

TOXICITY OF DANGEROUS PREY: VARIATION OF TETRODOTOXIN LEVELS WITHIN AND AMONG POPULATIONS OF THE NEWT *Taricha granulosa*

CHARLES T. HANIFIN,^{1,*} MARI YOTSU-YAMASHITA,²
TAKESHI YASUMOTO,^{2,4} EDMUND D. BRODIE, III,³
and EDMUND D. BRODIE, JR.¹

¹Department of Biology, Utah State University
Logan, Utah 84322-5305

²Faculty of Agriculture, Tohoku University
1-1 Tsutsumidori-Amamiya, Aoba-ku
Sendai 981-8555, Japan

³Department of Biology, Indiana University
Bloomington, Indiana 47405

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Abstract—The ability to identify and accurately measure traits at the phenotypic interface of potential coevolutionary interactions is critical in documenting reciprocal evolutionary change between species. We quantify the defensive chemical trait of a prey species, the newt *Taricha granulosa*, thought to be part of a coevolutionary arms race. Variation in newt toxicity among populations results from variation in levels of the neurotoxin tetrodotoxin (TTX). Individual variation in TTX levels occurs within populations. Although TTX exists as a family of stereoisomers, only two of these (TTX and 6-epi-TTX) are likely to be sufficiently toxic and abundant to play a role in the defensive ecology of the newt.

Key Words—*Taricha granulosa*, Caudata, coevolution, tetrodotoxin, predator-prey, arms race.

INTRODUCTION

Reciprocal selection and evolutionary change between two or more taxa are fundamental to the concept of coevolution (Janzen, 1980; Futuyma and Slatkin,

*To whom correspondence should be addressed.

⁴Present address: Japan Food Research Laboratories, Tama Laboratory, 6-11-10 Nagayama, Tama, Tokyo 206-0025, Japan.

1983; Vermeij, 1994; Thompson, 1994). Demonstrating this selection requires evidence that sufficient heritable variation exists in the relevant traits for both species to respond to selection and that changes in the trait values of one species directly drive changes in trait values of the partner species (Berenbaum and Zangerl, 1992, 1998). The primary requisite for obtaining evidence of reciprocal evolution is the ability to identify and quantify the specific traits that mediate the interaction between species.

When the characters at the phenotypic interface of coevolution are morphological or behavioral, this task is fairly straightforward. When the interaction involves a defensive chemical and corresponding resistance, investigating the core traits of the relationship is more problematic. Chemically mediated coevolution is an ideal context in which to document reciprocal evolutionary change (Spencer, 1988), but practical problems associated with its study have confounded progress. These difficulties are exemplified in plant-herbivore systems, where identifying the most critical defensive trait or traits is obfuscated by the diversity of potential defensive compounds that can be found in a single plant (Berenbaum and Zangerl, 1992; Rosenthal and Berenbaum, 1991; Bernays and Chapman, 1994). Phytophagous insects probably respond to suites of chemicals rather than individual compounds, further complicating the task of determining which chemicals are relevant to a given interaction (Berenbaum and Zangerl, 1992). Similarly, many putative defensive compounds can affect a broad range of exploiting taxa (Berenbaum and Zangerl, 1992). Determining the species composition of a given coevolutionary interaction can be difficult when this range of effect is coupled with the varying degrees of specialization present in phytophagous insects (Berenbaum and Zangerl, 1992; Bernays and Chapman, 1994; Thompson, 1994). Similar problems exist in chemically mediated exploitive interactions between animals. Predator-prey interactions usually involve multiple participants and animals also can produce a myriad of potential defensive compounds (Daly et al., 1984, 1987).

Even when the relevant species and chemicals are identified, the logistics of quantitatively sampling target chemicals is troublesome. Behavioral mechanisms allow many insect herbivores to reduce their exposure to defensive compounds (reviewed in Bernays and Chapman, 1994). Similarly, many predators have developed patterns of behavior allowing them to avoid prey toxins [e.g., shrikes and grasshoppers (Yosef and Whitman, 1992), raccoons and toads (Groves, 1980)]. The results of these behaviors are that the estimates obtained by a scientist sampling whole leaves or skin sections in the laboratory can differ widely from the actual concentrations experienced by the organisms being studied. Finally, errors in estimates of chemical concentrations can arise because of variance in toxicity assays (Martin and Martin, 1982).

An example of a chemically mediated exploiter-victim system that is not hampered by the above complications is the interaction between the toxic newt

Taricha granulosa and its resistant predator, the garter snake *Thamnophis sirtalis* (Brodie and Brodie, 1990, 1991). Unlike most insect-plant systems, the taxa that comprise the interaction are few and well understood. Only one predator (*Thamnophis sirtalis*) and a single genus of prey (the salamandrid genus *Taricha*) are involved, and the traits at the phenotypic interface have been clearly identified (Brodie and Brodie, 1990, 1991). Newts of the genus *Taricha* possess the potent neurotoxin tetrodotoxin (TTX) (Mosher et al., 1964; Wakely et al., 1966; Brodie et al., 1974; Yotsu et al., 1990), and *Taricha granulosa* is the most toxic species of the genus (Brodie et al., 1974). Tetrodotoxin is a low-molecular-weight, nonprotein toxin found in a large number of disparate taxa. Its mode of action is well understood; TTX binds to and blocks Na⁺ channels, thereby interfering with the propagation of nerve and muscle action potentials. This system is further simplified by the fact that TTX is unlikely to have synergistic interactions with its stereoisomers. Tetrodotoxin and tetrodotoxin analogs compete for the same binding site in Na⁺ channels, and analogs with low toxicity relative to TTX appear to have lower binding affinity for the binding site in known systems. (Kao and Walker, 1982; Kao and Yasumoto, 1985; Yang et al., 1992).

