The authors suggest that the long-term change in food preference was the result of the positive reward received by consuming crab. Some potential prey may not be immediately toxic if they acquire their defensive chemicals from their environment or if toxin load is developmentally dependent (Brodie et al. 1974; Yasumoto et al. 1986; Daly et al. 1994). If young predators are confronted with prey that are not yet toxic and they receive a positive reward (e.g., nutrients) from consuming such prey, then the predator may be inclined to consume the prey later in life. However, the predator will require some mechanism, such as resistance, to tolerate the toxin if it is to continue consuming toxic prey.

One benefit of consuming toxic resources that is commonly observed in plant-herbivore systems is the ability of the herbivore to utilize the consumed toxin for its own defense. For example, caterpillars of the monarch butterfly, *Danaus plexippus*, feed exclusively on milkweeds (*Asclepias spp.*) that contain highly toxic cardenolides. The larvae sequester the toxins and retain them in the adult form as a defense against predators (Nishida 2002). An example is also found in the natricine snake *Rhabdophis tigrinus*. This snake feeds on a variety of amphibians including toxic toads defended by bufadienolides. The snake consumes these toads and sequesters the toxin in its nuchal glands as a means of defense against predators. Further, mothers are able to provision their offspring with the toxin (Hutchinson et al. 2007; Mori et al. 2012) presumably to protect their neonate young from potential predators.

If predators consume toxic prey due to any of these ecological or behavioral reasons, they will have to evolve mechanisms for tolerating the toxic defenses of their

prey. Resistance to toxins can come in many forms (reviewed in Després et al. 2007; Glendinning 2007). Behavioral mechanisms such as food manipulation and intake regulation can be employed to minimize contact with the toxins or dilute their effects so they do not reach a toxic threshold (Freeland and Janzen 1974; Brower and Calvert 1985; Brower et al. 1988; Nyman and Julkunen-Tiitto 2000; Becerra et al. 2001; Cooper et al. 2002). Some organisms may exercise tolerance for the toxic effects at sub-lethal levels (Pfister 1999). A variety of physiological resistance mechanisms can be applied including inactivation of toxins by salivary proteins (Musser et al. 2002; Shimada 2006) or mutualistic gut microbiota (Allison et al. 1985). Absorption of the toxin may be hindered by efflux transporters and it may pass through the body (Sorensen and Dearing 2006). Toxins may also be metabolized through the production of detoxification enzymes (Berenbaum 2002; Glendinning 2002; Ratzka et al. 2002; Dearing et al. 2005; Hartmann et al. 2005) or actively sequestered into tissues for use in anti-predator defense (Brower et al. 1984; Isman 1992; Willinger and Dobler 2001; Ode 2006; Hutchinson et al. 2007). Organisms can also evolve specialized genetic mutations affecting the target site of the toxin that hinder or eliminate the toxic effects (Berenbaum 1986; Holzinger and Wink 1996; Labeyrie and Dobler 2004; Geffeney et al. 2005; Feldman et al. 2012).

Each of these resistance methods may have associated ecological or evolutionary costs (Freeland and Janzen 1974; Després et al. 2007; Glendinning 2007; Kliot and Ghanim 2012). Behavioral manipulation involves a time investment (Dussourd and Eisner 1987). Tolerance exposes the organism to the toxic effects, which may impact their behavior or physiological processes (Fowler 1983; Molyneux and Ralphs 1992).

Metabolic resistance and sequestration require the evolution of physiological or biochemical mechanisms that are triggered by the toxin and may be energetically expensive (Després et al. 2007). These modes of resistance tend to be inducible (i.e. the resistance mechanism is employed after contact with the toxin) and so cost is dependent on exposure. On the other hand, genetic mutations at the target site of the toxin are constant, the cost is fixed, and there may be persistent physiological trade-offs associated with these changes even in the absence of the toxin (Després et al. 2007). Because the benefits received must outweigh the costs incurred for a trait to evolve, understanding the potential costs associated with resistance mechanisms may help us to understand the relative importance of toxic prey for a predator.

In this dissertation, I investigate why predators engage with toxic prey as a means of understanding the evolution of toxin resistance. In a system where the predator has apparently evolved resistance to the defensive toxin of its prey, I explore the ecological and behavioral reasons why this predator consumes toxic prey and, therefore, has evolved resistance to prey toxin.

A predator needs to have some resistance mechanism for coping with the ingested toxin if it encounters toxic prey, recognizes it as food, and consumes it. Because resistance has not been fully characterized for the population being investigated, I measure physiological resistance to prey toxin in the predator and use data collected elsewhere about toxicity of the prey to understand how predator resistance compares with local prey toxicity. These results will confirm if the predator has evolved sufficient resistance to local prey toxin. I also examine the probable underlying mechanism of the

resistance phenotype, mutations at the target site of the toxin, and look for evidence of associated costs with toxin resistance. If the cost of resistance is persistent it may indicate that the benefit received by the predator from consuming toxic prey is great. I explore the ecological context in which predators consume toxic prey by examining the significance of toxic prey in the diet. I investigate sexual, ontogenetic, and temporal variation in diet of toxic prey because some prey may be more important for different classes of predators or at different times of the year (Arnold 1993; Thompson 1994). I expect to better understand under what circumstances the predator encounters toxic prey and potential ecological reasons why the predator eats this prey rather than avoids it. I test for behavioral biases for toxic prey to examine the behavioral attributes that may mediate toxic prey consumption. I investigate congenital preferences for prey as well as evidence for genetic correlations among preferences to see if the predator is predisposed to consume toxic prey either directly or indirectly. I test to see if early experience with toxic prey results in learned preference for that prey type, and I consider nutrient quality as a potential benefit of toxic prey. From these results I expect to understand how the predator recognizes toxic species as possible food and why the predator may attempt to consume these prey once encountered.

Study System

Thamnophis sirtalis, common garter snake (Natricinae), is native to North America and has the most extensive range of all *Thamnophis* species. *Thamnophis sirtalis* is found from Pacific to Atlantic coasts, from southern Canada to the Gulf of

Mexico in the east and to San Diego, California in the west. This species is notably absent from the southwestern United States except for a region in New Mexico and a small region in northern Chihuahua, Mexico (Stebbins 2003). *Thamnophis sirtalis* has been characterized as a generalist predator that consumes amphibians, invertebrates (earthworms, slugs, leeches), mammals, fish, and birds (Mushinsky 1987; Rossman et al. 1996; Conant and Collins 1998; Stebbins 2003), but exhibits considerable geographic, temporal, and ontogenetic variation in diet (White and Kolb 1974; Arnold 1981c; Kephart 1982; Kephart and Arnold 1982; Gregory and Nelson 1991). Among the prey species consumed by *Th. sirtalis* are *Taricha granulosa* (rough skinned newt) in the west (Fitch 1941b; Brodie 1968b; Nussbaum et al. 1983; Brodie and Brodie 1990; 1991, 1999a; Brodie et al. 2002) and *Notophthalmus viridescens* (red-spotted newt) in the east (Brodie1968a). These newts are defended by one of the most lethal natural toxins known, tetrodotoxin (TTX; Mosher et al. 1964; Wakely et al. 1966; Brodie et al. 1974; Yotsu-Yamashita and Mebs 2001, 2003; Daly 2004; Hanifin 2010).

For non-resistant organisms, consuming TTX results in paralysis, respiratory arrest, and often death (Brodie 1968b; Noguchi and Ebesu 2001; Hanifin 2010). Molecules of TTX bind to the outer pore region of voltage-gated nerve and muscle sodium channels blocking the passage of sodium ions and inhibiting the propagation of action potentials (Kao and Levinson 1986; Hille 2001). Resistance to TTX is conferred by mutations at the target site of the toxin. Amino acid substitutions at the binding sites of TTX in the outer pore region of the sodium channel alter binding affinity of the TTX molecule and reduce channel blockage (Lipkind and Fozzard 1994a; Lee and Ruben

2008; Fozzard and Lipkind 2010). In western *Th. sirtalis* amino acid substitutions in the $Na_v1.4$ skeletal muscle channel confer resistance to TTX (Geffeney et al. 2005; Feldman et al. 2009, 2012). Additional TTX-resistant amino acid substitutions have also been identified in the genes of peripheral nervous system sodium channels $Na_v1.6$ and $Na_v1.7$, and are thought to contribute to TTX resistance in these snakes (McGlothlin et al. 2014).

Thamnophis sirtalis is known to be resistant to TTX in the western part of its range. Resistance is variable within and among populations of *Th. sirtalis* (Brodie and Brodie 1990) and toxicity varies within and among populations of *Ta. granulosa* (Hanifin et al. 1999). Populations of highly resistant snakes tend to be sympatric with highly toxic newts, populations of moderately resistant snakes tend to be sympatric with less toxic newts, and populations of *Th. sirtalis* that are allopatric to newts are non-resistant (Brodie et al. 2002). It should be noted, however, that this geographic pattern does not imply that toxicity and resistance are functionally matched. Hanifin et al. (2008) found that the potential for strong reciprocal selection exists for only about half of populations studied in western North America.

In addition to *Th. sirtalis*, other garter snakes that prey on *Taricha* (*Th. atratus* and *Th. couchii*) exhibit elevated levels of TTX resistance. These species exhibit both convergent and unique mutations in the $Na_v1.4$ sodium channel (Feldman et al. 2009). Molecular evidence supports the hypothesis that this adaptive trait has independently evolved multiple times within Natricinae (incl. *Amphiesma pryeri* and *Rhabdophis tigrinus*; Feldman et al. 2012), within *Thamnophis*, and even among populations of *Th. sirtalis* (Feldman et al. 2009). Natricine snakes exhibit slightly elevated ancestral TTX

resistance (1-2 MAMU), which may act as a preadaptation for subsequent evolution of resistance (Motychak et al 1999). McGlothlin et al. (2014) suggest that ancestral resistance may be due to fixed mutations in the peripheral nervous system (sodium channels $Na_v1.6$ and $Na_v1.7$) and that independent adaptations to local levels of TTX experienced by garter snakes may be the result of mutations in the sodium channel expressed in skeletal muscle tissue ($Na_v1.4$).

Resistance to TTX is potentially costly. The sodium channels known to confer resistance in *Th. sirtalis* are expressed in skeletal muscle tissue ($Na_v1.4$; Geffeney et al. 2002) and these sodium channels exhibit slower kinetics when experimentally converted from TTX-sensitive to TTX-insensitive (Pappone 1980; Yoshida 1994). Locomotor performance trades-off with resistance in TTX resistant snakes, which means that snakes with high resistance are slower than less resistant snakes (Brodie and Brodie 1999a, b). In addition, TTX resistant mutations are found in the highly conserved pore region of the $Na_v1.4$ sodium channel (Geffeney et al. 2005; Feldman et al. 2009). Because the pore region serves a critical function for organisms (selectivity of ion passage) and so is highly conserved across taxa (Feldman et al. 2009), changes to this region are presumably costly. It has, therefore, been hypothesized that reduced crawl speed is a cost of resistance (Brodie and Brodie 1999b; Geffeney et al. 2002). However, no study has directly tested such costs of resistance. Ecologically, reduced crawl speeds may result in greater risk of predation for *Thamnophis* if they are unable to flee as quickly (Jayne and Bennett 1990), slower snakes may be unable to capture faster moving prey, and they may be unable to effectively thermoregulate (Brodie and Brodie 1999a).

While evolution of resistance in *Thamnophis* has been studied extensively in the Pacific Northwest, populations in eastern North America have not been studied with respect to TTX resistance, despite their pre-adapted potential to evolve resistance and sympatry with toxic *N. viridescens*. Exceptions include a study by Motychak et al. (1999) who tested TTX resistance in a litter of *Th. sirtalis* from York Co., Maine and found them to exhibit ancestral levels of resistance (1.8 MAMU). More recently, ten individuals from Mountain Lake Biological Station (MLBS) in Virginia were tested and demonstrated moderate levels of resistance to TTX (estimated population mean 7.29 MAMU, range 2.6 - 21.4 MAMU; Feldman et al. *unpublished*) indicating that toxic *N. viridescens* may be selecting for evolved resistance in these snakes. Additionally, four *Th. sirtalis* from this same population were found to exhibit amino acid substitutions in the $Na_v1.6$ and $Na_v1.7$ sodium channels that are predicted to be TTX-resistant (McGlothlin et al. 2014). The MLBS population of *Th. sirtalis* is the focal population of this dissertation.

Synopsis of Chapters

In this dissertation, I explore why predators engage with toxic prey as a means of understanding why they evolve resistance to toxins. I investigate a system in which the predator (*Thamnophis sirtalis*) has apparently evolved physiological resistance to the defense toxin of its prey (*Notophthalmus viridescens*). Because tetrodotoxin resistance in this population has not been fully characterized, I begin by measuring physiological resistance in *Th. sirtalis* and compare it to previously collected data on the toxicity of *N. viridescens* to determine if this population of garter snakes has evolved sufficient

resistance to consume local newts. I also investigate the mechanism of resistance and the associated costs of resistance as a means of understanding the relative benefit of consuming *N. viridescens* (Chapter 2). I then investigate if eastern *Th. sirtalis* are interacting with *N. viridescens* in a natural population (Chapter 3). I assess if toxic newts are important diet items during particular times of the year and at different life stages to understand the circumstances in which *Th. sirtalis* encounters *N. viridescens* and the possible ecological reason why garter snakes eat toxic newts rather than avoid them. I also evaluate the role that prey recognition and learning plays in the inclusion of toxic newts in the diet of *Th. sirtalis* (Chapter 4) to determine if *Th. sirtalis* are predisposed to consume *N. viridescens*, and why garter snakes may attempt to consume newts once encountered.

In Chapter 2, I measure physiological resistance to TTX in the MLBS population of *Th. sirtalis* because it has not been fully characterized, and I use previously collected data on the toxicity of *N. viridescens* at this site to determine if snakes are able to eat local newts. *Thamnophis sirtalis* at MLBS exhibit moderate resistance to TTX (7.66 MAMU) compared to other populations of *Th. sirtalis* surveyed in western North America. This level of TTX resistance is greater than ancestral levels observed in natricine snakes and is evidence that TTX resistance has evolved in this population. MLBS *Th. sirtalis* demonstrated a trade-off between locomotor ability and resistance as in other populations of *Thamnophis*. It is not clear that this persistent trade-off is a direct cost of resistance but if so, it may indicate that the benefit received from toxic newts is substantial. In general, MLBS *Th. sirtalis* demonstrate sufficient resistance to consume

most local newts, though life stage of both snakes and newts affects the functional relationship between resistance and toxicity in these species. Sub-adult snakes are more likely to be severely impacted by TTX if they consume toxic efts. The degree of mismatch calculated for snakes and newts at MLBS indicates the potential for these species to experience strong reciprocal selection. I also investigate the underlying mechanism for TTX resistance in the $Na_v1.4$ sodium channel. The amino acid substitutions in the $Na_v1.4$ sodium channel gene that have been shown to confer resistance in other *Thamnophis* are not present, suggesting a novel mechanism of resistance in this population.

Chapter 3 describes a three-year diet study of *Th. sirtalis* at Mountain Lake Biological Station. While general diet is described, special attention was paid to the frequency of *N. viridescens* observed in the diet of *Th. sirtalis*. This study shows that *Th. sirtalis* are interacting with sympatric toxic newts, though *N. viridescens* are consumed rarely (3.3% of the observed diet). Though the sample was small, newts were only consumed by female snakes. In four of the six cases newts were consumed by adult or juvenile snakes, one observation was of a newt consumed by a large yearling snake, and in one instance a newly metamorphosed eft was consumed by a neonate snake. There was no evidence that *N. viridescens* were important at a particular time of year. These data indicate that consuming toxic newts may be particularly important to females entering or in reproductive condition when individual energetic needs may be high.

In chapter 4, I tested three non-exclusive behavioral hypotheses for why *Th. sirtalis* have evolved to consume *N. viridescens*: congenital response to prey, correlated

responses to prey, and learned preference to determine if *Th. sirtalis* are predisposed to consume *N. viridescens* and to investigate the role that learning plays in the inclusion of toxic newt in *Th. sirtalis* diet once encountered. Food-naïve snakes exhibited congenital responsiveness to a water-based extract of *N. viridescens* but did not prefer it over other prey extracts tested. Snakes with stronger congenital responses for *N. viridescens* were more likely to consume them, and snakes fed only a diet of newts dramatically increased their responsiveness to them. Snakes fed only newts gained more weight than snakes that were fed only earthworms, suggesting that *N. viridescens* may be a more nutritious prey for *Th. sirtalis*. Congenital responsiveness to extracts of *N. viridescens* was correlated with other prey at the individual and family level. Some correlations persisted after experience with prey, but did not persist after consuming specific diets. Together these data suggest that a combination of congenital responsiveness to newt and learning from experience with this prey type contribute to the recognition of *N. viridescens* as prey and their inclusion in *Th. sirtalis* diet.

Summary

Why do *Th. sirtalis* consume toxic *N. viridescens* and evolve costly resistance to tetrodotoxin? Based on the data collected here, there are both behavioral and ecological reasons why *Th. sirtalis* consume toxic *N. viridescens*. Behaviorally, *Th. sirtalis* at MLBS are predisposed to recognizing newts as prey through congenital responsiveness. However, it is through experience with this prey that snakes learn to prefer newts over other prey types. Snakes may learn to prefer newts if they encounter them early in life

(for example, when neonates search for food before their first winter). One possible reason for learned preference is the nutrient benefit that snakes may receive from eating a vertebrate, such as a newt, compared to the most common prey in their diet, earthworms. This benefit may drive the evolution of congenital responsiveness for newts.

Although it happens infrequently, MLBS *Th. sirtalis* consume toxic *N. viridescens* and so they require resistance to TTX. These data confirm that this population of *Th. sirtalis* has evolved resistance to TTX and the level of resistance observed is sufficient for most snakes to consume most local newts with variable effects. Surprisingly, the mechanism of resistance for this population does not appear to involve the $Na_v1.4$ sodium channel gene as other resistant lineages have demonstrated. This population of *Th. sirtalis* displays the same trade-off between locomotor ability and resistance observed in other *Thamnophis* indicating that resistance may be costly for this population as well. Presumably the benefit received from consuming newts outweighs this cost in order for resistance to evolve. Because newts appear to be a more nutritious food resource for snakes than their most commonly consumed prey, earthworms, *Th. sirtalis* lacking the necessary resistance to exploit newts might experience reduced fitness compared to those snakes that can. This ability to exploit more nutritious prey when they become available may be one mechanism by which selection acts in favor of resistant phenotypes.

References

Abrams, P. A. 1986. Adaptive responses of predators to prey and prey to predators - the failure of the arms-race analogy. Evolution 40:1229-1247.

Allison, M., K. Dawson, W. Mayberry, and J. Foss. 1985. *Oxalobacter formigenes* gen. nov., sp. nov.: Oxalate-degrading anaerobes that inhabit the gastrointestinal tract. Arch. Microbiol. 141:1-7.

Arnold, S. J. 1981a. Behavioral variation in natural populations 1. Phenotypic, genetic and environmental correlations between chemoreceptive responses to prey in the garter snake, *Thamnophis elegans*. Evolution 35:489-509.

Arnold, S. J. 1981b. Behavioral variation in natural populations 2. The inheritance of a feeding response in crosses between geographic races of the garter snake, *Thamnophis elegans*. Evolution 35:510-515.

Arnold, S. J. 1981c. The microevolution of feeding behavior. Pp. 409-514 *in* A. C. Kamil, and T. D. Sargent, eds. Foraging Behavior: Ecological, Ethological, and Psychological Approaches. Garland Publishing, New York.

Arnold, S. J. 1993. Foraging theory and prey-size-predator-size relations in snakes. Pp. 87-116 *in* R. A. Seigel, and J. T. Collins, eds. Snakes: Ecology and Behavior. McGraw Hill, New York.

Aubret, F., G. M. Burghardt, S. Maumelat, X. Bonnet, and D. Bradshaw. 2006. Feeding preferences in 2 disjunct populations of tiger snakes, *Notechis scutatus* (Elapidae). Behavioral Ecology 17:716-725.

Barnett, C. A., M. Bateson, and C. Rowe. 2007. State-dependent decision making: Educated predators strategically trade off the costs and benefits of consuming aposematic prey. Behavioral Ecology 18:645-651.

Barnett, C. A., J. Skelhorn, M. Bateson, and C. Rowe. 2012. Educated predators make strategic decisions to eat defended prey according to their toxin content. Behavioral Ecology 23:418-424.

Becerra, J. X., D. L. Venable, P. H. Evans, and W. S. Bowers. 2001. Interactions between chemical and mechanical defenses in the plant genus *Bursera* and their implications for herbivores. American Zoologist 41:865-876.

Berenbaum, M. R. 1986. Target site insensivity in insect–plant interactions. Pp. 257–272 *in* L. B. Brattsten, and S. Ahmad, eds. Molecular Aspects of Insect–Plant Associations. Plenum Press, New York, NY.

Berenbaum, M. R. 2002. Postgenomic chemical ecology: From genetic code to ecological interactions. Journal of Chemical Ecology 28:873-896.

Brodie, E. D. III and E. D. Brodie, Jr. 1990. Tetrodotoxin resistance in garter snakes - an evolutionary response of predators to dangerous prey. Evolution 44:651-659.

Brodie, E. D. III and E. D. Brodie, Jr. 1991. Evolutionary response of predators to dangerous prey - reduction of toxicity of newts and resistance of garter snakes in island populations. Evolution 45:221-224.

Brodie, E. D. III and E. D. Brodie, Jr. 1999a. Predator-prey arms races. Bioscience 49:557-568.

Brodie, E. D. III and E. D. Brodie, Jr. 1999b. Costs of exploiting poisonous prey: Evolutionary trade-offs in a predator-prey arms race. Evolution 53:626-631.

Brodie, E. D., Jr. 1968a. Investigations on skin toxin of red-spotted newt *Notophthalmus viridescens viridescens*. American Midland Naturalist 80:276-280.

Brodie, E. D., Jr. 1968b. Investigations on the skin toxin of the adult rough-skinned newt, *Taricha granulosa*. Copeia 1968:307-313.

Brodie, E. D., Jr., J. L. Hensel, and J. A. Johnson. 1974. Toxicity of urodele amphibians *Taricha*, *Notophthalmus*, *Cynops* and *Paramesotriton* (Salamandridae). Copeia 1974:506-511.

Brodie, E. D., Jr., B. J. Ridenhour, and E. D. Brodie III. 2002. The evolutionary response of predators to dangerous prey: Hotspots and coldspots in the geographic mosaic of coevolution between garter snakes and newts. Evolution 56:2067-2082.

Brower, L. P. and W. H. Calvert. 1985. Foraging dynamics of bird predators on overwintering monarch butterflies in Mexico. Evolution 39:852-868.

Brower, L. P., C. J. Nelson, J. N. Seiber, L. S. Fink, and C. Bond. 1988. Exaptation as an alternative to coevolution in cardenolide-based chemical defense of monarch butterflies (*Danaus plexippus* L.) against avian predators. Pp. 447-475 *in* K. C. Spencer, ed. Chemical Mediation of Coevolution. Academic press, Inc., San Diego, CA.

Brower, L. P., J. N. Seiber, C. J. Nelson, S. P. Lynch, M. P. Hoggard, and J. A. Cohen. 1984. Plant-determined variation in cardenolide content and thin-layer chromatography profiles of monarch butterflies, *Danus-plexippus* (Lepidopters,

Danaidae) reared on milkweed plants in California. 3. *Asclepias-californica* (Apocynales, Asclepiadaceae). Journal of Chemical Ecology 10:1823-1857.

Burghardt, G. M. 1970. Chemical perception in reptiles. Pp. 241-308 *in* J. W. Johnston, D. G. Moulton, and A. Turk, eds. Advances in Chemoreception. Appleton-Century-Crofts, New York.

Burghardt, G. M. and J. P. Abeshahe. 1971. Responses to chemical stimuli of prey in newly hatched snakes of genus *Elaphe*. Animal Behaviour 19:486-.

Burghardt, G. M. and E. H. Hess. 1966. Food imprinting in the snapping turtle, *Chelydra serpentina*. Science 151:108-109.

Conant, R. and J. T. Collins. 1998. A Field Guide to Reptiles and Amphibians: Eastern and Central North America. Houghton Mifflin Co., New York, NY.

Cooper, W. E. 2007. Elevated tongue-flicking rate to cricket surface chemicals by the arthropodivorous rough green snake *Opheodrys aestivus*. Amphibia-Reptilia 28:413-417.

Cooper, W. E., G. M. Burghardt, and W. S. Brown. 2000. Behavioural responses by hatchling racers (*Coluber constrictor*) from two geographically distinct populations to chemical stimuli from potential prey and predators. Amphibia-Reptilia 21:103-115.

Cooper, W. E., V. Perez-Mellado, L. J. Vitt, and B. Budzinsky. 2002. Behavioral responses to plant toxins by two onmivorous lizard species. Physiol. Behav. 76:297-303.

Cooper, W. E. and S. Secor. 2007. Strong response to anuran chemical cues by an extreme dietary specialist, the eastern hog-nosed snake (*Heterodon platirhinos*). Canadian Journal of Zoology 85:619-625.

Daly, J., H. Martin Garraffo, T. Spande, C. Jaramillo, and A. Stanley Rand. 1994. Dietary source for skin alkaloids of poison frogs (Dendrobatidae). Journal of Chemical Ecology 20:943-955.

Daly, J. W. 2004. Marine toxins and nonmarine toxins: Convergence or symbiotic organisms. Journal of Natural Products 67:1211-1215.

Darmaillacq, A.-S., R. Chichery, R. Poirier, and L. Dickel. 2004. Effect of early feeding experience on subsequent prey preference by cuttlefish, *Sepia officinalis*. Developmental Psychobiology 45:239-244.

Dearing, M. D., W. J. Foley, and S. McLean. 2005. The influence of plant secondary metabolites on the nutritional ecology of herbivorous terrestrial vertebrates. Annual Review of Ecology, Evolution, and Systematics 36:169-189.

Després, L., J.-P. David, and C. Gallet. 2007. The evolutionary ecology of insect resistance to plant chemicals. Trends in Ecology & Evolution 22:298-307.

Drummond, H. and M. C. Garcia. 1995. Congenital responsiveness of garter snakes to a dangerous prey abolished by learning. Animal Behaviour 49:891-900.

Dussourd, D. E. and T. Eisner. 1987. Vein-cutting behavior: Insect counterploy to the latex defense of plants. Science 237:898-901.

Feldman, C. R., E. D. Brodie, Jr., E. D. Brodie III, and M. E. Pfrender. 2009. The evolutionary origins of beneficial alleles during the repeated adaptation of garter

snakes to deadly prey. Proceedings of the National Academy of Sciences of the United States of America 106:13415-13420.

Feldman, C. R., E. D. Brodie, Jr., E. D. Brodie III, and M. E. Pfrender. 2012. Constraint shapes convergence in tetrodotoxin-resistant sodium channels of snakes. Proceedings of the National Academy of Sciences of the United States of America 109:4556-4561.

Fitch, H. S. 1941. The feeding habits of California garter snakes. California Fish and Game 27:1-32.

Fowler, M. E. 1983. Plant poisoning in free-living wild animals: A review. Journal of Wildlife Diseases 19:34-43.

Fozzard, H. A. and G. M. Lipkind. 2010. The tetrodotoxin binding site is within the outer vestibule of the sodium channel. Marine Drugs 8:219-234.

Freeland, W. J. and D. H. Janzen. 1974. Strategies in herbivory by mammals - role of plant secondary compounds. American Naturalist 108:269-289.

Fuchs, J. L. and G. M. Burghardt. 1971. Effects of early feeding experience on the responses of garter snakes to food chemicals. Learning and Motivation 2:271-279.

Geffeney, S., E. D. Brodie, Jr., P. C. Ruben, and E. D. Brodie III. 2002. Mechanisms of adaptation in a predator-prey arms race: TTX-resistant sodium channels. Science 297:1336-1339.

Geffeney, S. L., E. Fujimoto, E. D. Brodie III, E. D. Brodie, Jr., and P. C. Ruben. 2005. Evolutionary diversification of TTX-resistant sodium channels in a predator-prey interaction. Nature 434:759-763.

Gelperin, A. 1968. Feeding behaviour of the praying mantis: A learned modification. Nature 219:399-400.

Glendinning, J. I. 2002. How do herbivorous insects cope with noxious secondary plant compounds in their diet? Entomol. Exp. Appl. 104:15-25.

Glendinning, J. I. 2007. How do predators cope with chemically defended foods? Biological Bulletin 213:252-266.

Glendinning, J. I. and L. P. Brower. 1990. Feeding and breeding responses of five mice species to overwintering aggregations of the monarch butterfly. Journal of Animal Ecology 59:1091-1112.

Gregory, P. T. and K. J. Nelson. 1991. Predation on fish and intersite variation in the diet of common garter snakes, *Thamnophis sirtalis*, on Vancouver Island. Canadian Journal of Zoology 69:988-994.

Hanifin, C. T. 2010. The chemical and evolutionary ecology of tetrodotoxin (TTX) toxicity in terrestrial vertebrates. Marine Drugs 8:577-593.

Hanifin, C. T., E. D. Brodie, Jr., and E. D. Brodie III. 2008. Phenotypic mismatches reveal escape from arms-race coevolution. PLoS. Biol. 6:471-482.

Hanifin, C. T., M. Yotsu-Yamashita, T. Yasumoto, E. D. Brodie, Jr., and E. D. Brodie III. 1999. Toxicity of dangerous prey: Variation of tetrodotoxin levels within and among populations of the newt *Taricha granulosa*. Journal of Chemical Ecology 25:2161-2175.

Hartmann, T., C. Theuring, T. Beuerle, N. Klewer, S. Schulz, M. S. Singer, and E. A. Bernays. 2005. Specific recognition, detoxification and metabolism of

pyrrolizidine alkaloids by the polyphagous arctiid *Estigmene acrea*. Insect Biochemistry and Molecular Biology 35:391-411.

Hileman, K. S., E. D. Brodie, Jr., and D. R. Formanowicz. 1995. Avoidance of unpalatable prey by predaceous diving beetle larvae - the role of hunger level and experience (Coleoptera, Dytiscidae). Journal of Insect Behavior 8:241-249.

Hille, B. 2001. Ion Channels of Excitable Membranes. Sinauer Associates, Sunderland, MA.

Holzinger, F. and M. Wink. 1996. Mediation of cardiac glycoside insensitivity in the monarch butterfly (*Danaus plexippus*): Role of an amino acid substitution in the ouabain binding site of Na+, K+-ATPase. Journal of Chemical Ecology 22:1921-1937.

Hutchinson, D. A., A. Mori, A. H. Savitzky, G. M. Burghardt, X. G. Wu, J. Meinwald, and F. C. Schroeder. 2007. Dietary sequestration of defensive steroids in nuchal glands of the Asian snake *Rhabdophis tigrinus*. Proceedings of the National Academy of Sciences of the United States of America 104:2265-2270.

Isman, M. B. 1992. A physiological perspective. Pp. 156–176 *in* B. D. Roitberg, and M. B. Isman, eds. Insect Chemical Ecology. Chapman & Hall, New York, NY.

Jayne, B. C. and A. F. Bennett. 1990. Selection on locomotor performance capacity in a natural population of garter snakes. Evolution 44:1204-1229.

Kao, C. Y. and S. R. Levinson. 1986. Tetrodotoxin, Saxitoxin, and the Molecular Biology of the Sodium Channel. New York Academy of Sciences, New York, NY.

Kephart, D. G. 1982. Microgeographic variation in the diets of garter snakes. Oecologia 52:287-291.

Kephart, D. G. and S. J. Arnold. 1982. Garter snake diets in a fluctuating environment - a 7-year study. Ecology 63:1232-1236.

Kingsbury, J. M. 1964. Poisonous Plants of the United States and Canada. Prentice-Hall,, Englewood Cliffs, NJ.

Kliot, A. and M. Ghanim. 2012. Fitness costs associated with insecticide resistance. Pest Management Science 68:1431-1437.

Labeyrie, E. and S. Dobler. 2004. Molecular adaptation of chrysochus leaf beetles to toxic compounds in their food plants. Molecular Biology and Evolution 21:218-221.

Lee, C. H. and P. C. Ruben. 2008. Interaction between voltage-gated sodium channels and the neurotoxin, tetrodotoxin. Channels 2:407-412.

Lipkind, G. M. and H. A. Fozzard. 1994. A structural model of the tetrodotoxin and saxitoxin binding-site of the Na+ channel. Biophysical Journal 66:1-13.

McGlothlin, J. W., J. P. Chuckalovcak, D. E. Janes, S. V. Edwards, C. R. Feldman, E. D. Brodie, Jr., M. E. Pfrender, and E. D. Brodie III. 2014. Parallel evolution of tetrodotoxin resistance in three voltage-gated sodium channel genes in the garter snake *Thamnophis sirtalis*. Molecular Biology and Evolution 31:2836-2846.

Molyneux, R. J. and M. H. Ralphs. 1992. Plant toxins and palatability to herbivores. Journal of Range Management 45:13-18.

Mori, A., G. Burghardt, A. Savitzky, K. Roberts, D. Hutchinson, and R. Goris. 2012. Nuchal glands: A novel defensive system in snakes. Chemoecology 22:187-198.

Mosher, H., F. Fuhrman, H. Bichwald, and H. Fischer. 1964. Tarichatoxin-tetrodotoxin: A potent neurotoxin. Science 144:1100-1110.

Mushinsky, H. R. 1987. Foraging ecology. Pp. 302-334 *in* R. A. Seigel, J. T. Collins, and S. S. Novak, eds. Snakes: Ecology and Evolutionary Biology. McGraw-Hill Publishing Co, New York, NY.

