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Quantifying tetrodotoxin levels in the California newt using a non-destructive sampling method

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ABSTRACT

Toxic or noxious substances often serve as a means of chemical defense for numerous taxa. However, such compounds may also facilitate ecological or evolutionary processes. The neurotoxin, tetrodotoxin (TTX), which is found in newts of the genus *Taricha*, acts as a selection pressure upon predatory garter snakes, is a chemical cue to conspecific larvae, which elicits antipredator behavior, and may also affect macroinvertebrate foraging behavior. To understand selection patterns and how potential variation might affect ecological and evolutionary processes, it is necessary to quantify TTX levels within individuals and populations. To do so has often required that animals be destructively sampled or removed from breeding habitats and brought into the laboratory. Here we demonstrate a non-destructive method of sampling adult *Taricha* that obviates the need to capture and collect individuals. We also show that embryos from oviposited California newt (*Taricha torosa*) egg masses can be individually sampled and TTX quantified from embryos. We employed three different extraction techniques to isolate TTX. Using a custom fabricated high performance liquid chromatography (HPLC) system we quantified recovery of TTX. We found that a newly developed micro-extraction technique significantly improved recovery compared to previously used methods. Results also indicate our improvements to the HPLC method have high repeatability and increased sensitivity, with a detection limit of 48 pg (0.15 pmol) TTX. The quantified amounts of TTX in adult newts suggest fine geographic variation in toxin levels between sampling localities isolated by as little as 3 km.

1. Introduction

An impressive diversity of taxa possess unique toxic or noxious substances (Yokoo, 1950; Noguchi and Hashimoto, 1973; Daly et al., 1987; Noguchi et al., 1982, 1986; Hwang et al., 1989; Berenbaum, 1995; Daly, 1995; Kubanek et al., 1995; Cimino and Ghiselin, 1998; Hartmann and Ober, 2000; Dumbacher et al., 1992, 2000; Fahey and Garson, 2002; Wood et al., 2012; Savitzky et al., 2012). Often these compounds serve as a means of chemical defense. However, they may also facilitate ecological and evolutionary processes (Elliott et al., 1993; Zimmer et al., 2006; Brodie and Brodie, 1990; Bucciarelli and Kats, in review). Prerequisite to determining how these compounds affect such processes, is the need to quantify their abundance within individuals and populations. Doing so provides the necessary foundation to understand their potential effect upon ecosystems at varied levels and time scales.

Newts in the family Salamandridae possess an extremely powerful neurotoxin, tetrodotoxin (TTX). Amounts of TTX found in newts have been quantified over

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the last 80 years to understand its occurrence and distribution across taxa (Twitty and Johnson, 1934, Mosher et al., 1964; Wakely et al., 1966; Shimizu and Kobayashi, 1983). Recently, broad geographical sampling and population estimates of TTX levels in newts of the genus *Taricha* have revealed its role as an agent of selection upon predatory garter snakes (Brodie et al., 2005; Hanifin et al., 2008). In other systems, larvae of the California newt, *Taricha torosa*, detect TTX, which elicits an antipredatory response. Larvae utilize TTX as a chemical cue to avoid cannibalistic adult *T. torosa* that will prey upon them when resources are scarce (Elliott et al., 1993; Zimmer et al., 2006). TTX from *T. torosa* may also affect macroinvertebrate foraging behavior (Bucciarelli and Kats, in review).

In order to quantify amounts of TTX in *Taricha*, whole newts have often been collected and sacrificed, or captured and brought into captivity to be sampled. Though populations of *Taricha* in central and northern California have historically been rather large, with upwards of 5000 newts found in breeding localities (Coates et al., 1970), not all populations are as substantial. Populations of *T. torosa* in southern California experience drastically different environmental selective pressures compared to congeners in northern locations, which has likely contributed to the decrease in breeding adults and low recruitment observed in their southern range (Jennings and Hayes, 1994). *T. torosa* found in the southern coastal areas of California (Santa Monica Mountains, Los Angeles, CA) breed in streams that are often ephemeral, in a heavily modified landscape that is arid, mountainous, and warmer than other parts of its range. Long term monitoring of amphibian populations across this landscape shows a negative trend in *T. torosa* populations. Currently, *T. torosa* is listed at the state level as a species of special concern (Jennings and Hayes, 1994; Thomson et al. in review).

