TRANSMISSIBILITY AND LOCALIZATION OF TETRODOTOXIN IN THE ROUGH-SKINNED NEWT, *TARICHA GRANULOSA*

By

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ABSTRACT

TRANSMISSIBILITY AND LOCALIZATION OF TETRODOTOXIN IN THE ROUGH-SKINNED NEWT, *TARICHA GRANULOSA*

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Tetrodotoxin (TTX) is a powerful neurotoxin that prevents the propagation of action potentials, leading to paralysis and sometimes death in nearly all animals. However, a diverse group of marine and freshwater animals possess TTX, which they use for offense, defense, and communication. One of most studied TTX-mediated interactions is the predator-prey arms race between the rough-skinned newt (*Taricha granulosa*) and common garter snake (*Thamnophis sirtalis*). Variation in toxicity among populations of newts matched by TTX-resistance in predatory snakes has captured the focus of much research centered on the hypothesis that the arms race is the sole driver of variation. Nevertheless, recent studies suggest a more complex dynamic. Explanations of the dramatic variation in TTX among different populations of newts can only be constructed once fundamental questions about the origin, function, and transmission of TTX in newts have been more thoroughly explored. In this study, I took two approaches to address the origin, function, and transmission of TTX: 1) a cohabitation experiment in which I paired toxic and non-toxic newts to test whether toxicity can be acquired through contact, and 2) an experiment to determine the distribution and concentration of TTX in different tissues. The cohabitation experiment revealed no detectable change in the toxicity of non-toxic male newts, suggesting that a physical or physiological impediment prevents non-toxic newts from becoming toxic. The tissue toxicity experiment demonstrated that TTX is present throughout the body in structurally and functionally diverse tissues, which has many implications for the involvement of TTX in communication and reproduction in addition to defense.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

LC-MS/MS	Liquid chromatography-tandem mass spectrometry
Na_v	Voltage-gated sodium channel
PA	Pyrrolizidine alkaloids
SVL	Snout-vent length
TTX	Tetrodotoxin

INTRODUCTION

Tetrodotoxin (TTX) is a highly potent neurotoxin that binds to voltage-gated sodium channels (Na_vs), preventing the generation of action potentials (Geffeney et al., 2002; Lee and Ruben, 2008). Most animals are susceptible to the effects of TTX even at very low concentrations (e.g., the LD_{50} in humans is 10.2µg/kg) (Hanifin, 2010); however, a diverse group of marine and freshwater animals including some Platyhelminthes, gastropods, cephalopods, annelids, echinoderms, teleosts, and amphibians have evolved adaptations that enable them to possess TTX and, in some instances, consume toxic prey (Miyazawa and Noguchi, 2001; Moczydlowski, 2013; Noguchi, et al., 2006). In marine species, TTX appears to originate as a bacterially-produced toxin that accumulates in animal hosts or is passed up the food chain, but the origin of TTX in freshwater animals, and newts in particular, is contested. Competing theories of endogenous origins and bacterial symbionts dominate the literature (Chau et al., 2011; Hanifin, 2010; Williams, 2010). Some authors assert that endogenous production is supported by regeneration of TTX after secretion (Cardall et al., 2004) and retention of toxicity in long-term captivity despite being fed non-toxic food (Gall et al., 2012; Hanifin et al., 2002), but the complicated potential biosynthetic pathways make endogenous origins unlikely (Chau et al., 2011; Hague et al., 2016). Production by bacterial symbionts seems more plausible based on observations in marine species. Bacteria cultured from newt skin in our lab produce TTX *in vitro* (PM Vaelli, personal communication), strengthening the case for a bacterial origin in freshwater animals.

The roles of toxicity in the ecology of TTX-bearing organisms include communication, offense, and defense (Hanifin, 2010; Williams, 2010; Moczydlowski, 2013). TTX acts as a male-attracting pheromone released by female pufferfish (*Takifugu niphobles*) (Matsumura, 1995), an

attractant in multiple species of toxic snails (Hwang et al., 2004), and a warning cue indicating the presence of cannibalistic adults for juvenile California newts (*Taricha torosa*) (Zimmer et al., 2006). Blue-ringed octopus (*Hapalochlaena maculosa*), as well as many species of arrowworms, flatworms, ribbon worms, and snails, sequester TTX and use it to envenomate their prey (Williams, 2010). The most notorious function of TTX is defense, which has been extensively studied in pufferfish and newts (Brodie, 1968; Kodama et al., 1986; Brodie III et al., 2005; Williams, 2010; Williams et al., 2010; Itoi et al., 2014). Of particular interest is the role of TTX in predator-prey arms races between sympatric populations of newts (*Taricha spp.*) and garter snakes (*Thamnophis sp.*) (Brodie III et al., 2005). The most well studied of these pairings is that of the rough-skinned newt (*Taricha granulosa*, hereafter newts) and common garter snake (*Thamnophis sirtalis*, hereafter snakes). Newt toxicity varies widely throughout their range, which extends from southern California to the Alaskan panhandle (Hanifin et al., 2008; Hague et al., 2016). Snakes, which have evolved varying degrees of resistance to TTX, live in sympatry with newts south of the Alaskan panhandle (Mebs et al., 2016). Newt toxicity and snake TTX-resistance appear to scale together in some locations (Brodie, Jr. et al., 2002); however, resistance in snakes sometimes outpaces newt toxicity (Hanifin et al., 2008). The correspondence between snake TTX-resistance and newt toxicity is attributed to an antagonistic predation-anti-predator dynamic (Brodie, Jr. et al., 2002; Williams et al. 2012). In this arms race, the toxicity of newts is thought to escalate as a result of predation by snakes with increasing resistance to TTX enabled by mutations in Na_vs (Geffeney et al., 2005). In addition to the variably toxic populations on the west coast, an inland population of newts in Moscow, ID possesses undetectable levels of TTX (hereafter referred to as non-toxic newts) and are sympatric with snakes that have low levels of TTX-resistance (Hanifin et al., 2008).

Recent work presents several challenges to the snake-driven predator-prey arms race hypothesis (Bucciarelli et al., 2016; Hague et al., 2016; Mebs et al., 2016). First, a study of the congener *T. torosa* demonstrates that the toxicity of individuals not only varies geographically, but also fluctuates considerably throughout the year (Bucciarelli et al., 2016). It is likely that the toxicity of *T. granulosa* shows similar patterns. Second, other animals, such as caddisfly larvae, *Limnephilus flavastellus*, (Gall et al., 2012b), great blue herons, *Ardea herodias* (Fellers et al., 2007), and otters, *Lontra canadensis* (Stokes et al., 2015), have been observed eating toxic newts without showing symptoms of intoxication. Whether these organisms are also resistant to TTX has not been examined. Additionally, non-toxic newts coexist with multiple potential predators, including garter snakes that have low levels of resistance (Hanifin et al., 2008), suggesting that chemical defenses could be beneficial to those populations as well (Hanifin et al., 2008; Hague et al., 2016). Conversely, snakes are absent in the Alaskan panhandle, but populations of newts possess TTX (Hague et al., 2016). Toxicity is generally low in Alaskan populations; however, a few individuals were observed to possess levels of TTX that rivaled the highly toxic populations found in Oregon where newts and highly resistant snakes are sympatric (Hague et al., 2016). The red-spotted newt (*Notophthalmus viridescens*), a TTX-bearing member of a clade that is sister to *Taricha*, also shows variability in toxicity throughout its range, but toxicity in red-spotted newts does not correlate with predation pressure (Yotsu-Yamashita et al., 2012). Taken together, predation pressure by snakes and other animals likely plays a role in newt toxicity but cannot be the only explanation.

The predator-prey arms race hypothesis presupposes that newt toxicity is a heritable trait that responds to selection, but the source of TTX in newts and the pathway for TTX biosynthesis have not been determined. Studies of newt population genetics using a small number of

fragments of the cytochrome oxidase I gene and 16s RNA (Mebs et al., 2016), microsatellites (Ridenhour et al., 2007; Hague et al., 2016), and allozymes and fragments of mitochondrial DNA (Kuchta and Tan, 2005) show that the degree of genetic differentiation among populations of newts ranging from northern California to the Alaskan panhandle does not match the amount of variation seen in toxicity. Low genetic differentiation is consistent with high gene flow among populations. Bucciarelli et al. (2016) observed that lower-toxicity *T. torosa* males exhibited weaker site fidelity than their higher-toxicity counterparts, and similar patterns of site fidelity could result in the observed genetic homogeneity in populations of *T. granulosa*. Although multiple studies suggest low levels of genetic differentiation among populations of newts throughout their range (Kuchta and Tan, 2005; Ridenhour et al., 2007; Mebs et al., 2016), the relationship between variation in toxicity and genetic differentiation is currently impossible to determine. These studies are based on a small number of sequences, which may be insufficient to capture genetic differentiation among populations. Furthermore, a genetic component of toxicity has not been established. Without identifying and comparing sequences of genes involved in toxicity, genetic differentiation associated with variation in toxicity cannot be assessed.