Musser, R. O., S. M. Hum-Musser, H. Eichenseer, M. Peiffer, G. Ervin, J. B. Murphy, and G. W. Felton. 2002. Herbivory: Caterpillar saliva beats plant defences - a new weapon emerges in the evolutionary arms race between plants and herbivores. Nature 416:599-600.

Nishida, R. 2002. Sequestration of defensive substances from plants by Lepidoptera. Annual Review of Entomology 47:57-92.

Noguchi, T. and J. S. M. Ebesu. 2001. Puffer poisoning: Epidemiology and treatment. Journal of Toxicology-Toxin Reviews 20:1-10.

Nussbaum, R. A., E. D. Brodie, Jr., and R. M. Storm. 1983. Amphibians and Reptiles of the Pacific Northwest. The University Press of Idaho, Moscow, ID.

Nyman, T. and R. Julkunen-Tiitto. 2000. Manipulation of the phenolic chemistry of willows by gall-inducing sawflies. Proceedings of the National Academy of Sciences of the United States of America 97:13184-13187.

Ode, P. J. 2006. Plant chemistry and natural enemy fitness: Effects on herbivore and natural enemy interactions. Pp. 163-185. Annual Review of Entomology. Annual Reviews, Palo Alto.

Pappone, P. A. 1980. Voltage-clamp experiments in normal and denervated mammalian skeletal muscle fibres. Journal of Physiology 306:377-410.

Pfister, J. A. 1999. Behavioral strategies for coping with poisonous plants. Pp. 45-59 *in* K. L. Launchbaugh, K. D. Sanders, and J. C. Mosley, eds. Grazing Behavior of Livestock and Wildlife. Idaho Forest, Wildlife, and Range Experiment Station Bulletin. No. 70, Moscow, ID.

Punzo, F. 2002. Food imprinting and subsequent prey preference in the lynx spider, *Oxyopes salticus* (Araneae: Oxyopidae). Behavioural Processes 58:177-181.

Ratzka, A., H. Vogel, D. J. Kliebenstein, T. Mitchell-Olds, and J. Kroymann. 2002. Disarming the mustard oil bomb. Proceedings of the National Academy of Sciences of the United States of America 99:11223-11228.

Rossman, D., N. Ford, and R. Seigel. 1996. The Garter Snakes: Evolution and Ecology. University of Oklahoma Press, Norman, OK.

Schall, J. J. and S. Ressel. 1991. Toxic plant-compounds and the diet of the predominantly herbivorous whiptail lizard, *Cnemidophorus-arubensis*. Copeia:111-119.

Sexton, O. J., C. Hoger, and E. Ortleb. 1966. *Anolis carolinensis* - effects of feeding on reaction to aposematic prey. Science 153:1140.

Shimada, T. 2006. Salivary proteins as a defense against dietary tannins. Journal of Chemical Ecology 32:1149-1163.

Sorensen, J. and M. D. Dearing. 2006. Efflux transporters as a novel herbivore counter mechanism to plant chemical defenses. Journal of Chemical Ecology 32:1181-1196.

Stebbins, R. C. 2003. A Field Guide to Western Reptiles and Amphibians. Houghton Mifflin Co., New York, NY.

Thompson, J. N. 1994. The Coevolutionary Process. The University of Chicago Press, Chicago.

Wakely, J. F., G. J. Fuhrman, F. A. Fuhrman, H. G. Fischer, and H. S. Mosher. 1966. The occurrence of tetrodotoxin (tarichatoxin) in amphibia and the distribution of the toxin in the organs of newts (*Taricha*). Toxicon 3:195-203.

White, M. and J. A. Kolb. 1974. A preliminary study of *Thamnophis* near Sagehen Creek, California. Copeia 1974:126-136.

Whiteman, D. W., M. S. Blum, and D. W. Alsop. 1990. Allomones: Chemicals for defense. Pp. 289-352 *in* L. Evans, and J. O. Schmidt, eds. Insect Defenses: Adaptive Mechanisms and Strategies of Prey and Predators. State University of New York Press, Albany.

Willinger, G. and S. Dobler. 2001. Selective sequestration of iridoid glycosides from their host plants in longitarsus flea beetles. Biochemical Systematics and Ecology 29:335-346.

Yasumoto, T., H. Nagai, D. Yasumura, T. Michishita, A. Endo, M. Yotsu, and Y. Kotaki. 1986. Interspecies distribution and possible origin of tetrodotoxin. Annals of the New York Academy of Sciences 479:44-51.

Yoshida, S. 1994. Tetrodotoxin-resistant sodium channels. Cell Mol Neurobiol 14:227-244.

Yotsu-Yamashita, M. and D. Mebs. 2001. The levels of tetrodotoxin and its analogue 6-epitetrodotoxin in the red-spotted newt, *Notophthalmus viridescens*. Toxicon 39:1261-1263.

Yotsu-Yamashita, M. and D. Mebs. 2003. Occurrence of 11-oxotetrodotoxin in the red-spotted newt, *Notophthalmus viridescens*, and further studies on the levels of tetrodotoxin and its analogues in the newt's efts. Toxicon 41:893-897.

Chapter 2:

Parallelism in a Coevolved System:

Tetrodotoxin Resistance in Eastern *Thamnophis sirtalis*

Abstract

Investigating parallelism in coevolved systems can help us understand how common selective forces generate similar evolutionary patterns. In coevolved systems, these evolutionary patterns are shaped by the interacting traits of the involved organisms. Tetrodotoxin (TTX, a potent neurotoxin) and physiological resistance to TTX are at the phenotypic interface of several coevolved systems exhibiting common evolutionary patterns. Among these are the well-studied populations of western *Thamnophis sirtalis* that consume toxic newts of the genus *Taricha*. Eastern populations of *Th. sirtalis* that interact with toxic *Notophthalmus viridescens*, however, are virtually unstudied. In this chapter, I examine TTX resistance in an eastern population of *Th. sirtalis* at Mountain Lake Biological Station (MLBS), Virginia, and test for evidence of parallelism. I examined whole animal and population level resistance to TTX to determine if resistance had evolved in this lineage. I tested for phenotypic trade-offs to look for potential costs of resistance and for evidence of selection on the resistance phenotype. I then analyzed the functional interaction between population level resistance and levels of TTX in sympatric newts, and calculated the degree of mismatch to determine the potential for strong reciprocal selection in this population. In addition, I investigated the underlying genetic mechanism known to confer resistance in other lineages. Like western populations of *Th. sirtalis* and other species of *Thamnophis*, TTX and resistance appear to be the driving force behind a common pattern of coevolution. *Thamnophis sirtalis* at MLBS have evolved resistance to TTX and resistance is potentially costly indicating that this trait is likely experiencing selection from sympatric toxic newts. Toxicity and resistance appear

to be matched and so have the potential to experience strong reciprocal selection indicative of coevolved systems. Surprisingly, the underlying mechanism of TTX resistance found in other snake species did not explain resistance variation in this population suggesting an alternate mechanism of TTX resistance for MLBS *Th. sirtalis*.

Key words: *parallelism, coevolution*, Notophthalmus viridescens, *Virginia, Mountain Lake Biological Station, tetrodotoxin*

Introduction

Investigating parallelism in coevolved systems is important because it provides the opportunity to understand how generalized the ecological context is for predicting similar evolutionary outcomes (Brodie et al. 2005). In coevolved systems the ecological context is defined by the phenotypic interface, the interacting traits upon which reciprocal selection acts (Brodie and Brodie 1999a; Brodie and Ridenhour 2003). Because selection is reciprocal, these traits are both the targets and agents of selection (Brodie and Brodie 1999a; Brodie and Ridenhour 2003; Brodie et al. 2005). In coevolved systems that exhibit parallelism, the phenotypic interface is, therefore, the driving force behind the common patterns observed in how traits covary (Brodie and Ridenhour 2003; Brodie et al. 2005; Feldman et al. 2009, 2012).

Toxin and resistance comprise one pair of traits observed at the phenotypic interface of coevolved systems. These traits have been selected for common outcomes in organisms engaged in the interaction. For example, one of the defenses of milkweed (*Asclepias spp.*) is toxic cardenolides that are used as defense against herbivores. Several herbivores, including *Danaus plexippus* (monarch butterfly; Holzinger et al. 1992), *Oncopeltus fasciatus* (milkweed bug; Moore and Scudder 1986), *Poekilocerus bufonius* (usher-hopper; Alrobai 1993), *Chrysochus auratus* and *Chrysochus cobaltinus* (chrysochus leaf beetles; Labeyrie and Dobler 2004), and *Liriomyza asclepiadis* (leaf miner fly; Dobler et al. 2011), have evolved mutations at the target site of the toxin reducing or eliminating the harmful effects of the chemicals (reviewed in Agrawal et al. 2012). This mechanism of resistance is thought to have evolved independently in each

lineage (Agrawal et al. 2012) because it encompasses several taxonomic orders (Heteroptera, Orthoptera, Coleoptera, Diptera). Similarly, physiological and genetic resistance (via target site mutations) to tetrodotoxin (TTX) found in amphibian prey has independently evolved in several lineages of snake predators including three species of *Thamnophis* (garter snakes; Brodie and Brodie 1990; Brodie et al. 2005; Geffeney et al. 2005; Feldman et al. 2009, 2010), *Liophis epinephelus* (northern swampsnake), *Amphiesma pryeri* (Pryer's keelback), and *Rhabdophis tigrinus* (tiger keelback; Feldman et al. 2012). These examples illustrate the generalized impact of these interacting phenotypes (toxin and resistance) as evidenced by similar evolutionary outcomes.

The phenotypic interface between TTX and resistance has been studied extensively in *Thamnophis* (garter snakes). In western North America, three species of garter snakes have coevolved with sympatric toxic prey. These garter snakes feed on toxic newts of the genus *Taricha* (Brodie 1968b; Nussbaum et al. 1983; Brodie 2002; Brodie et al. 2005; Wiseman and Pool 2007; Greene and Feldman 2009): *Th. atratus* prey on *Ta. granulosa* and *Ta. Torosa*, *Th. couchii* prey on *Ta. torosa* and *Ta. sierra*, and *Th. sirtalis* prey on *Ta. granulosa*. *Taricha* are chemically defended by TTX (Mosher et al. 1964; Wakely et al. 1966; Brodie 1968b; Brodie et al.1974) with toxicity varying among species as well as within and among populations (Hanifin et al. 1999; Brodie et al. 2002; Hanifin et al. 2008; Hanifin 2010).

Although resistance to TTX appears to be ancestral for natricine snakes (Motychak et al. 1999), elevated resistance to TTX has evolved in the three garter snake species: *Th. atratus, Th. couchii, and Th. sirtalis* (Brodie and Brodie 1990; Brodie et al.

2002; Brodie et al. 2005; Feldman et al. 2009, 2010). Resistance is variable within and among populations and species (Hanifin et al. 1999; Brodie et al. 2002; Brodie et al. 2005; Hanifin et al. 2008). Resistance in snakes and toxicity in newts varies geographically such that populations of highly toxic newts tend to be sympatric with highly resistant snakes, and populations of less toxic newts tend to be sympatric with moderate or low resistant snakes (Brodie et al. 2002). However, this phenotypic distribution does not signify reciprocal selection across all populations. By examining the level of mismatch between interacting phenotypes Hanifin et al. (2008) found that the potential for strong reciprocal selection exists for only about half of populations studied in western North America. In all other cases, resistance in snakes appears to far exceed that which is necessary to consume sympatric newts.

Resistance to TTX appears to be costly for *Thamnophis*. A trade-off exists between locomotor ability and resistance such that snakes with higher resistance are slower (Brodie and Brodie 1999b; Brodie et al. 2005). Reduced crawl speed may result in greater risk of predation for *Thamnophis* (Jayne and Bennett 1990), and slower snakes may be unable to effectively thermoregulate (Brodie and Brodie 1999a). It is unlikely that snakes would evolve or maintain such a costly trait unless they receive some benefit. Presence of such a trade-off, therefore, suggests that the resistance phenotype is experiencing selection (Brodie and Brodie 1999b).

Resistance to TTX is non-inducible (Brodie and Brodie 1990; Ridenhour et al. 1999) and ontogenetically stable (Ridenhour et al. 2004). Resistance is conferred, in part, by conformational changes in the $Na_v1.4$ sodium channel gene (*SCN4A*) expressed in

skeletal muscle tissue (Geffeney et al. 2005; Feldman et al. 2009). In non-resistant animals, the TTX molecule completely occludes the outer pore region of the channel, blocking action potentials (Kao and Levinson 1986; Hille 2001) resulting in paralysis, respiratory arrest, and death (Brodie 1968b; Noguchi and Ebesu 2001; Hanifin 2010). Changes in the amino acid sequence of the outer pore region of the channel in resistant snakes affects the binding affinity of the TTX molecule such that it no longer occludes the channel pore (Lipkind and Fozzard 1994a; Lee and Ruben 2008; Fozzard and Lipkind 2010). The level of resistance exhibited by an organism is dependent on both the binding affinity of TTX to the channel pore and the amount of TTX in the system (Brodie and Brodie 1990; Geffeney et al. 2005). "Resistant" individuals may still be affected by TTX if enough of the chemical is ingested, with affected animals exhibiting reduced locomotor ability (Brodie and Brodie 1990; 1999b).

The degree to which binding affinity of the TTX molecule is affected depends on the number and location of amino acid substitutions in the pore region (Geffeney et al. 2005; Feldman et al. 2009, 2012). The pore region is formed from the α-subunit of the sodium channel gene that consists of homologous 4 domains (DI–DIV). Each domain contains a highly conserved region (P-loop) that together form the outer pore of the channel. All four P-loop regions of the $Na_v1.4$ sodium channel gene have been investigated for variation in their amino acid sequence, but changes conferring TTX resistance have previously been detected only in Domains III & IV in snakes (Feldman et al. 2009, 2012).

Two additional sodium channel paralogs, $Na_v1.6$ (*SCN8A*) and $Na_v1.7$ (*SCN9A*) expressed in the peripheral nervous system also exhibit TTX resistant mutations in Domains III & IV (McGlothlin et al. 2014). Unlike the mutations in $Na_v1.4$, the mutations in these sodium channels appear to be fixed across lineages. McGlothlin et al. (2014) suggest that ancestral resistance in garter snakes may be due to these fixed mutations in sodium channels $Na_v1.6$ and $Na_v1.7$ and that independent adaptations to local levels of TTX are likely the result of mutations in $Na_v1.4$ found in skeletal muscle tissue.

Adaptive variation in the $Na_v1.4$ sodium channel has been observed in *Th. atratus*, *Th. couchii*, and *Th. sirtalis*. These garter snakes vary in the number, type, and location of amino acid substitutions in Domains III & IV (Feldman et al. 2009). Variation in the P-loop regions of $Na_v1.4$ has been also been investigated among western populations of *Th. sirtalis* exhibiting varying levels of TTX resistance. As observed among the different species of *Thamnophis*, these populations of *Th. sirtalis* vary in the number, type, and location of amino acid substitutions in Domains III & IV (Geffeney et al. 2005). Phylogenetic analysis of this variation concluded the independent origin of TTX resistance in these inter- and intra-species lineages (Geffeney et al. 2005; Feldman et al. 2009). Together, the independent origin of TTX resistance that is similarly characterized across species and populations along with the potential for strong reciprocal selection in many lineages provides compelling evidence for a repeated pattern of adaptive evolution in *Thamnophis* indicative of parallel coevolved systems.

Thamnophis sirtalis has an expansive geographic range that reaches across North America from the Pacific to Atlantic coasts. Even though *Th. sirtalis* has received the most attention with regard to investigation of the resistance phenotype, studies have been primarily restricted to the western part of their range (but see Motychak et al. 1999). This may be due to the range limits of *Taricha* as well as the other garter snakes of interest that are limited to western North America. However, *Th. sirtalis* is sympatric with another species of newt in the eastern part of its range that is also chemically defended by TTX, the red-spotted newt *Notophthalmus viridescens* (Brodie 1968b; Yotsu et al. 1990; Yotsu-Yamashita and Mebs 2001, 2003; Mebs et al. 2010). *Notophthalmus viridescens* has been observed in the diet of at least one population of eastern *Th. sirtalis* in Virginia at the Mountain Lake Biological Station (MLBS; Chapter 3), and the newts in this population are toxic (Yotsu-Yamashita and Mebs 2001; 2003; Stokes et al. *unpublished*). While moderate resistance has been previously reported in *Th. sirtalis* at MLBS (Feldman et al. 2009), a comprehensive study of resistance in this population has not yet been completed. The population of *Th. sirtalis* at MLBS presents an excellent opportunity to explore the resistance phenotype and underlying genetic mechanism in a geographically distinct population to test for additional evidence of parallelism in a coevolved system.

In this study I examined TTX resistance in the Virginia population of *Th. sirtalis* by measuring whole animal resistance and by investigating mutational variation in the sodium channel gene known to confer resistance in other populations of *Th. sirtalis*. I used an established bioassay to measure performance under controlled doses of toxin to

evaluate whole animal resistance to TTX and to characterize both individual and population level variation. I explored the potential costs of the resistance phenotype by evaluating performance trade-offs, and I assessed the functional interaction between snake resistance and toxicity of sympatric newts. In addition, I investigated the underlying molecular mechanism for resistance by sequencing the four P-loop regions of the *SCN4A* gene that codes for the $Na_v1.4$ sodium channel expressed in skeletal muscle tissue.

Methods

Study Specimens

I tested a combination of laboratory born and field captured snakes from the University of Virginia's Mountain Lake Biological Station (MLBS) in Giles County, Virginia (37°22'32"N, 80°31'20"W). Laboratory born snakes were born to wild-caught pregnant females captured 12 July – 5 August 2009 and 18 July – 3 August 2010. Pregnant females were housed at MLBS or the University of Virginia campus in Charlottesville (UVa) in a temperature controlled room (23°C), with a 13:11 L:D photoperiod, in plastic sweater boxes (58.5 cm x 42.5 cm x 11.5 cm) with a heat strip (32°C) under one end of the enclosure to provide a thermal gradient. Pregnant females were given free access to water and were offered earthworms twice per week. Adult females were released at their site of capture within three weeks of giving birth.

Neonates were born 9 August – 8 September 2009 and 1 – 12 August 2010 at MLBS or UVa. Post-parturition, neonates were weighed and measured for snout-vent

length (SVL) and total length. Those born at MLBS were transported to UVa on 14 August 2009 and 13 August 2010. All neonates were kept in a temperature controlled room (23°C, 45% humidity, 12:12 L:D). Neonates were initially individually housed in plastic tubs (11.5cm in diameter x 14cm in height) and watered daily. At approximately 2 weeks, they were moved to individual plastic terraria (Kritter Keeper, approx. 29.8 cm long x 19.7 cm wide x 20.3 cm high) with a heat strip under one end (32°C), paper towel as substrate, and free access to water. Neonates were offered food twice per week. For the first six weeks, some snakes were fed newly metamorphosed *N. viridescens* while others were fed cut pieces of earthworm (as part of a different experiment – Chapter 4). After this initial six week period all snakes were fed pieces of earthworm.

Field caught snakes consisted of yearlings (young born the previous fall season) captured in the field 13 June – 1 July 2010 and one neonate captured on 11 August 2010. Field caught snakes were kept in plastic terraria (as neonates above) for the duration of testing before being released at their site of capture.

Resistance Bioassay

Seventy snakes were assayed for resistance (20 lab-born 2009, 6 field caught in 2010, and 44 lab-born in 2010). Snakes born in 2009 were tested 6 – 16 July 2010, and snakes born in 2010 were tested 1 – 19 November 2010. Only neonate and yearling animals were used in this study due to size constraints of the testing mechanism.

I used an established bioassay of whole-animal sprint-speed performance to estimate TTX resistance (Brodie and Brodie 1990; Brodie et al. 2002; Ridenhour et al.

2004; Brodie et al. 2005; Feldman et al. 2009, 2010, 2012). Snakes were raced along a 9.5 cm wide by 2.5 m long track with indoor/outdoor carpet as substrate in a 26-27°C room. The central section of the track was equipped with 10 infrared sensors spaced 10 cm apart yielding nine segments for which speed was electronically measured. Snakes were chased down the track by gently and repeatedly tapping their tail.

Individual snakes were initially scored for speed twice in a single day (approx. 5 hrs. apart). The fastest 10 cm segment was recorded for each of the two trials and the average of these speed scores was used as the baseline sprint speed. Approximately 24 hours after the last baseline test, the mass of each snake was measured, the TTX dose calculated (see below), and toxin was administered via intra-peritoneal injection. Thirty minutes after injection, snakes were tested again and the fastest segment was recorded as "post-injection speed." Individuals were tested up to five times to measure resistance at different doses with a 48 hour recovery time between injections. For each dose of TTX, resistance was scored as the percent of individual baseline speed (100 X (post-injection speed/individual baseline speed)).

Snakes were fasted for 5 days prior to testing to ensure that their mass and crawl-speed were not altered by undigested food in the gastro-intestinal tract. Previous studies demonstrated that control injections of physiological saline have no effect on snake performance (Brodie and Brodie 1990).

Snakes were tested at effective doses. The effective dose of TTX administered to snakes was calculated in mass-adjusted mouse units (MAMU). Using effective doses controls for mass differences among individuals and gives a standardized measure for

comparing resistance with other populations and species. A mouse unit is the amount of TTX required to kill one gram of mouse in 10 min. (0.01429 ug TTX). The absolute amount of TTX given to an individual snake was determined by the following equation:

$$\mu g\ TTX = mass\ g\ \left(MAMU \times \frac{0.01429\ \mu g}{1\ g}\right) \quad \text{(eq. 1)}$$

where mass (in grams) is that of the individual snake tested (Brodie et al. 2002; Ridenhour et al. 2004; Brodie et al. 2005). The absolute doses given to individual snakes ranged from 0.02 μg – 6.42 μg TTX (1-100 MAMU) and were administered from known concentrations of TTX solution (mg TTX/ml amphibian ringer solution) using variable volumes (max 0.132 ml).

Effort was made to test all snakes at four common effective doses: 1, 5, 10, and 20 MAMU based on a preliminary estimate of population mean resistance of 7 MAMU (Feldman et al. 2009). However, during testing more resistant snakes received higher doses and some less resistant snakes received lower doses of TTX in order to more accurately estimate their individual level of resistance.

Population dose response curve

The 13 individuals that received all four common doses (1, 5, 10 and 20 MAMU) were included in the estimation of the population dose response curve Individual responses to each administered dose (52 observations) were used to estimate population resistance using a curvilinear regression. The linear regression $y' = \alpha + \beta x'$ was used applying the transforms $y' = \ln((1/y) - 1)$ and $x' = \ln(x + 1)$, where y is TTX resistance, x

is TTX dose, β is the slope, and α is the intercept (Brodie et al. 2002; Ridenhour et al. 2004). Population average resistance was estimated as the 50% dose (i.e. the amount of TTX required to reduce the average snake to half of its baseline speed). For additional details regarding these calculations refer to Brodie et al. (2002) and Ridenhour et al. (2004).

In addition, resistance level (50% dose) was calculated for each of the 70 snakes tested. Responses of an individual were regressed onto the doses (in MAMU) administered to determine individual level resistance. The resulting regression for an individual's responses was then used to calculate the individual's 50% dose. Family sizes were insufficient for testing to see if there were significant differences in resistance among families.

Phenotypic Tradeoffs

I tested for phenotypic tradeoff between TTX resistance and locomotor performance by regressing post-injection speed onto baseline speed for 57 snakes each tested at the 10 MAMU dose. The slope of the relationship was then compared to the null expectation. Two different methods for setting the null expectation have been used. Brodie and Brodie (1999a) argued that if TTX affects all individuals equally, then the slope is equal to one. Phillips et al. (2004) presented a more conservative approach in which the null slope is equal to the average proportion of baseline speed observed for the dose administered. This approach is more conservative because the null slope will always be less than one. In either case, a slope greater than the null indicates that snakes with

greater resistance are faster and a slope less than the null indicates that snakes with lower resistance are faster. Both null slopes were tested in this tradeoff analysis.

I also investigated the relationship between body size and resistance in this population as a comparison to other resistant populations of *Th. sirtalis* and other species of *Thamnophis*. Following the methods of Brodie and Brodie (1999a), post-injection and baseline speeds were each regressed onto mass. The residuals from each regression were used as mass-adjusted variables. Mass-adjusted post-injection speed was then regressed onto mass-adjusted baseline speed. I first compared the mass adjusted slope to the null expectation (slope = 1) to see if the results were comparable to the analysis using the unadjusted slope. I then compared the slope of the mass-adjusted regression to the slope of the unadjusted regression to see if they differed significantly. A difference in slope indicates that body size contributes to TTX resistance (Brodie and Brodie 1999b).

Functional interaction between snakes and newts

I modeled the predicted functional relationship between TTX resistance in *Th. sirtalis* and toxicity of *N. viridescens* using data on newt toxicity collected by Stokes et al. (2013, *unpublished*; Appendix 1) from sympatric newts at MLBS following the methods of Brodie et al. (2005). From those data on newt toxicity, I calculated mean TTX in adult *N. viridescens* = 0.028 mg (SE = 0.003, min = 0.006 mg, max = 0.076 mg, n = 33), and in efts = 0.067 mg (SE = 0.011, min < 0.001 mg, max = 0.353 mg, n = 51). I used these estimates of TTX to model the functional relationship between sympatric *Th. sirtalis* and *N. viridescens*.

The *Th. sirtalis* population dose response curve (see above) was used to interpolate the population average doses of TTX (in MAMU) for a given resistance. These were converted to absolute doses of injected TTX (in mg) relative to snake mass, and then adjusted to account for the relationship of injected to oral dose (Williams et al. 2002). The resulting data were used to estimate curves for 15%, 50%, and 85% resistance to TTX (in mg) in terms of snake mass (in grams). Data on snake mass in the population were obtained from census across three seasons 2008-2010 (Chapter 3).

The degree of functional matching between snake resistance and newt toxicity was estimated following the methods of Hanifin et al. (2008). Degree of mismatch (*d*) was calculated using the 50% dose of TTX in mg for the average mass of an adult post-parturition female snake in the population and total TTX in mg in the average adult newt in the population. The same calculation was made using the total TTX in mg in an average relevant sized eft (>2g) because efts are more toxic than adult newts on average. In addition, *d* was calculated for the different snake life stages (neonates, yearlings, juveniles) and for both adult males and females, because snake size affects the size of prey consumed and, therefore, the total amount of TTX consumed in appropriately sized prey. Degree of mismatch is defined by the following equation after Hanifin et al. (2008):

$$d = |(x - y) / \sqrt{2}| \qquad \text{(eq. 2)}$$

where x = log (50% dose) of snakes and y = log (avg. total skin TTX) of sympatric newts.

Genetic Resistance

I sequenced the p-loop regions of the $Na_v1.4$ sodium channel gene (*SCN4A*) in each of the four domains to investigate the genetic mechanism of resistance in this population. Snakes were assayed for resistance then tissue samples were collected by removing 3 mm – 5 mm of tail tip and preserving each sample in a 2 ml screw top microfuge tube containing 95% ethanol. 32 subjects were chosen for sequence analysis based on their individual level of resistance to ensure that the range of resistant phenotypes in the population was represented (50% MAMU = 1.5 – 72.7; Appendix 2).

Genomic DNA was extracted and purified using the DNeasy Tissue Kit (Qiagen). The four P-loop regions were amplified using primers designed by Feldman et al. (2009) and McGlothlin (*unpublished*). Regions DI, DII, and DIV were each amplified as a single segment and the DIII P-loop was amplified in two segments due to a large intron that interrupted the coding region. I used an ExoSap (Exonuclease 1 and Shrimp Alkaline Phosphatase) procedure to clean amplified products which were then either sequenced in-house or prepared and sent to the DNA Analysis Facility at Yale University for sequencing. In-house samples were cycle-sequenced using Big Dye 3.1 (Applied Biosystems) after which sequenced product was cleaned using Sephadex® G-50 and ran on an ABI 3130xl automated sequencer (Applied Biosystems). Primer was added to purified PCR product before samples were sent to the Yale DNA Analysis Facility for sequencing. There, samples were cycle sequenced using Big Dye Terminators, purified, and analyzed on the 3730xl automated sequencer (Applied Biosystems). I edited sequences by eye, then aligned and translated using Geneious Pro 5.3.6. Sequences of

additional populations and species were obtained from GenBank for comparison (Appendix 3).

Results

Resistance

The estimated 50% MAMU dose for the population of *Th. sirtalis* at Mountain Lake Biological Station, Virginia was calculated as 7.66 MAMU (Figure 1; 95% CI: 6.19 – 9.13 MAMU) from the regression: $y' = -4.46 + 2.07x'$ ($R^2 = 0.623$, $F_{1,50} = 82.49$, $p < 0.0001$). Individuals ranged in resistance from 1.5 MAMU to 72.7 MAMU (Figure 2).

Phenotypic Tradeoffs

There is a tradeoff between TTX resistance and locomotor performance such that more resistant snakes are slower than relatively less resistant snakes (Figure 3). The slope of the regression [post-injection speed = 10.96 - 0.035(baseline speed)] was significantly less than one ($t = -21.56$, $df = 55$, $p < 0.0001$), the null slope assuming that all snakes are equally affected by TTX. The slope of the regression of post-injection speed on baseline speed was also significantly less than the alternative null slope (0.29), which was equal to the average proportion of baseline speed observed at the 10 MAMU dose ($t = -6.7708$, $df = 55$, $p < 0.0001$). When adjusting for mass, the regression of the mass adjusted slope [mass-adjusted post-injection speed = 0.108 (mass-adjusted baseline speed)] was also significantly different from one ($t = -8.745$, $df = 55$, $p < 0.0001$) indicating that the trade-off is still apparent when adjusting for individual mass. Similar to other populations

tested, the mass adjusted slope did not differ from the unadjusted slope (t = 1.269, df = 110, p = 0.207) indicating that body size does not contribute to TTX resistance.

Functional interaction between snakes and newts

The model predicts that impairment from TTX toxicity is possible for some classes of snakes, primarily sub-adults (Figure 4). Juveniles (mean mass = 20.8 g) and yearling snakes (mean mass = 5.93 g) are predicted to be the most affected by toxic newts. Efts of average toxicity are predicted to reduce juvenile performance to approximately 50%, but the most toxic efts may be lethal. The amount of TTX in the average eft is predicted to reduce performance of the average sized yearling snake to approximately 15%, and more toxic efts are likely to be lethal. The model predicts that the amount of TTX in newts is less likely to impact adult snakes and neonates. Adult newts and efts are not expected to be lethal to adult snakes, though some efts may reduce performance below 50%. Neonate snakes are not expected to be affected by the toxin levels of efts because, due to their small size, neonates are most likely consuming newly metamorphosed efts that are essentially non-toxic (Figure 4B).

The degree of mismatch estimated based on the average mass of post-parturition females in the Virginia population is d = 0.60 (cf. Hanifin et al. 2008). Snake populations that are considered mismatched with newt toxicity have values of d > 0.6. This indicates that the Virginia population is at the edge of the phenotypic range where variable outcomes are likely to occur using the western populations as a basis for comparison. A closer look at degree of mismatch among the different life stages of snakes indicates that

the potential to experience reciprocal selection is highest among interactions with efts versus adult newts, especially those interactions involving younger, smaller snakes (Table 1).

Genetic Resistance

Despite relatively high levels of resistance observed in some individuals of the MLBS population, no amino acid sequence changes were observed in the P-loop regions of Domains I, II, III, or IV of the $Na_v1.4$ sodium channel gene, *SCN4A* (Figure 5). The P-loop regions of all four domains in the MLBS population showed complete homology with *Th. sirtalis* from the Bear Lake Co., ID population and *Th. elegans*, both of which exhibit ancestral levels of TTX resistance. Further investigation in the sections outside the P-loop but still within the S5-6 linker region revealed a single amino acid mutation in Domain II. This change from isoleucine to leucine at position 764 (I764L) varies from other *Th. sirtalis* populations and other *Thamnophis* species (Figure 6). Position 764 has not been specifically investigated for its role in TTX binding affinity in snakes, however, previous study of that region does not indicate significant involvement in TTX binding (Kontis and Goldin 1993).

Discussion

Thamnophis sirtalis at Mountain Lake Biological Station in Virginia have evolved moderate resistance to TTX compared to western populations and other resistant *Thamnophis*. Similar to other species of *Thamnophis* and other populations of *Th. sirtalis*,

a trade-off exists between resistance and locomotor ability suggesting that resistance may be costly. Analysis of the functional interaction between snake resistance and newt toxicity indicates that the ability of snakes to consume sympatric newts is dependent upon both the life stage of the snake and of the life stage of the newt. In addition, the degree of mismatch indicates the potential for strong reciprocal selection in these species especially when considering the different life stages. Together, these results argue for parallelism in a coevolved system. These data also demonstrate that snakes in this population lack mutations in the sodium channel gene known to confer resistance in other lineages, which suggests an alternate mechanism of TTX resistance.

Thamnophis sirtalis at MLBS exhibit population resistance that is higher than ancestral levels. Ancestral resistance for *Thamnophis* and other natricines is 1-2 MAMU (Motychak et al. 1999) and elevated levels of TTX resistance in *Thamnophis* is a derived condition (Feldman et al. 2009). Departure from the ancestral condition, therefore, indicates that this population has experienced selection for increased resistance to TTX. Evolved resistance in this population is in contrast to other eastern populations that have been examined. Populations in York Co., Maine and Whiteside Co., Illinois demonstrated ancestral resistance (1.8 and 1.7 MAMU, respectively; Motychak et al. 1999). These locations are sympatric with *Notophthalmus viridescens*, but toxicity in these populations of newts has not been measured. In comparison to western populations, MLBS *Th. sirtalis* exhibit moderate resistance and fall within the same level of resistance (5-10 MAMU; Appendix 2) as 20% of western populations (c.f. Brodie et al. 2002). Of the 40 populations tested by Brodie et al. (2002), 47.5% of western populations were less

resistant than the average of the MLBS population and 32.5% of western populations were more resistant than the MLBS population.