In order to minimally disturb and help preserve *T. torosa* populations, we speculated that a smaller tissue sample than previously used to quantify TTX in *Taricha* (e. g. Hanifin et al., 2002) could be collected from adult newts in the wild. This would obviate the need to 1) collect animals, 2) disrupt breeding, 3) sacrifice animals, and 4) inflict a large wound. Few methods designed to sample TTX from animals have used non-lethal protocols (Khor et al., 2013; Hanifin et al., 2002), however these methods still required animals to be removed from natural habitat. As previous studies have documented low to non-existent amounts of TTX in *Taricha* populations (Hanifin et al., 1999), we improved upon an existing high performance liquid chromatography (HPLC) method to increase sensitivity and detect lesser amounts of TTX, should *T. torosa* in southern California have extremely low levels of TTX. Finally, we sought to detect and quantify TTX from wild embryos collected from egg masses oviposited late in the breeding season.

2. Methods

2.1. Materials

TTX solutions were prepared using commercial TTX (citrate salt, Fisher Scientific) and stored at 2–4 °C. All other reagents were ACS reagent grade or better (Fisher Scientific). Water was purified to >16.5 MΩ-cm and filtered through a 0.22 μm nylon membrane filter (Barnstead Nanopure II).

2.2. Instrumentation

2.2.1. High performance liquid chromatography (HPLC) system

A high performance liquid chromatography system coupled with fluorescence detection (HPLC-FLD) was adapted from a previous design (Yasumoto and Michishita, 1985). The chromatography system consisted of a Thermo Separation Products pump (P4000), a manual injector with a 50 μL loop (Rheodyne 7125), and a fluorescence detector (Waters model 474) equipped with a 16 μL flow cell. Fluorescence was observed at 505 nm with 381 nm excitation. The analytical column was a Sphericlone ODS 5 μm d_p 4.6 × 150 mm (Phenomenex, Torrance, CA, USA) protected with a SecurityGuard™ C18 guard cartridge (Phenomenex). The mobile phase consisted of 2 mM heptanesulfonic acid in 50 mM phosphate buffer, pH 7. The optimal flow rate was 0.35 mL/min. All components were connected via PEEK tubing and fittings.

2.2.2. Post-column derivatization

A second HPLC pump, identical to the analytical pump, supplied 4 M NaOH at 0.35 mL/min to facilitate post-column derivatization. We utilized a post-column reactor (Analytical Scientific Instruments, model 310) equipped with a 500 μL reactor cartridge thermostatted to 120 ± 0.5 °C. Eluant from the analytical column was mixed with 4 M NaOH at a PEEK mixing-Tee then passed through the post-column reactor (PCR). The analytical mobile phase and NaOH solution were continuously helium-degassed during analysis.

To provide sufficient backpressure to inhibit cavitation and cool the mixture prior to entering the detector, the eluant was passed through a 2 m coil of PEEK tubing (0.13 mm i.d.), which provided about 20 bar backpressure and acted as a heat exchanger to cool the eluant. A 75 psi (5 bar) backpressure regulator was placed on the detector waste outlet to further inhibit cavitation in the flow cell.

2.2.3. High performance liquid chromatography optimization

To test the optimization of this system, we ran TTX standards ranging from 5 to 200 ng/mL at temperatures from 100° to 140 °C. Furthermore, we tested the repeatability of our system by completing serial injections of a standard. At least 20 injections per standard were performed. An ANOVA was used to test injection to injection repeatability.

2.3. Animal sampling and intra-individual variation

Adult male California newts (*T. torosa*) were collected by hand or dip net from watersheds throughout the Santa Monica Mountains (Los Angeles, CA, USA). We improved a non-lethal sampling technique adapted from Hanifin et al. (1999) whereby a 2 mm skin biopsy tool (Acu-Punch, Acuderm Inc. Fort Lauderdale, FL) is used to collect tissue

from wild caught individuals. This method requires approximately 84% less tissue from animals.

Given the reduced tissue sample size, we tested variation within an individual by collecting three dorsal skin samples from adult male newts during the breeding season. Prior to collecting tissue, newts were anesthetized in a 300 ppm methanesulfonic acid (MS222) solution. Dorsal skin is known to have a uniform distribution of toxin glands (Hanifin et al., 2004), but collecting tissue too close to the vertebrae risks harming animals. Therefore, the sample was collected 1 cm away from the vertebrae, near the posterior dorsolateral area. Tissue was collected from the same area of an adult for each newt, followed by two subsequent samples collected randomly in the same proximity on either side of the newt. Sampling tools were sterilized between individuals. All samples were immediately placed in 300 μL of 0.1 M aqueous acetic acid and stored on dry ice before being transported to the laboratory. Anesthesia usually wore off within 5 min and newts were returned to their original habitat.