In all of the previously-discussed studies, skin toxicity was used as a proxy for newt toxicity, as presence of TTX in the skin is often associated with defense (Noguchi et al., 2006); however, the distribution of TTX goes beyond the skin (Wakely et al., 1966). In newts, skin is the most toxic tissue, but ovary, testes, blood, viscera, and liver contain small amounts of TTX (Wakely et al., 1966). The location of TTX can relate to its function and origin. In pufferfish, the distribution of TTX throughout the body is species dependent; however, in some species, location of TTX corresponds with its function (Noguchi et al., 2006). For example, ovaries of female pufferfish (*Takifugu niphobles*) contain concentrations of TTX that far exceed that of the

skin (Noguchi et al., 2006), and the oocytes produced by these females are laden with TTX. In this species, TTX from ovulated oocytes acts as a pheromone that attracts males (Matsumura, 1995), so the location of TTX in the ovaries is tied to its reproductive and communicative functions. Additionally, studies of the pufferfish *Takifugu rubipes* showed that TTX is absorbed from the intestines and is selectively transported to the liver, where it is found in large quantities (Matsumoto et al., 2007). The presence of TTX in the intestine and liver, paired with studies demonstrating a gain of toxicity when reared on toxic food, provides evidence that TTX comes from an exogenous source in this species of pufferfish (Matsumoto et al., 2007). Learning more about the distribution of TTX in newt tissues could provide insight into its source as well as the role of TTX in newt biology and factors that impact variation in toxicity.

Understanding the function(s) of TTX in newt biology is an essential step in explaining the substantial variability in toxicity. To address this need, I conducted two experiments: 1) I cohoused toxic and non-toxic newts to examine possible links among the origin of TTX, the transmissibility of toxicity via direct contact, and variation in toxicity between individuals; and 2) I described the distribution and concentration of TTX in a large group of tissues to provide greater insight into the origin and non-defensive roles TTX may play in newts. In the first experiment, I tested the hypothesis that TTX can be transferred between cohabitating individuals, which I assessed by cohousing toxic and non-toxic newts and measuring changes in skin toxicity at intervals of at least one month. In the second experiment, I measured the concentration of TTX in tissues involved in diverse processes, focusing on the potential functional significance of location.

METHODS

Subjects

Male and female rough-skinned newts (*Taricha granulosa*) were collected from an unnamed pond (hereafter Sandpiper Pond) between the Pacific Coast Highway and the Sandpiper Village subdivision in Waldport, Oregon (44° 26’ 53.10" N, 124° 4' 26.60" W) on July 13, 2016 (Figure 1), a section of the Elk River in Port Orford, Oregon (42° 47’ 14.64” N, 124° 28’ 50.58” W) on July 16, 2016 (Figure 2), and a pond (hereafter Phillips Farm Pond) at Virgil Phillips Farm Park in Moscow, Idaho (46° 48’ 49.6” N, 117° 0’ 55.86” W) on November 1, 2016 (Figure 3). Sandpiper Pond is approximately 185 km NE of Elk River and 610 km SW of Phillips Farm Pond. Phillips Farm Pond is approximately 930 km NE of Elk River (Figure 4).

All collection activities and experiments were conducted in accordance with Protocol 10/12-195-00 authorized by Michigan State University’s Institutional Animal Care and Use Committee. Collection of newts from Oregon (Sandpiper Pond: n = 10 males, 4 females; Elk River: n = 16 males, 4 females) was conducted in accordance with Oregon Department of Fish and Wildlife Scientific Taking Permit #104-15. Collection of newts from Idaho (Phillips Farm Pond: n = 10 males, 4 females) was conducted in accordance with the Idaho Department of Fish and Game Wildlife Bureau Wildlife Collection/Banding/Possession Permit #150521.

Subject Collection

Newts were collected with dip nets or in partially submerged minnow traps (Promar Collapsible Minnow Traps, size small and medium, Gardena, CA) baited with worms and glow sticks. Minnow traps were placed in the water in the evening and checked the next morning. Animals were removed from the traps and all males were collected. To minimize the impact on the breeding population, only a small number of females was removed from each location. All

juvenile newts and non-target organisms were immediately released. Adult newts used in this study were packed in moss-filled containers placed inside a cooler with ice packs and shipped to Michigan State University.

Animal Maintenance

All newts were housed in 10-gallon aquaria in an animal facility at Michigan State University. Aquaria were filled with Holtfreter's solution (Armstrong et al., 1989) and included a platform that allowed newts to climb out of the water. Newts were fed live California black worms (*Lumbriculus variegatus*) three days per week *ad libitum*.

In the cohabitation experiment, each aquarium contained two male newts in one of three combinations: both from Sandpiper Pond (toxic control), both from Phillips Farm Pond (non-toxic control), or one from Sandpiper Pond and one from Phillips Farm Pond (mixed-toxicity treatment). For the tissue toxicity experiment, newts from Elk River, Sandpiper Pond, and Phillips Farm Pond were housed in aquaria containing four to six same-sex individuals from the same population. All aquaria were cleaned daily using different equipment for newts in different treatments or from different populations. Both rooms were maintained between 18.3 and 22.2 °C with a 12:12 light-dark cycle for the newts in the cohabitation experiment and a 13:11 light-dark cycle for those in the tissue toxicity experiment.

Set-up and Sample Collection for the Cohabitation Experiment

Newts from Sandpiper and Phillips Farm Ponds were randomly assigned to one of three conditions: the toxic control, comprised of two males from Sandpiper Pond (n = 2 aquaria); the non-toxic control comprised of two males from Phillips Farm Pond (n = 2 aquaria), and the mixed-toxicity treatment comprised of one male from Sandpiper Pond and one male from Phillips Farm Pond (n = 6 aquaria).

Unfortunately, over the course of this 10-month-long experiment, four newts from Phillips Farms Pond died. Three were in non-toxic control aquaria and one was from a mixed-toxicity treatment aquarium. One non-toxic control newt died before the March sampling date, so there was only one non-toxic control aquarium from March until May. Two other non-toxic control newts died before the June sampling date, so for the final two sampling dates, there were no non-toxic control aquaria. The Phillips Farm Pond newt from a mixed-toxicity treatment aquarium died before the June sampling date, so $n = 6$ mixed-toxicity treatment aquaria from February to May and $n = 5$ in June and December. Data presented here only include aquaria with a pair of newts.

Approximately every four weeks between February and June and once in December of 2016, each newt was anesthetized in methanesulfonic acid (MS222, pH 7.2-7.4) before collecting skin samples. Following the skin biopsy (described below), newts were placed in individual containers of Holtfreter's solution until the effects of anesthesia wore off, which was usually within 15 min. Newts were then returned to their assigned aquaria.

Sample Collection for the Tissue Toxicity Experiment

Post-mortem tissue samples were collected from newts from Elk River ($n = 9$ males, 4 females), Sandpiper Pond ($n = 2$ females), and Phillips Farm Pond ($n = 1$ male, 3 females) to determine how TTX is distributed throughout the body. Newts were anesthetized in methanesulfonic acid (MS222, pH 7.2-7.4) for at least 30 min before I measured the newts' mass, total length, snout-vent length (SVL), head width from the tip of the left quadrate to the tip of the right quadrate, and head length from snout to quadrate. Two skin samples were collected from each individual and combined for further analysis. Newts were then decapitated and a small sample of blood was immediately collected. Brain, feces, heart, kidney, and pieces of fat, skeletal

muscle, spleen, and liver were collected from all newts. Additionally, testes and vas deferens were collected from males and a small sample of unfertilized eggs and piece of oviduct were collected from females.

Skin Sampling and Tissue Processing

Using a sampling protocol modified from Bucciarelli et al., (2014), one skin sample was removed from each side of the posterior dorsolateral area approximately 1 cm below the vertebral column using a 2 mm skin biopsy punch (Acu-punch, Acuderm Inc. Fort Lauderdale, FL). Skin samples and all other tissues collected from newts were weighed before being placed in individual microcentrifuge tubes with 300 µl 0.1M aqueous acetic acid and homogenized using a single-use disposable tissue grinder (Pellet Pestle®, Kimble® Kontes®, Rockwood, TN). Samples were stored at -80 °C until further analysis.

Toxin Extraction from Tissue

All tissue samples collected in the cohabitation and tissue toxicity experiments were processed to extract TTX, which was then quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS). To do so, I followed the extraction protocol described in Bucciarelli et al. (2014). Specifically, frozen samples were thawed at room temperature, then the macerated samples were placed in a boiling water bath for 5 min, followed by 5 min in an ice bath. Samples were then centrifuged for 20 min at 13,000 x g and the supernatants were transferred to centrifugal filters (Ultrafree-MC GV 0.22 µm pore size, EMD Millipore Corporation, Billerica, MA) and centrifuged for 20 min at 13,000 x g. An additional 100 µl of 0.1M aqueous acetic acid was added to the centrifugal filters before a final 20-min centrifugation at 13,000 x g. The extracts were then diluted with 600 µl 0.1M aqueous acetic acid and either frozen immediately at -80 °C or prepared for LC-MS/MS.