As observed in other *Thamnophis* tested (Brodie and Brodie 1999b; Brodie et al. 2005), a trade-off appears to exist between resistance and locomotor ability. The presence of a trade-off indicates that resistance is potentially costly for snakes in this population because slower snakes may be more susceptible to predation (Jayne and Bennett 1990). Further, potential costs associated with resistance suggest that snakes and newts are coevolving (Brodie and Brodie 1999b). Snakes are not expected to exhibit elevated resistance if resistance is costly unless there is some benefit, such as prey exploitation. Similarly, snakes are expected to reduce their costs and become less resistant in the absence of selection by toxic newts. The observed trade-off between resistance and locomotor ability has, therefore, been offered as an explanation for the pattern of geographic covariation among these interacting phenotypes in western populations (Brodie and Brodie 1999b).

Overall, snake resistance and newt toxicity appear to be matched in the MLBS population, similar to approximately half of other *Th. sirtalis* populations previously tested in western North America (Hanifin et al. 2008), as evidenced by both the functional interaction assessment and the degree of mismatch calculated. That is, variable fitness consequences exist for both snakes and newts due to the variance in their interacting phenotypes; resistance and toxicity, respectively (Brodie and Ridenhour 2003; Hanifin et al. 2008). A closer look at snakes and newts at different life stages illustrates a more variable picture of the functional interaction than was indicated by the initial

assessment of the mismatch coefficient (*d*). Efts in this population are more toxic on average than adult newts, so interactions with efts are likely to be more impactful to snakes. In addition, since efts tend to be smaller than adult newts, they are more likely to be consumed by smaller snakes. The resulting interaction (smaller snakes consuming larger absolute quantities of TTX) can have more severe consequences for sub-adult snakes. Neonate snakes may be an exception as gape-limitations restrict the size of newt that can be taken by these small snakes (Shine 1991). As a result, neonates are most likely to take only newly metamorphosed efts that contain the lowest levels of TTX (Gall et al. 2011). The significance of the matching phenotypes observed in this population is its potential to generate strong reciprocal selection and, therefore, drive coevolution of these traits (Janzen 1980; Thompson 1994; Hanifin et al. 2008). In the West, this has resulted in a coevolutionary arms race and the evolution of escalated resistance and toxicity in some populations (Brodie et al. 2002). The reciprocal selection that appears to be occurring in the MLBS population indicates potential for a similar coevolutionary arms race.

The molecular analysis of the $Na_v1.4$ sodium channel gene revealed that the P-loop regions of the four domains were no different than ancestral lineages. Previous investigation of the genetic architecture of TTX resistance revealed that even snakes that are homozygous for the ancestral allele (i.e. the ancestral $Na_v1.4$ sequence) exhibit some level of elevated resistance (Feldman et al. 2010). For *Th. atratus*, snakes that are homozygous for the ancestral allele exhibited on average 7.41 MAMU (SD = 3.99), and *Th. sirtalis* exhibited on average 18.97 MAMU (SD = 8.58). While MLBS *Th. sirtalis*

demonstrate population resistance at 7.66 MAMU, some individuals within the population exhibit resistance up to 72.7 MAMU. Observed resistance in these individuals cannot be explained by demonstrated resistance in homozygous ancestral alleles in the $Na_v1.4$ sodium channel.

Variation in other sodium channel genes does not appear to explain the high resistance observed in some MLBS snakes. McGlothlin et al. (2014) found that sodium channels $Na_v1.6$ and $Na_v1.7$, expressed in the peripheral nervous system (Goldin 2001; Catterall et al. 2005), exhibited substitutions of the type and location expected to confer TTX resistance. These substitutions were fixed across *Th. sirtalis* populations (western and eastern) and are expected to contribute only to ancestral levels of TTX resistance (i.e. protection from very small amounts of ingested TTX; McGlothlin et al. 2014). The other sodium channels are either shielded from TTX exposure (CNS: $Na_v1.1$, $Na_v1.2$, $Na_v1.3$; Zimmer 2010), likely to be ancestrally TTX resistant in all snakes (cardiac muscle: $Na_v1.5$; Vornanen et al. 2011), or may not contribute significantly to organismal resistance (small-diameter sensory neurons: $Na_v1.8$, $Na_v1.9$; McGlothlin et al. 2014).

While variation in the $Na_v1.4$ sodium channel gene is strongly correlated with whole animal resistance to TTX in snakes, it does not account for all the phenotypic variation observed (Geffeney et al. 2002; Geffeney et al. 2005; Feldman et al. 2010). A single mutation observed in the $Na_v1.4$ channel for the population of *Th. sirtalis* in Warrenton (Clatsop Co., OR; 15.2 MAMU) explained only a small portion of the observed resistance (Geffeney et al. 2005). Likewise, allelic variation in the $Na_v1.4$ channel accounted for only 23% of TTX resistance in *Th. atratus* (> 100 MAMU;

Feldman et al. 2010). In addition, not all the variation in whole animal resistance is explained by skeletal muscle resistance when skeletal muscle tissue was isolated, and so it has been previously suggested that additional underlying mechanisms contribute to TTX resistance in *Thamnophis* (Geffeney et al. 2002).

Even so, it is surprising that there is no observed variation in $Na_v1.4$ given that such high levels of TTX resistance were observed among individuals within the MLBS population. The absence of common substitutions in $Na_v1.4$ is especially surprising given that six species exhibit TTX resistant mutations in the pore regions of this gene, including three species of *Thamnophis* (*Th. atratus, Th. couchii, Th. sirtalis*), two other natricines (*Amphiesma pryeri*, *Rhabdophis tigrinus*), and one distantly related colubrid (*Liophis epinephelus*; Feldman et al. 2012). The lack of variation in $Na_v1.4$ as well as the apparent and predicted absence of genetic variation in other TTX sensitive channels suggests that MLBS *Th. sirtalis* are achieving resistance differently than populations previously studied, and that there is likely an undiscovered mechanism for low/moderate resistance.

TTX and resistance appear to be the driving force behind a common pattern of coevolution. The results of this investigation suggest that *Th. sirtalis* and *N. viridescens* are engaged in a coevolutionary interaction in a region not previously investigated that parallels that observed in other TTX resistant lineages. The lines of evidence that support this conclusions include (1) observed resistance to TTX that is elevated above ancestral levels demonstrating that this trait has evolved, (2) a trade-off indicating the potential cost of TTX resistance and suggesting that snakes are experiencing selection from local newts, and (3) matched levels of resistance and toxicity signifying the potential for strong

reciprocal selection at the phenotypic interface. In addition, the molecular analysis of resistance revealed that what appeared to be a ubiquitous mechanism of TTX resistance in other snake lineages apparently does not explain within population variation in this eastern population.

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References

Agrawal, A. A., G. Petschenka, R. A. Bingham, M. G. Weber, and S. Rasmann. 2012. Toxic cardenolides: Chemical ecology and coevolution of specialized plant-herbivore interactions. New Phytologist 194:28-45.

Alrobai, A. A. 1993. Different ouabain sensitivities of Na+/K+-ATPase from *Poekilocerus bufonius* tissues and a possible physiological cost. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 106:805-812.

Brodie, E. D. III and E. D. Brodie, Jr. 1990. Tetrodotoxin resistance in garter snakes - an evolutionary response of predators to dangerous prey. Evolution 44:651-659.

Brodie, E. D. III and E. D. Brodie, Jr. 1999a. Predator-prey arms races. Bioscience 49:557-568.

Brodie, E. D. III and E. D. Brodie, Jr. 1999b. Costs of exploiting poisonous prey: Evolutionary trade-offs in a predator-prey arms race. Evolution 53:626-631.

Brodie, E. D. III, C. R. Feldman, C. T. Hanifin, J. E. Motychak, D. G. Mulcahy, B. L. Williams, and E. D. Brodie, Jr. 2005. Parallel arms races between garter snakes and newts involving tetrodotoxin as the phenotypic interface of coevolution. Journal of Chemical Ecology 31:343-356.

Brodie, E. D. III and B. J. Ridenhour. 2003. Reciprocal selection at the phenotypic interface of coevolution. Integrative and Comparative Biology 43:408-418.

Brodie, E. D., Jr. 1968. Investigations on the skin toxin of the adult rough-skinned newt, *Taricha granulosa*. Copeia 1968:307-313.

Brodie, E. D., Jr., J. L. Hensel, and J. A. Johnson. 1974. Toxicity of urodele amphibians *Taricha*, *Notophthalmus*, *Cynops* and *Paramesotriton* (Salamandridae). Copeia 1974:506-511.

Brodie, E. D., Jr., B. J. Ridenhour, and E. D. Brodie III. 2002. The evolutionary response of predators to dangerous prey: Hotspots and coldspots in the geographic mosaic of coevolution between garter snakes and newts. Evolution 56:2067-2082.

Catterall, W. A., A. L. Goldin, and S. G. Waxman. 2005. International Union of Pharmacology. XLVII. Nomenclature and structure-function relationships of voltage-gated sodium channels. Pharmacological Reviews 57:397-409.

Dobler, S., G. Petschenka, and H. Pankoke. 2011. Coping with toxic plant compounds - the insect's perspective on iridoid glycosides and cardenolides. Phytochemistry 72:1593-1604.

Feldman, C. R., E. D. Brodie, Jr., E. D. Brodie III, and M. E. Pfrender. 2009. The evolutionary origins of beneficial alleles during the repeated adaptation of garter snakes to deadly prey. Proceedings of the National Academy of Sciences of the United States of America 106:13415-13420.

Feldman, C. R., E. D. Brodie, Jr., E. D. Brodie III, and M. E. Pfrender. 2010. Genetic architecture of a feeding adaptation: Garter snake (*Thamnophis*) resistance to tetrodotoxin bearing prey. Proceedings of the Royal Society B-Biological Sciences 277:3317-3325.

Feldman, C. R., E. D. Brodie, Jr., E. D. Brodie III, and M. E. Pfrender. 2012. Constraint shapes convergence in tetrodotoxin-resistant sodium channels of snakes.

Proceedings of the National Academy of Sciences of the United States of America 109:4556-4561.

Fozzard, H. A. and G. M. Lipkind. 2010. The tetrodotoxin binding site is within the outer vestibule of the sodium channel. Marine Drugs 8:219-234.

Gall, B. G., A. N. Stokes, S. S. French, E. A. Schlepphorst, E. D. Brodie III, and E. D. Brodie, Jr. 2011. Tetrodotoxin levels in larval and metamorphosed newts (*Taricha granulosa*) and palatability to predatory dragonflies. Toxicon 57:978-983.

Geffeney, S., E. D. Brodie, Jr., P. C. Ruben, and E. D. Brodie III. 2002. Mechanisms of adaptation in a predator-prey arms race: TTX-resistant sodium channels. Science 297:1336-1339.

Geffeney, S. L., E. Fujimoto, E. D. Brodie III, E. D. Brodie, Jr., and P. C. Ruben. 2005. Evolutionary diversification of TTX-resistant sodium channels in a predator-prey interaction. Nature 434:759-763.

George, A. L., J. Komisarof, R. G. Kallen, and R. L. Barchi. 1992. Primary structure of the adult human skeletal muscle voltage-dependent sodium channel. Annals of Neurology 31:131-137.

Goldin, A. L. 2001. Resurgence of sodium channel research. Annual Review of Physiology 63:871-894.

Greene, R. R. and C. R. Feldman. 2009. *Thamnophis atratus atratus* diet. Herpetological Review 40:103-104.

Hanifin, C. T. 2010. The chemical and evolutionary ecology of tetrodotoxin (TTX) toxicity in terrestrial vertebrates. Marine Drugs 8:577-593.

Hanifin, C. T., E. D. Brodie, Jr., and E. D. Brodie III. 2008. Phenotypic mismatches reveal escape from arms-race coevolution. PLoS. Biol. 6:471-482.

Hanifin, C. T., M. Yotsu-Yamashita, T. Yasumoto, E. D. Brodie, Jr., and E. D. Brodie III. 1999. Toxicity of dangerous prey: Variation of tetrodotoxin levels within and among populations of the newt *Taricha granulosa*. Journal of Chemical Ecology 25:2161-2175.

Hille, B. 2001. Ion Channels of Excitable Membranes. Sinauer Associates, Sunderland, MA.

Holzinger, F., C. Frick, and M. Wink. 1992. Molecular-basis for the insensitivity of the monarch (*Danaus plexippus*) to cardiac-glycosides. Febs Letters 314:477-480.

Janzen, D. H. 1980. When is it coevolution? Evolution 34:611-612.

Jayne, B. C. and A. F. Bennett. 1990. Selection on locomotor performance capacity in a natural population of garter snakes. Evolution 44:1204-1229.

Kao, C. Y. and S. R. Levinson. 1986. Tetrodotoxin, Saxitoxin, and the Molecular Biology of the Sodium Channel. New York Academy of Sciences, New York, NY.

Kontis, K. J. and A. L. Goldin. 1993. Site-directed mutagenesis of the putative pore region of the rat IIa sodium-channel. Molecular Pharmacology 43:635-644.

Labeyrie, E. and S. Dobler. 2004. Molecular adaptation of chrysochus leaf beetles to toxic compounds in their food plants. Molecular Biology and Evolution 21:218-221.

Lee, C. H. and P. C. Ruben. 2008. Interaction between voltage-gated sodium channels and the neurotoxin, tetrodotoxin. Channels 2:407-412.

Lipkind, G. M. and H. A. Fozzard. 1994. A structural model of the tetrodotoxin and saxitoxin binding-site of the Na+ channel. Biophysical Journal 66:1-13.

Lipkind, G. M. and H. A. Fozzard. 2000. KcsA crystal structure as framework for a molecular model of the Na+ channel pore. Biochemistry 39:8161-8170.

McGlothlin, J. W., J. P. Chuckalovcak, D. E. Janes, S. V. Edwards, C. R. Feldman, E. D. Brodie, Jr., M. E. Pfrender, and E. D. Brodie III. 2014. Parallel evolution of tetrodotoxin resistance in three voltage-gated sodium channel genes in the garter snake *Thamnophis sirtalis*. Molecular Biology and Evolution 31:2836-2846.

Mebs, D., O. Arakawa, and M. Yotsu-Yamashita. 2010. Tissue distribution of tetrodotoxin in the red-spotted newt *Notophthalmus viridescens*. Toxicon 55:1353-1357.

Moore, L. V. and G. G. E. Scudder. 1986. Ouabain-resistant Na, K-ATPases and cardenolide tolerance in the large milkweed bug, *Oncopeltus fasciatus*. Journal of Insect Physiology 32:27-33.

Mosher, H., F. Fuhrman, H. Bichwald, and H. Fischer. 1964. Tarichatoxin-tetrodotoxin: A potent neurotoxin. Science 144:1100-1110.

Motychak, J. E., E. D. Brodie, Jr., and E. D. Brodie III. 1999. Evolutionary response of predators to dangerous prey: Preadaptation and the evolution of tetrodotoxin resistance in garter snakes. Evolution 53:1528-1535.

Noguchi, T. and J. S. M. Ebesu. 2001. Puffer poisoning: Epidemiology and treatment. Journal of Toxicology-Toxin Reviews 20:1-10.

Nussbaum, R. A., E. D. Brodie, Jr., and R. M. Storm. 1983. Amphibians and Reptiles of the Pacific Northwest. The University Press of Idaho, Moscow, ID.

Phillips, B. L., G. P. Brown, and R. Shine. 2004. Assessing the potential for an evolutionary response to rapid environmental change: Invasive toads and an Australian snake. Evolutionary Ecology Research 6:799-811.

Ridenhour, B. J., E. D. Brodie, Jr., and E. D. Brodie III. 1999. Repeated injections of TTX do not affect TTX resistance or growth in the garter snake *Thamnophis sirtalis*. Copeia:531-535.

Ridenhour, B. J., E. D. Brodie, Jr., and E. D. Brodie III. 2004. Resistance of neonates and field-collected garter snakes (*Thamnophis* spp.) to tetrodotoxin. Journal of Chemical Ecology 30:143-154.

Shine, R. 1991. Why do larger snakes eat larger prey items? Functional Ecology 5:493-502.

Thompson, J. N. 1994. The Coevolutionary Process. The University of Chicago Press, Chicago.

Vornanen, M., M. Hassinen, and J. Haverinen. 2011. Tetrodotoxin sensitivity of the vertebrate cardiac Na+ current. Marine Drugs 9:2409-2422.

Wakely, J. F., G. J. Fuhrman, F. A. Fuhrman, H. G. Fischer, and H. S. Mosher. 1966. The occurrence of tetrodotoxin (tarichatoxin) in amphibia and the distribution of the toxin in the organs of newts (*Taricha*). Toxicon 3:195-203.

Williams, B. L., E. D. Brodie, Jr., and E. D. Brodie III. 2002. Comparisons between toxic effects of tetrodotoxin administered orally and by intraperitoneal injection to the garter snake *Thamnophis sirtalis*. Journal of Herpetology 36:112-115.

Wiseman, K. D. and A. C. Pool. 2007. *Thamnophis couchii* (sierra garter snake): Predator-prey interaction. Herpetological Review 38:344.

Yotsu-Yamashita, M. and D. Mebs. 2001. The levels of tetrodotoxin and its analogue 6-epitetrodotoxin in the red-spotted newt, *Notophthalmus viridescens*. Toxicon 39:1261-1263.

Yotsu-Yamashita, M. and D. Mebs. 2003. Occurrence of 11-oxotetrodotoxin in the red-spotted newt, *Notophthalmus viridescens*, and further studies on the levels of tetrodotoxin and its analogues in the newt's efts. Toxicon 41:893-897.

Yotsu, M., M. Iorizzi, and T. Yasumoto. 1990. Distribution of tetrodotoxin, 6-epitetrodotoxin, and 11-deoxytetrodotoxin in newts. Toxicon 28:238-241.

Zimmer, T. 2010. Effects of tetrodotoxin on the mammalian cardiovascular system. Marine Drugs 8:741-762.

Table 1: Degree of mismatch for different classes of *Th. sirtalis* and *N. viridescens*. For each snake class, the average mass (g), relevant class of newt, and degree of mismatch (*d*) are given. In some cases, both adult newts and efts may be relevant prey for the snake class and *d* is calculated for both.

Snake Class	mass (g)	Newt Class	*d*	Newt Class	*d*
postpartum female	44.73	adult newt	0.60	eft (>2g)	0.22
adult	56.18	adult newt	0.67	eft (>2g)	0.29
adult female	64.58	adult newt	0.71	eft (>2g)	0.33
adult male	36.58	adult newt	0.54	eft (>2g)	0.16
juvenile	20.80	adult newt	0.36	eft (>2g)	0.02
yearling	5.93			eft (0.5g - 2g)	0.28
neonate	2.09			eft (<0.5g)	0.31

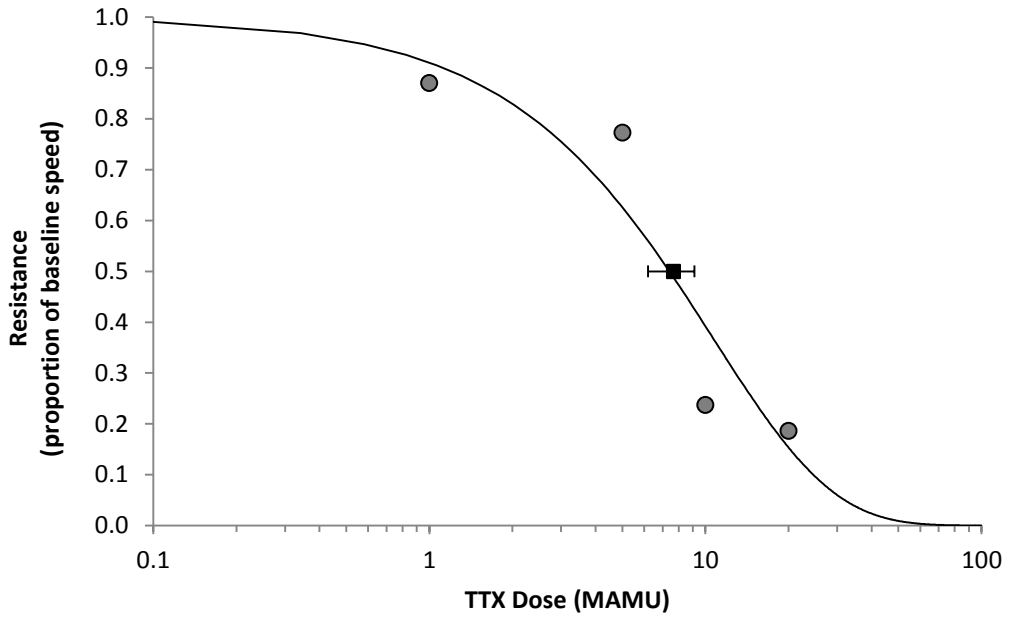


Figure 1. Population dose response curve. The population dose response curve, based on 52 observations (13 individual snakes each tested at 4 common doses: 1, 5, 10, and 20 MAMU), estimated from curvilinear regression is shown. Grey circles are the mean resistance of individuals tested at that dose (all SE < 0.14). The 50% dose for the population (7.66±1.47 MAMU), as estimated from the curvilinear regression, is illustrated with the black square. Error bars denote 95% confidence interval.

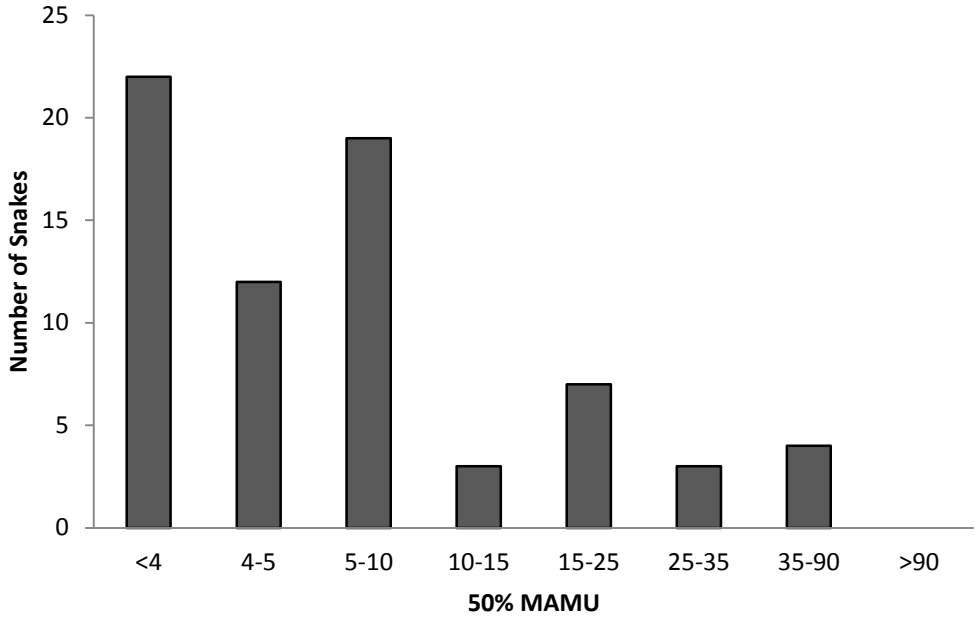


Figure 2. Distribution of TTX resistance in *Thamnophis sirtalis* at MLBS. The number of snakes exhibiting levels of resistance is shown for $n = 70$ snakes. Resistance levels are binned according to those reported in Brodie et al. (2002).

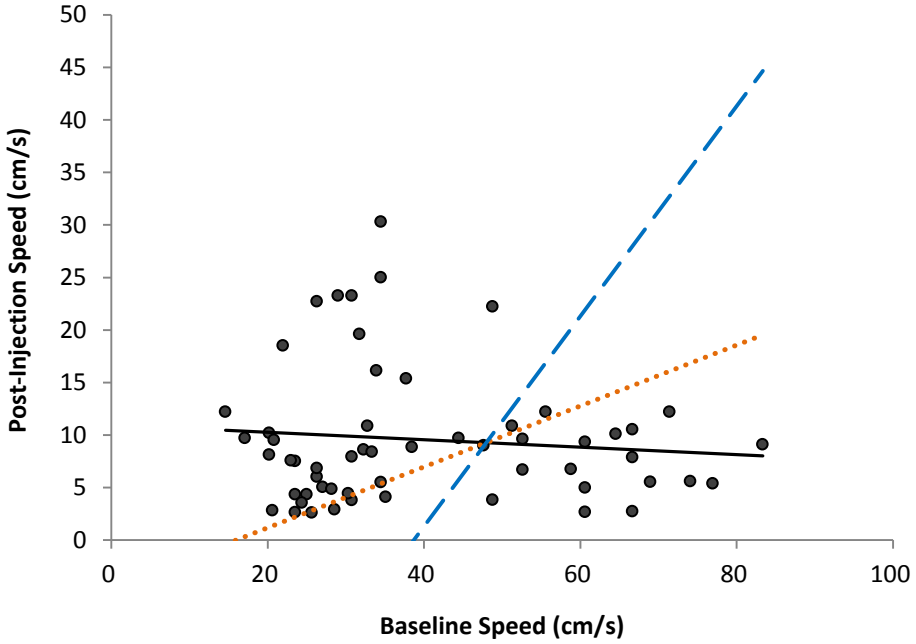
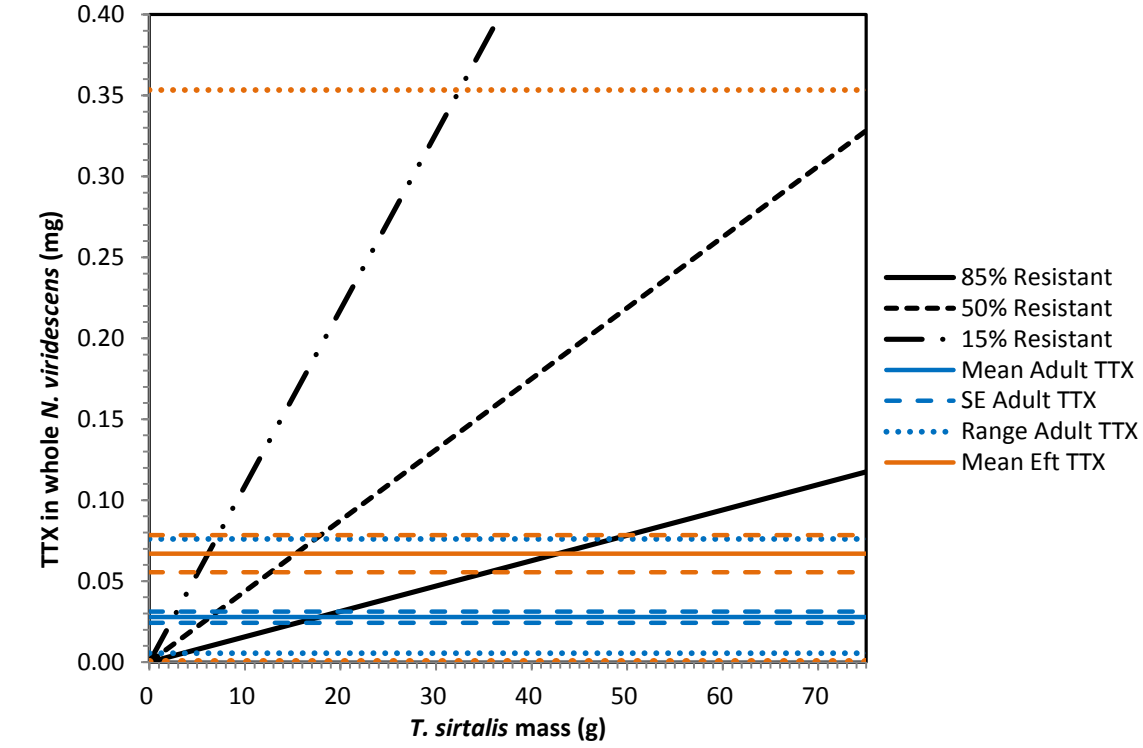


Figure 3. Tradeoff of TTX resistance and crawl speed. Data points represent individuals (n = 57) tested at a common dose (10 MAMU). The regression of post-injection speed on baseline speed is shown with the solid black regression line (y = -0.035x + 10.96). The blue dashed line represents the null expectation assuming that TTX affects all individuals equally (slope = 1; Brodie and Brodie 1999b). The orange dotted line represents an alternate null expectation in which the slope is equal to the average proportion of baseline speed observed at the administered dose (slope = 0.29; Phillips et al. 2004). The slope of the regression is significantly lower than both null expectations indicating that slower snakes are more resistant.

A.


0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
TTX in whole N. viridescens (mg)
0
10
20
30
40
50
60
70
T. sirtalis mass (g)
85% Resistant
50% Resistant
15% Resistant
Mean Adult TTX
SE Adult TTX
Range Adult TTX
Mean Eft TTX

B.

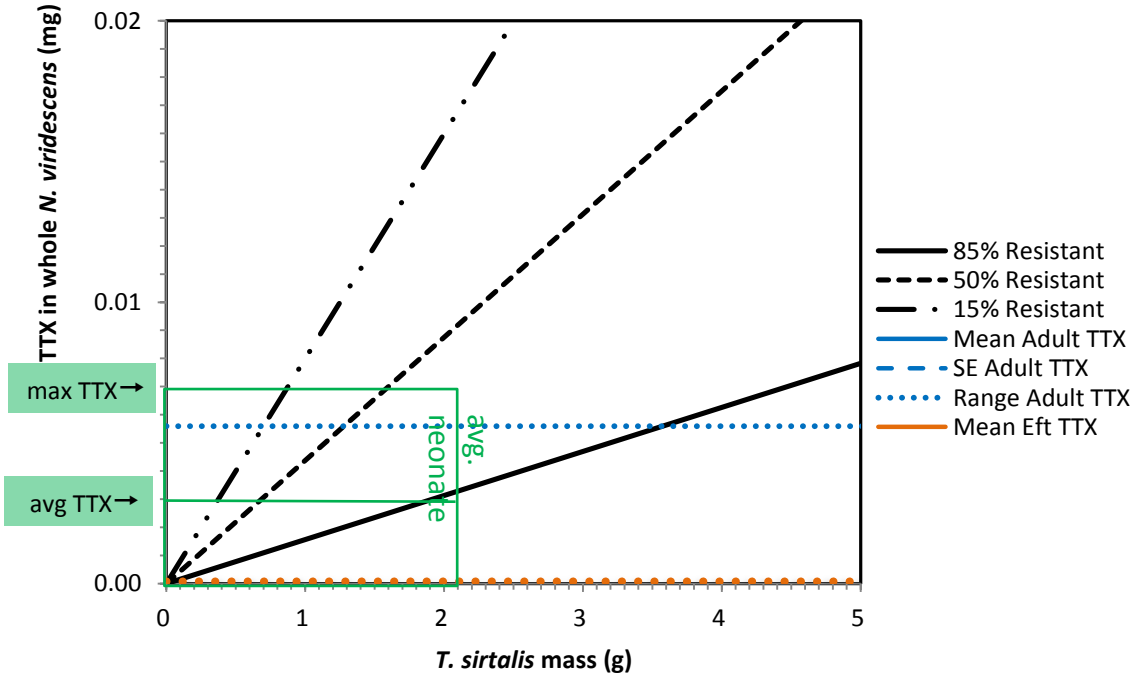

0.02
0.01
0.00
TTX in whole N. viridescens (mg)
max TTX
avg TTX
avg. neonate
0
1
2
3
4
5
T. sirtalis mass (g)
85% Resistant
50% Resistant
15% Resistant
Mean Adult TTX
SE Adult TTX
Range Adult TTX
Mean Eft TTX

Figure 4. Functional interaction between snakes and newts (cf. Brodie et al. 2005). (A) Average population resistance, as determined by the population dose response curve, is plotted as a function of snake mass in grams (horizontal axis) and milligrams of TTX in whole *N. viridescens* (vertical axis) at MLBS. Average population resistance at the 15% (near immobility), 50%, and 85% levels are shown in black. Blue (adult newts) and orange (efts) horizontal lines show the mean, standard error, and range of whole animal TTX in *N. viridescens* at MLBS. At the intersection of performance line and newt toxicity is the estimated predicted effect for a snake of a given size in the MLBS population. (B) Magnified view of the functional interaction of neonate snakes and relevant sized efts (<0.5 g). Vertical green line identifies the average sized neonate, and horizontal green lines identify the average and maximum amounts of TTX neonates are likely to encounter. Predicted effect for the average neonate is observed at the intersection of vertical and horizontal green lines in relation to average population resistance levels (black lines).