Embryos were collected from late-season oviposited egg masses found in local watersheds of the Santa Monica Mountains. Only egg masses that were detached from vegetation and found in shallow, drying pools were sampled. In the field, a scalpel was used to dissect an egg within the mass. The embryo was collected, rinsed with water, placed in 0.1 M aqueous acetic acid, and stored on dry ice. Entire egg masses were not compromised during collection, and the remaining egg mass was returned to the pool from where it was collected. A mixed model with a random intercept was used to test intra-individual variation of TTX levels between physical sampling locations on an individual newt.

2.4. Toxin extraction

2.4.1. Original method

To extract TTX from newt skin and embryo samples, we followed the method outlined by Hanifin et al. (1999). Briefly, an entire skin sample or embryo was weighed to the nearest 0.1 mg and macerated with the 300 μL of 0.1 M acetic acid in a glass tissue grinder. Suspensions were heated in a boiling water bath for 5 min then cooled in an ice bath for 5 min. Samples were sedimented and separated at 13,000 × *g* for 20 min. Supernatant was then collected and placed in centrifugal filters (Amicon Ultra, 10,000 MWCO) and centrifuged for 20 min. The remaining filtrate was diluted to 1 mL with 0.1 M acetic acid.

2.4.2. Modified method

Following the published method described in 2.4.1, we performed a simple modification to the procedure. Prior to the final dilution to 1 mL, the supernatant was transferred to the centrifugal filter a second time and centrifuged for 20 min at 13,000 × *g* to determine if a second pass over the pellet bed in the centrifugal filter would wash remaining TTX from any solids.

2.4.3. Micro-extraction method

We speculated that polar and ionic surfaces on proteins from newt tissue could be binding TTX through the centrifuging process and as a result reduce amounts of TTX recovered. To test this, we developed an experimental procedure, optimized for the 2 mm skin sample, whereby extractions were macerated, boiled, and cooled as described in 2.4.1. Samples were suspended in centrifugal filters and sedimented once for 20 min. Any solids remaining in the centrifugal filter were then washed by adding 100 μL of 0.1 M acetic acid to each filter and centrifuging for 20 min. Samples were diluted to 1 mL with 0.1 M acetic acid. For each extraction method used, extracts were immediately analyzed by HPLC or stored at −80 °C for later analysis.

2.5. Recovery studies of TTX from newt skin samples and embryos

To evaluate the recovery of the toxin from samples by the different extraction methods, a sample was macerated in a glass tissue grinder and separated into equal volumes in two vials. One vial was spiked with standard TTX and 100 μL of 0.1 M acetic acid was added to the other vial as a control. Spiked samples and the corresponding controls were then prepared following one of the extraction methods. Because of the limited number of embryos collected, embryos were prepared following only the modified and micro-extraction methods.

3. Results

3.1. Temperature optimization and injection to injection repeatability

Chromatograms in Fig. 1 show the separation of TTX from other epimers in two different adult skin samples. TTX epimers are clearly separated. We tested the sensitivity of the HPLC system by running TTX standards (5–200 ng/mL) at a range of PCR temperatures (100 °C–140 °C at 10° intervals). We observed an increase in sensitivity at 130 °C, but beyond 130 °C a loss of sensitivity was observed. The effect of PCR temperature upon sensitivity is presented in

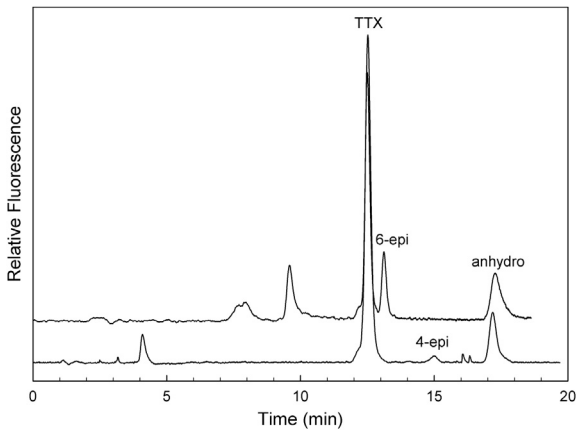

Fig. 1. HPLC-FLD chromatograms from adult male newts show the separation of TTX from other epimers. Observed epimers included 4-epi-, 6-epi-, and anhydro- TTX.

calibration curves in Fig. 2. The linearity of each calibration curve was high ($r^2 > 0.98$).