Solid Phase Extraction of Samples

Oasis MCX 1 cc Vac Cartridges (30 µm particle size, Waters, Milford, MA) were washed with 1 ml methanol and then 1 ml of deionized water. 1 ml of the extracted sample was filtered through the prepared Vac Cartridge over 30 sec followed by washes with 1 ml acetonitrile, 1 ml methanol, and 1 ml deionized water. The Vac Cartridges were transferred to a manifold (VacMaster^{TM} 10 Sample Processing Manifold, Biotage®, Charlotte, NC) and eluted twice with 125 µl 0.2M hydrochloric acid/20% methanol with a 30-sec soak time for each elution. The processed extracts were then dried in a vacuum concentrator (Savant SpeedVac SC110, Thermo Fisher Scientific, Waltham, MA), reconstituted with 100 µl 1:1 acetonitrile: 0.1M acetic acid, and transferred to vials (2 ml Screw Top Autosampler Vial and 100 µl inserts, Agilent, Santa Clara, CA).

TTX Quantification

The concentration of TTX in tissue samples was measured in MSU's Mass Spectrometry and Metabolomics Core using a QTRAP 3200 liquid chromatography-tandem mass spectrometry (LC-MS/MS) system with electron spray ionization (Sciex, Framingham, MA). 5 µl of each sample was passed through an Xbridge Amide filter (3.5 µm, Waters, Milford, MA) using a 50:50 mix of 10mM ammonium acetate (pH 3.5-4.0): acetonitrile for the mobile phase at a flow rate of 0.4 ml/min for 5 min. TTX standards ranging from 0 ng/ml to 3,000 ng/ml were run alongside the samples and used to calculate the concentration of TTX in tissues. TTX standards below 5 ng/ml were not consistently detected or they decreased the linearity of the standard curve when included in the calculations and therefore were excluded from the standard curve. The ability to quantify the concentration of TTX in a tissue sample is constrained by the highest and lowest values of the standard curve, which comprise the limit of quantification, a range of

concentrations in which samples can reliably be calculated using the standard curve. Following standard practice in the field, any tissue sample with a concentration detected as greater than 0 ng/ml but less than 5 ng/ml was excluded from the data set (Keizer et al., 2015). Removal of these samples from the dataset did not alter the interpretations of the results. The LC-MS/MS measurements were reported in ng TTX/ml sample and then converted to μg TTX/g tissue using the amount of 0.1M acetic acid used to reconstitute the sample after vacuum concentration (100 μl) and the mass of the tissue.

Statistical Analysis

Descriptive statistics, ANOVA, and t-tests were calculated using Microsoft Excel and regressions were calculated using the Data Analysis ToolPack in Excel (Microsoft Office 365 ProPlus, Microsoft, Redmond, Washington). Post-hoc analyses were performed using QuickCalcs (GraphPad Software, Inc., La Jolla, California).

RESULTS

Cohabitation Experiment

The toxicity of Phillips Farm Pond newts remained at 0 µg/g during the 10 months of the experiment. As illustrated in Figure 5, this result was observed both in the newts cohoused with toxic Sandpiper Pond newts (mixed-toxicity treatment) and those cohoused with another newt from Phillips Farm Pond (non-toxic control). Although most skin biopsy samples showed no TTX, in four instances different Phillips Farm Pond newt samples had amounts of TTX that ranged from the equivalent of 0 to <5 ng/ml (the units of the standard curve), which falls below the level where the concentration can be determined with confidence. Because the amount of TTX present could not be quantified accurately, the samples were excluded from analyses. Three of the four of these newts were cohoused with toxic newts in treatment aquaria and one was cohoused with another non-toxic newt in a low-toxicity control aquarium. None of these individuals possessed trace amounts of TTX more than once during the experiment.

All Sandpiper newts consistently possessed TTX; however, the average amount of TTX varied significantly over time when the treatment and control newt toxicities were pooled (ANOVA, $F = 4.92$, $df = 59$, $p = 0.00088$; Figure 6). Specifically, the toxicity of newts in December was significantly greater than in April, May, and June (Tukey Kramer post-hoc: $p = <0.05$ for comparisons between December and April, May, and June). The variation in mixed-toxicity treatment newts showed the same significant trend where toxicity of the December samples was significantly greater than in samples from April, May, and June (ANOVA, $F = 1.98$, $df = 35$, $p = 0.00630$; Tukey Kramer post-hoc: $p = <0.05$ for comparisons between December and April, May, and June; Figure 7), but the trend was not significant when the control newts were analyzed separately (ANOVA, $F = 1.98$, $df = 23$, $p = 0.13$; Figure 7).

Tissue Toxicity Experiment

TTX was not detected in the skin or any of the other organs test in newts from Phillips Farm Pond with one exception: a very small quantity of TTX was detected in a heart sample from the single Phillips Farm Pond male. The concentration of TTX found in this sample (2.34 ng/ml) was below the level of quantification (5 ng/ml), therefore the sample was excluded from the data set.

All newts from Elk River and Sandpiper Pond possessed TTX. Of the tissues sampled, the concentration of TTX in the skin in every toxic individual was significantly greater than the toxicity of any other tissue (ANOVA, $F = 7.81$, $df = 13$, $p = 3.07\text{x}10^{-11}$; Tukey Kramer post-hoc: $p = <0.05$ for all tissue compared to skin). Highly variable amounts of TTX were also detected in brain, heart, skeletal muscle, ova, oviduct, testes, vas deferens, kidney, liver, spleen, blood, fat, and feces in some female and male newts from Elk River and Sandpiper Pond (Tables 1-8). However, of the newts with toxic skin, only skin, skeletal muscle, brain, ova, and vas deferens were toxic in all individuals. For blood, fat, feces, heart, kidney, liver, spleen, and testes, at least one sample from one Elk River or Sandpiper Pond individual had 0 μg/g TTX. Additionally, a subset of brain, fat, feces, heart, kidney, liver, skeletal muscle, spleen, testes, and vas deferens samples contained TTX; however, the concentration was between 0 ng/ml—5 ng/ml TTX, which is below the level of quantification (Table 9), so these samples were excluded from the analyses. Toxicity of individual organs did not follow a consistent hierarchy based the amount of TTX found in each organ: the relative toxicities of different organs varied among individuals with no patterns discernable based on sex or population.

Body condition, the ratio of mass to SVL, is an index of reproductive condition, movement, and survival in amphibians (MacCracken and Stebbings, 2012; Bucciarelli et al.,

2016). Morphological measurements varied between males and females (Table 10). Toxic males had a significantly greater mean mass (t-test, $t_{crit} = 2.20$, $df = 11$, $p = 0.000168$) and longer average SVL than toxic females (t-test, $t_{crit} = 2.20$, $df = 11$, $p = 0.0000442$), but the mean for body condition was significantly lower in males than females ($t_{crit} = 2.36$, $df = 7$, $p = 0.0113$). Toxicity was not significantly different between males and females ($t_{crit} = 2.57$, $df = 5$, $p = 0.173$).

To determine if toxicity was correlated with body condition, mass, and SVL and toxicity, I performed regression analyses for all toxic newts ($n = 14$), all Elk River newts ($n = 12$), all toxic females ($n = 6$), Elk River males ($n = 8$), and Elk River females ($n = 4$) (Table 11). The "all toxic females" grouping includes both Elk River ($n = 4$) and Sandpiper Pond females ($n = 2$). Due to small sample size, a regression analysis was not performed on only Sandpiper Pond females.

No significant correlation between toxicity and body condition existed for all toxic newts ($R^2 = 0.022$, $p = 0.61$), all Elk River newts ($R^2 = 0.030$, $p = 0.59$), all toxic females ($R^2 = 0.15$, $p = 0.44$), Elk River males ($R^2 = 0.0010$, $p = 0.94$), and Elk River females ($R^2 = 0.22$, $p = 0.53$). Similarly, no significant relationship between toxicity and mass existed for all toxic newts ($R^2 = 0.12$, $p = 0.23$), all Elk River newts ($R^2 = 0.11$, $p = 0.30$), all toxic females ($R^2 = 0.05$, $p = 0.67$), Elk River males ($R^2 = 0.026$, $p = 0.70$), and Elk River females ($R^2 = 0.17$, $p = 0.59$). In contrast, as illustrated in Figure 8, the relationship between toxicity and SVL was significant when all toxic newts were included in the analysis ($R^2 = 0.37$, $p = 0.022$). When the two Sandpiper Pond females, which were significantly more toxic than Elk River newts ($F = 30.0$, $df = 1$, $p = 0.000142$), were excluded from the analysis, the result was not significant for all Elk River newts $R^2 = 0.31$, $p = 0.059$), Elk River males ($R^2 = 0.25$, $p = 0.21$), and Elk River females ($R^2 = 0.073$,

$p = 0.73$). The relationship between SVL and toxicity was also not significant when all females were considered ($R^2 = 0.38$, $p = 0.20$).

DISCUSSION

The data presented here for the cohabitation and tissue toxicity experiments have important implications for understanding the relationship between variation in toxicity and the origin, transmissibility, and function of TTX in newts, which I will discuss in turn below.