$Na_v1.4$ P-loops	Domain I	Domain II	50% MAMU
	-380 -390 -400	-780 -790 -800	
T. sirtalis MLBS	SYDTFSWAFLSLFRLMTQDYWENL	HMHDFFHSFLIVFRILCGEWIETM	7.7
T. sirtalis Bear Lake	SYDTFSWAFLSLFRLMTQDYWENL	HMHDFFHSFLIVFRILCGEWIETM	3.6
T. sirtalis Warrenton	SYDTFSWAFLSLFRLMTQDYWENL	HMHDFFHSFLIVFRILCGEWIETM	15.2
T. sirtalis Benton	SYDTFSWAFLSLFRLMTQDYWENL	HMHDFFHSFLIVFRILCGEWIETM	34.1
T. sirtalis Willow Creek	SYDTFSWAFLSLFRLMTQDYWENL	HMHDFFHSFLIVFRILCGEWIETM	730
T. atratus	SYDTFSWAFLSLFRLMTQDYWENL	HMHDFFHSFLIVFRILCGEWIETM	>100
T. couchii	SYDTFSWAFLSLFRLMTQDYWENL	HMHDFFHSFLIVFRILCGEWIETM	86.5
T. elegans	SYDTFSWAFLSLFRLMTQDYWENL	HMHDFFHSFLIVFRILCGEWIETM	2
Homo sapiens	SYDTFSWAFLALFRLMTQDYWENL	HMHDFFHSFLIVFRILCGEWIETM	n/r
	----------α---------- * ----β---	----------α---------- * ----β---	

$Na_v1.4$ P-loops	Domain III	Domain IV	50% MAMU
	-1260 -1270	-1550 -1560 -1570	
T. sirtalis MLBS	NFDNVGLGYLSLLQVATFKGWMDI	NFETFGNSIICLFEITTSAGWDGL	7.7
T. sirtalis Bear Lake	NFDNVGLGYLSLLQVATFKGWMDI	NFETFGNSIICLFEITTSAGWDGL	3.6
T. sirtalis Warrenton	NFDNVGLGYLSLLQVATFKGWMDI	NFETFGNSIICLFEVTTSAGWDGL	15.2
T. sirtalis Benton	NFDNVGLGYLSLLQVATFKGWMDI	NFETFGNSIICLFEVTTSAAWDGL	34.1
T. sirtalis Willow Creek	NFDNVGLGYLSLLQVATFKGWMDI	NFETFGNSILCLFEVTTSAGWNVL	730
T. atratus	NFDNVGLGYLSLLQVATFKGWMEI	NFETFGNSIICLFEITTSAGWNGL	>100
T. couchii	NFDNVGLGYLSLLQVATFKGWTDI	NFETFGNSIICLFEITTSAGWDGL	86.5
T. elegans	NFDNVGLGYLSLLQVATFKGWMDI	NFETFGNSIICLFEITTSAGWDGL	2
Homo sapiens	NYDNVGLGYLSLLQVATFKGWMDI	NFETFGNSIICLFEITTSAGWDGL	n/r
	----------α---------- * ----β---	----------α---------- * ----β---	

Figure 5. Variation in the P-loop regions of the SCN4A sodium channel gene. Amino acid sequence of the P-loop regions of SCN4A for the *Thamnophis sirtalis* population at Mountain Lake Biological Station (MLBS) are shown in comparison to four western populations of *Th. sirtalis* of varying resistance (Geffeney et al. 2005) as well three additional species of *Thamnophis* (Feldman et al. 2009). Sequence for *Homo sapiens* is shown for comparison. P-loop structure indicated below the sequence (α = α-helix, * = selectivity filter, β = β-strand; Lipkind and Fozzard 2000). Population level resistance indicated at the 50% MAMU level to the right of each row of sequence (n/r = not resistant). Sequence from MLBS snakes is noted at the top and compared to previously published sequences of *Th. sirtalis*, other species of *Thamnophis*, and *Homo sapiens*.

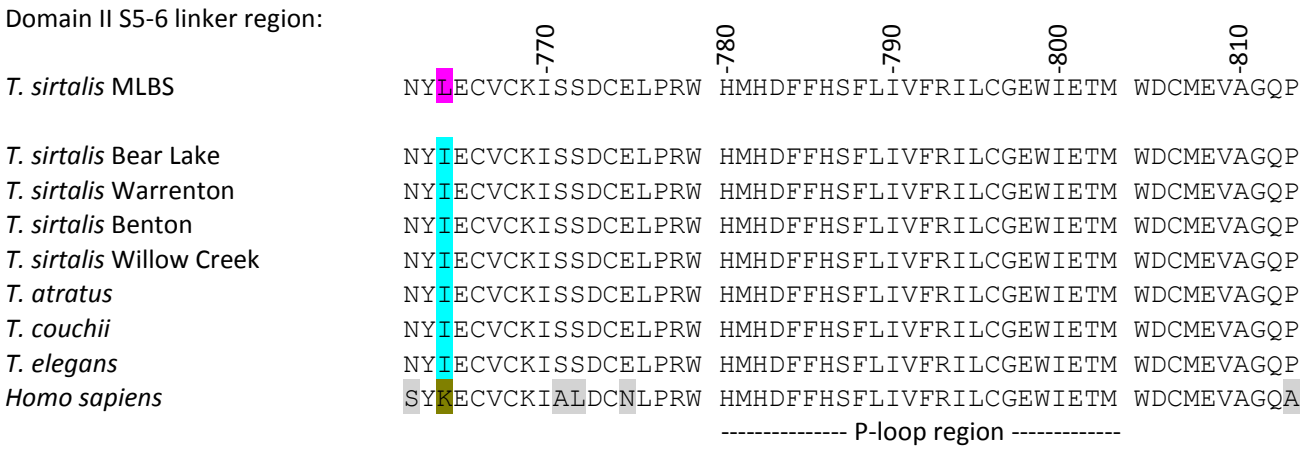


Figure 6. S5-S6 linker regions for Domain II of sodium channel $Na_v1.4$ is shown. P-loop region is designated by spaces and denoted below *Homo sapiens*. Shaded amino acid residues indicate sequence variation. Proposed S5-6 linker regions for the domain based on the human sequence (George et al. 1992). MLBS sequence is noted at the top and compared to previously published sequences of *Th. sirtalis*, other species of *Thamnophis*, and *Homo sapiens*.

Chapter 3:

Diet Partitioning in an Eastern Population of *Thamnophis sirtalis* (Natricinae)

"In any study of evolutionary ecology, food relations appear as one of the most important aspects of the system of animate nature." Hutchinson 1959

"...it is on a common fabric of available food that all patterns of animal activities are worked out." Weatherley 1963

Abstract

Thamnophis sirtalis is a wide-ranging Natricine snake that consumes a variety of prey. However, a more detailed examination of their diet reveals partitioning of that diet variation, as different prey may be important for different life stages, sexes, seasons, or localities. Previous studies of *Th. sirtalis* have shown that diet can be locally specific, but the majority of studies on *Th. sirtalis* diet have occurred in the western part of its range. This study investigates the feeding ecology of an eastern population of *Th. sirtalis* to examine annual, seasonal, micro-geographic, intersexual, and ontogenetic variation in diet. Special attention was paid to the presence of sympatric *Notophthalmus viridescens*, an abundant and toxic newt, in the diet of snakes. During a three-year study, earthworms were the primary prey for this population of *Th. sirtalis*, with salamanders, *Plethodon cinereus* in particular, being secondarily important. *Notophthalmus viridescens* were consumed rarely and only by female snakes. Diet varied annually and ontogenetically, as well as between sexes and among microhabitats. Weather was important in predicting the likelihood of observing snakes with recently consumed food, and recently consumed salamanders, but not other prey types. Contrasts with the diets of other *Th. sirtalis* populations, as well as observed annual variation in diet, highlight the importance of conducting long-term population specific studies of feeding ecology.

Key words: *foraging,* Thamnophis sirtalis, Notophthalmus viridescens, *Virginia*

Introduction

Understanding diet variation in natural populations of snakes is important for understanding their ecology and evolution (Hutchinson 1959; Weatherley 1963; Glaudas et al. 2008) because diet directly affects growth, survival, and reproduction (Andrews 1982; Seigel and Ford 1987; Greene et al. 1994; Bronikowski and Arnold 1999; Madsen and Shine 2000; Brito 2004). Species are often highly variable in diet, but may exhibit less variation within subgroups (e.g., populations, life stages, sex) due to differences in prey availability, metabolic needs, or size of individuals in a given class. Studies of the partitioning of diet variation, therefore, allow us to better understand life history evolution (Fitch 1987; Mushinsky 1987; Shine and Slip 1990; Wallace and Diller 1990; Shine 1996), habitat use (Reinert et al. 1984), community structure (Vitt 1983; Cadle and Greene 1993), trophic relationships (Greene et al. 1994), and predator and prey coevolution (Downes and Shine 1998; Brodie and Brodie 1999a). Because variation among subgroups can result in extensive intraspecific diet variation, comprehensive and long-term studies are required to gain a complete picture of dietary habits in order to partition the observed diet variation, and may be a reason why detailed studies remain relatively rare (but see Greene and Rodriguez-Robles 2003; Brito 2004; Glaudas et al. 2008).

Within the subfamily Natricinae (Colubridae) feeding ecology varies at every level. Geographic and inter-population variation has been observed within species of *Natrix, Nerodia,* and *Thamnophis* (Gregory and Stewart 1975; Arnold 1981c; Kephart and Arnold 1982; King 1993; Bronikowski and Arnold 1999; Gregory and Isaac 2004;

Luiselli et al. 2007), and microgeographic variation in diet has been observed in *Nerodia sipedon, Thamnophis elegans, Th. ordinoides,* and *Th. sirtalis* (White and Kolb 1974; Kephart 1982; Gregory 1984; Gregory and Nelson 1991; King 1993). Seasonal variation in diet has also been observed among several Natricine species including *Natrix natrix, N. tessellate, Th. elegans, Th. eques, Th. radix,* and *Th. sirtalis* (Gregory and Stewart 1975; Kephart and Arnold 1982; Seigel 1984; García and Drummond 1988; Rossman et al. 1996; Gregory and Isaac 2004; Luiselli et al. 2007). Broad ranging generalist species, like many Natricine snakes, are expected to vary their diet based on local prey availability (Gregory and Isaac 2004); this explains much of the geographic and seasonal variation in diet in the above examples. Feeding ecology also varies among age and reproductive classes within populations. For example, ontogenetic differences in diet have been described in *Nerodia, Natrix,* and *Thamnophis* (White and Kolb 1974; Seigel 1984; García and Drummond 1988; Greene et al. 1994; Rossman et al. 1996), as well as among sexes and reproductive condition of females within species of *Natrix* (Gregory and Isaac 2004; Luiselli et al. 2007) and *Thamnophis* (Ford and Hampton 2009; Tuttle and Gregory 2009).

Thamnophis sirtalis (the common garter snake) is characterized as a generalist consuming amphibians, fish, earthworms, leeches, slugs, mammals, and birds (Nussbaum et al. 1983; Mushinsky 1987; Rossman et al. 1996; Stebbins 2003). Previous work has shown that diet in *Th. sirtalis* varies extensively among populations across its range (throughout most of North America). For example, some populations consume amphibians (toads, frogs and salamanders; Kephart 1982), other populations most

frequently consume anurans (White and Kolb 1974; Kephart and Arnold 1982), fish (White and Kolb 1974; Gregory and Nelson 1991), or earthworms (Carpenter 1952). The majority of diet studies of *Th. sirtalis* have been conducted in the western part of its range (Fitch 1941a; Hebard 1951; Fouquette 1954; White and Kolb 1974; Gregory and Stewart 1975; Arnold and Wassersug 1978; Kephart 1982; Kephart and Arnold 1982; Gregory 1984; Gregory and Nelson 1991), with some older studies in the Midwest (Lagler and Salyer 1945; Carpenter 1952; Fitch 1965; Seigel 1984) and few in the East (Uhler et al. 1939; Barbour 1950; Hamilton 1951). Regardless of geographic location, observations of seasonal, annual, microhabitat, and individual variation in diet have been observed.

One understudied component of variation that may be essential for understanding the ecology and evolution of *Th. sirtalis* is its interaction with toxic prey. Western *Th. sirtalis* are known to consume newts of the genus *Taricha* (Brodie 1968b; Nussbaum et al. 1983; Brodie et al. 2002) a newt that secretes tetrodotoxin (TTX) as an anti-predator defense (Mosher et al. 1964; Brodie 1968b; Brodie et al. 1974). *Thamnophis sirtalis* has evolved resistance to this toxin (Brodie and Brodie 1990, 2002; Brodie et al. 2002; Geffeney et al. 2002; Ridenhour et al. 2004; Geffeney et al. 2005; McGlothlin et al. 2014). In addition, other western species of *Thamnophis* prey on *Taricha* and are resistant to TTX (Brodie et al. 2005; Wiseman and Pool 2007; Feldman et al. 2009; Greene and Feldman 2009; Feldman et al. 2010, 2012); *Th. couchii* prey on *Ta. torosa* and *Ta. sierra*, and *Th. atratus* prey on *Ta. granulosa* and *Ta. torosa*. Resistance to prey toxin has evolved despite newts only rarely appearing as diet items in any of these species. However, no season-long studies have examined the possibility that newts are important

components of the diet in some seasons or life stages. In the eastern part of its range, *Th. sirtalis* is sympatric with *Notophthalmus viridescens* (red-spotted newt), a sister genus to *Taricha* that is also defended by TTX (Brodie et al. 1974; Yotsu-Yamashita and Mebs 2001, 2003). Because *Th. sirtalis* in this region have evolved resistance to TTX (Feldman et al. 2009; Chapter 2) it is reasonable to assume that they are consuming this toxic prey.

The goal of this study was to examine variation in diet within a single population of *Th. sirtalis*. By following a single population across multiple years, I was able to investigate how diet varies within and across multiple years and seasons, something that rarely has been considered in other studies. Based on the variation observed in other populations, and the broadly generalist nature of *Th. sirtalis*, I predicted that diet would reflect local prey availability. I also predicted that diet items would be partitioned among classes of *Th. sirtalis*, and that diet would vary spatially (between microhabitats), temporally, and climatically. Special attention was given to describe the frequency of, and circumstances in which *N. viridescens* were observed because understanding the ecological relationship between *Th. sirtalis* and toxic prey could inform our understanding of the evolution of toxin resistance in this and other species.

Methods

The diet of free-ranging *Th. sirtalis* was surveyed across three years, 2008-2010, at Mountain Lake Biological Station (MLBS), University of Virginia in Giles County, Virginia (37°22’32”N, 80°31’20”W). In each year, snakes were captured repeatedly throughout their active season beginning at their first emergence above ground in the

spring (7 May 2008, 11 Apr 2009, and 4 Apr 2010) until they retreated for their winter hibernation in the fall (2 Nov 2008, 5 Oct 2009, 18 Oct 2010).

Cover boards were used as the primary method for capturing snakes, though subjects encountered haphazardly in the area at other times were also captured. Most cover boards were made of black plastic polyethylene cut to size (approximately 76 cm x 61 cm x 0.65 cm). In 2008, however, a few large pieces of aluminum and small pieces of 19mm plywood were also used. These were subsequently changed to the standard polyethylene cover boards prior to the 2009 season. Eighty-eight cover boards were placed in 2008 throughout the MLBS property near ponds and streams, as well as in open grassy areas in an effort to capture the maximum number of snakes and the greatest diversity of food items consumed by *Th. sirtalis* in this population. In 2009, additional cover boards (bringing the total to 220) were more regularly deployed around the study area to provide systematic sampling of microhabitat variation in diet (Figure 1). Microhabitats were classified as either aquatic (within 10 meters of either a pond or stream) or terrestrial (greater than 10 meters from water) for analysis. *Thamnophis sirtalis* in this population were never directly observed foraging in water, but had the potential to consume amphibians living near these aquatic habitats. There were 64 boards in the aquatic microhabitat and 156 boards in the terrestrial microhabitat.

All cover boards were sampled two to three times per week. Snakes were captured by hand and placed into a standard cotton snake bag for holding. The bags were tagged with the board number and time of capture. The time of day that boards were sampled

varied to avoid biasing results toward snakes that were morning versus afternoon feeders. The collection circuit was also started at different boards to vary the order of visitation.

Snakes with a snout-vent length (SVL) of 200mm or greater were marked with a PIT tag (AVID, Microchip ID Systems, Inc.) by injecting the transponder with a hypodermic needle sub-cutaneously on the right side of the venter approximately 20 scale rows anterior to the cloaca. For snakes less than 200mm in length, a unique code was clipped into the ventral scales (Brodie 1992).

Diet was assessed by inducing snakes to regurgitate their gut contents by gently palpating the food from the stomach toward the mouth. Once regurgitated, prey items were identified to genus or species whenever possible. Two groups could not be regularly identified to species and were, therefore, identified only by genus (*Lithobates*) or family (*Lumbricidae*).

Location and time of capture, sex, life stage, SVL, total length, weight, and reproductive condition were also recorded. Location of capture was recorded as the board number under which the snake was captured or the closest board if the snake was not located under a board. Sex was determined by either everting the hemipenes in young males or by tail shape. Life stage was based on a snake's SVL, time of year it was captured, and data from previous captures: neonates (born that year) ≤ 285mm, yearlings (born the previous year) 140-340mm, juveniles/adults ("juv/adult"; greater than 1 year) ≥ 288mm. Because diet was evaluated across years, snakes that were captured prior to the juv/adult stage in the first two years of the study shifted life stage across years. Pregnancy was assessed by palpating the ovaries of juv/adult females.

Gross variation in diet was not statistically different from year to year (Table 1); therefore, data collected across the 2008-2010 seasons were pooled for analyses of sex and life stage variation. Microhabitat differences were analyzed using pooled data for the 2009-2010 seasons once the number of boards were expanded and designed to capture this variation. For these analyses, proportion of observed diet made up by each prey species was calculated and variation in sex, life stage, and microhabitat was examined. Fisher's Exact tests were used to test observed number of prey items consumed against the null hypothesis (equal numbers consumed among classifications of snakes) using IBM SPSS Statistics 22. Post-hoc pairwise comparisons were performed using Fisher's Exact test to test for statistical differences among life stages.

Logistic regression was used to assess the likelihood of observing snakes with food or with a particular class of prey in a given year, month (season), or weather condition. Presence (1) or absence (0) of food in an observation was the dependent variable used to assess general prey consumption and included observations of snakes with and without recently consumed prey. To assess the likelihood of observing snakes with a particular prey class, only observations of snakes with recently consumed prey were included in the analysis, and presence (1) or absence (0) of each prey class of interest (earthworm, salamander, or frog) was used as the dependent variable for each analysis. Because there were only two observations of recently consumed mammals, this prey class was omitted from the prey class analyses.

In these analyses, each capture of a subject was treated as an independent observation if the capture occurred more than 5 days after the most recent capture. If the

same subject was captured within 5 days of the last observation, it was removed from analysis to avoid sampling bias from frequently captured snakes that were unlikely to have fed between rapid recaptures. Gut clearance time for *Th. sirtalis* has not been published; however, a gut clearance time of ~4 days is estimated based on a study of *Heterodon* (Smith 1976). It should be noted however that Hamilton (1951) found that *Th. sirtalis* would take food again one to eight or more days after their previous meal depending on whether they had been fed a single small prey item or been allowed to gorge themselves. Seventy-eight of the 991 observations were excluded due to rapid recapture, and 11 observations were excluded due to incomplete data.

Year and month (as a proxy for season) were the independent variables used in a simple logistic regression model to assess temporal variation in diet. Because the months of April and November were not represented in all three years and because there were few total observations during these months (n = 11 and n = 2, respectively), April and November were removed from the analysis of season. Because the combination of temperature and precipitation is likely to explain foraging activity, measurements of both weather components were assessed in a multiple regression model. All temperature and precipitation measurements were obtained from the MLBS weather station. Maximum daily temperature (°C), daily mean temperature (°C), and total daily precipitation (mm) were calculated for each capture date. In addition, days since last precipitation event (≥ 5mm), total precipitation (mm) over the last 5 days (including the date of capture), and the average maximum daily temperature over the last 5 days (including the date of capture) were calculated for each capture day, and were included because some prey are

less likely to be active during warm, dry periods which may result in increased active foraging by snakes. In all logistic regression analyses, the full model was tested against a constant only model. Nagelkerke's R^2 was used to assess the relationship between the predictors and the dependent variable and each variable was assessed for significance in contributing to the model. All analyses were conducted using IBM SPSS Statistics 20.

Results

Sample sizes

Four hundred and forty one (441) individual snakes (208 males, 230 females, 3 of unknown sex) were captured a total of 991 times during the study period (mean number of captures per snake = 2.25, SD = 2.27). One hundred and forty seven (33.3%) of these snakes (80 males and 67 females) were captured a total of 179 times with food ("food capture"). Nineteen per cent of snakes were captured with food more than once; (24 snakes were captured with food twice and four snakes were captured with food three times). Six of the snakes with single food captures were observed with two different species of prey. Of the snakes with two food captures, two were observed with two different prey species in one of the two captures. On all other occasions when a snake was captured with food only a single prey type was observed.

Four snakes that were captured with food were omitted from observed diet, sex, life stage, and microhabitat analyses for the following reasons. One snake (yearling male) had a single food capture in 2008 with prey that could only be identified as plethodontid tail and was excluded due to prey non-specificity. In 2008, a juv/adult male had a single

food capture in which a small mammal, possibly a vole, was regurgitated and in 2009 a juv/adult male had a single food capture in which a small mammal, possibly a mouse, was regurgitated. Prey type was not assessed for one snake (neonate male in 2010) because regurgitation was not induced. Each prey type represented less than 1% of the observed diet and, therefore, these snakes were excluded from these diet analyses. In addition, one snake that was captured with food twice in 2010 was not induced to regurgitate in one of the two captures, so this observation, but not the snake, was omitted from analysis. The final sample sizes for the observed diet, sex, and life stage analyses were n = 143 snakes (76 males and 67 females) with 174 total food capture observations, and 181 diet items.

Life stage classification did not change across years for 138 of the snakes captured with food; however, it did change for five snakes due to multiple food captures across years in which their life stage also changed. One snake was captured with food as a yearling and again as an juv/adult, and four snakes were captured with food as neonates and again as yearlings. One snake from 2008 that consumed an earthworm was omitted from the life stage analysis because SVL was not recorded for that individual and it was never recaptured so life stage could not be assessed. This resulted in diet data for 44 juv/adults, 64 yearlings, and 40 neonates [138 snakes assessed at a single life stage class + (5 snakes assessed at * 2 life stages classes) = 148 snakes observed across life stages] with 173 total food capture observations and 180 diet items.

Eighty-six of the 143 snakes were included in the microhabitat analysis that was restricted to 2009-2010 observations. Eighteen of these snakes were captured with food

multiple times, seven of which were captured under boards in both the aquatic and terrestrial microhabitats. Thirty four snakes were captured with food in the aquatic habitat and 59 snakes were captured with food in the terrestrial habitat [79 snakes assessed at a single microhabitat + (7 snakes assessed at * 2 microhabitats) = 93 observations].

In the analyses of year, season, and weather as predictors of capturing a snake that had recently consumed a meal, 902 of the 991 total capture observations were included. Unlike the previous analyses, the four snakes that were previously omitted (one not induced to regurgitate, one that consumed a salamander, and 2 that consumed mammals), were included in the analyses examining general prey consumption, as was the additional observation of the snake not induced to regurgitate on its second food capture. In addition, the snake that consumed the prey only identified as "plethodontid tail" was also included in the analyses of prey class. In total, the analyses of year, season, and weather as predictors of capturing a snake that consumed a particular prey class included 113 observations with earthworm, 54 observations with salamander, and 13 observations with frog.

Observed Diet

At MLBS, *Th. sirtalis* diets included the following taxa: *Lumbricidae* (earthworms), *Eurycea cirrigera* (southern two-lined salamander), *Notophthalmus viridescens* (red-spotted newt), *Plethodon cinereus* (red-backed salamander), *Plethodon glutinosus* (northern slimy salamander), *Pseudacris crucifer* (spring peeper), and *Lithobates spp.* (*L. catesbeianus* and *L. clamitans*). In addition, two small mammals were

observed, but their specific identities were not confirmed. *Lumbricus terrestris* and *L. rubellus* were among earthworms consumed by *Th. sirtalis* when identifications could be made. *Lumbricidae* made up the largest proportion of the diet (62.4%), followed by *P. cinereus* (19.9%). *Notophthalmus viridescens* comprised 3.3% of the observed diet (Figure 2). Amphibians combined accounted for less than half (37.6%) of the observed diet of *Th. sirtalis* at MLBS with salamanders accounting for 30.4% and frogs 7.2%.

Sex and Life Stage

Only female snakes were observed with recently consumed *N. viridescens* (Table 2). Three of the four juv/adult snakes that consumed newts were carrying young. Males and females did not differ in the proportion of any other prey type in their observed diet (Table 2). Only adult snakes consumed *Lithobates spp*. Life stage did not differ significantly in the proportion of other recently consumed prey (Table 3).

Microhabitat

A significantly greater proportion of snakes in terrestrial habitats were found with recently consumed *P. cinereus,* and a significantly greater proportion of snakes in aquatic habitats were found with recently consumed *Lithobates spp* (Table 4).

Year and Season

The generalized linear model indicated that year was a significant predictor of capturing a snake that had recently consumed prey (Table 5 and Figure 3). Each year

significantly contributed to the model, but the likelihood of capturing snakes that had recently consumed prey was significantly lower in 2010. In a separate model, year was also a significant predictor of capturing snakes that had recently consumed earthworms.

Table 6 shows seasonal observations of total captures as well as the number of observations of snakes that had recently consumed prey and recently consumed prey of each class. The generalized linear model indicated that season was a significant predictor of capturing a snake that had recently consumed prey (Table 7). Early and late months of the active season significantly contributed to the model. Analysis of separate models indicated that season was not a significant predictor for capturing snakes that had eaten a particular prey class.

Weather

A summary of the weather statistics is presented in Table 8. A test of the full generalized linear model with all weather variables included as predictors against a constant only model was significant indicating that weather significantly explained the likelihood of capturing a snake with food (Table 9A). Among the variables included in the model, maximum daily temperature was the only significant predictor. In the analysis of weather variables as predictors for capturing snakes that had recently consumed salamanders, number of days since the last precipitation event and total precipitation over the preceding 5 days were significant predictors (Table 9C) indicating that snakes are more likely to consume salamanders after it has rained. No weather variables predicted the presence of earthworms or frogs in the diet (Table 9B & 9D, respectively).

Discussion

The diet of *Th. sirtalis* in southwest Virginia included a broad range of prey types, but was characterized by a high frequency of earthworms. Salamanders were also common in the diet, followed by several less common prey types. Weather variables predicted the overall frequency of having eaten any item (temperature) and the prevalence of salamanders (precipitation) in particular. Microhabitat and life stage variables predicted some of the variation in diet, but only of less common prey types. Toxic newts (*N. viridescens*) were preyed upon by *Th. sirtalis*, but did not represent a major portion of the diet for any life stage, microhabitat, or season.

This study supports previous research that shows earthworms are an important prey for *Th. sirtalis* populations in the Midwest and East (Uhler et al. 1939; Lagler and Salyer 1945; Barbour 1950; Hamilton 1951; Carpenter 1952; Fitch 2005), in contrast to western populations in which amphibians (particularly anurans) are the most important food resource (Fitch 1941a; White and Kolb 1974; Arnold 1978; Kephart 1982; Gregory and Nelson 1991). The high proportion of earthworms in the diet of *Th. sirtalis* in this study may be due to the high density of earthworms at the site. Several species of native and non-native earthworms are found at MLBS, with local densities estimated at 7.33 native earthworms/m^2 in the forest (Rearick et al. 2011) and 40.11 invasive earthworms/m^2 in and near open lawns (Billak and Ransom, *unpublished*). An earlier study by Uhler et al. (1939) in the nearby George Washington National Forest found that diets of *Th. sirtalis* were more evenly comprised by earthworms (27%), salamanders (24%), toads (18%), and insects (18%). MLBS is only ~140km south-west of that study

site, and while toads are not common, other anurans and insects are abundant throughout much of the active season of garter snakes. This disparity in diet highlights the scale of local specificity of diet variation for apparently generalist predators.

Within-population variation in diet was limited primarily to the less common prey items, with earthworms representing a substantial component of the diet for all groups, seasons, and conditions. Differences in prey types consumed by different life stages of *Th. sirtalis* may be due to gape-limitations and alternate foraging strategies. The larger head and body size of adult snakes enables them to consume larger prey such as *Lithobates spp.*, whereas younger, smaller snakes may be incapable of doing so (Shine 1991). Larger snakes are known to drop smaller food items from the diet in favor of larger prey (reviewed in Arnold 1993) as observed in *Th. proximus* (Ford and Hampton 2009), *Th. sirtalis* (Fitch 1965), and *Nerodia sipedon* (King 2002). Alternatively, earthworms (up to 20cm in length; Curry et al. 1989) and *P. cinereus* (up to 12.5cm total length; Petranka 1998) range greatly in size and are consumed by all snake life stages in this population.

Microhabitat differences in diet in this study likely reflect the differential availability of a few uncommonly exploited prey in aquatic versus terrestrial locations. Microgeographic variation in diet has been observed in other populations of *Th. sirtalis*. For example, *Th. sirtalis* at fish hatcheries consumed primarily fish, while at natural sites fish were rarely consumed (Lagler and Salyer 1945; Gregory and Nelson 1991). Kephart (1982) also found that diet of *Th. sirtalis* was site specific and related to prey availability, taking leeches and worms at some sites and exclusively amphibians or fish at others.

Differential availability of prey resulting in fine scale geographic variation in diet is fairly common among other snakes as well including *N. sipedon* (King 1993), *Notechis ater* (Shine 1987), and *Vipera berus* (Forsman 1991).

The frequency of snakes with diet items varied both annually and monthly within years indicating that extended sampling periods are important to understanding local diet variation. Increased feeding frequency in early summer is common for snakes as they are likely to be more intensely foraging after they emerge from hibernation (Fitch and Twining 1946; Gregory and Stewart 1975; Beavers 1976; Filippi et al. 1996; Reading and Davies 1996; Weatherhead et al. 2003; Gregory and Isaac 2004). However, an additional increase in feeding prior moving to overwinter retreats has rarely been observed. The additional increase in feeding frequency in *Natrix natrix* at the end of the active season may be due to a milder weather than in previous studies (e.g., Manitoba; Gregory and Stewart 1975) allowing for a longer active season and may be the case for the MLBS population as well.

While variation in feeding frequency among months was anticipated, annual variation in diet was not necessarily expected. In the two previous studies in which annual variation of *Th. sirtalis* diet was examined, one found no differences over a two year study in Manitoba (Gregory and Stewart 1975), and the other found that diet varied annually over a seven year census in California (Kephart and Arnold 1982). Annual variation in diet was also observed in a 13-year study of *Liasis fuscus* (Madsen and Shine 2000) in which fluctuating diet corresponded with the availability of their primary prey.

In the present investigation, foraging in year three differed from the first two years in that the relative importance of earthworms and salamanders as prey differed.

Recent weather conditions influenced the diet variation observed among snakes in this population. Temperature predicted the probability of having eaten any diet item, whereas recent precipitation correlated positively with the probability that snakes had eaten a salamander. These results were not unexpected as *Th. sirtalis* increase their food intake at higher temperatures (Aleksiuk 1976). In addition, because terrestrial salamanders are more active when soil moisture is high (Heatwole 1962; Feder 1983; Grover 1998), snakes are more likely to encounter salamanders after a precipitation event. The pattern of precipitation in this location affecting the availability of salamanders may be one reason why *Th. sirtalis* do not consume salamanders with greater frequency. Across longer time scales, amphibian availability in wetter years may be important for the life history of these snakes. In a California population of *Th. elegans*, annual precipitation was an important factor in the availability of anuran prey which in turn affected growth and reproduction of snakes (Bronikowski and Arnold 1999). In Australia, the availability of dusky rats, the main prey of water pythons, varied with annual rainfall and significantly affected growth rates of snakes, especially females (Madsen and Shine 2000). Consumption of vertebrate prey has also been shown to increase growth rates in *Th. sirtalis* (Scudder-Davis and Burghardt 1987; Lyman-Henley and Burghardt 1995; Burghardt et al. 2000). Therefore, salamander availability mediated by precipitation, may be important for the life history of this population of *Th. sirtalis*.

Notophthalmus viridescens appear infrequently in the diet of *Th. sirtalis* even though they are very abundant at the study site (approx. 7600-12,700 adult individuals in Riopel Pond during the breeding season; Grayson and Wilbur 2009). This low frequency of newts in the diet is consistent with the results of shorter term surveys in western populations of *Th. sirtalis. Taricha* have been found in the diet of *Th. sirtalis* throughout their sympatric ranges, but are never a common food item (Arnold 1992; Brodie unpublished; Nussbaum et al. 1983). Despite the rarity with which they consume newts, both western and eastern *Th. sirtalis* have evolved resistance to TTX (Feldman et al. 2009; Chapter 2). With other prey abundantly available and more commonly eaten it is unclear why *Th. sirtalis* do not avoid toxic newts altogether. Perhaps it is related to the consumption of *N. viridescens* by females, particularly those that are approaching or are in reproductive condition. With the higher energetic costs associated with reproduction (Charland 1995) there may be strong selection to obtain a meal during this reproductive period despite potential costs of eating toxic prey such as reduced locomotor performance (Brodie and Brodie 1990, 1999a, b). Further, investing more energetic resources in her young may improve their survival (Kissner and Weatherhead 2005). Alternatively, avoidance may not be possible for *Th. sirtalis* due to underlying genetic correlations in the prey recognition response for salamanders or amphibians in general (Arnold 1981a, b; Brodie and Brodie 1999a). Understanding why *Th. sirtalis* eat rather than avoid toxic prey is an area that merits further investigation.

Overall, eastern *Th. sirtalis* are fairly typical generalist snakes with respect to their feeding ecology. As expected, this population of *Th. sirtalis* appears to be

opportunistic based on local prey availability, consuming plentiful earthworms and red-backed salamanders with greater frequency than other prey that may be less abundant, less available, or less palatable. Less available or less palatable prey items may only be consumed when encountered (e.g., *Lithobates spp.* consumed near water) or when needed (e.g., *N. viridescens* consumed by females). Precipitation may play an important role in the life history of this population as it affects the availability of salamanders which, like other vertebrate prey, may be important for growth.

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References

Aleksiuk, M. 1976. Metabolic and behavioural adjustments to temperature change in the red-sided garter snake (*Thamnophis sirtalis parietalis*): An integrated approach. Journal of Thermal Biology 1:153-156.

Andrews, R. M. 1982. Patterns of growth in reptiles. Pp. 273-312 *in* C. Gans, and F. H. Pough, eds. Biology of the Reptilia. Academic Press, London.