At a PCR temperature of 100 °C a detection limit of approximately 1 ng (3 pmol) TTX (S/N > 3) was measured, indicating no significant improvement in detection limit over other previous reports utilizing HPLC-FLD (Jen et al., 2008). At 120 °C we measured a detection limit of 48 pg (0.15 pmol) TTX (S/N > 3) and at 130 °C 41 pg (0.13 pmol). Operating the PCR at 120 °C offered the optimal compromise between stability, sensitivity, and detection limit.

Greater than 20 injections per standard, from a range of 5–200 ng TTX per mL, were used to test our HPLC method. Detected amounts of TTX from serial injections of standards was not significantly different (*ANOVA*, $p = 0.94$, $F = 0.059$, $df = 90$).

3.2. Intra-individual variation

Individual amounts of TTX in each of the three skin samples from ten adult male newts were derived using the peak area from HPLC chromatograms. TTX in all three skin samples from an individual are presented in Fig. 3. Results from the analysis showed no significant variation from injection to injection ($p = 0.613$) and no significant difference in variation of TTX levels between pairs of sampling locations on a newt ($p > 0.1$).

3.3. Toxin recovery from skin and embryo samples

Percentages of recovered TTX differed between extraction methods in both adult skin and embryo samples (Fig. 4). These differences were significant for embryo (*t test*, $p = 0.01$, $df = 6$, $t = 3.409$) and adult skin samples (*ANOVA*, $p < 0.00$, $t = 3.4$, $df = 23$). A post hoc analysis using a Tukey HSD test showed that recovered amounts of TTX were significantly different between the micro-extraction method and the original and modified methods ($p < 0.05$), but recovered amounts were not significantly different between the original and modified methods. TTX extracted from embryos using the modified method showed an average recovery of 73.4% ($n = 4$, percentage recovery SD = 22.1%, range = 62.5%–80.9%), whereas TTX extracted from embryos using the micro-extraction method showed considerably greater recovery at an average of 92.9% ($n = 4$, percentage recovery SD = 12.8%, range = 81%–99.4%). Skin samples extracted using the original method recovered on average 74.9% of TTX ($n = 4$, percentage recovery SD = 21.7%, range = 8.4%–90.5%). The modified method recovered on average 60.4% of TTX ($n = 17$, percentage recovery SD = 11.9%, range = 25.3%–96.1%). However, samples prepared with the micro-extraction methods recovered 95.9% of TTX ($n = 5$, percentage recovery SD = 8.9%, range = 84.3%–99.8%).

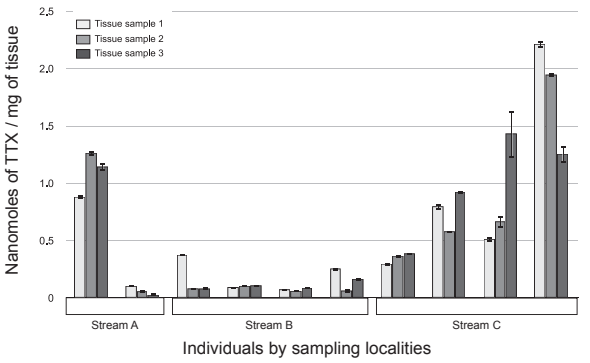

Fig. 3. Intra-individual variation of TTX levels of ten adult newts from three different watersheds. TTX levels between pairs of sampling locations on a newt were not significantly different.

4. Discussion

We modified a non-lethal biopsy method to collect smaller dorsal skin samples from wild-caught newts to quantify amounts of TTX in adult tissue. Because of the current state-level conservation status of *T. torosa*, this method mitigates any negative consequences that previous tissue sampling methods could have upon already greatly reduced populations in the Santa Monica Mountains. Unlike previous methods where newts were collected (e.g. Hanifin et al., 1999, 2002, 2004), this method ensures that

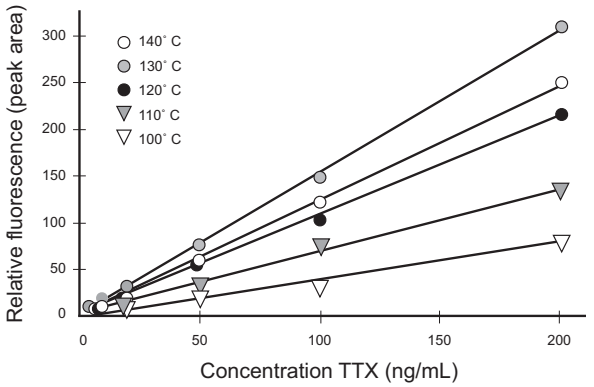

Fig. 2. Calibration curves of increasing concentrations of TTX at five temperatures. Sensitivity increases with increasing post column reactor (PCR) temperature, but decreases beyond 130 °C.