Non-toxic Newts in the Cohabitation Study

Phillips Farm Pond newts did not become toxic during the cohabitation study; however, garter snakes were present at the field site (personal observation). It also seems likely that they face other predators, as their toxic counterparts can be successfully consumed by non-snake predators (Stokes et al., 2015; Gall et al., 2011; Fellers et al., 2007). This raises questions about why newts are not toxic if predation is a relevant selection pressure and why they do not become toxic with exposure to other toxic individuals. Approaching these questions requires consideration of the trade-offs involved in being toxic, and therefore resistant to the toxin, and the source of the toxin.

TTX resistance in other organisms is due to mutations in Na_Vs that decrease the binding affinity for TTX (Lee and Ruben, 2008; Chau et al., 2011). Because being toxic requires being resistant to the toxin (Hanifin, 2010), newts also possess mutations in Na_Vs (Hanifin and Gilly, 2015). Resistance-conferring mutations occur in portions of Na_Vs to which TTX binds, but these portions are also essential to ion selectivity and flow; thus, changes that increase resistance also likely impede channel function (Jost et al., 2008). Therefore, being toxic could be very costly, and, in some environments, the benefits of being toxic may be outweighed by the costs, favoring a lack of TTX. If populations of toxic and non-toxic newts were separated before some of the resistance-conferring mutations occurred, toxic and non-toxic newts may not have followed parallel evolutionary trajectories. Predation pressure likely accounts for only some of the

variation in newt toxicity; identifying other selective pressures and understanding the function of TTX in newt biology could provide further insight into the trade-offs that govern toxicity.

In addition to the uncertain ecological and physiological significance of non-toxic populations of newts, the source of TTX has not been determined. The lack of a detectable change in non-toxic newt toxicity does not provide additional support for a particular hypothesis. However, given the production of TTX by bacteria cultured from the skin of toxic newts (PM Vaelli, personal communication) in addition to TTX-producing bacteria isolated from marine organisms (Chau et al., 2011), one possible scenario is that newts acquire TTX from bacterial symbionts. Because toxic and non-toxic newts are geographically separated, TTX-producing bacteria may not occur in the environment of non-toxic newts. Cohousing toxic and non-toxic newts exposes non-toxic newts to TTX-producing bacteria, providing many opportunities for the exchange of microbes. Exchange of microbes between cohabitating conspecifics and even heterospecifics is well documented in the literature (Archie and Theis, 2011; Leclaire et al., 2014; Song et al., 2013, Whittaker et al., 2016). For example, in studies of human families living together, the skin microbiomes of cohabitating individuals, even those that are not related, show greater similarity than the microbiomes of individuals from different households (Song et al., 2013). Additionally, microbiota appear to be shared between humans and their dogs (Song et al., 2013). Skin microbiomes showed the greatest similarity among individuals, and the convergence appears to be due to the amount of contact between individuals living together (Song et al., 2013).

Cohabitating newts interacted regularly and were often seen in amplexus (personal observation), but even if microbes were transferred between toxic and non-toxic newts, no TTX was detected in non-toxic newts during the experiment. (We have not analyzed the microbiomes

of these newts, so the degree of similarity between cohoused newts cannot be assessed.) Assuming that microbes were shared between cohabitating newts, it is very curious that non-toxic newts did not become toxic. Toxic newts may produce a substrate required by the TTX-producing bacteria that non-toxic newts do not. The absence of such a substrate could inhibit the growth or survival of TTX-producing bacteria or the production of TTX. If the substrate is a protein or the product of enzymatic activity, toxicity could have a genetic component, as the coevolutionary arms race presupposes. Morphological comparisons of toxic Washington and Oregon populations with the non-toxic Idaho population have led to the suggestion that toxic newts were introduced to Idaho (Nussbaum and Brodie, 1971); however, the mtDNA of non-toxic Idaho newts suggests that they are genetically distinct from toxic populations (Kuchta and Tan, 2005). Thus, not only could genetic differences in Na_Vs limit resistance as suggested earlier, but genetic differences among populations could also limit TTX production by microbes.

Based on these data, endogenous production cannot be ruled out as a possible mechanism underlying toxicity. To date, biosynthesis of TTX has only been identified in bacteria and the pathway is likely complex (Chau et al., 2011). However, the lack of evidence supporting endogenous production does not preclude it as a possibility. Additionally, some marine organisms acquire TTX from their diet, but this is not a likely source of TTX in newts because they are able to retain and regenerate TTX in the absence of a TTX-containing food source (Cardall et al., 2004), unlike pufferfish whose toxicity is dependent on consuming toxic food (Noguchi et al., 2006).

In all, the results of the cohabitation experiment demonstrated that prolonged contact between toxic and non-toxic individuals was insufficient to induce detectable levels of toxicity in non-toxic newts.

Toxic Newts in the Cohabitation Experiment

Similar to Bucciarelli et al.'s (2016) results with *T. torosa*, the toxicity measured in the Sandpiper Pond males used in the cohabitation experiment varied widely over time: average toxicity of Sandpiper males increased from February to March, decreased between March and June, and then increased again between June and December (Figure 6). Three possible mechanisms that could account for these fluctuations are: 1) season or breeding condition; 2) temporal changes in the microbiome; and 3) repeated injury leading to secretion of TTX with an insufficient inter-sampling interval for complete TTX recovery.

First, newts are semi-aquatic, moving into ponds and streams during the breeding season. The timing of the breeding season varies based on location, but it generally starts in late winter/early spring. In addition to changes in their environment, newts undergo concomitant morphological and physiological changes (Mccurdy, 1931; Jones et al., 2002a, 2002b; Mills and Rombough, 2008), and toxicity could vary in response to any of these factors. In *T. torosa*, toxicity cycles annually, reaching the highest levels during the peak of the breeding season (Bucciarelli et al., 2016). The fluctuations in toxicity of Sandpiper Pond males observed during the cohabitation experiment may relate to their breeding season, and the timing of the breeding season can be inferred from reports of other Oregon populations at similar elevations. Toxicity of the Sandpiper Pond males increased between February and March, which corresponds to the breeding season of a population located approximately 65 km NE of Sandpiper Pond (Jones et al., 2002a) and then declines through June before increasing again in December. If TTX levels are tied to breeding, then it is likely that newts have a strong internal annual rhythm because the newts used in this experiment were kept on a constant 12:12 light-dark cycle and had been isolated from females since July 2015.

Second, temporal variability in skin TTX levels may not be independent of changes in the microbiome. Microbial communities are not stagnant; they change or differ based on environment, genetics, age, and diet (Archie and Theis, 2011; Song et al., 2013; Leclaire et al., 2014; Whittaker et al., 2016). Changes in the newt microbiome in response to temporal changes in environment, morphology, and physiology could all account for variation in toxicity. If newts are viewed as an environment for bacteria, a newt's location or physiological state could influence the resources it provisions for bacteria, making different times of year more or less conducive to TTX production.

Finally, taking skin biopsy samples, while not lethal, can be traumatic and the newts are left with two small wounds. Secreting TTX could be a response to the trauma, and while newts have been shown to regenerate TTX after expelling it in response to trauma, recovery took many months (Cardall et al., 2004). The newts in this experiment were sampled every four weeks for five months and then again six months later. Wounds healed completely between sampling events, but if newts secreted TTX during a previous sampling event, the intervening month may have been insufficient to allow for regeneration of the expelled TTX. Thus, repeated sampling at such close intervals may have contributed to a cumulative decline in toxicity from March to June. TTX levels were much higher after a six-month break from sampling, but the December sampling event was also close to the start of the next breeding season. The suggestion of a cycle in toxicity in Sandpiper Pond newts and the apparent annual pattern observed in *T. torosa* by Bucciarelli et al. (2016) are intriguing, but establishing the occurrence of a cyclical pattern in toxicity that might parallel breeding events in *T. granulosa* requires multi-year repetition of measurements with greater consideration given to the impact of sampling.

Temporal variation in toxicity has other implications for our understanding of the role of TTX in newt biology. Previous studies of toxicity in newts have used the amount of TTX in the skin measured at a single point in time to compare and characterize the toxicity of newts in different locations (Hanifin et al., 2008; Hague et al., 2016; Mebs et al., 2016). In these studies, comparisons were made among samples taken at different times of the year and, in some cases, with very few animals, which could confound the results. Understanding variation in toxicity may also impact our interpretation of the newt-snake arms race: parity between toxicity and TTX resistance may vary depending on the timing of toxicity measurements. Rather than describe populations as toxic or non-toxic based on a sample at a single time point, more extensive studies that characterize the toxicity profile throughout the entire geographic range of newts over the course of many years are necessary to fully understand the potentially complex dynamics underscoring variation in toxicity and the functional significance of TTX.

Tissue Toxicity Experiment

The distribution of TTX throughout the body of newts found in this experiment reinforces and expands upon that described in previous studies of toxic newts (Wakely et al., 1966; Hanifin et al., 2003; Lehman et al., 2004). In addition to skin, oviduct, ova, skeletal muscle, blood, liver, and testes, which were previously known to be toxic (Wakely et al. 1966), I detected TTX in fat, feces, kidney, vas deferens, brain, and heart, as shown in Tables 1-8. Toxicity varied greatly among tissues, which may be related to the tissue's affinity for TTX (Mebs et al., 2016) or the ability of TTX to pass through cell membranes (Matsumoto et al., 2007).