Arnold, S. J. 1978. Some effects of early experience on feeding responses in common garter snake, *Thamnophis sirtalis*. Animal Behaviour 26:455-462.

Arnold, S. J. 1981a. Behavioral variation in natural populations 1. Phenotypic, genetic and environmental correlations between chemoreceptive responses to prey in the garter snake, *Thamnophis elegans*. Evolution 35:489-509.

Arnold, S. J. 1981b. Behavioral variation in natural populations 2. The inheritance of a feeding response in crosses between geographic races of the garter snake, *Thamnophis elegans*. Evolution 35:510-515.

Arnold, S. J. 1981c. The microevolution of feeding behavior. Pp. 409-514 *in* A. C. Kamil, and T. D. Sargent, eds. Foraging Behavior: Ecological, Ethological, and Psychological Approaches. Garland Publishing, New York.

Arnold, S. J. 1992. Behavioural variation in natural populations. 4. Prey responses by two species of garter snakes in three regions of sympatry. Animal Behaviour 44:705-719.

Arnold, S. J. 1993. Foraging theory and prey-size-predator-size relations in snakes. Pp. 87-116 *in* R. A. Seigel, and J. T. Collins, eds. Snakes: Ecology and Behavior. McGraw Hill, New York.

Arnold, S. J. and R. J. Wassersug. 1978. Differential predation on metamorphic anurans by garter snakes (*Thamnophis*): Social behavior as a possible defense. Ecology 59:1014-1022.

Barbour, R. W. 1950. The reptiles of Big Black Mountain, Harlan County, Kentucky. Copeia 1950:100-107.

Beavers, R. A. 1976. Food habits of the western diamondback rattlesnake, *Crotalus atrox*, in Texas (Viperidae). The Southwestern Naturalist 20:503-515.

Brito, J. C. 2004. Feeding ecology of *Vipera latastei* in northern Portugal: Ontogenetic shifts, prey size and seasonal variations. Herpetolog. J. 14:13-19.

Brodie, E. D. III. 1992. Correlational selection for color pattern and antipredator behavior in the garter snake *Thamnophis ordinoides*. Evolution 46:1284-1298.

Brodie, E. D. III and E. D. Brodie, Jr. 1990. Tetrodotoxin resistance in garter snakes - an evolutionary response of predators to dangerous prey. Evolution 44:651-659.

Brodie, E. D. III and E. D. Brodie, Jr. 1999a. Predator-prey arms races. Bioscience 49:557-568.

Brodie, E. D. III and E. D. Brodie, Jr. 1999b. Costs of exploiting poisonous prey: Evolutionary trade-offs in a predator-prey arms race. Evolution 53:626-631.

Brodie, E. D. III and E. D. Brodie, Jr. 2002. Recovery of garter snakes (*Thamnophis sirtalis*) from the effects of tetrodotoxin. Journal of Herpetology 36:95-98.

Brodie, E. D. III, C. R. Feldman, C. T. Hanifin, J. E. Motychak, D. G. Mulcahy, B. L. Williams, and E. D. Brodie, Jr. 2005. Parallel arms races between garter snakes and newts involving tetrodotoxin as the phenotypic interface of coevolution. Journal of Chemical Ecology 31:343-356.

Brodie, E. D., Jr. 1968. Investigations on the skin toxin of the adult rough-skinned newt, *Taricha granulosa*. Copeia 1968:307-313.

Brodie, E. D., Jr., J. L. Hensel, and J. A. Johnson. 1974. Toxicity of urodele amphibians *Taricha*, *Notophthalmus*, *Cynops* and *Paramesotriton* (Salamandridae). Copeia 1974:506-511.

Brodie, E. D., Jr., B. J. Ridenhour, and E. D. Brodie III. 2002. The evolutionary response of predators to dangerous prey: Hotspots and coldspots in the geographic mosaic of coevolution between garter snakes and newts. Evolution 56:2067-2082.

Bronikowski, A. M. and S. J. Arnold. 1999. The evolutionary ecology of life history variation in the garter snake *Thamnophis elegans*. Ecology 80:2314-2325.

Burghardt, G. M., D. G. Layne, and L. Konigsberg. 2000. The genetics of dietary experience in a restricted natural population. Psychological Science 11:69-72.

Cadle, J. E. and H. W. Greene. 1993. Phylogenetic patterns, biogeography, and the ecological structure of neotropical snake assemblages. Pp. 281-293 *in* R. E. Ricklefs, and D. Schluter, eds. Species Diversity in Ecological Communities: Historical and Geographical Perspectives. University of Chicago Press, Chicago.

Carpenter, C. C. 1952. Comparative ecology of the common garter snake (*Thamnophis s. sirtalis*), the ribbon snake (*Thamnophis s. sauritus*), and butler's garter snake (*Thamnophis butleri*) in mixed populations. Ecological Monographs 22:236-258.

Charland, M. B. 1995. Thermal consequences of reptilian viviparity: Thermoregulation in gravid and nongravid garter snakes (*Thamnophis*). Journal of Herpetology 29:383-390.

Curry, W. J., I. Fairweather, C. F. Johnston, D. W. Halton, and K. D. Buchanan. 1989. Immunocytochemical demonstration of vertebrate neuropeptides in the earthworm *Lumbricus terrestris* (Annelida, Oligochaeta). Cell Tissue Res. 257:577-586.

Downes, S. and R. Shine. 1998. Sedentary snakes and gullible geckos: Predator-prey coevolution in nocturnal rock-dwelling reptiles. Animal Behaviour 55:1373-1385.

Feder, M. E. 1983. Integrating the ecology and physiology of plethodontid salamanders. Herpetologica 39:291-310.

Feldman, C. R., E. D. Brodie, Jr., E. D. Brodie III, and M. E. Pfrender. 2009. The evolutionary origins of beneficial alleles during the repeated adaptation of garter snakes to deadly prey. Proceedings of the National Academy of Sciences of the United States of America 106:13415-13420.

Feldman, C. R., E. D. Brodie, Jr., E. D. Brodie III, and M. E. Pfrender. 2010. Genetic architecture of a feeding adaptation: Garter snake (*Thamnophis*) resistance to tetrodotoxin bearing prey. Proceedings of the Royal Society B-Biological Sciences 277:3317-3325.

Feldman, C. R., E. D. Brodie, Jr., E. D. Brodie III, and M. E. Pfrender. 2012. Constraint shapes convergence in tetrodotoxin-resistant sodium channels of snakes. Proceedings of the National Academy of Sciences of the United States of America 109:4556-4561.

Filippi, E., M. Capula, L. Luiselli, and U. Agrimi. 1996. The prey spectrum of *Natrix natrix* (Linnaeus, 1758) and *Natrix tessellata* (Laurenti, 1768) in sympatric populations. Herpetozoa 8:155-164.

Fitch, H. S. 1941. The feeding habits of California garter snakes. California Fish and Game 27:1-32.

Fitch, H. S. 1965. An Ecological Study of the Garter Snake, *Thamnophis sirtalis*. University of Kansas Publications, Museum of Natural History 15:493-564.

Fitch, H. S. 1987. Collecting and life history techniques. Pp. 143-164 *in* R. A. Seigel, J. T. Collins, and S. S. Novak, eds. Snakes: Ecology and Evolutionary Biology. Macmillan Publishing Co., New York.

Fitch, H. S. 2005. Amphibian and reptile responses on a Kansas natural area. Journal of Kansas Herpetology 15:10-15.

Fitch, H. S. and H. Twining. 1946. Feeding habits of the Pacific rattlesnake. Copeia 1946:64-71.

Ford, N. B. and P. M. Hampton. 2009. Ontogenetic and sexual differences in diet in an actively foraging snake, *Thamnophis proximus*. Canadian Journal of Zoology 87:254-261.

Forsman, A. 1991. Variation in sexual size dimorphism and maximum body size among adder populations - effects of prey size. Journal of Animal Ecology 60:253-267.

Fouquette, M. J. J. 1954. Food competition among four sympatric species of garter snakes, genus *Thamnophis*. The Texas Journal of Science 6:172-188.

García, C. M. and H. Drummond. 1988. Seasonal and ontogenetic ariation in the diet of the Mexican Garter Snake, *Thamnophis eques*, in Lake Tecocomulco, Hidalgo. Journal of Herpetology 22:129-134.

Geffeney, S., E. D. Brodie, Jr., P. C. Ruben, and E. D. Brodie III. 2002. Mechanisms of adaptation in a predator-prey arms race: TTX-resistant sodium channels. Science 297:1336-1339.

Geffeney, S. L., E. Fujimoto, E. D. Brodie III, E. D. Brodie, Jr., and P. C. Ruben. 2005. Evolutionary diversification of TTX-resistant sodium channels in a predator-prey interaction. Nature 434:759-763.

Glaudas, X., T. Jezkova, and J. A. Rodriguez-Robles. 2008. Feeding ecology of the Great Basin rattlesnake (*Crotalus lutosus*, Viperidae). Canadian Journal of Zoology 86:723-734.

Grayson, K. L. and H. M. Wilbur. 2009. Sex- and context-dependent migration in a pond-breeding amphibian. Ecology 90:306-312.

Greene, B. D., J. R. Dixon, J. M. Mueller, M. J. Whiting, and O. W. Thornton. 1994. Feeding ecology of the concho water-snake, *Nerodia harteri paucimaculata*. Journal of Herpetology 28:165-172.

Greene, H. W. and J. A. Rodriguez-Robles. 2003. Feeding ecology of the California mountain kingsnake, *Lampropeltis zonata* (Colubridae). Copeia:308-314.

Greene, R. R. and C. R. Feldman. 2009. *Thamnophis atratus atratus* diet. Herpetological Review 40:103-104.

Gregory, P. T. 1984. Habitat, diet, and composition of assemblages of garter snakes (*Thamnophis*) at eight sites on Vancouver Island. Canadian Journal of Zoology 62:2013-2022.

Gregory, P. T. and L. A. Isaac. 2004. Food habits of the grass snake in southeastern England: Is *Natrix natrix* a generalist predator? Journal of Herpetology 38:88-95.

Gregory, P. T. and K. J. Nelson. 1991. Predation on fish and intersite variation in the diet of common garter snakes, *Thamnophis sirtalis*, on Vancouver Island. Canadian Journal of Zoology 69:988-994.

Gregory, P. T. and K. W. Stewart. 1975. Long-distance dispersal and feeding strategy of the red-sided garter snake (*Thamnophis sirtalis parietalis*) in the interlake of Manitoba. Canadian Journal of Zoology 53:238-245.

Grover, M. C. 1998. Influence of cover and moisture on abundances of the terrestrial salamanders *Plethodon cinereus* and *Plethodon glutinosus*. Journal of Herpetology 32:489-497.

Hamilton, W. J., Jr. 1951. The food and feeding behavior of the garter snake in New York state. American Midland Naturalist 46:385-390.

Heatwole, H. 1962. Environmental factors influencing local distribution and activity of the salamander, *Plethodon cinereus*. Ecology 43:460-472.

Hebard, W. B. 1951. Notes on the ecology of garter snakes in the Puget Sound region. Herpetologica 7:61-62.

Hutchinson, G. E. 1959. Homage to Santa Rosalia or why are there so many kinds of animals? The American Naturalist 93:145-159.

Kephart, D. G. 1982. Microgeographic variation in the diets of garter snakes. Oecologia 52:287-291.

Kephart, D. G. and S. J. Arnold. 1982. Garter snake diets in a fluctuating environment - a 7-year study. Ecology 63:1232-1236.

King, R. B. 1993. Microgeographic, historical, and size-correlated variation in water snake diet composition. Journal of Herpetology 27:90-94.

King, R. B. 2002. Predicted and observed maximum prey size - snake size allometry. Functional Ecology 16:766-772.

Kissner, K. J. and P. J. Weatherhead. 2005. Phenotypic effects on survival of neonatal northern watersnakes *Nerodia sipedon*. Journal of Animal Ecology 74:259-265.

Lagler, K. F. and J. C. I. Salyer. 1945. Influence of availability on the feeding habits of the common garter snake. Copeia 1945:159-162.

Luiselli, L., D. Capizzi, E. Flippi, C. Anibaldi, L. Rugiero, and M. Capula. 2007. Comparative diets of three populations of an aquatic snake (*Natrix tessellata*, Colubridae) from Mediterranean streams with different hydric regimes. Copeia:426-435.

Lyman-Henley, L. P. and G. M. Burghardt. 1995. Diet, litter, and sex effects on chemical prey preference, growth, and site selection in two sympatric species of *Thamnophis*. Herpetological Monographs 9:140-160.

Madsen, T. and R. Shine. 2000. Silver spoons and snake body sizes: Prey availability early in life influences long-term growth rates of free-ranging pythons. Journal of Animal Ecology 69:952-958.

McGlothlin, J. W., J. P. Chuckalovcak, D. E. Janes, S. V. Edwards, C. R. Feldman, E. D. Brodie, Jr., M. E. Pfrender, and E. D. Brodie III. 2014. Parallel evolution of tetrodotoxin resistance in three voltage-gated sodium channel genes in the garter snake *Thamnophis sirtalis*. Molecular Biology and Evolution 31:2836-2846.

Mosher, H., F. Fuhrman, H. Bichwald, and H. Fischer. 1964. Tarichatoxin-tetrodotoxin: A potent neurotoxin. Science 144:1100-1110.

Mushinsky, H. R. 1987. Foraging ecology. Pp. 302-334 *in* R. A. Seigel, J. T. Collins, and S. S. Novak, eds. Snakes: Ecology and Evolutionary Biology. McGraw-Hill Publishing Co, New York, NY.

Nussbaum, R. A., E. D. Brodie, Jr., and R. M. Storm. 1983. Amphibians and Reptiles of the Pacific Northwest. The University Press of Idaho, Moscow, ID.

Petranka, J. W. 1998. Salamanders of the United States and Canada. Smithsonian Institution Press, Washington, D.C.

Reading, C. J. and J. L. Davies. 1996. Predation by grass snakes (*Natrix natrix*) at a site in southern England. Journal of Zoology 239:73-82.

Rearick, D., L. Kintz, K. L. Burke, and T. S. Ransom. 2011. Effects of white-tailed deer on the native earthworm, *Eisenoides carolinensis*, in the southern Appalachian Mountains, USA. Pedobiologia 54, Supplement:S173-S180.

Reinert, H. K., D. Cundall, and L. M. Bushar. 1984. Foraging behavior of the timber rattlesnake, *Crotalus horridus*. Copeia:976-981.

Ridenhour, B. J., E. D. Brodie, Jr., and E. D. Brodie III. 2004. Resistance of neonates and field-collected garter snakes (*Thamnophis* spp.) to tetrodotoxin. Journal of Chemical Ecology 30:143-154.

Rossman, D., N. Ford, and R. Seigel. 1996. The Garter Snakes: Evolution and Ecology. University of Oklahoma Press, Norman, OK.

Scudder-Davis, R. M. and G. M. Burghardt. 1987. Diet and growth in juveniles of the garter snakes *Thamnophis sirtalis infernalis* and *Thamnophis radix radix*. Growth 51:74-85.

Seigel, R. A. 1984. The Foraging Ecology and Resource Partitioning Patterns of Two Species of Garter Snakes (Doctoral dissertation). University of Kansas, Lawrence.

Seigel, R. A. and N. B. Ford. 1987. Reproductive ecology. Pp. 210-252 *in* R. A. Seigel, J. T. Collins, and S. S. Novak, eds. Snakes: Ecology and Evolutionary Biology. Macmillan, New York.

Shine, R. 1987. Ecological comparisons of island and mainland populations of Australian tigersnakes (*Notechis*, Elapidae). Herpetologica 43:233-240.

Shine, R. 1991. Why do larger snakes eat larger prey items? Functional Ecology 5:493-502.

Shine, R. 1996. Life-history evolution in Australian snakes: A path analysis. Oecologia 107:484-489.

Shine, R. and D. J. Slip. 1990. Biological aspects of the adaptive radiation of Australasian pythons (Serpentes, Boidae). Herpetologica 46:283-290.

Smith, G. C. 1976. Ecological energetics of three species of ectothermic vertebrates. Ecology 57:252-264.

Stebbins, R. C. 2003. A Field Guide to Western Reptiles and Amphibians. Houghton Mifflin Co., New York, NY.

Tuttle, K. N. and P. T. Gregory. 2009. Food habits of the plains garter snake (*Thamnophis radix*) at the northern limit of its range. Journal of Herpetology 43:65-73.

Uhler, F. M., C. Cottam, and T. E. Clarke. 1939. Food of snakes of the George Washington National Forest, Virginia. Pp. 605-622. Transactions of the North American Wildlife Conference.

Vitt, L. J. 1983. Ecology of an anuran-eating guild of terrestrial tropical snakes. Herpetologica 39:52-66.

Wallace, R. L. and L. V. Diller. 1990. Feeding ecology of the rattlesnake, *Crotalus viridis oreganus*, in northern Idaho. Journal of Herpetology 24:246-253.

Weatherhead, P. J., G. Blouin-Demers, and K. M. Cavey. 2003. Seasonal and prey-size dietary patterns of black ratsnakes (*Elaphe obsoleta obsoleta*). The American Midland Naturalist 150:275-281.

Weatherley, A. H. 1963. Notions of the niche and competition among animals, with special reference to freshwater fish. Nature 197:14-17.

White, M. and J. A. Kolb. 1974. A preliminary study of *Thamnophis* near Sagehen Creek, California. Copeia 1974:126-136.

Wiseman, K. D. and A. C. Pool. 2007. *Thamnophis couchii* (sierra garter snake): Predator-prey interaction. Herpetological Review 38:344.

Yotsu-Yamashita, M. and D. Mebs. 2001. The levels of tetrodotoxin and its analogue 6-epitetrodotoxin in the red-spotted newt, *Notophthalmus viridescens*. Toxicon 39:1261-1263.

Yotsu-Yamashita, M. and D. Mebs. 2003. Occurrence of 11-oxotetrodotoxin in the red-spotted newt, *Notophthalmus viridescens*, and further studies on the levels of tetrodotoxin and its analogues in the newt's efts. Toxicon 41:893-897.

Table 1: Annual differences in diet items. The top section of the table shows the total number of observations across the three years (2008-2010) as well as the proportion (and number) of total observations with recently consumed prey ("Obs. With Prey") for each year. In the remainder of the table, for a given year, the proportions (and numbers) of specific prey items (middle) or classes of prey (bottom) that were observed from the total observations of recently consumed prey are given. The *p*-value (*p*) for the Fisher's Exact test is given for specific prey items. One additional observation in 2008 of a snake that consumed an item only identified as "plethodontid" is not included in the species observations, but is included in the combined "salamander" prey class for the analyses of year, season, and weather, and is denoted by †.

2008-2010	2008	2009	2010	*p*
# of observations	290	382	230	
Obs. with Prey	0.23 (67)	0.2 (77)	0.13 (30)	
Lumbricidae	0.67 (45)	0.7 (54)	0.47 (14)	0.068
E. cirrigera	0.09 (6)	0.04 (3)	0.07 (2)	0.453
N. viridescens	0.01 (1)	0.03 (2)	0.1 (3)	0.146
P. cinereus	0.22 (15)	0.17 (13)	0.27 (8)	0.463
P. glutinosus	0.01 (1)	0 (0)	0.03 (1)	0.162
Lithobates spp.	0.01 (1)	0.05 (4)	0.1 (3)	0.148
P. crucifer	0.03 (2)	0.03 (2)	0.03 (1)	1.000
Salamander	0.34† (23)†	0.23 (18)	0.44 (14)	
Frog	0.04 (3)	0.08 (6)	0.13 (4)	

Table 2: Diet differences among male and female *Th. sirtalis*. Proportion (and number; *N*) of females and males observed with recently consumed diet items are given. The *p*-value (*p*) for the Fisher Exact test is given for each prey type. $p < 0.05$ denoted by an asterisk (*). Only female snakes were observed with recently consumed *N. viridescens*. Males and females did not differ in the proportion of any other prey type in their observed diet.

2008-2010	Female (N = 67)	Male (N = 76)	*p*
Lumbricidae	0.69 (46)	0.67 (51)	0.860
E. cirrigera	0.06 (4)	0.09 (7)	0.542
N. viridescens	0.09 (6)	0 (0)	0.009*
P. cinereus	0.21 (14)	0.28 (21)	0.436
P. glutinosus	0.01 (1)	0.01 (1)	1.000
Lithobates spp.	0.09 (6)	0.03 (2)	0.147
P. crucifer	0.04 (3)	0.03 (2)	0.665

Table 3: Diet differences among life stages of *Th. sirtalis*. Proportion (and number; *N*) of observations of diet items at each life stage are given. The *p*-value (*p*) for the exact test is given for each prey type. $p < 0.05$ denoted by an asterisk (*). Life stages differed significantly in the proportions of *Lithobates spp.* consumed.

2008-2010	Neonate ($N = 44$)	Yearling ($N = 79$)	Juv/Adult ($N = 50$)	*p*
Lumbricidae	0.71 (31)	0.7 (55)	0.52 (26)	0.094
E. cirrigera	0.02 (1)	0.09 (7)	0.06 (3)	0.443
N. viridescens	0.02 (1)	0.01 (1)	0.08 (4)	0.112
P. cinereus	0.3 (13)	0.2 (16)	0.14 (7)	0.195
P. glutinosus	0 (0)	0.01 (1)	0.02 (1)	1.000
Lithobates spp.	0 (0)	0 (0)	0.16 (8)	< 0.001*
P. crucifer	0 (0)	0.03 (2)	0.06 (3)	0.213

Table 4: Diet differentiation among microhabitats. Proportion (and number; *N*) of snakes observed with each prey type within a microhabitat are given. The *p*-value (*p*) for the Fisher Exact test is given for each diet item. $p < 0.05$ denoted by an asterisk (*). A significantly greater proportion of snakes in terrestrial habitats were found with recently consumed *P. cinereus,* while a significantly greater proportion of snakes in aquatic habitats were found with recently consumed *Lithobates spp.*

2009-2010	Aquatic (N = 34)	Terrestrial (N = 59)	*p*
Lumbricidae	0.74 (25)	0.61 (36)	0.262
E. cirrigera	0 (0)	0.08 (5)	0.154
N. viridescens	0.09 (3)	0.03 (2)	0.351
P. cinereus	0.09 (3)	0.29 (17)	0.035*
P. glutinosus	0 (0)	0.02 (1)	1.000
Lithobates sp.	0.18 (6)	0.02 (1)	0.009*
P. crucifer	0.03 (1)	0.03 (2)	1.000

Table 5: The effect of year on the likelihood of capturing a snake that had recently consumed prey. Tables provide a statistical summary of the generalized linear models from simple logistic regression analysis. Year is the categorical predictor variable in which individual comparisons are made in relation to the last year, 2010. In each model, the dependent variable differs as follows: presence or absence of (A) food, (B) earthworm, (C) salamander, and (D) frog. $p < 0.05$ denoted by an asterisk (*). Year significantly predicted capturing a snake that had recently consumed prey (A) and earthworm (B). See Figure 3 for among year differences in the likelihood of capturing snakes with recently consumed prey and prey types.

A. Food Observations	χ^2	*df*	*p*	Nagelkerke R^2
Model	8.34	2	0.015*	0.020
Predictors	Wald	*df*	*p*	e^{β}
Year (main effect)	7.87	2	0.020*	
2008	7.84	1	0.005*	1.93
2009	4.08	1	0.043*	1.59

B. Earthworm Observations	χ^2	*df*	*p*	Nagelkerke R^2
Model	6.35	2	0.042*	0.048
Predictors	Wald	*df*	*p*	e^{β}
Year (main effect)	6.27	2	0.043*	
2008	4.06	1	0.044*	2.41
2009	6.03	1	0.014*	2.89

C. Salamander Observations	χ^2	*df*	*p*	Nagelkerke R^2
Model	4.68	2	0.096	0.037

Predictors	Wald	*df*	*p*	e^{β}
Year (main effect)	4.64	2	0.098	
2008	1.33	1	0.249	0.60
2009	4.56	1	0.033*	0.39

D. Frog Observations	χ^2	*df*	*p*	Nagelkerke R^2
Model	2.12	2	0.347	0.029

Predictors	Wald	*df*	*p*	e^{β}
Year (main effect)	2.07	2	0.355	
2008	2.07	1	0.150	0.32
2009	0.62	1	0.430	0.58

Table 6: Summary of monthly capture observations. Total number (and percent) of observations for each month, observations with recently consumed food, and observations with recently consumed prey by class are given in the table below.

Observation Months	May	June	July	August	Sept	Oct
Total Observations	79	261	250	179	97	23
Obs. with Food	11	69	33	29	28	8
% of total observations	*14%*	*26%*	*13%*	*16%*	*29%*	*35%*
Obs. with Earthworm	4	44	23	20	16	5
% of food observations	*36%*	*64%*	*70%*	*69%*	*57%*	*63%*
Obs. with Salamander	5	20	10	6	10	2
% of food observations	*46%*	*29%*	*30%*	*21%*	*36%*	*3%*
Obs. with Frog	2	4	1	4	2	0
% of food observations	*18%*	*6%*	*3%*	*14%*	*7%*	*0%*

Table 7: The effect of season (month) on the likelihood of capturing a snake that had recently consumed prey. Tables provide a statistical summary of the generalized linear model from simple logistic regression analysis. Month is the categorical predictor variable in which individual comparisons are made in relation to the first month, May. Dependent variable differs as follows: presence or absence of (A) food, (B) earthworm, (C) salamander, and (D) frog. $p < 0.05$ denoted by an asterisk (*). Season significantly predicted capturing a snake that had recently consumed prey, but was not a significant predictor of capturing snakes that had eaten any particular class of prey. See Figure 4 for significant differences among season in the likelihood of capturing snakes with recently consumed prey and prey types.

A. Food Observations	χ^2	*df*	*p*	Nagelkerke R^2
Model	25.03	5	< 0.001*	0.044
Predictors	Wald	*df*	*p*	e^{β}
Month (main effect)	24.43	5	< 0.001*	
June	5.08	1	0.024*	2.22
July	0.03	1	0.869	0.94
August	0.22	1	0.642	1.20
September	5.43	1	0.020*	2.51
October	4.79	1	0.029*	3.30

B. Earthworm Observations	χ^2	*df*	*p*	Nagelkerke R^2
Model	4.73	5	0.450	0.036
Predictors	Wald	*df*	*p*	e^{β}
Month (main effect)	4.53	5	0.476	
June	2.78	1	0.096	3.08
July	3.62	1	0.057	4.03
August	3.33	1	0.068	3.89
September	1.33	1	0.248	2.33
October	1.24	1	0.266	2.92

C. Salamander Observations	χ^2	*df*	*p*	Nagelkerke R^2
Model	3	5	0.700	0.024
Predictors	Wald	*df*	*p*	e^{β}
Month (main effect)	2.94	5	0.709	
June	1.17	1	0.280	0.49
July	0.83	1	0.362	0.52
August	2.34	1	0.126	0.31
September	0.32	1	0.575	0.67
October	0.81	1	0.367	0.40

D. Frog Observations	χ^2	*df*	*p*	Nagelkerke R^2
Model	5.45	5	0.364	0.074
Predictors	Wald	*df*	*p*	e^{β}
Month (main effect)	4.11	5	0.534	
June	1.88	1	0.170	0.28
July	2.34	1	0.126	0.14
August	0.12	1	0.729	0.72
September	0.98	1	0.322	0.35
October	< 0.001	1	0.999	<0.001

Table 8: Summary of annual weather statistics. Mean (and range) for temperature and precipitation variables on capture days during the active season of each year.

Year	**2008**	**2009**	**2010**
Active Season	May 7 – Nov 2	Apr 11 – Oct 5	Apr 4 – Oct 18
# Capture Days	N = 46	N = 52	N = 47
Avg Daily Temp (°C)	17 (9.14 - 22.48)	16.61 (4.76 - 21.09)	18.45 (12.13 - 23.47)
Max Daily Temp (°C)	21.78 (14.4 - 28.03)	21.01 (7.66 - 25.61)	23.3 (14.58 - 28.56)
Avg 5 Day Max Temp (°C)	21.22 (11.34 - 27.35)	20.19 (7.41 - 23.93)	22.57 (10.20 - 27.01)
Days Since Last Precip Event (≥ 5 mm)	5.7 (0 - 22)	3.87 (0 - 18)	5.62 (0 - 29)
Total Daily Precip (mm)	1.09 (0 - 20.06)	3.23 (0 - 53.59)	2.47 (0 - 32.77)
5 Day Total Precip (mm)	12.99 (0 - 63.5)	23.36 (0 - 109.22)	17.27 (0 - 84.83)

Table 9: The effect of weather variables on the likelihood of capturing a snake that had recently consumed prey. Tables provide a statistical summary of the generalized linear models from multiple logistic regression analysis using temperature (temp) and precipitation (precip) indicator variables. Dependent variables differ as follows: presence or absence of (A) food, (B) earthworm, (C) salamander, or (D) frog. $p < 0.05$ denoted by an asterisk (*). Temperature significantly predicted the presence of prey generally in the diet but not of specific prey classes, while precipitation significantly predicted the presence of salamanders in the diet.

A. Food Observations	χ^2	*df*	*p*	Nagelkerke R^2
Model	14.53	6	0.024*	0.025
Predictors	Wald	*df*	*p*	e^{β}
Avg. Daily Temp	2.12	1	0.145	0.88
Max Daily Temp	8.68	1	0.003*	1.26
Avg 5 Day Max Temp	3.77	1	0.052	0.92
Total Daily Precip	0.03	1	0.859	1.00
Days Since Last Precip Event	0.28	1	0.596	1.01
5 Day Total Precip	0.03	1	0.858	1.00

B. Earthworm Observations	χ^2	*df*	*p*	Nagelkerke R^2
Model	8.78	6	0.186	0.065
Predictors	Wald	*df*	*p*	e^{β}
Avg. Daily Temp	1.03	1	0.311	1.17
Max Daily Temp	2.86	1	0.091	0.77
Avg 5 Day Max Temp	0.04	1	0.841	1.02
Total Daily Precip	1.21	1	0.272	1.05
Days Since Last Precip Event	3.03	1	0.082	0.93
5 Day Total Precip	2.05	1	0.152	0.99

C. Salamander Observations	χ^2	*df*	*p*	Nagelkerke R^2
Model	13.61	6	0.034*	0.104
Predictors	Wald	*df*	*p*	e^{β}
Avg. Daily Temp	0.66	1	0.416	0.87
Max Daily Temp	2.42	1	0.120	1.30
Avg 5 Day Max Temp	0.00	1	0.979	1.00
Total Daily Precip	0.57	1	0.451	0.97
Days Since Last Precip Event	5.82	1	0.016*	1.13
5 Day Total Precip	6.20	1	0.013*	1.03

D. Frog Observations	χ^2	*df*	*p*	Nagelkerke R^2
Model	4.49	6	0.611	0.061
Predictors	Wald	*df*	*p*	e^{β}
Avg. Daily Temp	0.82	1	0.367	0.76
Max Daily Temp	0.72	1	0.397	1.30
Avg 5 Day Max Temp	0.00	1	0.985	1.00
Total Daily Precip	0.06	1	0.809	1.01
Days Since Last Precip Event	1.72	1	0.190	0.83
5 Day Total Precip	0.34	1	0.562	0.99

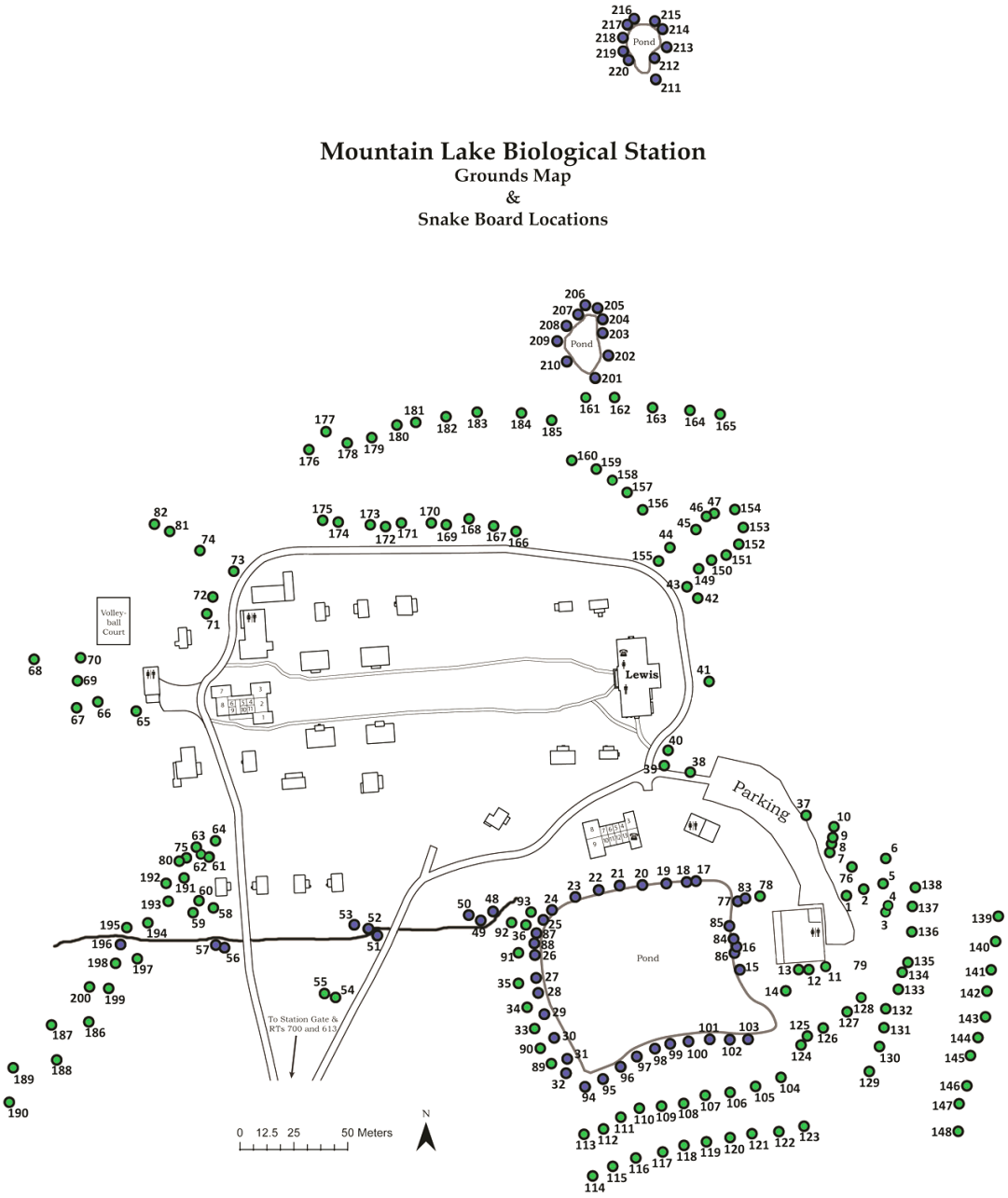

Figure 1. Map of MLBS grounds and the location of snake boards based on GPS data. Terrestrial boards in green and aquatic boards in blue.