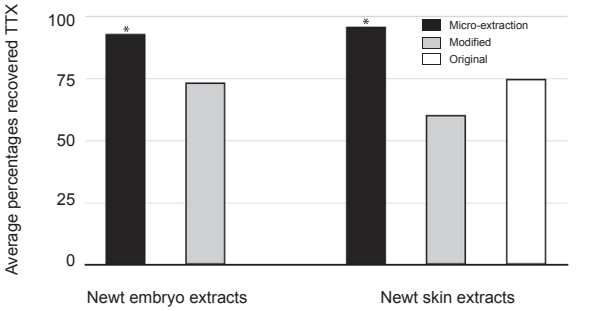

Fig. 4. Average recovered amounts of TTX from newt embryo extracts and newt skin extracts using micro, modified, or original extraction methods. Significantly greater amounts of TTX were recovered for both newt embryo and newt skin extracts using the micro-extraction method. Amounts of TTX recovered between original and modified methods were not significantly different.

live animals are not sacrificed nor removed from breeding localities. No fatalities occurred during the sampling of newts, and in subsequent sampling events, adult newts previously sampled were recaptured and showed signs of nearly complete recovery in as little as 14 days.

The variability of our non-lethal sampling method was tested by taking additional tissue samples from adult males in relatively the same region of the body immediately after an initial sample was collected. Though variation in amounts of TTX detected between sampling locations on the dorsal region of a newt was observed, the overall variation in amounts of TTX within an individual was generally consistently high or consistently low relative to other individuals. It does not appear that sampling causes animals to immediately release more toxin. Furthermore, our data show that skin samples taken along the length of a newt in relatively the same dorsal area can be used to describe amounts of TTX in the skin of adult newts. Given the small tissue size, individuals could be sampled repeatedly through time at fine temporal scales to better understand the chemical ecology of *Taricha*, but how repeated sampling of wild newts at such scales would affect individuals and TTX levels is unclear. Previous studies show that TTX levels increase in captive individuals, yet these individuals were only sampled three times throughout a year (Hanifin et al., 2002). How TTX levels change within populations throughout a regular breeding season remains undetermined.

Toxin was also successfully extracted from embryos and quantified. Because embryos were rinsed, it is not likely that embryonic fluid or egg mass material contributed to the detection of TTX in the embryo samples. Larvae and eggs are known to have TTX, but to our knowledge this is the first experiment to isolate embryos in the wild and extract and quantify amounts of TTX from them. Over the last five breeding seasons, the frequency of observing adults in streams has gradually declined and the breeding window has significantly decreased (Bucciarelli and Kats personal observations), but egg masses can often be found in streams later in the season. Though congeneric TTX levels appear to correlate with female TTX levels (Hanifin et al., 2003), whether TTX levels in embryos correlates with adult male TTX levels remains untested. In this study, only male *T. torosa* were sampled since females are almost always gravid when encountered during the breeding season. It is entirely possible that embryos could provide a snap shot of population level adult TTX levels, and describing and comparing patterns of TTX in embryos and adults could clarify whether TTX in *Taricha* is genetically controlled or the result of endosymbionts (Lehman et al., 2004).

Recovery experiments indicate that the original and modified TTX extraction methods did not recover considerable amounts of TTX, however, we should note the original method was for approximately six times more tissue, and this could affect recovery. The results of our recovery experiments show that washing the centrifugal filter with acetic acid can greatly increase the percentage of TTX recovered, leaving on average less than 10% TTX returned, as opposed to nearly 26%–40% with the other methods. These results suggest that the efficiency of a single extraction is relatively poor and is only marginally improved, if at all, by a second pass of the original extractant across the tissue bed in the centrifugal filter. Performing a second extraction by washing the tissue bed with fresh acetic acid improves the extraction yield. In addition, a small amount of TTX may be retained in the tissue bed in the centrifugal filter when only a single extraction is performed, even when washed with the original extractant, thereby contributing to the lower extraction yield. The micro-extraction method appears to be extremely reliable with improved yield and high precision. Inadequate recovery in earlier studies utilizing the original extraction method could be a partial explanation of previously observed levels of phenotypic mismatch between *Taricha* predator resistance and *Taricha* toxicity. As a result, overall toxicity of some populations may be underestimated, and at evolutionary scales contribute to the degree of mismatch between less toxic newt populations and predator populations with disproportionately greater resistance.