Additionally, my experiment provides the first analysis of internal organs in non-toxic newts: in all instances, if TTX was absent from the skin, as was the case for all the Phillips Farm

Pond newts, the internal organs also lacked TTX. Conversely, if TTX was present in the skin, as observed in all Elk River and Sandpiper Pond newts (Table 1), at least some of the internal organs also contained TTX in each individual (Tables 2-8). The distribution of TTX in tissues with diverse functions adds further evidence that TTX is involved in more than just defense in toxic newts.

As noted in previous studies (e.g., Bucciarelli et al., 2016; Hanifin et al., 1999, 2008) and confirmed in this study, the amount of TTX in skin varies widely among individuals. Aside from temporal variation, differences in individual resistance and the trade-offs between costs and benefits of toxicity could contribute to variation. Resistance to TTX is a function of the number, location, and type of mutations present in Na_vs and individual toxicity may be limited by the mutations that individual possesses (Brodie III and Brodie Jr., 1999; Lee and Ruben, 2008). Therefore, it seems reasonable to speculate that less toxic individuals can survive in mixed-toxicity populations because being surrounded by more toxic individuals could provide protection in a manner similar to herd immunity. Newts aggregate in large groups in breeding ponds (Janzen and Brodie, 1989), so less toxic individuals could be protected from predators by the presence of other highly toxic individuals without incurring the costs that come with being toxic (Bucciarelli et al., 2016).

The presence of TTX in the blood (Table 2) suggests a means of regulating internal levels of TTX, which could play a role in mitigating the physiological costs of being toxic and preventing self-toxicity. As documented in other tetrodotoxic species, possessing TTX imposes physiological costs through compromises in Na_vs function, although specific costs in newts have not been studied (Bucciarelli et al., 2016). Newts may balance the trade-off between toxicity and

these costs by transporting TTX through the blood from some tissues to others for sequestration or excretion.

Newts may also regulate toxicity by eliminating TTX in feces, which varied by an order of magnitude among individuals (Table 3). The amount of TTX I measured in feces was up to an order of magnitude greater than TTX found in blood, so perhaps TTX accumulates in the intestines for incorporation into feces until it can be excreted. Newts may need to excrete TTX because the amount of TTX an individual can possess is likely constrained by its physiology. As TTX levels increase during the year, newts may need to excrete more TTX. Further studies examining temporal variation in fecal TTX relative to skin toxicity, which is the only tissue that can be collected is non-lethally, are needed to gain insight into whether the presence of TTX in the feces presents a means of general excretion and toxicity regulation, safeguarding newts against self-intoxication.

Presence of TTX in feces of toxic newts also indicates that newts release TTX in multiple ways. Newts secrete TTX from the granular glands in their skin as a defense mechanism (Cardall et al., 2004), but little consideration has been given to other functions of TTX secretion. In addition to regulating TTX levels, the TTX excreted in feces could play a role in communication. In the congener *T. torosa*, TTX is used by larvae as a cue that cannibalistic adults are nearby, and the presence of TTX elicits antipredator behavior (Zimmer et al., 2006). Although cannibalism has not been documented in *T. granulosa*, TTX may communicate similar or even other information among newts in toxic populations. TTX secreted from the skin and excreted in feces could indicate where newts are located, attracting others to the area for mating or feeding. TTX is a pheromone in pufferfish (Matsumura, 1995) and may also act in newts as an aggregation pheromone during the breeding season, when levels peak (Itoi et al., 2016). Like pufferfish

(Okita et al., 2013; Itoi et al., 2016), newts can smell TTX (*T. torosa*: Zimmer et al., 2006; *T. granulosa*: HL Eisthen, unpublished data) and *T. granulosa* are attracted to it (JD Merkel and HL Eisthen, unpublished data). Additionally, the amount of TTX in feces could function as an indicator of overall toxicity and thus play a role in mate selection, as toxicity does in *T. torosa* (Bucciarelli et al., 2016).

The presence of TTX in both female and male reproductive tissues in toxic newts (Tables 4-6) provides further evidence that TTX could be involved in reproduction. However, I found that body condition, a commonly used measure of reproductive status in amphibians given by the ratio of body mass to SVL, does not correlate with toxicity. The lack of relationship holds true regardless of whether all toxic newts from this experiment are grouped together, separated by population, separated by sex, or separated by population and sex. My result is consistent with Bucciarelli et al.'s (2016) finding of no relationship between body condition and TTX level in *T. torosa*. The time of year could explain the lack of correlation: I collected most of the samples between June and August, which is likely well after the height of the breeding season. Males cease to court females once they begin ovipositing (Jones et al., 2002a), which likely occurs earlier in the year, so it is possible that the newts are no longer in peak breeding condition. Alternatively, body condition may not be a good metric of reproductive condition and other indices may be more appropriate (MacCracken and Stebbings, 2012). The correlation between mass and toxicity was also non-significant. However, I found a significant negative correlation between SVL and toxicity when all male and female newts were combined (Figure 8). The mean SVL of females was shorter than that of males, but females were more toxic (Table 10). Small size or ovipositing behavior may make females more vulnerable to predators, offsetting the potential costs of high levels of toxicity. Although I observed sexually dimorphic trends in size

and toxicity, I found no correlation between toxicity and SVL when the analysis was performed separately on males and females (Table 11). Small sample sizes, lack of comparisons among multiple populations, and measurements taken at a single time point may obscure correlations. Despite the lack of correlation I found in most of the regressions, the presence of TTX in reproductive tissues suggests that a relationship between toxicity and reproduction exists; therefore, the role of TTX in reproduction should not be dismissed.

A previous study has shown that TTX is maternally provisioned in the yolk and cytoplasm of fertilized eggs and that female and egg toxicity are positively correlated, although the R^2 value (0.48) indicates that the correlation does not fully describe the relationship (Hanifin et al., 2003). Maternally provisioned TTX most likely protects the eggs from predators (Brodie, 1968; Hanifin et al., 2003). As shown in Table 4, whole ova from Elk River and Sandpiper Pond females were found to contain substantial amounts of TTX, in agreement with the maternal endowment hypothesis (Hanifin et al., 2003; Gall et al., 2012a). The jelly coat typically contains small amounts of TTX (Gall et al., 2012; Hanifin et al., 2003), and my results show that the oviduct, which contributes the jelly coat, contains little or no TTX (Table 4). All toxic females had large quantities of mature oocytes, so it is possible that the very low levels of TTX I observed in the oviduct result from depletion due to deposition, but it is also possible that TTX in the jelly coat is contributed by another source.

In contrast to maternal endowment, little attention has been paid to potential contributions of TTX to eggs by males. Sperm is transferred to females via a spermatophore, which is composed of a gelatinous stalk with branching fibrils and a sperm cap on top (Davis and Twitty, 1964). During courtship, the male attaches the jelly stalk to the substrate at the end of a lengthy ritual, and the receptive female positions her cloaca over the spermatophore to pick up

the sperm cap for internal fertilization of the eggs (Propper, 1991). Although the presence of TTX in spermatophores has not been studied, my measurements of TTX in the testes and vas deferens of Elk River newts (Table 5) suggest that spermatophores could contain TTX. The testes had very little TTX, so the sperm cap may not be toxic, but the vas deferens had large quantities of TTX and plays a role in the development of the stalk of the spermatophore in concert with cloacal glands (Uzzell, 1969; Hopkins, 1978).

Understanding the potential contribution of TTX from males to offspring requires knowledge of the distribution of TTX in the spermatophore, which has not been studied. However, parental endowment of defensive chemicals in other species, such as the ornate moth (*Utetheisa ornatrix*), is well documented (Dussourd et al., 1988; González et al., 1999; LaMunyon and Eisner, 1993). Male and female *Utetheisa* possess pyrrolizidine alkaloids (PA) derived from food (Dussourd et al., 1991), and both sexes contribute PA to the eggs, where they function in defense (Dussourd et al., 1988). Males contribute PA via the spermatophore, and, along with nutrients, PA constitute a nuptial gift. Females use the nutrients and PA from the spermatophore to replenish their own stores and also incorporate some of the male's PA into the eggs. The concentration of PA in spermatophores also play a role in the females' postcopulatory judgment of male quality (Eisner and Meinwald, 1995). *Utetheisa* males are not alone in the provisioning offspring with defensive compounds: male assassin bugs (*Apiomerus flaviventris*), beetles in the families Meloidae and Pyrochroidae, and danaine butterflies, such as the queen butterfly (*Danaus gilippus*), contribute compounds that are included in eggs, and in many cases females use the concentrations of these compounds as an indicator of male quality (Eisner and Meinwald, 1995).

Female newts use multiple cues to assess mates, such as body size and tailfin height (Janzen and Brodie, 1989), but because of the costliness of being toxic and defensive benefits of TTX (Bucciarelli et al., 2016), TTX could be a particularly useful indicator of male quality. In addition, the presence of TTX in male newt reproductive organs found in this study suggests that TTX may also be transmitted to females in spermatophores as a nuptial gift that can be incorporated into the egg. Like *Utetheisa*, female newts mate multiple times and can store and use sperm selectively (*Utetheisa*: Eisner and Meinwald, 1995; LaMunyon and Eisner, 1993; newts: Jones et al., 2002). Female choice in *Utetheisa* is important for increasing offspring fitness because choosing males with large amounts of PA enable females to increase the load of defensive PA in eggs (Iyengar and Eisner, 1999). Preference for toxic spermatophores has also been observed in toxic and non-toxic moths (Iyengar et al., 2001). A parallel scenario in which TTX plays a substantial role in the reproductive biology of newts seems possible.