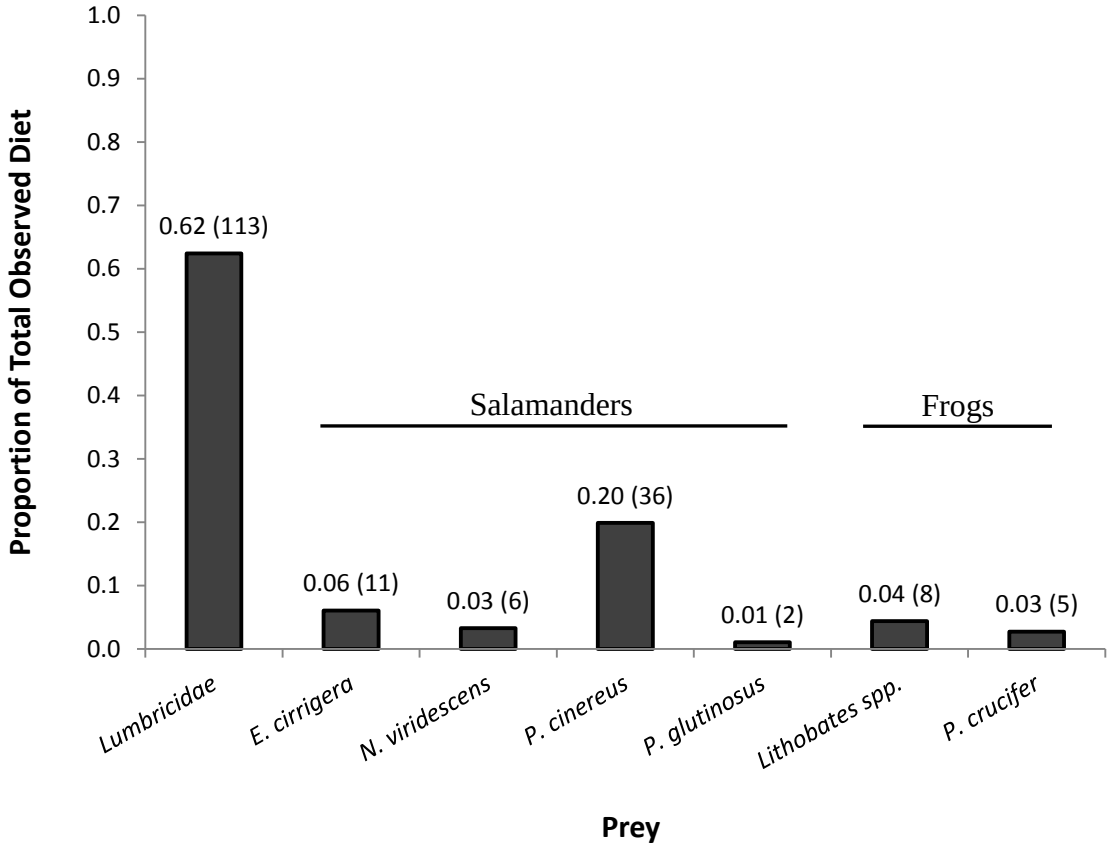

Figure 2. Observed diet of Th. sirtalis at MLBS 2008-2010 displayed as the proportion of each prey type in total observed diet (181 observed diet items). Number of observed diet items is shown in parentheses next to the proportion value. Data represent 174 observations from 143 snakes (7 observations yielded 2 different prey types).

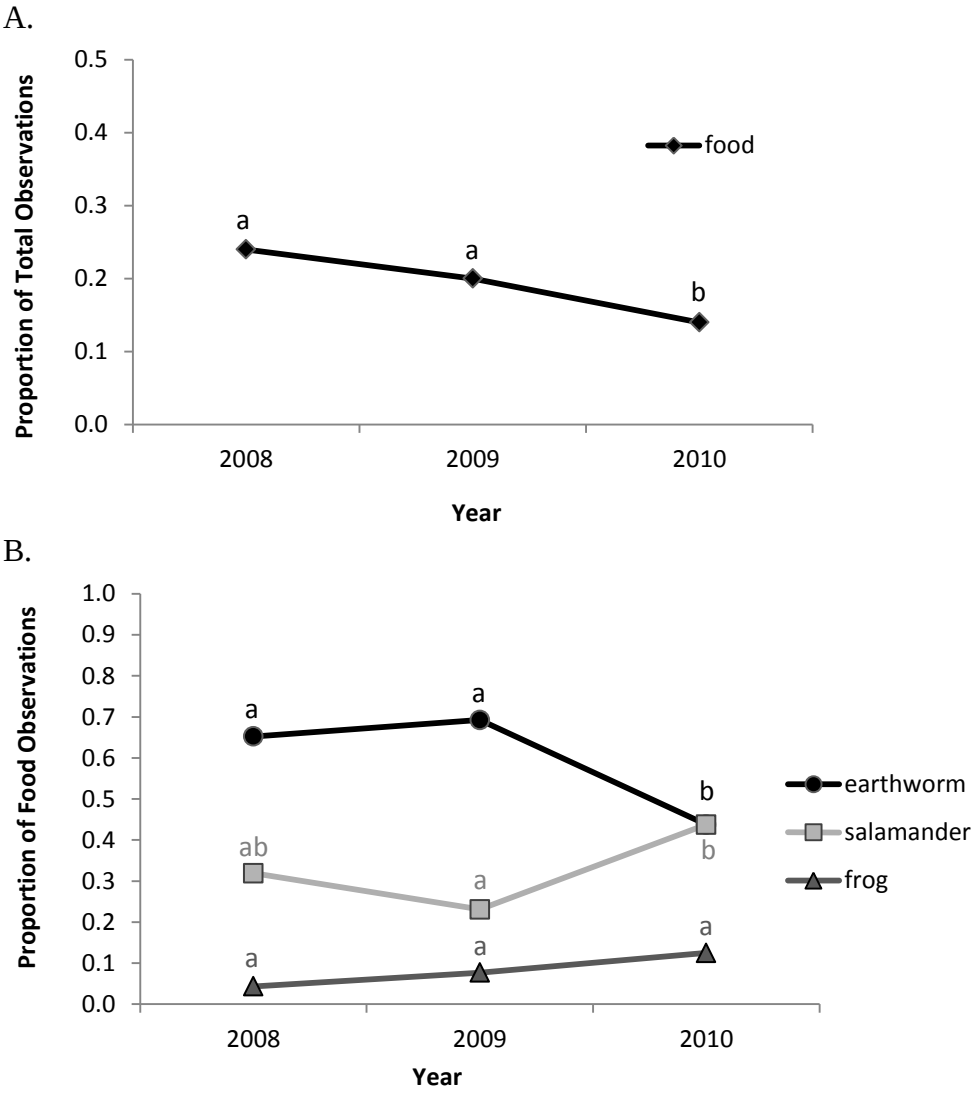

Figure 3. For years 2008-2010, (A) the proportion of total capture observations in which snakes had recently consumed food and (B) the proportion of food observations in which snakes had recently consumed earthworms, salamanders, and frogs are illustrated. Letters a and b indicate significantly similar years for a given prey group. Year significantly affected the likelihood of capturing a snake that had recently consumed prey and, more specifically, the likelihood of capturing a snake that had recently consumed earthworm.

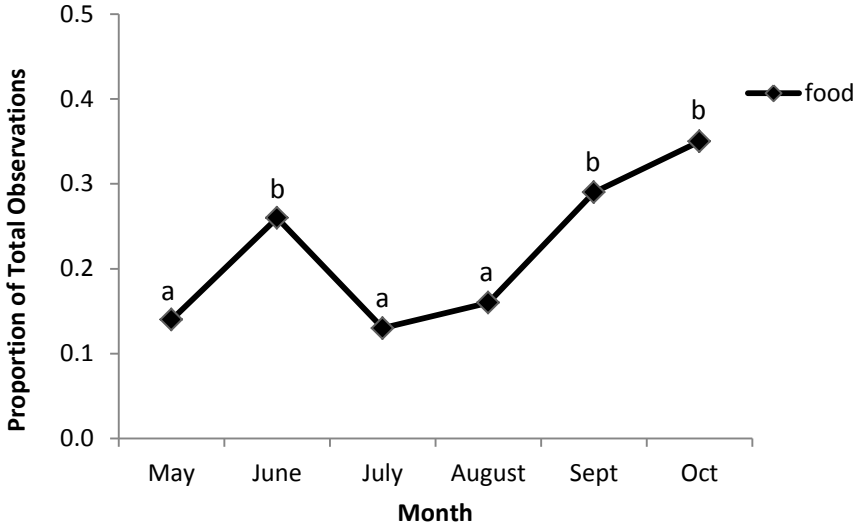

Figure 4. For months May-October, the proportion of total capture observations in which snakes had recently consumed food is illustrated. Letters a and b indicate significantly similar months. Season significantly affected the likelihood of capturing a snake that had recently consumed prey, but not the likelihood of capturing a snake that had recently consumed a particular prey class.

Chapter 4:

Eastern *Thamnophis sirtalis* Demonstrate Congenital Response to and Learned Preference for Toxic Prey

Abstract

Predators have two evolutionary paths to adapt to potential prey that are toxic: resistance to the prey toxin or avoidance of that prey. Despite apparent costs, many predators have evolved resistance that allows them to consume toxic prey rather than avoid them. Behavioral factors might constrain predators to consume toxic prey through congenital preference for prey types, learned preference, and congenital correlations in responsiveness to prey. Here, I explore these behavioral explanations in *Thamnophis sirtalis* (common garter snake), a predator known to consume toxic *Notophthalmus viridescens* (red-spotted newt), by assessing prey recognition behavior and responsiveness to natural prey extracts by food-naïve and food-experienced neonate snakes. Snakes exhibited congenital responsiveness to *N. viridescens* extract, but did not prefer the extract over alternative prey types. The decision to consume or to refuse newts was dependent upon congenital responsiveness to *N. viridescens,* and neonate snakes fed only newts dramatically increased their preference for this prey. Responsiveness to *N. viridescens* was correlated with that to other prey at the individual and family level. Some correlations persisted after experience with prey generally, but did not persist after consuming specific diets. Additionally, the magnitude of change in responsiveness to *N. viridescens* was not matched by responsiveness to other prey species. Although *Th. sirtalis* are born recognizing *N. viridescens* as prey, snakes do not prefer newts over other prey types. However, snakes with stronger congenital responses for *N. viridescens* are more likely to consume them, and then learn to prefer them as prey. A combination of

congenital response for *N. viridescens* and learned preference for newt appears to explain the inclusion of toxic *N, viridescens* in the diet of *Th. sirtalis* in this eastern population.

Key words: *toxic prey, prey recognition, congenital response, correlated response, behavioral plasticity, learned preference,* Thamnophis sirtalis, Notophthalmus viridescens, *tetrodotoxin*

Introduction

Predators often co-occur with potential prey that are toxic, and predators have two alternative paths when faced with such prey: avoidance or evolved ability to consume toxic prey (Brodie and Brodie 1999a). Ingesting toxic prey can be problematic for predators because of costs associated with consuming the toxins: feeding inhibition (Demott et al. 1991), reduced locomotor performance (Brodie and Brodie 1999b; Llewelyn et al. 2009), reduced growth rates (Fisker and Toft 2004), impeded development (Paradise and Stamp 1993; Weiser and Stamp 1998), and neurological and reproductive impairment (Freeland and Janzen 1974). Consuming toxic prey, therefore, requires the predator to evolve some mechanism, such as resistance, for dealing with toxins they encounter, but resistance itself (behavioral, physiological, or genetic), is also costly (reviewed in Després et al. 2007) and does not necessarily mitigate all toxic effects. Alternatively, avoidance of toxic prey can be learned or can evolve as innate avoidance that precludes the need to evolve mechanisms to tolerate prey toxins. However, avoidance also incurs a cost: relinquishing a common or under-exploited food source. Even so, avoidance may be expected because it circumvents the challenges presented by toxin consumption and resistance. Some predators consume toxic prey rather than avoid them despite the costs of consuming toxins and of evolving abilities such as resistance (Mori and Moriguchi 1988; Daly et al. 1997; Dumbacher et al. 2004; Robbins et al. 2013). However, it is unclear what drives some species to evolve resistance rather than avoidance.

There are a variety of possible causes that may result in predators consuming toxic prey rather than avoiding them. Ecological factors such as alternative prey availability and ease of capture will influence the rate at which predators encounter and interact with toxic prey. These factors, in addition to nutritional quality and other potential benefits, will shape the selective advantages to the predator from eating such prey. Toxin levels should correlate with the prey's nutritional value (Speed and Ruxton 2014), so there may be a selective advantage to exploiting such a resource. The ability to consume nutritious prey during critical periods even though it is defended by toxin(s) may drive predators to evolve resistance rather than avoidance.

Physiological needs for energetic resources drive predators to search for food, and while searching, chance encounters allow the predator to behaviorally assess potential prey. Behavioral responses to potential prey could lead to inclusion in the diet in three distinct ways. First, congenital responses to potential prey can influence which species are included in the diet of predators because of an evolved response by the predator to that prey type (Burghardt and Pruitt 1975; Arnold 1978; 1981a, b, c; Burghardt 1993; Darmaillacq et al. 2004). Congenital prey recognition can be genetically based and subject to selection (Arnold 1977, 1981a, b; Burghardt 1993), and evolved congenital responses correspond to a predator's inclination for feeding on sympatric prey (Burghardt 1967; Arnold 1977, 1978; 1981a, b, c). This type of behavioral response toward prey might have evolved by chance or because of an ecological advantage provided by the prey (e.g., it may be more nutritious). Regardless, if a predator is born with congenital

preference for a toxic prey species, then the predator would be predisposed to eating that prey upon encounter rather than avoiding it. In some cases, congenital responses are fixed, causing prey to be included in the diet regardless of experience (Burghardt et al. 1973; Arnold 1978), thus trapping the predator into consuming the prey throughout its lifetime.

Second, positive experience with a given prey type can result in a heightened response to that prey in the future (Hess 1962). This type of experiential enhancement of prey preference has been demonstrated in a variety of predators including snapping turtles (Burghardt and Hess 1966), garter snakes (Fuchs and Burghardt 1971; Arnold 1978; Burghardt 1992; Burghardt et al. 2000), lynx spiders (Punzo 2002), and cuttlefish (Darmaillacq et al. 2004). Experiences early in life, such as the first prey encountered, can have disproportionate effects on subsequent prey preferences (Burghardt and Hess 1966; Punzo 2002; Darmaillacq et al. 2004). Negative experiences may lead to aversive responses and exclusion of some taxa from the diet of the predator (Burghardt et al. 1973; Arnold 1981c). Conversely, positive experiences with specific prey can lead to increased preference and inclusion in the diet (Burghardt and Hess 1966; Fuchs and Burghardt 1971; Arnold 1978; Punzo 2002). Such canalization of responses can result in some individual predators developing positive responses to particular prey types thereby hindering the evolution of avoidance of that species.

Third, patterns of correlated responses to prey can constrain predators to attack suites of taxa and prevent exclusion of a single species from the diet. Congenital

responses to alternative prey types are often strongly correlated, such that predators respond similarly (either preferentially or aversively) to multiple species (Arnold 1981a, c), due to similar signals (e.g., color, shape, chemical) among prey that prevent discrimination among species. Associated signals or signal reception can manifest as genetic correlations in predators that link the inheritance of responses to multiple prey in which an inherited response to one prey type accompanies an inherited response to another. In this scenario, the genetic coupling of prey response could prevent the predator from evolving avoidance of one prey without excluding the other from the diet. Strong selection to feed on one prey may thereby lead to the correlated inclusion of other taxa in the diet. Such a correlated response has been observed in coastal populations of *Thamnophis elegans* where a positive response to frequently consumed slugs is correlated with response to hazardous leeches resulting in their inclusion in the diet (Arnold 1981c).

I investigated responses to alternative prey in the common garter snake, *Thamnophis sirtalis* to evaluate these three alternative explanations for why predators evolve to consume toxic prey rather than avoid them. *Thamnophis sirtalis* is a generalist predator that consumes a variety of prey including amphibians and terrestrial invertebrates (Mushinsky 1987; Rossman et al. 1996; Stebbins 2003). Throughout their range *Th. sirtalis* is known to consume small frequencies of toxic newts (Brodie 1968b; Nussbaum et al. 1983; Brodie et al. 2002; Chapter 3). In the western part of its range, *Th. sirtalis* eats newts of the genus *Taricha* and in the eastern part of its range *Th. sirtalis* eats *Notophthalmus viridescens* (red-spotted newt). Newts of both genera secrete tetrodotoxin

(TTX), a potent neurotoxin that is fatal to most vertebrates (Kao 1966; Narahashi et al. 1967; Brodie 1968b; Lipkind and Fozzard 1994b). In many localities, *Th. sirtalis* exhibits physiological resistance to TTX (Brodie and Brodie 1990; 1999b; Brodie et al. 2002; Chapter 2), but resistance does not mitigate all effects of the toxin and consuming toxic newts can have severe consequences (Brodie and Brodie 1990, 1999b), including locomotor impairment, immobilization, or death (Brodie and Brodie 1990, 1999b; Williams et al. 2003; Williams et al. 2010).

Most snakes, including garter snakes, recognize prey primarily through chemosensory channels. Variable responses to chemical cues are easily measured by scoring tongue-flicking behavior, which increases with interest in a stimulus and attacks of putative prey (Burghardt 1969, 1975, 1992, 1993; Arnold 1977, 1978 , 1981a, b, c; Ayres and Arnold 1983; Cooper et al. 2000; Shepard et al. 2004; Aubret et al. 2006; Cooper 2007; Cooper and Secor 2007; Llewelyn et al. 2010). Previous studies in garter snakes have demonstrated that the tongue-flicking prey recognition response varies inter- and intraspecifically depending on the diet of the snake species and local prey abundance (Burghardt 1970b; Arnold 1977; 1981b, c; Kephart 1982; Kephart and Arnold 1982; Burghardt 1993). The behavior is congenital (Burghardt and Pruitt 1975; Arnold 1981a, b) as well as behaviorally plastic (Fuchs and Burghardt 1971; Burghardt 1973; Arnold 1978). Arnold (1981a) demonstrated both high heritability and an integrated structure of genetic correlations among responses to different prey types in *Th. elegans*.

This investigation tested three non-exclusive behavioral hypotheses for why predators evolve to consume toxic prey:

1. The *congenital response* hypothesis: predators are born with a preference for toxic prey. If congenital preference explains non-avoidance, neonate *Th. sirtalis* would be expected to exhibit a positive response to *N. viridescens*.
2. The *learned preference* hypothesis: predators exhibit enhanced response to a prey type after experience. If learned preference explains *Th. sirtalis* consumption of toxic prey, an increase in response to *N. viridescens* after experience with it is expected.
3. The *correlated response* hypothesis: predators are constrained to eat toxic prey because of correlated recognition of multiple prey types. If correlated response explains non-avoidance, strong correlations between responsiveness to *N. viridescens* and other prey species are expected.

These behavioral hypotheses were studied in an eastern population of *Th. sirtalis*. I was able to investigate responsiveness to toxic *N. viridescens* and a variety of natural prey by measuring tongue-flicking behavior. I measured congenital responsiveness to several prey species in food-naive neonate *Th. sirtalis*, to identify if snakes are born with a preference for toxic newt. Neonate *Th. sirtalis* were then reared on exclusive diets of *N. viridescens* or earthworms for six weeks, after which responses to prey extracts were re-evaluated to ascertain if learned preference plays a role in non-avoidance of toxic newt.

Finally, congenital and experienced responses to prey were evaluated for individual and family level correlations to determine if correlated response explains the inclusion of toxic newt in *Th. sirtalis* diet.

Methods

Prey Recognition Behaviors

Response to prey was tested using the common method of measuring tongue-flick and attack behaviors to assess differential responses to prey extracts (Burghardt 1969, 1970a, 1970b, 1975, 1992, 1993; Burghardt and Abeshahe 1971; Fuchs and Burghardt 1971; Burghardt and Pruitt 1975; Arnold 1977, 1978, 1981a, b, c; Ayres and Arnold 1983; Cooper et al. 2000; Greene et al. 2002; Shepard et al. 2004; Aubret et al. 2006; Cooper 2007; Cooper and Secor 2007; Llewelyn et al. 2010). Each extract was presented to the snake for 60 seconds, with a minimum of 20 minutes between each extract presentation. Measured behaviors included number of tongue flicks toward the applicator tip, nosing, latency to nosing, attack, and latency to attack. A tongue-flick (TF) was any protrusion of the tongue from the mouth in the direction of the applicator tip. Nosing was defined as contact between the snake's snout and the applicator tip with a subsequent rub of the snout on the applicator. Nosing was considered a stronger response to prey than tongue-flicking as snakes often perform this behavior prior to consuming dead prey. Nosing behavior was observed and the latency to perform this behavior in seconds was recorded. The timer was stopped if the snake attacked the applicator (opened mouth

contact with the applicator tip), trial presentation ended with the attack, and the latency to attack was measured in seconds. Attack was considered the strongest response. Defensive behaviors and attempts to escape were also recorded during each one-minute presentation.

Tongue-Flick Score (TFS)

Tongue-Flick Score (TFS) was measured for each presentation of an extract to an individual snake. If a snake did not nose or attack the applicator then they were given a basic Tongue-Flick Score (TFS_{basic}) that was equal to the number of tongue flicks toward the applicator during the 60 second presentation.

$$TFS_{basic} = TF \quad \text{(eq. 1)}$$

If a snake exhibited a nosing or attack behavior, then an alternate Tongue-Flick Score (TFS_{alt}), based on the previously used "tongue-flick attack score" (Burghardt 1969; Cooper and Burghardt 1990), was used to calculate the response to prey extract for that presentation. TFS_{alt} was defined by the following equation:

$$TFS_{alt} = TF_{max} + [(TL - LN)/2] + (TL - LA) \quad \text{(eq. 2)}$$

where TF_{max} is the maximum number of tongue flicks exhibited toward the applicator by any individual in any presentation or trial for a given study (either the congenital study or food-experienced study, see below), *TL* is the trial length in seconds in the absence of an attack (*TL = 60sec.*), *LN* is the latency to nose the applicator in seconds, and *LA* is the latency to attack the applicator in seconds. The nosing term (*TL-LN*) is divided by 2

whereas the attack term (*TL-LA*) is not in order to give greater weight to an attack versus a nosing behavior.

Because nosing and attack behaviors were considered to be stronger responses than tongue-flicking alone, it was necessary to ensure that individuals exhibiting nosing or attack behaviors were scored stronger than individuals exhibiting only tongue-flicks. This was accomplished by elevating scores that included nosing and attack behaviors above the maximum *TF* for any snake. A comparison of response variables by Cooper and Burghardt (1990) found that scoring the stronger response (in this case nosing and attack) above TF_{max} to be the best indicator of responsiveness in squamates for this type of chemoreceptive study.

Prey Extracts

Prey recognition response of neonate *Th. sirtalis* was tested with prey species most commonly found in their natural diet based on field data collected in 2008-2010 (Chapter 3): *Lumbricus terrestris* (nightcrawler), *Plethodon cinereus* (red-backed salamander), *Notophthalmus viridescens* (red-spotted newt), *Pseudacris crucifer* (spring peeper frog), and *Lithobates catesbeianus* (bullfrog). Samples of prey species were collected from MLBS in 2009 and 2010. Prey extracts were prepared in advance and sufficient quantities were made to test all subjects in all trials (congenital and experienced) in a given year (i.e. one batch was prepared in 2009 for all testing performed in that year, and another batch was prepared in 2010 for testing performed in

that year). Extract preparation was based on methods described by Burghardt (1966, 1969) and Sheffield et al. (1968). Extracts were frozen in small aliquots until needed, thawed to room temperature just before trials, and presented to snakes on cotton tipped applicators. Extracts were placed in the refrigerator between trials and no tube of extract was used after 3 days of being thawed as the potency of the extract may diminish after this time (Burghardt and Hess 1968). Because several individuals of each prey type were used in the preparation of extracts, I was able to mitigate some of the possible individual variation in skin molecules of prey.

Trials

A trial consisted of the presentation of each of six different extracts (*L. terrestris*, *P. cinereus*, *N. viridescens*, *P. crucifer*, and *L. catesbeianus* as well as a negative control (applicator tip dipped in sterile H_2O). Extracts were presented in randomized order. Two trials were administered on successive days, and the average score of the two trials used to increase accuracy of the measurements (Falconer and Mackay 1996).

Subjects were tested at 28+/-1°C (Burghardt 1970a). Test chambers (plastic enclosures 7.75”L X 11.5”W X 8”H) were covered with an opaque sheet with a small observer window to prevent the snake from being distracted by external activities during response testing. Snakes were placed into test enclosures a minimum of 24 hours before the first trial, and were undisturbed at least 30 minutes before the trial began to avoid

eliciting defensive behaviors immediately before testing. Between trials snakes were offered water *ad libitum* and held at 22+/-1ºC.

If any presentation within a trial elicited defensive behaviors, or if the snake was not participating and instead trying to escape for the entire presentation, then the trial was excluded from data analysis. Trials in which snakes did not respond to the applicator for any of the six presentations were also excluded on the basis that the snake was not actively participating in the trial. In the event that one of the trials was excluded, snakes remained in the overall analysis, but were evaluated on the TFS score from a single trial instead of the average of two trials. Repeatability of chemoreceptive behavior was estimated for each prey type using intraclass correlation coefficient in SPSS (v.18; Table 1) to determine if individual snakes were consistent in their responsiveness to a prey type across the two trials. Repeatabilities were estimated for both the congenital responses and the experienced responses.

Congenital Responses (CongTFS)

To examine congenital responses to potential prey, 205 food-naïve neonate *Th. sirtalis* from 31 families (5-10 individuals per family) were tested. Pregnant *Th. sirtalis* were collected in 2009 and 2010 from Mountain Lake Biological Station (MLBS), University of Virginia in Giles County, Virginia (37°22’32”N, 80°31’20”W). Pregnant females were subsequently transported to the laboratory at the University of Virginia where they were individual housed, given constant access to fresh water, and offered

earthworms twice per week. Adult females were returned to MLBS and released at their site of capture within three weeks post-parturition. Neonate snakes were born in July and August from these wild-caught females. Neonates were separated within 24 hours of birth, housed individually, and were given access to fresh water daily. Food-naïve neonates were tested on days 15 and 16 after birth to allow time for absorption of yolk sacs. Of the 205 food-naïve snakes tested, 10 snakes had one of the two trials excluded because of defensive behavior or lack of engagement with stimuli.

Food-Experienced Responses (ExpTFS)

Neonates from the 2010 sample were assigned to two treatment groups after congenital response testing to examine how experience with food alters response to prey cues. One group was fed a piece of worm (*L. terrestris*, obtained commercially), whereas the second group was fed a newly metamorphosed newt (*N. viridescens*, captured as larvae from a pond at the snake collection site and metamorphosed in captivity). Effort was made to standardize mass of food items by selecting uniform sized newts to feed to snakes in the newt treatment and cutting sections of worm in the worm treatment to approximately match the mass of newts. Snakes were fed twice per week for six weeks (12 feedings). Seven days after the final feeding, snakes were re-tested for responsiveness to prey.

Of the 80 neonates that began this experiment, 47 survived to food-experienced prey response testing. This general level of neonate survival is consistent with that

observed in previous laboratory rearing studies of this species (Brodie, *unpublished data*). Of the 47 food-experienced snakes tested, five snakes had one of the two trials excluded due to defensive behaviors elicited during one or more presentation in the trial, and one snake was excluded completely due to unresponsiveness in both trials. The resulting sample sizes were $N = 46$ individuals from 10 families (2-8 individuals per family, mean family size 4.6); 30 *Th. sirtalis* in the worm treatment group and 16 *Th. sirtalis* in the newt treatment group.

Mortality & Food Quality

Some snakes persistently refused their prey (refused to eat the first two or more meals). The relationships between food refusal and congenital response, as well as between amount and type of food consumed and mass gained were examined. Fisher's Exact tests and Independent Samples t-test using PAWS Statistics (SPSS) v. 18 were used to investigate proportional and mean differences, respectively. One-tailed significance tests were used to evaluate the relationship of food refusal and congenital prey response as there was no reason to expect that snakes with a high congenital response to a prey type would be persistent refusers of that prey; two-tailed significance tests were used in all other cases.

Hypothesis Testing

Congenital Response

To test if *Th. sirtalis* is born with preference for toxic newt, congenital responses (CongTFS) were analyzed to determine if neonate snakes exhibited a greater response to *N. viridescens* when compared to other prey tested. This was accomplished using a linear mixed model (Restricted Maximum Likelihood - REML) to assess differences in mean congenital responsiveness to prey types. This analysis included other fixed factors that may affect congenital responses including sex, family, trial, and order of prey presentation. Interaction effects with prey type were also included as they were potentially meaningful in understanding congenital responses to prey. Individual was treated as a random variable. The final model describing CongTFS excluded all non-significant interaction effects. Post-hoc pair-wise comparisons for significant main effects were analyzed with Bonferroni corrections. Univariate ANOVA was used to investigate significant differences within interaction terms. All statistical analyses were performed using PAWS Statistics (SPSS) v. 18.

Learned Preference

To evaluate the role of learning in non-avoidance, experienced responses (ExpTFS) were analyzed to see if naïve responses predicted experienced responses or if responses to chemical prey cues changed in response to specific diet treatments. Multivariate analysis of variance (MANOVA) was used to compare the ExpTFS profile

of each treatment group (newt and worm) with the 2010 CongTFS profile to see if overall responsiveness to prey had changed. Additionally, MANOVA was used to assess if the ExpTFS profile of the newt treatment group differed significantly from the ExpTFS profile of snakes in the worm treatment group to see if a particular diet altered overall responsiveness to prey. Differences in ExpTFS to particular prey types among the two treatment groups were analyzed with univariate ANOVA to determine if treatment affected response to any particular type of prey.

ExpTFS within each treatment group (newt and worm) were then analyzed separately to determine if snakes exhibited a greater response to the prey type on which they were raised when compared to other prey types. Number and sizes of families (N = 8 families in the newt treatment & N = 10 in the worm treatment with 1-4 individuals per family) precluded analysis at the family level. As with the congenital responses, REML was used to assess differences in ExpTFS to prey types within each treatment. These analyses also included sex, trial, and order of prey presentation as fixed factors, as well as interaction effects with prey type. Again, individual was treated as a random variable. Post-hoc pair-wise comparisons within significant main effects were analyzed with Bonferroni corrections.

In addition to absolute experienced responses (ExpTFS), change in responsiveness to prey types (ΔTFS) was also investigated because individuals were expected to vary in their baseline response to prey cues (i.e. congenital responses). ΔTFS was, therefore, able to capture how much individuals adjusted their responsiveness to

prey cues after specific diet treatments. Change in responsiveness was calculated for individual snakes and was defined as ΔTFS = average ExpTFS - average CongTFS for each prey extract tested. Similar to the analysis of ExpTFS above, MANOVA was used to compare ΔTFS treatment profiles to each other, and REML was used to investigate differences within each treatment group. All statistical analyses were performed using PAWS Statistics (SPSS) v. 18.

Correlated Response

To investigate the correlated response hypothesis, CongTFS were used to investigate correlations in chemosensory responses to prey types as well the potential for underlying genetic correlations. While the correlation matrices produced using individual responses as data points describe phenotypic correlations, correlation of family means provides an approximation of underlying genetic correlations (Arnold 1981a; Via 1984). In addition, correlations among ExpTFS were analyzed to understand how labile these behaviors were. Pearson's correlation was used for all analyses. ExpTFS from each diet treatment were also analyzed separately. Family sizes were too small to consider correlations of family means in experienced responses for individual treatment groups. In addition, correlations among individual ΔTFS were investigated to determine whether a change in one response is accompanied by others. All statistical analyses were performed using PAWS Statistics (SPSS) v. 18.

Results

Congenital Response

Individual congenital responses to prey extracts were repeatable across the two trials (Table 1A). The final model describing CongTFS for *Th. sirtalis* is in Table 2A. Food-naïve snakes differed significantly in their responses to prey ($F_{5,2034} = 146.354$, $p < 0.001$). Specifically, all responses to prey were significantly stronger than responses to the water control ($p < 0.001$), and responsiveness to *P. cinereus* was significantly greater than to *N. viridescens* ($p = 0.003$) and to *L. catesbeianus* ($p = 0.001$; Figure 1).