Interestingly, the patterns of TTX variation between watersheds (Fig. 3) suggest that *T. torosa* throughout the Santa Monica Mountains may exhibit fine scale geographic variation in TTX levels. Given the minimal amount of sampling to describe the chemical ecology of *Taricha*, our sampling methods allow researchers to collect data on populations of newts in the wild and across broad or fine scale geographical ranges. Other species known to possess TTX could also be sampled at similar scales using these methods. A relationship between concentrations of TTX in skin samples and total amounts of TTX in newt skin does exist (Hanifin et al., 2004). A correlation between concentrations of TTX in organs of an invertebrate and overall TTX concentrations has also been observed in an invertebrate (Khor et al., 2013). Future studies could quantitate overall amounts of TTX in *T. torosa*, different organs, or blood.

Use of fluorescence spectroscopy to detect TTX has become one the most common methods of analysis (Yasumoto and Michishita, 1985; Hanifin et al., 1999; Asakawa et al., 2000; O'Leary et al., 2004; Mebs et al., 2012; Sup) and though a number of other methods are also in use (Alcaraz et al., 1999; Kawatsu et al., 1999; Ito et al., 2006; Diener et al., 2007; Jen et al., 2008; Huang et al., 2008; Man et al., 2010; McNabb et al., 2010, Fong et al., 2011; Kudo et al., 2012; Cho et al., 2012; Stokes et al., 2012; Suprasert, 2013), each with its distinct advantages, HPLC with fluorescence detection remains popular, fast, and economical because of its simple and flexible sample preparation. Furthermore, our developed HPLC system demonstrates high repeatability, obviating repeated injections of samples. Thus, a small single tissue sample from an individual and a single HPLC injection can reliably be used to characterize TTX, thus conserving specimens, time, and funds.

Various TTX stereoisomers and epimers occur in TTX laden newts, and in some populations of *Taricha*, newts possess the epimer 6-epi TTX. When performing chromatography this epimer can be problematic because its peak is very close to TTX and separation of the two peaks does not always occur. Furthermore, HPLC-FLD systems can be approximately 20 times more sensitive to this epimer (Yasumoto and Michishita, 1985). Therefore, separation of

6-epi TTX from TTX is essential to determining actual amounts of TTX in samples. From the population of newts we sampled, 4-epi-, 6-epi-, and anhydro- TTX were observed and successfully separated from the TTX peak (Fig. 1). Other epimers did not occur in sufficient concentrations to be unequivocally identified.

To quantitate much smaller amounts of TTX and improve detection limits, our system was operated at temperatures above the commonly maintained 100 °C (Yasumoto and Michishita, 1985; O'Leary et al., 2004; Yotsu-Yamashita et al., 2012). Although the system is relatively stable for analysis up to 130 °C, we operated at 120 °C to achieve a higher level of stability. As a result, the mobile phase solutions needed to be continuously sparged with helium during analysis to prevent outgassing and cavitation. This made it necessary to have backpressure regulation before and after the detector to inhibit bubble formation.

Our results broadly contribute to the ongoing efforts to quantitate TTX for human health purposes, but also begin to characterize the chemical ecology of newts. Relative to the current understanding of how newt predator resistance plays out at evolutionary scales, an understanding of *Taricha* and their chemical ecology across landscapes is absent. Our sampling methods and the developed HPLC system allow for such a study. Given the current status of *T. torosa* in southern California, quantifying TTX levels in wild populations may also have long term implications for their conservation. Currently, no TTX-laden organisms is known to produce TTX without endosymbionts, but if the *Taricha* TTX phenotype is uniquely genetically controlled, then determining the landscape genetics of *Taricha*, and *T. torosa* specifically, may be used by land managers to conserve *Taricha* populations, as well as explain patterns of TTX variation at both fine and broad geographical scales.

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Conflict of interest

None declared.

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