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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