In addition, the family by prey interaction effect was significant ($F_{150,2034} = 2.866$, $p < 0.001$) indicating that families significantly differed in their responsiveness to prey types. Specifically, from post hoc analyses, families significantly differed in their response to worm ($F_{30,174} = 1.998$, $p = 0.003$), newt ($F_{30,174} = 1.564$, $p = 0.040$), red-backed salamander ($F_{30,174} = 1.651$, $p = 0.025$), and frog ($F_{30,174} = 3.041$, $p < 0.001$), but did not significantly differ in their response to spring peeper ($F_{30,174} = 1.192$, $p = 0.240$) or control ($F_{30,174} = 65.451$, $p = 0.092$).

Learned Preference

Individual experienced responses to prey extracts were repeatable across the two trials except for responsiveness to *L. catesbeianus* (Table 1B). ExpTFS profiles for both the worm and the newt treatments were significantly greater than the CongTFS profile (worm treatment: $F_{6,103} = 4.89$, $p < 0.001$, and newt treatment: $F_{6,89} = 7.07$, $p < 0.001$;

Figure 2). The difference between the newt treatment profile and the worm treatment profile was only marginally significant ($F_{6,39} = 2.30$, $p = 0.054$). Pairwise comparisons between responses to specific types of prey cues in the newt and worm treatments show that the difference in these profiles is driven primarily by an increase in responsiveness to a *N. viridescens* cue by snakes in the newt treatment, relative to those in the worm treatment ($p = 0.005$; Figure 2).

The models describing ExpTFS for *Th. sirtalis* in the newt and worm treatment groups are given in Table 2B and 2C, respectively. There was a significant effect of prey type on ExpTFS in the worm treatment, and response to all prey extracts were significantly greater than to the water control ($p < 0.001$). Also in the worm treatment, response to *N. viridescens* extract was significantly lower than all other prey tested (Figure 2). There was also an effect of prey type in the newt treatment group, and response to all prey extracts were significantly greater than to the water control ($p < 0.001$). There were no other significant differences among the responses to prey extracts within the newt treatment group.

ΔTFS profiles for the newt and worm treatments were not significantly different from each other ($F_{6,39} = 1.859$, $p = 0.620$). There was an effect of prey type on ΔTFS within both the newt and worm treatment groups (Tables 2D and 2E, respectively). In the newt treatment, only ΔTFS to *N. viridescens* extract was significantly greater than ΔTFS to the control ($p = 0.015$, $df = 75$; Figure 3). ΔTFS to *N. viridescens* was also significantly greater than that toward *L. terrestris* ($p = 0.008$, $df = 75$). In the worm

treatment, ΔTFS to *L. terrestris* ($p = 0.045$, $df = 145$), *N. viridescens* ($p = 0.012$, $df = 145$), and *P. crucifer* ($p = 0.006$, $df = 145$) were significantly greater than control (Figure 3). In addition, ΔTFS to *N. viridescens* ($p = 0.019$, $df = 145$) and *P. crucifer* ($p = 0.011$, $df = 145$) were significantly greater than to *L. catesbeianus*.

Correlated Responses

Correlations among all CongTFS responses were significant, but low to moderate in magnitude. Family means were based on a smaller sample, but exhibited slightly elevated correlations between *N. viridescens* and all prey types tested, including a relatively strong correlation between response to *N. viridescens* and *P. cinereus* (Table 3).

Some significant correlations of individual level responses and family mean responses persisted after experience with food (ExpTFS), primarily among amphibian prey (Table 4). Among them were an individual correlation and a strong family mean correlation between response to *N. viridescens* and *P. cinereus*. Most individual ExpTFS to prey types (but not to water control) were significantly correlated within the newt treatment, including a strong correlation between responsiveness to *N. viridescens* and *P. cinereus* (Table 5). Conversely, very few individual responses to prey types were significantly correlated within the worm treatment (Table 5). Specifically, individual ExpTFS between *N. viridescens* and *P. cinereus* by snakes in the worm treatment were

not significantly correlated. There were no significant individual correlations in ΔTFS in either the newt or worm treatment groups (Table 6).

Even though correlations were observed among family means, caution should be taken when inferring underlying genetic structure from these data. While family sizes of 10-11 individuals have been used to approximate genetic correlations (Arnold 1981a), Roff and Preziosi (1994) reported that for family sizes less than ~20 individuals the true genetic correlation is correctly estimated only a small percentage of the time unless the genetic and phenotypic correlations are very similar. That is, the estimate of the family mean correlation may be inaccurate due to the small numbers of individuals within families especially when the genetic and phenotypic correlations are very different. In the present study, mean family size for the congenital analysis was 7 individuals (*SD* = 1.33, range 5-10 snakes per family) and for the analysis of experienced responses (data from combined treatment groups) was 4.6 individuals (*SD* = 1.78, range 2-8 snakes per family), making the interpretation of genetic correlation from family mean correlation in this investigation questionable.

Mortality and Food Quality

A significantly greater proportion of snakes in the worm treatment group survived (0.78) than in the newt treatment group (0.40; $p = 0.001$). More snakes in the newt treatment persistently refused food ($p = 0.005$) and persistent refusers were less likely to survive ($p = < 0.001$; Figure 4). In the newt treatment group, persistent refusers were

those individuals with lower CongTFS to offered prey (t = 2.063, df = 38, one-tailed p = 0.023; Figure 5). In the worm treatment group, CongTFS to offered prey did not differ between persistent refusers and those that were not (t = 1.47, df = 38, one-tailed p = 0.075).

Not all of the surviving snakes consumed all 12 meals; on occasion snakes would refuse a meal. Snakes in the two treatments did not significantly differ in the number of meals consumed (mean worm meals = 10.97, mean newt meals = 10.50, t = 1.52, df = 45, p = 0.135). Though effort was made to standardize the amount of food offered to snakes in both groups, the mean total mass of prey consumed per snake was greater in the worm treatment (mean = 3g) than in the newt treatment (mean = 2.5g; t = 5.518, df = 45, p < 0.001). On average, snakes in the newt treatment gained significantly more mass (0.17g) than snakes in the worm treatment (t = -4.026, df = 45, p < 0.001; Figure 6).

Discussion

In this investigation three non-exclusive behavioral explanations for why predators eat toxic prey were tested: congenital preference, learned preference, and correlated response. The results demonstrate that a combination of congenital preference for and individual experience with *N. viridescens* elevates newts as a preferred food for *Th. sirtalis* in this population. Correlations between responses to newts and other common food types are not strong, suggesting that correlated responses do not explain the inclusion of toxic prey in the diet of eastern *Th. sirtalis*. Further, *N. viridescens*

appear to have a higher per gram nutrient quality than the more commonly eaten earthworms. Nutrient benefits received from consuming *N. viridescens* may explain, at least in part, why predators have not evolved avoidance of these toxic prey.

Congenital Response

The congenital preference hypothesis requires that an elevated positive response to a prey item is exhibited by naïve predators. In this study, *Th. sirtalis* exhibited a positive congenital response for *N. viridescens,* but on average the response was not as strong as to some other prey types tested. This result indicates that while *N. viridescens* are considered food by neonate *Th. sirtalis*, they may not be viewed as a preferred food type relative to other prey species.

The relatively weaker congenital response to newts compared to other prey is consistent with observations of wild snake diets in this population (Chapter 3). In the study population, *N. viridescens* make up about 3% of the observed diet of *Th. sirtalis,* while *P. cinereus* compose 20% and earthworms 62% of the diet (Chapter 3). However, the discrepancy between congenital response to newt and congenital response to *P. cinereus* is not as great as the observed diet suggests, and there was no significant difference in congenital responsiveness to newts and earthworms. This disparity between realized diet and chemosensory responsiveness of naïve predators may be due to a variety of factors such as prey abundance and ease of capture. For example, earthworms are terrestrial and occur in high densities at MLBS: 7.33 native earthworms/m^2 (Rearick et al.

2011) and 40.11 invasive earthworms/m^2 (Billak and Ransom, *unpublished*). *Plethodon cinereus* are exclusively terrestrial and are abundant at this site (~2.8/m2; Mathis 1991). *Notophthalmus viridescens*, while also very abundant (approx. 7600-12,700 individuals in Riopel Pond; Grayson & Wilbur 2009), are primarily found clumped in local ponds during the active season of *Th. sirtalis*. Because *Th. sirtalis* in this population do not appear to forage in water (personal observation), earthworms and *P. cinereus* may be the most abundantly encountered prey for *Th. sirtalis* and easier for *Th. sirtalis* to capture than adult *N. viridescens*.

An alternative explanation for the disparity between congenital response and observed diet is that exposure to TTX leads to rejection of individual prey. *Thamnophis sirtalis* are unable to detect TTX by chemoreception (Avila et al. 2012) but they do employ taste-rejection of toxic prey. In laboratory studies, Williams et al. (2003, 2010) found that while virtually all *Th. sirtalis* attack and begin to swallow newts, prey with higher TTX levels were behaviorally rejected by predators with lower resistance. Eastern *Th. sirtalis* may recognize *N. viridescens* as prey, attack, and attempt to consume them, but subsequently reject individual newts of relatively high toxicity. Based on the assessment of snake resistance and newt toxicity in this population (Chapter 2), behavioral rejection of newts is unlikely to occur for neonate *Th. sirtalis*. Neonate snakes should be able to consume all or most newts in their relative size class (newly metamorphosed efts) with little to no impairment (Brodie 1968a; Yotsu-Yamashita and Mebs 2003; Chapter 2). Similarly, adult snakes should be relatively unaffected by the

average sized eft or adult newt but may be somewhat more affected by the most toxic efts, though not lethally so. On the other hand, yearling and sub-adult *Th. sirtalis* are predicted to experience more severe effects of TTX intoxication if they attack toxic efts. If behavioral rejection were to explain the limited appearance of *N. viridescens* in the diet of *Th. sirtalis*, then it would likely be due to snake-newt interactions at the yearling and sub-adult stages.

Learned Preference

Although congenital prey preferences can predispose predators to attack and eat some potential prey and not others, such preferences are not necessarily fixed. An early favorable experience can lead to increased responsiveness to that prey item, thereby increasing the likelihood of feeding on it in the future. The increase in responsiveness to newt extract observed in snakes that fed on newts early in life suggests that learned preferences contribute to the inclusion of toxic prey in the diet of garter snakes. These results indicate that experience with any food type increased responsiveness to all prey species tested. However, snakes that experienced *N. viridescens* as prey exhibited increased responsiveness to newt extract more than snakes that experienced worm as prey (Figure 2). In addition, the greatest positive change in responsiveness to any prey extract was observed in snakes responding to *N. viridescens* after experience with this prey (Figure 3). These results contrast the findings of another study in which eastern *Th. sirtalis* from Michigan were fed a diet of either fish or worm and showed elevated

responsiveness to both prey stimuli regardless of specific experience (Lyman-Henley and Burghardt 1995). This difference in plasticity suggests that early experience with *N. viridescens* may be an especially important factor influencing diet of *Th. sirtalis* in this population.

The opportunity for a chance encounter between *Th. sirtalis* and *N. viridescens* early in life and during a critical period for *Th. sirtalis* is likely to be high. Neonate *Th. sirtalis* in this population emerge in August - early September and go into hibernation late October - early November (Chapter 3). Finding food during this short period when neonates are active is crucial for their winter survival (Gregory 1982; Bronikowski 2000). Newly metamorphosed *N. viridescens* emerge from local ponds onto land at this time of year (Aug – Sept, Grayson *unpublished*) and these small efts are a potentially abundant food resource for neonate *Th. sirtalis* (Arnold and Wassersug 1978; Drummond and Garcia 1989). In the MLBS population, I observed a neonate *Th. sirtalis* that had recently consumed a newly metamorphosed newt (Chapter 3), providing evidence that young *N. viridescens* are a relevant prey for neonate *Th. sirtalis*.

During this early exposure to *N. viridescens*, the potential for a negative experience resulting from TTX exposure is essentially non-existent for *Th. sirtalis*. *Notophthalmus viridescens* larvae contain much lower levels of TTX than efts or adults (0.0006-0.00361µg/mg; Brossman et al. 2014) and the amount of TTX may be at its lowest level when larvae undergo metamorphosis (Gall et al. 2011). Measures of young eft toxicity at MLBS confirm that they are an effectively non-toxic food for neonate *Th.*

sirtalis (Yotsu-Yamashita and Mebs 2003; Chapter 2). Without the negative effects associated with TTX, the benefits of consuming *N. viridescens* are more likely to outweigh any potential costs. This interaction between neonate snakes and newly metamorphosed efts may provide the early experience for *Th. sirtalis* that results in an elevated preference for toxic newt, thereby increasing the probability that a snake will eat a more functionally toxic newt later in life.

It is important to note that while these findings provide evidence for an elevated preference for *N. viridescens*, it is unknown how long the effect will last or if a single exposure to newt, rather than repeated experience, would have the same effect. One study on cuttlefish demonstrated a learned preference for alternative prey with a single early exposure (Darmaillacq et al. 2004), so it is possible that a single experience between neonate snake and newly metamorphosed eft may have lasting effects. Even though evidence supports some ontogenetic stability of congenital responses to prey (Arnold 1981c; Drummond and Burghardt 1983), the long-term stability of experienced responses has not been established. For example, in an Illinois population, Fuchs and Burghardt (1971) found that responsiveness to guppies was heightened in *Th. sirtalis* after being fed them as an initial diet, but responsiveness to guppies returned to original levels once their diet changed to a consistent regimen of redworms and instead responsiveness to redworm became elevated. On the other hand, snapping turtles continue to prefer the food on which they were raised after being fed an alternative diet (Burghardt and Hess 1966).

Further investigation into the extent of experience required and the stability of learned preference for prey is needed.

Correlated Response

If predators retain toxic prey in their diets as an indirect consequence of responding to other species, then we would expect to observe strong phenotypic and family level correlations between the congenital responses to *N. viridescens* and other quantitatively important prey. Moreover, correlations should persist after experience with prey, indicating that correlated responses are not labile over the life of a predator.

Although some correlation structure was observed among chemosensory responses of *Th. sirtalis* to different prey types, correlations were generally not strong or persistent enough to clearly explain inclusion of newts in the diet.

A phenotypic correlation between the congenital response to *N. viridescens* and another important food item, *P. cinereus*, was observed and this correlation persisted after experience with *N. viridescens* prey (but not after experience with *L. terrestris*). This correlation appears to have a genetic basis, as suggested by the family mean correlation (Arnold 1981a; Via 1984). In fact, the magnitude of the family mean correlation between responsiveness to *N. viridescens* and *P. cinereus* increased after experience (though the number of families represented was small). Together, these data appear to support the correlated response hypothesis. However, after being fed a diet of newts, responsiveness to *N. viridescens* increased dramatically (Figure 2) with no

accompanying increase in response to *P. cinereus*. In addition, no significant correlation in responsiveness to newt and *P. cinereus* was observed in the *L. terrestris* treatment group. Together, these results suggest that the correlations among chemosensory responses are somewhat labile and are unlikely to represent a strong behavioral constraint that couples feeding responses to newts and other prey over the life of an individual snake.

Nutrient Quality of Prey

Despite the greater mortality in the newt treatment group, *N. viridescens* appear to be a more nutritious food than the most common prey type, earthworms, for neonate *Th. sirtalis*. This effect may be due to earthworms having a higher per-gram water content than vertebrate prey (Scudder-Davis and Burghardt 1987) and having a digestive tract containing soil which is undigestible for a snake. Alternatively, the increased growth rate of snakes that consumed newts may be due to the relative nutritional benefits of vertebrates when compared to worms (Scudder-Davis and Burghardt 1987, Lyman-Henley and Burghardt 1995, Burghardt et al. 2000). Scudder-Davis and Burghardt (1987) found that snakes fed fish grew faster than worm-fed snakes even though worm-fed snakes consumed a greater a amount of total food. This effect has been attributed to the higher levels of calcium and phosphorus in vertebrate prey (Torrey 1971; Scudder-Davis and Burghardt 1987; Burghardt 1990; Lyman-Henley and Burghardt 1995) and was supported by experiments demonstrating that snakes fed worms supplemented with

calcium and phosphorus grew faster than snakes fed fish (Scudder-Davis and Burghardt 1987).

The nutritional benefit of eating a vertebrate such as *N. viridescens* may explain why congenital preferences have evolved for other salamanders and frogs in MLBS *Th. sirtalis*. The increase in responsiveness to *N. viridescens* after experiencing newts as prey seems to be contingent upon an individual's congenital preference for newt. These results suggest that if a snake is born with a positive congenital response to *N. viridescens* it will be more likely to eat it upon encounter, and that experience leads to a subsequent increase in responsiveness to newts. If consuming newts provides some fitness benefit to the snake (e.g., greater overwinter survival or decreased predation risk due to larger body size), then there may be a selective advantage to those snakes possessing higher congenital responses to *N. viridescens.* In addition, if some neonate *Th. sirtalis* persistently refuse *N. viridescens*, presumably because congenital response to this prey is lower, and no other food is available to them prior to hibernation, then this may be one mechanism of selection against *Th. sirtalis* who do not respond strongly to *N. viridescens*.

The nutritional benefit of *N. viridescens* may also be important to *Th. sirtalis* at critical periods later in life. In a three-year diet study, freshly consumed *N. viridescens* were only observed in the diets of female *Th. sirtalis*, and primarily in juvenile/adults that were either carrying young or that were likely to be entering their reproductive phase (Chapter 3). Reproduction is energetically expensive and viviparous *Th. sirtalis* tend to fast during the majority of their pregnancy. In addition, calcium (as well as a significant

proportion of water and other nutrients) are provisioned directly by the mother to the developing embryos (Stewart et al. 1990). The need to provide developing embryos with calcium and other nutrients may drive reproductively active females to take more nutritious prey, such as *N. viridescens*, when they are available. Adult *Th. sirtalis* in the MLBS population should be minimally affected by the amount of toxin present in adult newts (Chapter 2). The nutrient benefit received from consuming this vertebrate prey is, therefore, likely to outweigh the costs associated with the effects of the toxin itself (Brodie and Brodie 1999a).

Conclusion

The results of this work suggest that a combination of experience and congenital predisposition shape the predatory responses of *Th. sirtalis* to toxic *N. viridescens*. Snakes born with a congenital preference for *N. viridescens* are more likely to feed on newts early in life when the newts have very limited toxicity. This experience results in a learned preference for newts and may render some snakes more likely to prey on newts at a later age. The relatively high nutritional value of vertebrates such as newts further represents a selection pressure that could contribute to the maintenance of congenital preferences for this prey type.

Congenital responsiveness, learned preference, and nutritional benefit are factors contributing to non-avoidance of toxic prey for *Th. sirtalis*. Congenital responsiveness predisposes snakes to consume toxic prey upon encounter, learned preference may act to

maintain toxic prey in *Th. sirtalis* diet throughout its lifetime, and nutritional quality of *N. viridescens* may provide a selective advantage for snakes that consume them. *Thamnophis sirtalis* must, therefore, exhibit a level of resistance to TTX allowing them to prey upon *N. viridescens* in this population. The ability or inability to consume high quality food when they become available may be an indirect mechanism by which toxin resistance has evolved in this system.

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References

Arnold, S. J. 1977. Polymorphism and geographic variation in feeding-behavior of garter snake *Thamnophis elegans*. Science 197:676-678.

Arnold, S. J. 1978. Some effects of early experience on feeding responses in common garter snake, *Thamnophis sirtalis*. Animal Behaviour 26:455-462.

Arnold, S. J. 1981a. Behavioral variation in natural populations 1. Phenotypic, genetic and environmental correlations between chemoreceptive responses to prey in the garter snake, *Thamnophis elegans*. Evolution 35:489-509.

Arnold, S. J. 1981b. Behavioral variation in natural populations 2. The inheritance of a feeding response in crosses between geographic races of the garter snake, *Thamnophis elegans*. Evolution 35:510-515.

Arnold, S. J. 1981c. The microevolution of feeding behavior. Pp. 409-514 *in* A. C. Kamil, and T. D. Sargent, eds. Foraging Behavior: Ecological, Ethological, and Psychological Approaches. Garland Publishing, New York.

Arnold, S. J. and R. J. Wassersug. 1978. Differential predation on metamorphic anurans by garter snakes (*Thamnophis*): Social behavior as a possible defense. Ecology 59:1014-1022.

Aubret, F., G. M. Burghardt, S. Maumelat, X. Bonnet, and D. Bradshaw. 2006. Feeding preferences in 2 disjunct populations of tiger snakes, *Notechis scutatus* (Elapidae). Behavioral Ecology 17:716-725.

Avila, L. A., R. Wiggins, E. D. Brodie, Jr., E. D. Brodie III. 2012. Garter snakes do not respond to TTX via chemoreception. Chemoecology 22:263-268.

Ayres, F. A. and S. J. Arnold. 1983. Behavioral variation in natural-populations 4. Mendelian models and heritability of a feeding response in the garter snake, *Thamnophis elegans*. Heredity 51:405-413.

Brodie, E. D. III and E. D. Brodie, Jr. 1990. Tetrodotoxin resistance in garter snakes - an evolutionary response of predators to dangerous prey. Evolution 44:651-659.

Brodie, E. D. III and E. D. Brodie, Jr. 1999a. Predator-prey arms races. Bioscience 49:557-568.

Brodie, E. D. III and E. D. Brodie, Jr. 1999b. Costs of exploiting poisonous prey: Evolutionary trade-offs in a predator-prey arms race. Evolution 53:626-631.

Brodie, E. D., Jr. 1968a. Investigations on skin toxin of red-spotted newt *Notophthalmus viridescens viridescens*. American Midland Naturalist 80:276-280.

Brodie, E. D., Jr. 1968b. Investigations on the skin toxin of the adult rough-skinned newt, *Taricha granulosa*. Copeia 1968:307-313.

Brodie, E. D., Jr., B. J. Ridenhour, and E. D. Brodie III. 2002. The evolutionary response of predators to dangerous prey: Hotspots and coldspots in the geographic mosaic of coevolution between garter snakes and newts. Evolution 56:2067-2082.

Bronikowski, A. M. 2000. Experimental evidence for the adaptive evolution of growth rate in the garter snake *Thamnophis elegans*. Evolution 54:1760-1767.

Brossman, K. H., B. E. Carlson, A. N. Stokes, and T. Langkilde. 2014. Eastern newt (*Notophthalmus viridescens*) larvae alter morphological but not chemical defenses in response to predator cues. Canadian Journal of Zoology 92:279-283.

Burghardt, G. M. 1966. Stimulus control of the prey attack response in naïve garter snakes. Psychonomic Science 4:37-38.

Burghardt, G. M. 1967. Chemical-cue preferences of inexperienced snakes: Comparative aspects. Science 157:718-721.

Burghardt, G. M. 1969. Comparative prey-attack studies in newborn snakes of the genus *Thamnophis*. Behaviour 33:77-114.

Burghardt, G. M. 1970a. Chemical perception in reptiles. Pp. 241-308 *in* J. W. Johnston, D. G. Moulton, and A. Turk, eds. Advances in Chemoreception. Appleton-Century-Crofts, New York.

Burghardt, G. M. 1970b. Intraspecific geographical variation in chemical food cue preferences of newborn garter snakes (*Thamnophis sirtalis*). Behaviour 36:246-257.

Burghardt, G. M. 1973. Instinct and innate behavior: Toward an ethological psychology. Pp. 322-400 *in* J. A. Nevin, and G. S. Reynolds, eds. The Study of Behavior: Learning, Motivation, Emotion, and Instinct. Scott Foresman and Co., Glenview.

Burghardt, G. M. 1975. Chemical prey preference polymorphism in newborn garter snakes *Thamnophis sirtalis*. Behaviour 52:203-225.

Burghardt, G. M. 1990. Chemically mediated predation in vertebrates: Diversity, ontogeny, and information. Pp. 477-499 *in* D. McDonald, D. Miiller-Schwarze, and S. Natynczuk, eds. Chemical Signals in Vertebrates. Oxford University Press, Oxford, U.K.

Burghardt, G. M. 1992. Prior exposure to prey cues influences chemical prey preference and prey choice in neonatal garter snakes. Animal Behaviour 44:787-789.

Burghardt, G. M. 1993. The comparative imperative - genetics and ontogeny of chemoreceptive prey responses in natricine snakes. Brain Behavior and Evolution 41:138-146.

Burghardt, G. M. and J. P. Abeshahe. 1971. Responses to chemical stimuli of prey in newly hatched snakes of genus *Elaphe*. Animal Behaviour 19:486-.

Burghardt, G. M. and E. H. Hess. 1966. Food imprinting in the snapping turtle, *Chelydra serpentina*. Science 151:108-109.

Burghardt, G. M. and E. H. Hess. 1968. Factors influencing the chemical release of prey attack in newborn snakes. Journal of Comparative and Physiological Psychology 66:289-295.

Burghardt, G. M., D. G. Layne, and L. Konigsberg. 2000. The genetics of dietary experience in a restricted natural population. Psychological Science 11:69-72.

Burghardt, G. M. and C. H. Pruitt. 1975. Role of tongue and senses in feeding of naive and experienced garter snakes. Physiol. Behav. 14:185-194.

Burghardt, G. M., H. C. Wilcoxon, and J. A. Czaplicki. 1973. Conditioning in garter snakes: Aversion to palatable prey induced by delayed illness. Animal Learning & Behavior 1:317-320.

Cooper, W. E. 2007. Elevated tongue-flicking rate to cricket surface chemicals by the arthropodivorous rough green snake *Opheodrys aestivus*. Amphibia-Reptilia 28:413-417.

Cooper, W. E. and G. M. Burghardt. 1990. A comparative-analaysis of scoring methods for chemical-descrimination of prey by squamate reptiles. Journal of Chemical Ecology 16:45-65.

Cooper, W. E., G. M. Burghardt, and W. S. Brown. 2000. Behavioural responses by hatchling racers (*Coluber constrictor*) from two geographically distinct populations to chemical stimuli from potential prey and predators. Amphibia-Reptilia 21:103-115.

Cooper, W. E. and S. Secor. 2007. Strong response to anuran chemical cues by an extreme dietary specialist, the eastern hog-nosed snake (*Heterodon platirhinos*). Canadian Journal of Zoology 85:619-625.

Daly, J. W., H. Martin Garraffo, G. S. E. Hall, and J. F. Cover Jr. 1997. Absence of skin alkaloids in captive-raised Madagascan mantelline frogs (*Mantella*) and sequestration of dietary alkaloids. Toxicon 35:1131-1135.

Darmaillacq, A.-S., R. Chichery, R. Poirier, and L. Dickel. 2004. Effect of early feeding experience on subsequent prey preference by cuttlefish, *Sepia officinalis*. Developmental Psychobiology 45:239-244.

Demott, W. R., Q. X. Zhang, and W. W. Carmichael. 1991. Effects of toxic cyanobacteria and purified toxins on the survival and feeding of a copepod and 3 species of daphnia. Limnol. Oceanogr. 36:1346-1357.

Després, L., J.-P. David, and C. Gallet. 2007. The evolutionary ecology of insect resistance to plant chemicals. Trends in Ecology & Evolution 22:298-307.

Drummond, H. and G. M. Burghardt. 1983. Geographic-variation in the foraging behavior of the garter snake, *Thamnophis elegans*. Behavioral Ecology and Sociobiology 12:43-48.

Drummond, H. and C. M. Garcia. 1989. Limitations of a generalist: A field comparison of foraging snakes. Behaviour 108:23-43.

Dumbacher, J. P., A. Wako, S. R. Derrickson, A. Samuelson, T. F. Spande, and J. W. Daly. 2004. Melyrid beetles (*Choresine*): A putative source for the batrachotoxin alkaloids found in poison-dart frogs and toxic passerine birds. Proceedings of the National Academy of Sciences of the United States of America 101:15857-15860.

Falconer, D. S. and F. C. Mackay. 1996. Introduction to Quantitative Genetics, Ed. 4. Longmans Green, Harlow, Essex, UK.

Fisker, E. N. and S. Toft. 2004. Effects of chronic exposure to a toxic prey in a generalist predator. Physiological Entomology 29:129-138.

Freeland, W. J. and D. H. Janzen. 1974. Strategies in herbivory by mammals - role of plant secondary compounds. American Naturalist 108:269-289.

Fuchs, J. L. and G. M. Burghardt. 1971. Effects of early feeding experience on the responses of garter snakes to food chemicals. Learning and Motivation 2:271-279.

Gall, B. G., A. N. Stokes, S. S. French, E. A. Schlepphorst, E. D. Brodie, III, and E. D. Brodie, Jr. 2011. Tetrodotoxin levels in larval and metamorphosed newts (*Taricha granulosa*) and palatability to predatory dragonflies. Toxicon 57:978-983.

Greene, M. J., S. L. Stark, and R. T. Mason. 2002. Predatory response of brown tree snakes to chemical stimuli from human skin. Journal of Chemical Ecology 28:2465-2473.

Gregory, P. T. 1982. Reptilian hibernation. Pp. 53-154 *in* C. Gans, and F. H. Pough, eds. Biology of the Reptilia. Academic Press, New York.

Hess, E. H. 1962. Imprinting and the 'critical period' concept. Pp. 254–267 *in* E. L. Bliss, ed. Roots of Behavior: Genetics, Instinct, and Socialization in Animal Behavior. Harper, New York.

Kao, C. Y. 1966. Tetrodotoxin, saxitoxin, and their significance in the study of excitation phenomena. Pharmacological Review 18:997-1049.

Kephart, D. G. 1982. Microgeographic variation in the diets of garter snakes. Oecologia 52:287-291.

Kephart, D. G. and S. J. Arnold. 1982. Garter snake diets in a fluctuating environment - a 7-year study. Ecology 63:1232-1236.

Lipkind, G. M. and H. A. Fozzard. 1994. A structural model of the tetrodotoxin and saxitoxin binding site of the Na+ channel. Biophysical Journal 66:1-13.

Llewelyn, J., L. Schwarzkopf, R. Alford, and R. Shine. 2010. Something different for dinner? Responses of a native Australian predator (the keelback snake) to an invasive prey species (the cane toad). Biological Invasions 12:1045-1051.

Llewelyn, J. S., B. L. Phillips, and R. Shine. 2009. Sublethal costs associated with the consumption of toxic prey by snakes. Austral Ecology 34:179-184.

Lyman-Henley, L. P. and G. M. Burghardt. 1995. Diet, litter, and sex effects on chemical prey preference, growth, and site selection in two sympatric species of *Thamnophis*. Herpetological Monographs 9:140-160.

Mathis, A. 1991. Territories of male and female terrestrial salamanders: Costs, benefits, and intersexual spatial associations. Oecologia 86:433-440.

Mori, A. and H. Moriguchi. 1988. Food habits of snakes in Japan: A critical review. Snake 20:98-113.

Mushinsky, H. R. 1987. Foraging ecology. Pp. 302-334 *in* R. A. Seigel, J. T. Collins, and S. S. Novak, eds. Snakes: Ecology and Evolutionary Biology. McGraw-Hill Publishing Co, New York, NY.

Narahashi, T., M. J.W., and R. N. Poston. 1967. Tetrodotoxin derivatives: Chemical structure and blockage of nerve membrane conductance. Science 156.

Nussbaum, R. A., E. D. Brodie, Jr., and R. M. Storm. 1983. Amphibians and Reptiles of the Pacific Northwest. The University Press of Idaho, Moscow, ID.

Paradise, C. J. and N. E. Stamp. 1993. Episodes of unpalatable prey reduce consumption and growth of juvenile praying mantids. Journal of Insect Behavior 6:155-166.

Punzo, F. 2002. Food imprinting and subsequent prey preference in the lynx spider, *Oxyopes salticus* (Araneae: Oxyopidae). Behavioural Processes 58:177-181.

Rearick, D., L. Kintz, K. L. Burke, and T. S. Ransom. 2011. Effects of white-tailed deer on the native earthworm, *Eisenoides carolinensis*, in the southern Appalachian Mountains, USA. Pedobiologia 54, Supplement:S173-S180.

Robbins, T., N. Freidenfelds, and T. Langkilde. 2013. Native predator eats invasive toxic prey: Evidence for increased incidence of consumption rather than aversion-learning. Biological Invasions 15:407-415.

Roff, D. A. and R. Preziosi. 1994. The estimation of the genetic correlation: The use of the jackknife. Heredity 73:544-548.

Rossman, D., N. Ford, and R. Seigel. 1996. The Garter Snakes: Evolution and Ecology. University of Oklahoma Press, Norman, OK.

Scudder-Davis, R. M. and G. M. Burghardt. 1987. Diet and growth in juveniles of the garter snakes *Thamnophis sirtalis infernalis* and *Thamnophis radix radix*. Growth 51:74-85.

Sheffield, L. P., J. M. Law, and G. M. Burghard. 1968. On the nature of chemical food sign stimuli for newborn garter snakes. Communications in Behavioral Biology 2:7-12.

Shepard, D. B., C. A. Phillips, M. J. Dreslik, and B. C. Jellen. 2004. Prey preference and diet of neonate eastern massasaugas (*Sistrurus c. catenatus*). American Midland Naturalist 152:360-368.

Speed, M. P. and G. D. Ruxton. 2014. Ecological pharmacodynamics: Prey toxin evolution depends on the physiological characteristics of predators. Animal Behaviour 98:53-67.

Stebbins, R. C. 2003. A Field Guide to Western Reptiles and Amphibians. Houghton Mifflin Co., New York, NY.

Stewart, J. R., D. G. Blackburn, D. C. Baxter, and L. H. Hoffman. 1990. Nutritional provision to embryos in a predominantly lecithotrophic placental reptile, *Thamnophis ordinoides* (Squamata: Serpentes). Physiological Zoology 63:722-734.