The existence of a naturally occurring non-toxic population of newts opens the possibility to elucidate the role of TTX in reproduction. Determining whether males contribute TTX to offspring could be accomplished by mating non-toxic females with toxic males. Given that TTX is not transferred from toxic to non-toxic male newts through contact and cohabitation, reproduction could be a means of transmission among adults although non-toxic females may prefer not to mate with toxic males. Furthermore, if mating does occur, offspring may not be viable if TTX is contributed from the male and the offspring lack resistance-conferring adaptations. In either case, TTX could be a barrier to reproduction between toxic and non-toxic newts, which could provide an explanation for populations devoid of TTX.

The bright yellow bilateral fat bodies, a reproductive structure overlying the gonads in both males and females, are toxic (Table 6). Fat bodies synthesize steroid hormones (Lupo di

Prisco et al., 1971) and store lipids that are essential for reproductive function in many amphibians (Jorgensen, 1986; Girish and Saidapur, 2000). Energy resources from fat bodies are involved in gametogenesis and maintaining the reproductive organs (Girish and Saidapur, 2000; Madelaire and Gomes, 2016). I found that TTX is present in the fat bodies of both males and females at levels similar to blood (Tables 2 and 6). The fat bodies could function as TTX repositories that supply TTX to the gonads, resulting in levels of TTX that fluctuate with reproductive state. Chieffi et al. (1980) described a portal system linking the fat bodies with testes in male edible frogs (*Rana esculenta*), and if a similar portal system exists in newts it is possible that TTX from the fat bodies could be transferred directly to the gonads.

I detected small amounts of TTX in the kidney, liver, and spleen in only about half of the toxic newts (Table 7). In many toxic marine species, the presence of TTX in digestive tissues results from accumulation of TTX from dietary sources or bacterial symbionts (Hanifin, 2010; Lago et al., 2015; Noguchi et al., 2006). For example, non-toxic pufferfish gain toxicity after ingesting toxic food (Noguchi et al. 2006) and the grey side-gilled sea slug (*Pleurobranchaea maculata*) gains and loses toxicity based on the toxicity of its diet (Salvitti et al., 2017). In contrast, newts do not seem to acquire TTX from their food: newts reared in captivity on non-toxic diets do not lose toxicity, which can even increase over time (Hanifin et al., 2003; Cardall et al., 2004; Gall et al., 2012a) and captive newts regenerate TTX following secretion (Cardall et al., 2004). Additionally, the relatively low levels of TTX in digestive tissues observed in this experiment support the idea that newts do not derive TTX from their food. The low level of TTX that I found in the liver is particularly notable because the liver is responsible for detoxifying and metabolizing compounds and is one of the most toxic organs in pufferfish (Noguchi et al., 2006). If the liver were involved in regulating toxicity in newts, larger amounts of TTX would be

expected. The average concentrations of TTX I found in digestive tissues are near or below levels in the blood, which was not drained from the tissues prior to analysis, suggesting that TTX does not accumulate in digestive tissues in newts.

Finally, my analysis of excitable tissues corroborates earlier accounts of TTX in skeletal muscle (Wakely et al., 1966), but they also show for the first time that TTX is present in both the brain and heart muscle (Table 8). This result demonstrates that TTX crosses the blood-brain barrier and also could affect cells in both heart and skeletal muscle, but to date direct evidence has been lacking. The average concentration of TTX in the brain and heart muscle exceeds that found in the blood by one to two orders of magnitude (Tables 2 and 8), demonstrating that TTX is transported into these tissues. It is entirely unclear whether TTX may serve a function in excitable tissues. Alternatively, perhaps TTX does not serve a function in these tissues; if Na_vs in newts are incompletely resistant to TTX and allow partial binding, the toxin could simply accumulate in tissues that possess Na_vs. Given that all isoforms need to be resistant to TTX for a TTX-possessing organism to survive (Jost et al., 2008), a comparative study of Na_v sequences in toxic and non-toxic newts could provide a physiological explanation for the existence of non-toxic populations if mutations are absent in these animals.

CONCLUSION

My experiments provide a solid foundation for many future studies examining the function of TTX in newts extending beyond the popular notion of defense against predators. Non-toxic newts poses a challenge: if TTX is so effective as a defense, why would an entire population of individuals that is still vulnerable to predation lose their toxicity? Although my study does not offer an answer to that question, the demonstration that long-term cohabitation cannot induce toxicity in non-toxic individuals suggests the existence of a physical or physiological cost or impediment. In addition, I found that toxicity is not a static trait: the amount of TTX present in skin varies among populations and temporally within individuals, and TTX is found in many tissues with dissimilar functions throughout the body. My experiments join the growing body of evidence that toxicity in newts is more complicated than once conceived. Although defense is certainly a benefit derived from toxicity, the role that TTX plays is likely multifaceted. Further studies of newts' reproduction, behavior, microbiota, and sodium channels are necessary for the explication of this complex and dynamic trait.

APPENDICES

APPENDIX A

TABLES

Table 1. Toxicity of Skin

		Skin	
Population	*n*	*Toxicity*	*Range*
ER Male	8	20 ± 6.0	2.7 - 50
ER Female	4	37 ± 11	22 - 69
PF Male	1	0	
PF Female	3	0 ± 0	0 - 0
SP Female	2	240 ± 120	120 - 360

Mean ± standard error (SE) for toxicity ("Toxicity") and range of toxicities ("Range") observed for skin samples collected in the tissue toxicity experiment. Toxicity is measured in µg TTX/g skin. Newts are separated by sex and population; ER = Elk River, PF = Phillips Farm Pond, and SP = Sandpiper Pond.

Table 2. Toxicity of Blood

		Blood	
Population	*n*	*Toxicity*	*Range*
ER Male	8	0.057 ± 0.027	0 - 0.20
ER Female	3	0.032 ± 0.028	0 - 0.088
PF Female	2	0 ± 0	0 - 0
SP Female	1	0.78	

Mean ± SE and range of toxicities observed for blood samples collected in the tissue toxicity experiment. Toxicity is measured in µg TTX/g blood. Newts are separated by sex and population; ER = Elk River, PF = Phillips Farm Pond, and SP = Sandpiper Pond.

Table 3. Toxicity of Feces

		Feces	
Population	*n*	*Toxicity*	*Range*
ER Male	4	0.14 ± 0.066	0 - 0.28
ER Female	2	0.025 ± 0.025	0 - 0.050
PF Female	3	0 ± 0	0 - 0
SP Female	1	0.14	

Mean ± SE and range of toxicities observed for feces collected in the tissue toxicity experiment. Toxicity is measured in µg TTX/g feces. Newts are separated by sex and population; ER = Elk River, PF = Phillips Farm Pond, and SP = Sandpiper Pond.

Table 4. Toxicity of Female Reproductive Tissues

		Ova			Oviduct	
Population	*n*	*Toxicity*	*Range*	*n*	*Toxicity*	*Range*
ER Female	4	1.1 ± 0.63	0.064 - 3.0	3	0.0032 ± 0.0032	0 - 0.0095
PF Female	3	0 ± 0	0 - 0	3	0 ± 0	0 - 0
SP Female	2	6.8 ± 0.29	6.5 - 7.1	2	0.10 ± 0.052	0 - 0.16

Mean ± SE and range of toxicities observed for ova and oviduct collected in the tissue toxicity experiment. Toxicity is measured in µg TTX/g tissue. Newts are separated by population; ER = Elk River, PF = Phillips Farm Pond, and SP = Sandpiper Pond.

Table 5. Toxicity of Male Reproductive Tissues

		Testes			Vas deferens	
Population	*n*	*Toxicity*	*Range*	*n*	*Toxicity*	*Range*
ER Male	8	0.013 ± 0.0053	0 – 0.044	8	0.11 ± 0.044	0.014 – 0.37
PF Male	1	0		1	0	

Mean ± SE and range of toxicities observed for testes and vas deferens collected in the tissue toxicity experiment. Toxicity is measured in µg TTX/g tissue. Newts are separated by population; ER = Elk River and PF = Phillips Farm Pond. No males from Sandpiper Pond were included in this experiment.

Table 6. Toxicity of Fat Body

		Fat Body	
Population	*n*	*Toxicity*	*Range*
ER Male	6	0.058 ± 0.028	0 - 0.19
ER Female	4	0.044 ± 0.012	0.032 - 0.089
PF Female	3	0 ± 0	0 - 0
SP Female	1	0.32	

Mean ± SE and range of toxicities observed for fat bodies collected in the tissue toxicity experiment. Toxicity is measured in µg TTX/g tissue. Newts are separated by sex and population; ER = Elk River, PF = Phillips Farm Pond, and SP = Sandpiper Pond.