Torrey, T. W. 1971. Morphogenesis of the Vertebrates. John Wiley and Sons, Inc., New York.

Via, S. 1984. The quantitative genetics of polyphagy in an insect herbivore. II. Genetic correlations in larval performance within and among host plants. Evolution 38:896-905.

Weiser, L. A. and N. E. Stamp. 1998. Combined effects of allelochemicals, prey availability, and supplemental plant material on growth of a generalist insect predator. Entomol. Exp. Appl. 87:181-189.

Williams, B. L., E. D. Brodie, Jr., and E. D. Brodie III. 2003. Coevolution of deadly toxins and predator resistance: Self-assessment of resistance by garter snakes leads to behavioral rejection of toxic newt prey. Herpetologica 59:155-163.

Williams, B. L., C. T. Hanifin, E. D. Brodie, Jr., and E. D. Brodie III. 2010. Tetrodotoxin affects survival probability of rough-skinned newts (*Taricha granulosa*) faced with TTX-resistant garter snake predators (*Thamnophis sirtalis*). Chemoecology 20:285-290.

Yotsu-Yamashita, M. and D. Mebs. 2003. Occurrence of 11-oxotetrodotoxin in the red-spotted newt, *Notophthalmus viridescens*, and further studies on the levels of tetrodotoxin and its analogues in the newt's efts. Toxicon 41:893-897.

Table 1: Estimated repeatabilities for prey recognition responses to each extract presented. (A) neonate congenital responses in 2009-2010 and (B) experienced responses in 2010.

A. Congenital Responses

Prey Extract	Intraclass Correlation Coefficient	*df*	*p*
Control (dH_2O)	0.14	194	0.026
L. terrestris	0.54	194	< 0.001
N. viridescens	0.49	194	< 0.001
P. cinereus	0.42	194	< 0.001
P. crucifer	0.43	194	< 0.001
L. catesbeianus	0.48	194	< 0.001

B. Experienced Responses

Prey Extract	Intraclass Correlation Coefficient	*df*	*p*
Control (dH_2O)	0.65	40	< 0.001
L. terrestris	0.42	40	0.003
N. viridescens	0.58	40	< 0.001
P. cinereus	0.68	40	< 0.001
P. crucifer	0.45	40	0.001
L. catesbeianus	-0.029	40	0.571

Table 2: Models describing individual differences in response to prey extracts from the linear mixed model analyses. Models describing (A) congenital responses to prey extracts (CongTFS), (B) experienced responses to prey extracts (ExpTFS) for snakes in the worm treatment, (C) experienced responses to prey extracts (ExpTFS) for snakes in the newt treatment, (D) change in responsiveness (ΔTFS) for snakes in the worm treatment, and (E) change in responsiveness (ΔTFS) for snakes in the newt treatment. Main effects and significant interaction terms are listed.

A. CongTFS				
Model Terms	*F*	Num *df*	Den *df*	*p*
Prey	146.354	5	2034	< 0.001
Family	1.294	30	173	0.156
Sex	1.514	1	173	0.220
Trial	0.054	1	2057	0.815
Order	1.598	5	2035	0.157
Family * Prey	2.866	150	2034	< 0.001

B. ExpTFS: Newt Treatment				
Model Terms	*F*	Num *df*	Den *df*	*p*
Prey	46.427	5	165	< 0.001
Sex	1.272	1	14	0.278
Trial	0.115	1	165	0.735
Order	1.997	5	165	0.082

C. ExpTFS: Worm Treatment				
Model Terms	*F*	Num *df*	Den *df*	*p*
Prey	46.427	5	165	< 0.001
Sex	1.272	1	14	0.278
Trial	0.115	1	165	0.735
Order	1.997	5	165	0.082

D. ΔTFS: Newt Treatment				
Model Terms	*F*	Num *df*	Den *df*	*p*
Prey	3.839	5	14	0.004
Sex	0.72	1	14	0.410

E. ΔTFS: Worm Treatment				
Model Terms	*F*	Num *df*	Den *df*	*p*
Prey	5.234	5	145	< 0.001
Sex	2.716	1	28	0.111

Table 3: Correlations of congenital prey recognition responses to prey extracts and water control. Correlations for individuals (N = 205) are above the diagonal and for families (N = 31) are below the diagonal for 2009-2010 seasons. Pearson correlation statistic (r) and two-tailed significance value (p) are given for significant correlations. Non-significant correlations are noted with "*ns*".

Family \ Individual		Control	*L. terrestris*	*N. viridescens*	*P. cinereus*	*P. crucifer*	*L. catesbeianus*
Control	*r*		0.244	0.173	0.288	0.219	0.302
	p		< 0.001	0.013	< 0.001	0.002	< 0.001
L. terrestris	*r*	-0.045		0.336	0.477	0.379	0.416
	p	*ns*		< 0.001	< 0.001	< 0.001	< 0.001
N. viridescens	*r*	0.296	0.409		0.437	0.446	0.383
	p	*ns*	0.022		< 0.001	< 0.001	< 0.001
P. cinereus	*r*	0.519	0.335	0.597		0.476	0.465
	p	0.003	*ns*	< 0.001		< 0.001	< 0.001
P. crucifer	*r*	0.038	0.352	0.455	0.025		0.284
	p	*ns*	*ns*	0.010	*ns*		< 0.001
L. catesbeianus	*r*	0.244	0.113	0.442	0.455	0.348	
	p	*ns*	*ns*	0.013	0.010	*ns*	

Table 4: Correlations of experienced prey recognition responses to prey extracts and water control for all *Th. sirtalis* tested after diet treatment. Correlations for individuals (N = 46) are above the diagonal and for families (N = 10) are below the diagonal for the 2010 season. Pearson correlation statistic (r) and two-tailed significance value (p) are given for significant correlations. Non-significant correlations are noted with "*ns*".

Family \ Individual		Control	*L. terrestris*	*N. viridescens*	*P. cinereus*	*P. crucifer*	*L. catesbeianus*
Control	*r*		0.199	0.217	0.221	0.199	0.234
	p		*ns*	*ns*	*ns*	*ns*	*ns*
L. terrestris	*r*	0.552		0.128	0.194	0.392	0.169
	p	*ns*		*ns*	*ns*	0.007	*ns*
N. viridescens	*r*	0.293	0.515		0.304	0.280	0.496
	p	*ns*	*ns*		0.040	*ns*	< 0.001
P. cinereus	*r*	0.198	0.197	0.738		0.588	0.526
	p	*ns*	*ns*	0.015		< 0.001	< 0.001
P. crucifer	*r*	0.485	0.466	0.641	0.488		0.272
	p	*ns*	*ns*	0.046	*ns*		*ns*
L. catesbeianus	*r*	0.008	0.291	0.410	0.652	0.127	
	p	*ns*	*ns*	*ns*	0.041	*ns*	

Table 5: Correlations of experienced prey recognition responses to prey extracts and water control for *Th. sirtalis* fed newt diet (above the diagonal; $N = 16$) and worm diet (below the diagonal; $N = 30$) in the 2010 season. Pearson correlation statistic (r) and two-tailed significance value (p) are given for significant correlations. Non-significant correlations are noted with "*ns*".

Worm \ Newt		Control	*L. terrestris*	*N. viridescens*	*P. cinereus*	*P. crucifer*	*L. catesbeianus*
Control	*r*		0.206	0.178	0.247	0.243	0.232
	p		*ns*	*ns*	*ns*	*ns*	*ns*
L. terrestris	*r*	0.264		0.670	0.435	0.690	0.609
	p	*ns*		0.004	*ns*	0.003	0.012
N. viridescens	*r*	0.277	0.110		0.726	0.428	0.944
	p	*ns*	*ns*		0.001	*ns*	< 0.001
P. cinereus	*r*	0.257	-0.071	0.340		0.599	0.835
	p	*ns*	*ns*	*ns*		0.014	< 0.001
P. crucifer	*r*	0.193	0.065	0.359	0.579		0.418
	p	*ns*	*ns*	*ns*	0.001		*ns*
L. catesbeianus	*r*	0.267	0.020	0.400	0.496	0.269	
	p	*ns*	*ns*	0.029	0.005	*ns*	

Table 6: Correlations of the change in prey recognition responses to prey extracts and water control for *Th. sirtalis* fed newt diet (above the diagonal; $N = 16$) and worm diet (below the diagonal; $N = 30$) in the 2010 season. Pearson correlation statistic (r) and two-tailed significance value (p) are given for significant correlations. Non-significant correlations are noted with "*ns*".

Worm \ Newt		Control	*L. terrestris*	*N. viridescens*	*P. cinereus*	*P. crucifer*	*L. catesbeianus*
Control	*r*		0.104	-0.285	0.018	-0.116	0.036
	p		*ns*	*ns*	*ns*	*ns*	*ns*
L. terrestris	*r*	-0.050		-0.055	0.099	0.470	-0.049
	p	*ns*		*ns*	*ns*	*ns*	*ns*
N. viridescens	*r*	0.155	0.102		0.317	0.088	0.096
	p	*ns*	*ns*		*ns*	*ns*	*ns*
P. cinereus	*r*	0.043	0.347	0.117		0.172	0.122
	p	*ns*	*ns*	*ns*		*ns*	*ns*
P. crucifer	*r*	-0.113	0.098	0.050	0.260		-0.290
	p	*ns*	*ns*	*ns*	*ns*		*ns*
L. catesbeianus	*r*	0.045	0.095	-0.085	0.182	-0.072	
	p	*ns*	*ns*	*ns*	*ns*	*ns*	

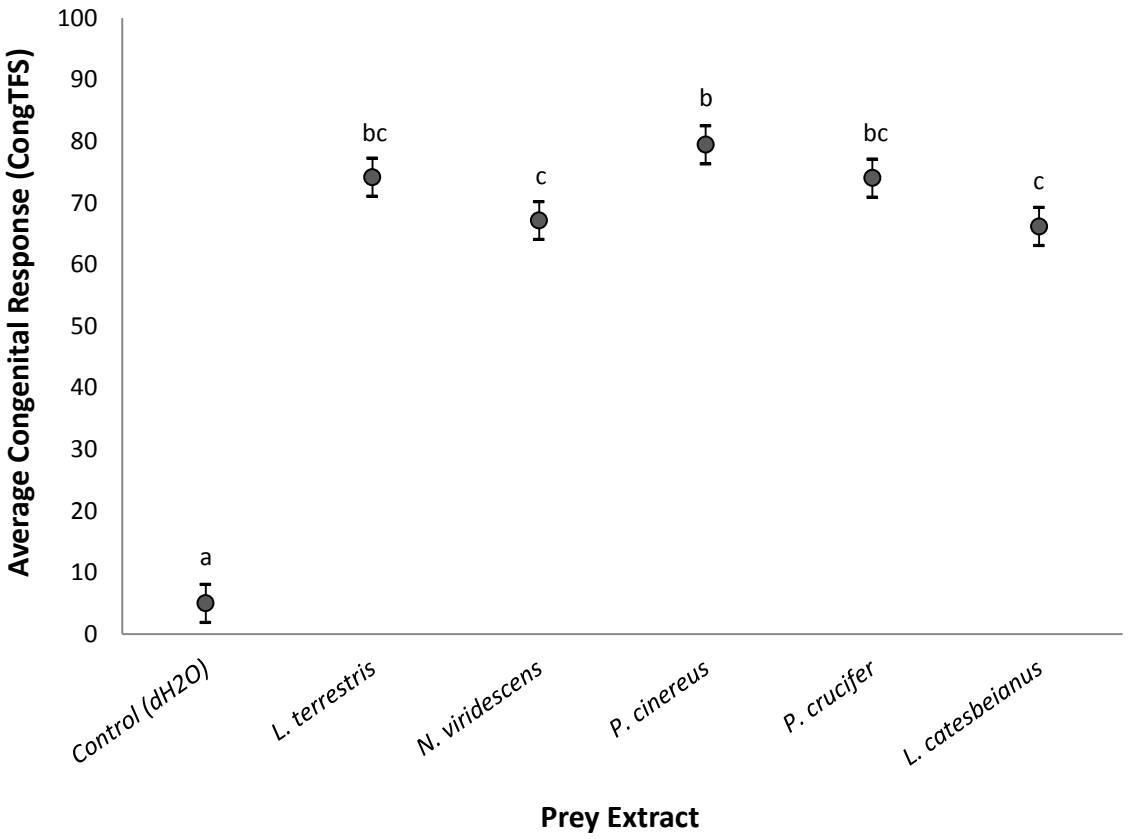

Figure 1. Mean congenital responses to prey presented to neonate *Th. sirtalis* in combined years, 2009 and 2010. Responses, measured as tongue-flick score (TFS) for five prey species extracts and a water control, are plotted as estimated marginal means (circles) ± the standard error (whiskers). Letters (a, b, and c) indicate significantly similar groups of responses. Food naïve *Th. sirtalis* exhibited a positive congenital response for *N. viridescens,* but on average the response was not as strong as to some other prey types tested.

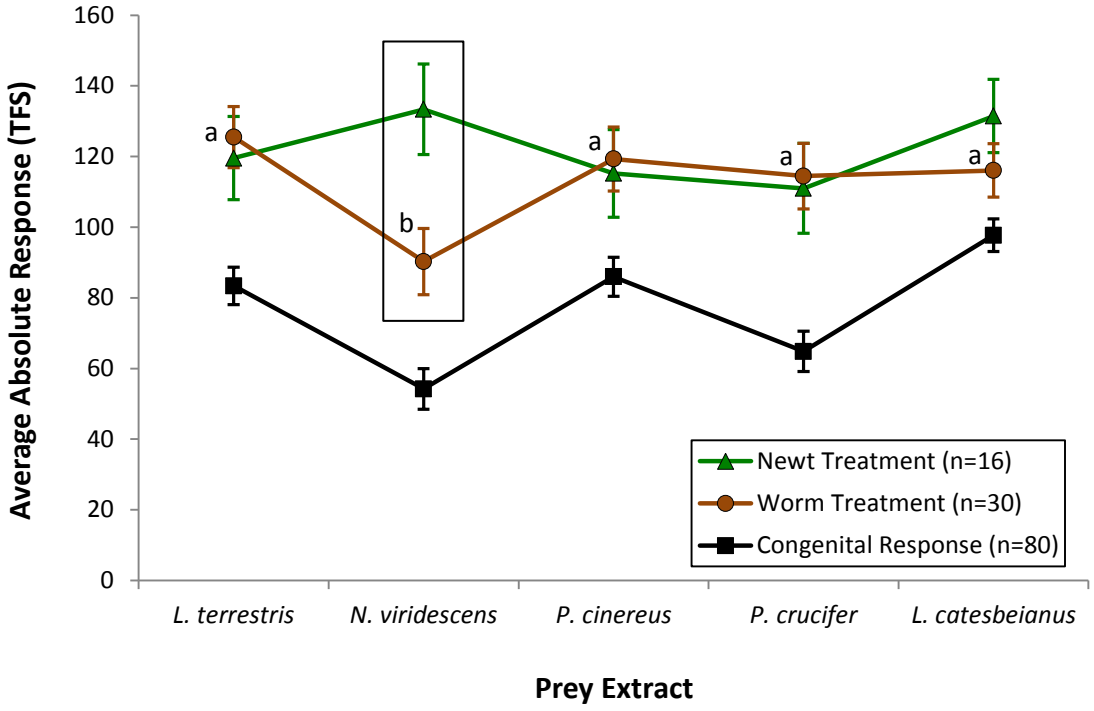

Figure 2. Prey response profiles of MLBS *Th. sirtalis* during the 2010 season. Average responses are plotted for each prey type tested with the congenital profile plotted in black squares, the food-experienced profile for snakes in the worm treatment plotted in brown circles, and the food-experienced profile for snakes in the newt treatment plotted in green triangles. Whiskers show ± standard error. Lines are for visualization of response profiles only and do not reflect functions relating the points to each other. Experienced responses to *N. viridescens* among treatment groups significantly differed (black rectangle). Within the worm treatment, letters a and b indicate significantly similar responses to prey types. No significant differences among experienced responses to prey types were observed in the newt treatment group outside of the water control.

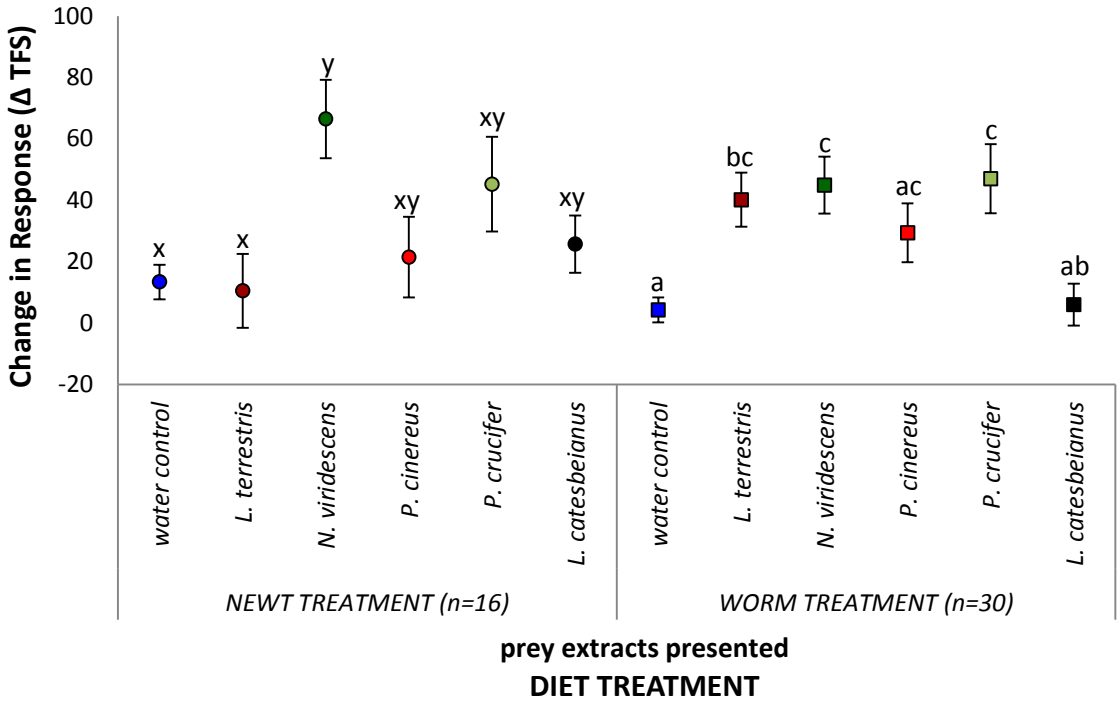

Figure 3. Change in prey recognition responses to prey extracts for individuals in the newt diet treatment (circles) and individuals in the worm diet treatment (squares). Change in prey recognition response (ΔTFS) = food experienced response – congenital response; zero values indicate no change in response. Plot based on estimated marginal means and standard errors from MANOVA. Significantly similar differences among mean responses to prey extracts within a food group are noted by letters x, y (newt treatment) and a, b, c (worm treatment). Colors indicate the type of prey extract presented. In the newt treatment, only change in responsiveness toward *N. viridescens* was significantly greater than change in responsiveness to the control.

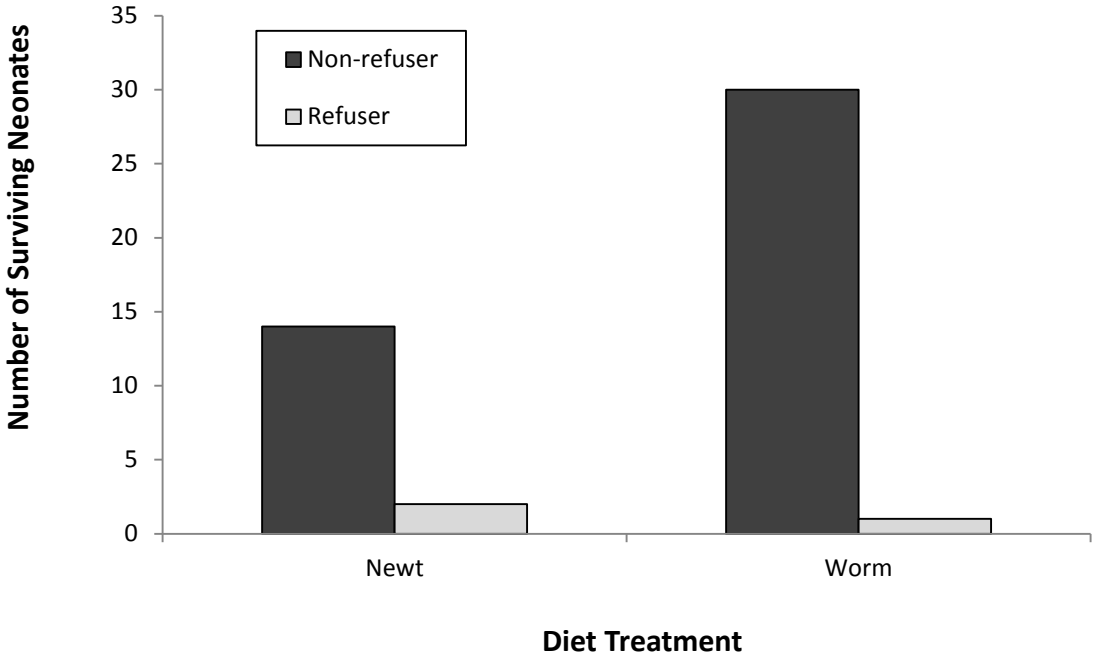

Figure 4. Numbers of surviving food refuser and non-refuser neonate *Th. sirtalis* in each of the two diet treatments, those fed newt and those fed worm. Surviving non-refusers are shown in dark gray and surviving persistent refusers in light gray.

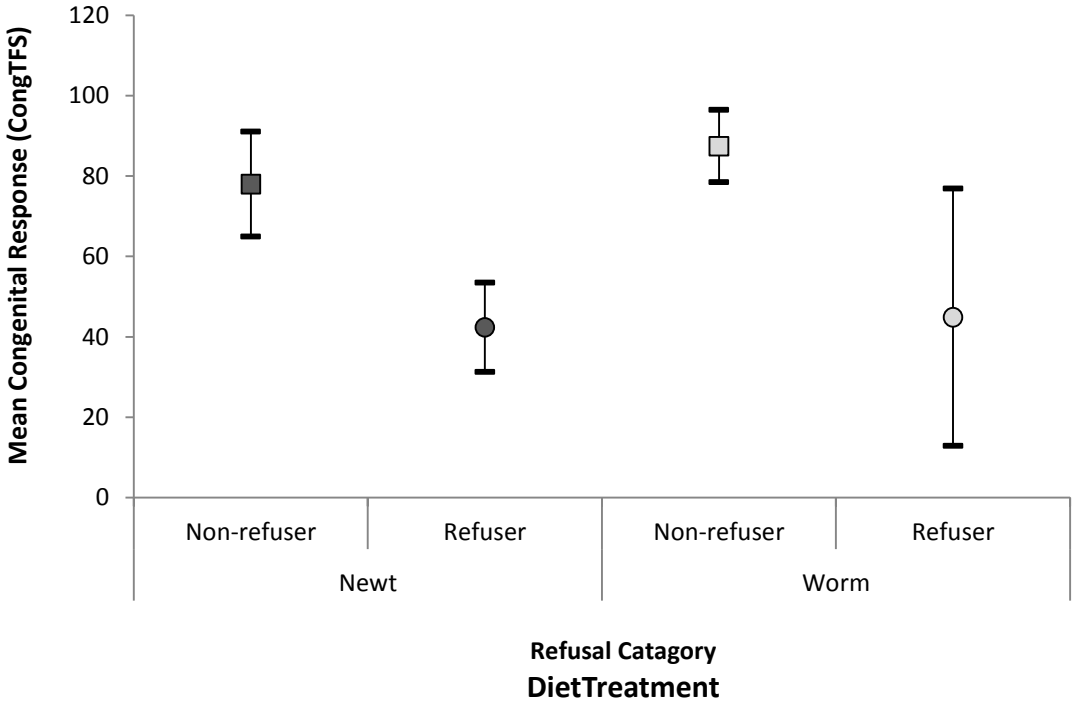

Figure 5. Mean congenital response of food refuser and non-refuser neonate *Th. sirtalis*. Mean congenital tongue-flick scores of neonates in each of the two food groups to the food they were offered are plotted: response to *N. viridescens* for neonates in the newt treatment group (dark gray) and response to *L. terrestris* for neonates in the worm treatment group (light gray). Mean response of non-refusers plotted as squares and mean response of refusers plotted as circles.

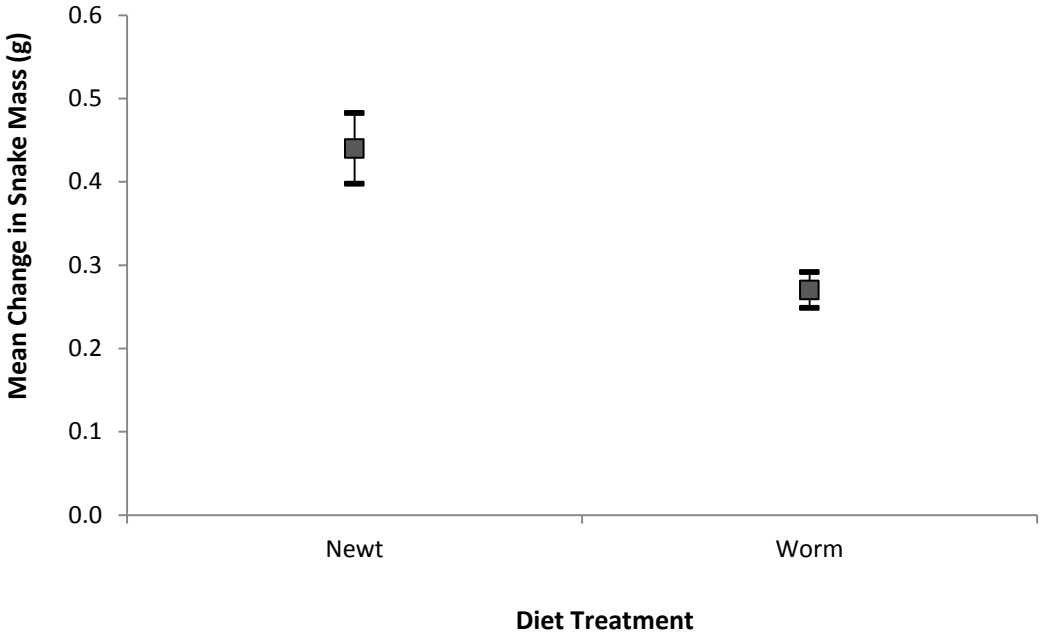

Figure 6. Mean change in mass (grams) for neonate *Th. sirtalis* fed either newt or worm is plotted ± the standard error. Mean mass gained by neonates in the worm treatment = 0.27g, SE = 0.04, *N* = 31. Mean mass gained by neonates in the newt treatment = 0.44g, SE = 0.02, *N* = 16.

Appendix 1: Data collected by Stokes, A., L. Sacco, E.D. Brodie III (*unpublished*). Quantification of whole animal TTX (in milligrams) in adult (A) and eft (E) *Notophthalmus viridescens viridescens* collected from Mountain Lake Biological Station, Giles County, Virgina, June 2013.

Samples	mass (g)	Whole Newt TTX (mg)
A1	2.42	0.0362
A2	2.28	0.0167
A4	1.7	0.0149
A5	1.86	0.0612
A6	1.95	0.0513
A8	2.41	0.0167
A9	2.03	0.0279
A10	2.51	0.0385
A11	1.79	0.0171
A12	1.77	0.0293
A13	1.84	0.0136
A15	1.67	0.0373
A17	2.17	0.0081
A19	2.58	0.0072
A20	2.79	0.0158
A21	1.49	0.0320
A23	1.91	0.0573
A24	1.9	0.0090
A25	1.58	0.0100
A26	1.83	0.0761
A28	1.73	0.0057
A29	2.13	0.0204
A30	1.86	0.0356
A31	2.47	0.0121
A32	2.45	0.0056
A33	2.57	0.0440
A34	1.94	0.0375
A35	2.95	0.0111
A36	1.83	0.0166
A37	2.14	0.0463
A38	1.75	0.0722
A39	1.99	0.0122
A40	1.87	0.0206

Samples	mass (g)	Whole Newt TTX (mg)
E1	0.73	0.0027
E2	1.82	0.0757
E3	1.69	0.0127
E4	0.22	0.0001
E6	2.38	0.0628
E7	1.88	0.0270
E8	1.3	0.0435
E9	1.66	0.0691
E10	0.24	0.0018
E11	1.15	0.0583
E12	1.62	0.0741
E13	1.03	0.0438
E14	1.03	0.0575
E15	0.26	0.0073
E16	0.33	0.0014
E17	1.8	0.0956
E18	0.38	0.0061
E19	1.41	0.0478
E20	1.41	0.0907
E21	1.99	0.0155
E22	1.2	0.0221
E23	0.86	0.0133
E24	2.67	0.2175
E25	0.93	0.0126
E26	2.13	0.0234
E27	1.76	0.1720
E28	1.94	0.0749
E30	1.77	0.0129
E31	1.77	0.3275
E33	2.32	0.3533
E34	2.25	0.0329
E35	0.77	0.0067
E36	1.98	0.1242
E39	1.68	0.0494
E40	1.76	0.2394
E41	2.46	0.0867
E42	1.65	0.0060
E43	0.97	0.0451
E44	1.92	0.0557
E45	2.23	0.0527

Samples	mass (g)	Whole Newt TTX (mg)
E46	1.34	0.0961
E47	0.99	0.0482
E48	0.88	0.0404
E49	3.23	0.0979
E50	2.36	0.0104
E51	1.89	0.0170
E52	2.91	0.2660
E53	2.01	0.0131
E55	2.14	0.0073
E56	1.96	0.0699
E57	2.02	0.0278

Appendix 2: Subjects sampled for sequencing of the $Na_v1.4$ sodium channel gene (*SCN4A*) and their individual resistance to TTX. For each subject identification number (ID) and individual resistance (MAMU) given as the mass adjusted dose that will reduce locomotor speed by 50%. Colors denote resistance levels as per Brodie et al. (2002; see legend).

ID	MAMU
HH 636	1.47
HH 637	2.36
JJ 666	2.77
FF596	3.15
BB572	3.69
HH643	4.49
AA554	4.85
II 647	4.88
FF598	5.03
KK 673	5.13
II 653	6.26
SC691	7.01
AA553	7.61
GG 625	8.88
JJ 663	9.75
DD579	11.19
AA548	12.99
BB563	14.41
CC576	15.03
FF597	15.50
E272	16.44
BB561	18.19
JJ 658	20.57
CC578	21.85
FF599	22.69
DD588	25.15
DD583	27.60
017-885-270	33.01
DD582	40.33
FF595	41.78
GG 619	43.83
KK 674	72.67

MAMU's
<4
4-5
5-10
10-15
15-25
25-35
35-90
>90

Appendix 3: $Na_v1.4$ sodium channel gene (*SCN4A*) sequences obtained from GeneBank for comparison.

Species	Location	Gene Region	Accession #	Citation
Th. sirtalis	Bear Lake Co., ID	complete cds	AY851743	Geffeney et al. 2005
Th. sirtalis	Benton Co., OR	complete cds	AY851744	Geffeney et al. 2005
Th. sirtalis	Warrenton (Clatsop Co.), OR	complete cds	AY851745	Geffeney et al. 2005
Th. sirtalis	Willow Creek (Sonoma Co.), CA	complete cds	AY851746	Geffeney et al. 2005
Th. couchii	Cold Springs Creek (Tulare Co.), CA	partial cds - exons 8-9 (DI)	FJ570828	Feldman et al. 2009
Th. couchii	Cold Springs Creek (Tulare Co.), CA	partial cds - exon 13 (DII)	FJ570879	Feldman et al. 2009
Th. couchii	Cold Springs Creek (Tulare Co.), CA	partial cds - exon 19 (DIIIa)	FJ570930	Feldman et al. 2009
Th. couchii	Cold Springs Creek (Tulare Co.), CA	partial cds - exon 20 (DIIIb)	FJ570978	Feldman et al. 2009
Th. couchii	Cold Springs Creek (Tulare Co.), CA	partial cds - exon 24 (DIV)	FJ571029	Feldman et al. 2009
Th. atratus	Molino Creek (Santa Cruz Co.), CA	partial cds - exons 8-9 (DI)	FJ570821	Feldman et al. 2009
Th. atratus	Molino Creek (Santa Cruz Co.), CA	partial cds - exon 13 (DII)	FJ570872	Feldman et al. 2009
Th. atratus	Molino Creek (Santa Cruz Co.), CA	partial cds - exon 19 (DIIIa)	FJ570923	Feldman et al. 2009
Th. atratus	Molino Creek (Santa Cruz Co.), CA	partial cds - exon 20 (DIIIb)	FJ570971	Feldman et al. 2009
Th. atratus	Molino Creek (Santa Cruz Co.), CA	partial cds - exon 24 (DIV)	FJ571022	Feldman et al. 2009
Th. elegans	Nacimiento Creek (Monterey Co.), CA	partial cds - exons 8-9 (DI)	FJ570840	Feldman et al. 2009
Th. elegans	Nacimiento Creek (Monterey Co.), CA	partial cds - exon 13 (DII)	FJ570891	Feldman et al. 2009
Th. elegans	Nacimiento Creek (Monterey Co.), CA	partial cds - exon 19 (DIIIa)	FJ570941	Feldman et al. 2009
Th. elegans	Nacimiento Creek (Monterey Co.), CA	partial cds - exon 20 (DIIIb)	FJ570990	Feldman et al. 2009
Th. elegans	Nacimiento Creek (Monterey Co.), CA	partial cds - exon 24 (DIV)	FJ571041	Feldman et al. 2009
Homo sapiens		complete cds	NM_000334	George et al. 1992