Table 7. Toxicity of Digestive Tissues

	Kidney			Liver			Spleen	
Population	*n*	*Toxicity*	*Range*	*n*	*Toxicity*	*Range*	*n*	*Toxicity*	*Range*
ER Male	9	0.021 ± 0.011	0 - 0.098	7	0.0097 ± 0.0031	0 - 0.019	6	0.012 ± 0.011	0 - 0.070
ER Female	3	0.0089 ± 0.0089	0 - 0.027	3	0.025 ± 0.025	0 - 0.075	2	0 ± 0	0 - 0
PF Male	1	0		1	0		0		
PF Female	3	0 ± 0	0 - 0	2	0 ± 0		3	0 ± 0	0 - 0
SP Female	2	0.064 ± 0.031	0.033 - 0.095	2	0.0079 ± 0.0079	0 - 0.016	1	0.13	

Mean ± SE and range of toxicities observed for kidney, liver, and spleen collected in the tissue toxicity experiment. Toxicity is measured in µg TTX/g tissue. Newts are separated by sex and population; ER = Elk River, PF = Phillips Farm Pond, and SP = Sandpiper Pond.

Table 8. Toxicity of Excitable Tissues

	Brain			Heart Muscle			Skeletal Muscle		
Population	*n*	*Toxicity*	*Range*	*n*	*Toxicity*	*Range*	*n*	*Toxicity*	*Range*
ER Male	6	1.7 ± 0.71	0.045 – 3.7	9	0.36 ± 0.27	0 – 2.4	6	0.23 ± 0.094	0.012 – 0.61
ER Female	4	1.9 ± 0.92	0.13 – 4.5	2	0.11 ± 0.11	0 – 0.21	4	0.94 ± 0.6	0.025 – 2.6
PF Male	1	0		0			1	0	
PF Female	3	0 ± 0	0 - 0	3	0 ± 0	0 – 0	3	0 ± 0	0 - 0
SP Female	2	3.5 ± 0.84	2.7 – 4.4	1	0.84		2	2.0 ± 0.57	1.4 – 2.5

Mean ± SE and range of toxicities observed for brain, heart muscle, and skeletal muscle collected in the tissue toxicity experiment. Toxicity is measured in µg TTX/g tissue. Newts are separated by sex and population; ER = Elk River, PF = Phillips Farm Pond, and SP = Sandpiper Pond.

Table 9. Number of Tissue Samples from Toxic Newts with No Quantifiable TTX

	Elk River Males			Elk River Females			Sandpiper Females		
Tissue	*Total*	*0µg/g*	*0-5ng/ml*	*Total*	*0µg/g*	*0-5ng/ml*	*Total*	*0µg/g*	*0-5ng/ml*
Blood	8	1	0	3	1	0	1	*n/a*	0
Brain	8	0	2	4	0	0	2	0	0
Fat	6	1	0	3	0	1	1	0	0
Feces	5	0	1	3	1	1	1	0	0
Heart	9	5	0	3	1	1	2	0	1
Kidney	9	4	0	4	2	1	2	0	0
Liver	9	2	2	4	2	1	2	1	0
Muscle	8	0	0	4	0	2	2	0	0
Ova	*n/a*	*n/a*	*n/a*	4	0	0	2	0	0
Oviduct	*n/a*	*n/a*	*n/a*	3	2	0	2	0	0
Skin	8	0	0	4	0	0	2	0	0
Spleen	7	4	1	3	2	1	1	0	0
Testes	9	3	1	*n/a*	*n/a*	*n/a*	*n/a*	*n/a*	*n/a*
Vas deferens	9	0	1	*n/a*	*n/a*	*n/a*	*n/a*	*n/a*	*n/a*

Skin from all Elk River (ER) and Sandpiper Pond (SP) newts contained TTX; however, other tissues from some individuals with toxic skin contained extremely low levels of TTX or lacked it altogether. The total number of samples collected (*Total*), and the number of tissue samples that had either no TTX (*0 ng/ml*) or levels below the limit of detection (*0-5 ng/ml*) is indicated above. The limit of quantification is reported in ng/ml because it is based on the units of the TTX standards used to generate the standard curve. The concentration of TTX in newt tissues was converted using the mass of the tissue sample and reported in µg/g. *n/a* = not applicable.

Table 10. Mean Toxicity, Body Condition, Mass, and SVL of Toxic Newts

		Toxicity		Body Condition		Mass		SVL	
Population	*n*	*Mean ± SE*	*Range*	*Mean ± SE*	*Range*	*Mean ± SE*	*Range*	*Mean ± SE*	*Range*
All Toxic Newts	14	57 ± 25	2.7 - 360	4.2 ± 0.25	3.1 - 6.3	20 ± 1.4	12 - 28	79 ± 1.7	69 - 87
All Elk River Newts	12	26 ± 5.6	2.7 – 69	4.1 ± 0.26	3.1 - 6.3	21 ± 1.4	12 - 28	80 ± 1.6	73 - 87
All Toxic Females	6	110 ± 53	22 – 360	4.9 ± 0.36	4.1 - 6.3	15 ± 1.1	12 - 18	73 ± 1.3	70 -79
Elk River Males	8	20 ± 6.0	2.7 – 50	3.6 ± 0.16	3.1 - 4.3	24 ± 1.0	18 - 28	84 ± 1.1	78 - 87
Elk River Females	4	37 ± 11	22 – 69	5.0 ± 0.47	4.2 - 6.3	15 ± 1.4	12 - 18	74 ± 14	73 - 79

Mean ± SE and range of skin toxicity, body condition, mass, and snout-vent length (SVL) observed for toxic newts in the tissue toxicity experiment. Toxicity is measured in µg TTX/g tissue, body condition is measured in g/mm, mass is measured in g, and SVL is measured in mm. All Toxic Newts = male and female Elk River Newts and female Sandpiper Pond newts, All Elk River Newts = male and female Elk River Newts, All Toxic Females = Elk River and Sandpiper Pond newts.

Table 11. Regression Analysis of Toxicity on Body Condition, Mass, and SVL

		Toxicity on Body Condition		Toxicity on Mass		Toxicity on SVL	
Population	*n*	R^2	*p*	R^2	*p*	R^2	*p*
All Toxic Newts	14	0.022	0.61	0.12	0.23	0.37	0.022
All Elk River Newts	12	0.030	0.59	0.11	0.30	0.17	0.059
All Toxic Females	6	0.15	0.44	0.05	0.67	0.38	0.20
Elk River Males	8	0.001	0.59	0.026	0.70	0.25	0.21
Elk River Females	4	0.22	0.53	0.17	0.59	0.073	0.73

R^2 and *p*-values ($\alpha = 0.05$) for regressions of toxicity on body condition, mass, and snout-vent length (SVL) observed for toxic newts in the tissue toxicity experiment. All Toxic Newts = male and female Elk River Newts and female Sandpiper Pond newts, All Elk River Newts = male and female Elk River Newts, All Toxic Females = Elk River and Sandpiper Pond newts.

APPENDIX B

FIGURES

Figure 1. Aerial and Ground View of Sandpiper Pond. Top panel: Aerial view of Sandpiper Pond. Sandpiper Pond (indicated by arrows) is divided by the Oregon Coast Highway (Highway 101) in Waldport, OR. The part of the pond on the west side of the highway is bordered by Sandpiper Village subdivision and the east side (partially obscured by tree cover) is bounded by a wooded area. Newts were caught on both sides of the highway. Lower panel: Photograph of the west side of Sandpiper Pond. Vegetation buffers the pond on all sides. Floating vegetation is found at the edge of the pond and fallen trees are floating throughout. Sandpiper Pond has gentle slope with a soft bottom. Photo credits: Google Earth Pro 2017.

Figure 2. Aerial View of Elk River. Newts were captured in a calm, shallow, soft-bottomed inlet branching off from the Elk River (indicated by the arrow) located approximately 1 km east of the Oregon Coast Highway (Highway 101). The field site was flanked by a wooded area on the west and a sandy surface on the right. Part of the field site had floating vegetation, while other parts were open. The main part of the Elk River, which has a rocky bottom and moderate current, is separated from the field site by a sandy area. Multiple garter snakes (*Thamnophis sirtalis*) were observed on the shore while sampling. Photo credit: Google Earth Pro 2017.

Figure 3. Aerial View of Phillips Farm Pond. Phillips Farm Pond (indicated by the arrow) is located in Virgil Phillips Farm Park, a public park in Moscow, ID operated by Latah County Parks and Recreation, with unpaved hiking trails, picnic areas, wetlands, forest, and open fields. The western side of the pond is deep and permanent whereas the eastern side is shallower and was partially dried out when we were collecting newts. The pond is surrounded by vegetation, with some floating vegetation around the edge, open water in the middle, and a generally soft bottom with rocks closer to the edge. Photo credit: Google Earth Pro 2017.

Figure 4. Relative Locations of Field Sites. Sandpiper Pond and Elk River were both located along the Oregon Coast Highway (Highway 101), approximately 185 km apart. Sandpiper Pond and Phillips Farm Pond are approximately 610 km apart and Elk River and Phillips Farm Pond are approximately 930 km apart. Photo credit: Google Earth Pro 2017.

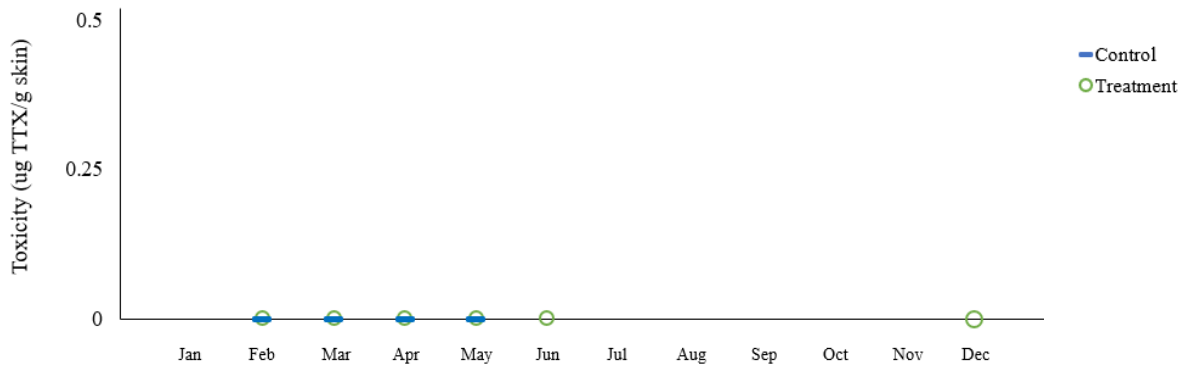

Figure 5. Toxicity of Philips Farm Pond Male Newts in the Cohabitation Experiment. The toxicity of non-toxic males in mixed treatment tanks and non-toxic control tanks remained at 0 µg TTX/g skin over the course of the experiment. Values shown are means.

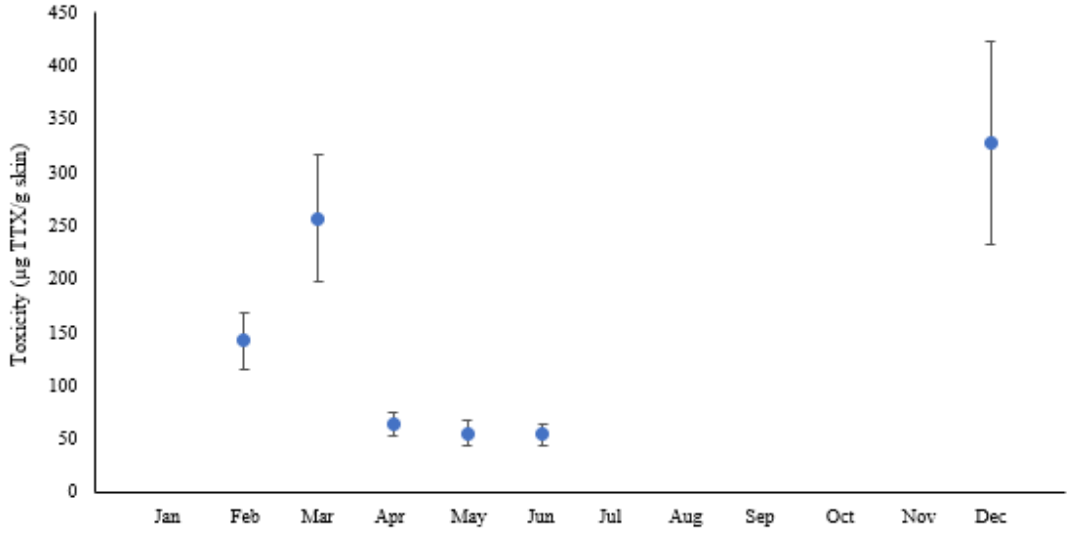

Figure 6. Toxicity of All Sandpiper Pond Male Newts in the Cohabitation Experiment. Data were collected February – June and again in December. Values shown are means with standard errors. 6a. Toxicity of all toxic males varied significantly over the course of the experiment (p = 0.00088, df = 59, F = 4.9).

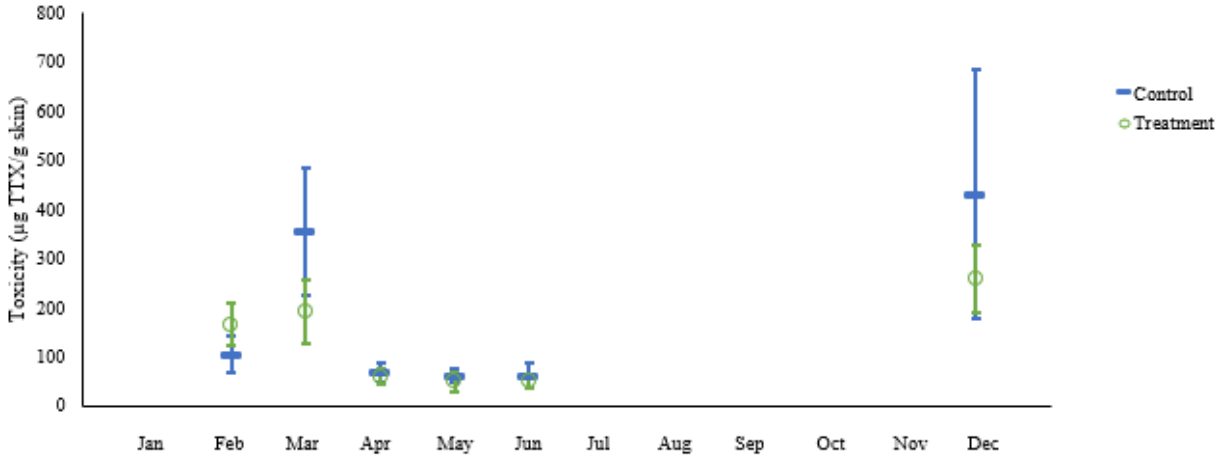

Figure 7. Toxicity of Treatment and Control Sandpiper Pond Male Newts in the Cohabitation Experiment. Data were collected February – June and again in December. Values shown are means with standard errors. Significant temporal variation in toxicity was observed in the toxic newts in the mixed-toxicity treatment (ANOVA, $p = 0.00630$, $df = 35$, $F = 1.98$, but not in the control treatment (ANOVA, $p = 0.13$, $df = 23$, $F = 1.98$).

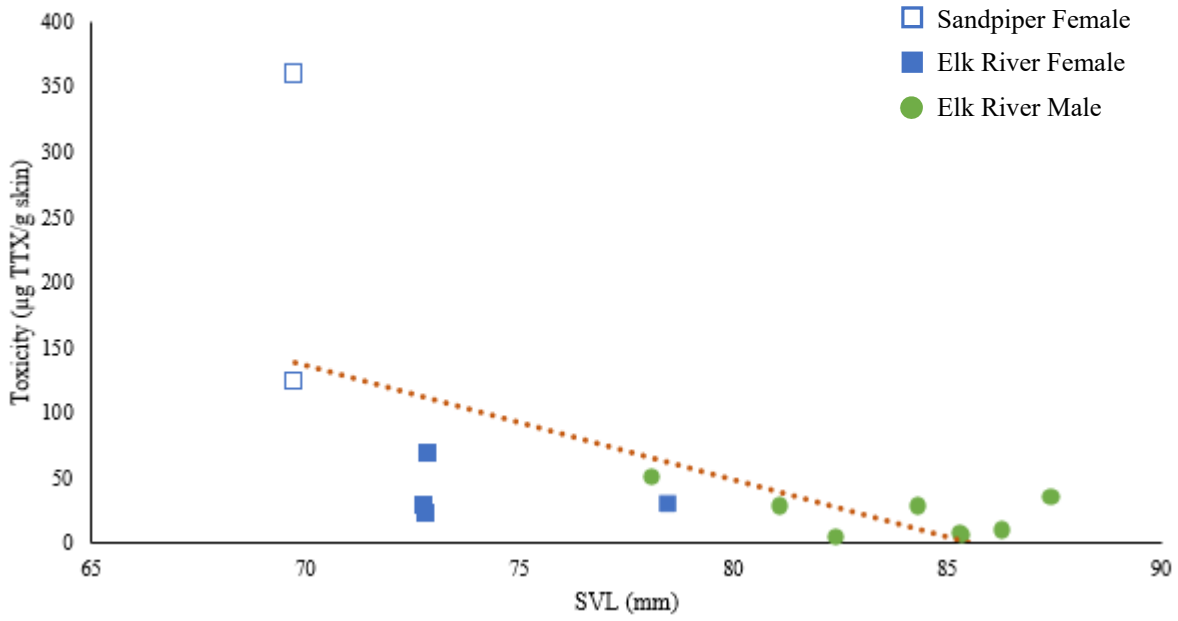

Figure 8. Correlation between SVL and Toxicity in Toxic Newts. SVL and toxicity were significantly correlated ($R^2 = 0.37$, $p = 0.022$) for toxic newts in the tissue toxicity experiment. Data shown here include Sandpiper Pond females ($n = 2$), Elk River females ($n = 4$), and Elk River males ($n = 8$).

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