Tetrodotoxin is known to be lethal to most potential predators (Brodie, 1968) and is an important antipredator mechanism for *Taricha granulosa* (Brodie, 1968; Brodie et al., 1974; Daly et al., 1987). *Thamnophis sirtalis* is the only known potential predator of *Taricha granulosa* that can survive ingestion of TTX (Brodie, 1968). This species has evolved resistance to TTX, allowing it to exploit newts in regions where the two are sympatric (Brodie and Brodie, 1990). Although *Taricha granulosa* also may possess an additional high-molecular-weight toxin (Brandon and Huheey, 1981, 1985), most of their toxicity likely results from TTX and its stereoisomers (Brodie et al., 1974). Additionally, because snake predators swallow their prey whole, they are unable to perform the sort of limited sampling of prey chemicals that complicates the relationship between the amounts of compounds found in prey and the amounts experienced by predators.

The limited number of defensive chemicals in this system makes the identification of the relevant defensive trait (TTX toxicity) straightforward, but quantifying variation in the trait has been more problematic. Previous studies have examined total toxicity of newts rather than TTX levels (Wakely et al., 1966; Brodie et al., 1974; Brodie and Brodie, 1991). This inability to quantify TTX itself has prevented investigation of the linkage between the defensive trait (TTX) in prey and the exploitative trait (TTX resistance) of predators.

In this study we used high-performance liquid chromatography (HPLC) techniques to quantify the TTX levels of *Taricha granulosa* from seven populations in the Pacific Northwest of North America. High-performance liquid chromatography provides an accurate and highly sensitive assay for TTX and TTX analogs (Yasumoto and Michishita, 1985; Yotsu et al., 1989; Wu et al., 1996). This technique has been used to study TTX in *Taricha granulosa* and

other salamandrids (Yotsu et al., 1990; Kotaki and Shimizu, 1993), as well as other Amphibia (Yotsu-Yamashita et al., 1992; Daly et al., 1994, 1997; Mebs et al., 1995). We tested the hypothesis that previously documented population variation in newt toxicity results from variation in TTX levels by sampling from populations where total toxicity had already been assayed (Brodie, 1968; Brodie et al., 1974). In addition, we explicitly tested the hypothesis that populations of *Taricha granulosa* from Vancouver Island, British Columbia, do not possess TTX (Brodie and Brodie, 1991) by sampling populations from this area. We also sampled three other populations from Oregon and Washington to investigate patterns of interpopulational variation in TTX levels of *Taricha granulosa*. Finally, because multiple analogs of TTX have been found in *Taricha granulosa* (Yotsu et al., 1990; Kotaki and Shimizu, 1993), we examined the stereoisomers of TTX to determine which analogs are both toxic and abundant enough to be biologically important in the interaction between *Taricha granulosa* and *Thamnophis sirtalis*.

METHODS AND MATERIALS

Samples. Seventeen adult *Taricha granulosa* were collected from six sites in Oregon, Washington, and the Vancouver Island region during 1996 and 1997 (Figure 1). Specimens from Oregon collected in 1996 or 1997 came from one of two populations: a coastal population in Lane County [Tenmile ($N = 5$)], and a Willamette Valley population [Benton County ($N = 1$)]. Specimens from Washington came from a coastal site [Grays Harbor ($N = 4$)] and the Olympic Peninsula [Beaver Lake ($N = 4$)]. Of the animals collected on or around Vancouver Island, one site is on Vancouver Island [Qualicum ($N = 1$)]; the other site is on an island adjacent to Vancouver Island [Texada Island ($N = 2$)]. These animals were either frozen immediately at -80°C or tissue was removed and frozen in liquid nitrogen. Preserved specimens from two sites [Reid Island, British Columbia ($N = 4$), and Benton County, Oregon ($N = 4$)] were also used. These preserved animals were collected in 1989, preserved in 95% ethanol and stored at 4°C . Preserved animals from Reid Island, the third site from the Vancouver Island region, came from an island near the southern end of Vancouver Island. Because preserved and wild-caught animals from Benton County were from physically proximate sites, samples from these two groups were pooled as "Benton County." Previous research on TTX (Brodie, 1968; Brodie et al., 1974) indicated that TTX toxicity was stable for at least one year when stored at low temperatures. Data collected for this manuscript indicated that samples from the same population (Benton County) did not significantly differ in TTX levels regardless of their method or length of storage (fresh frozen vs. storage in ethanol for eight years), indicating that neither the length nor method of tissue storage in this study altered

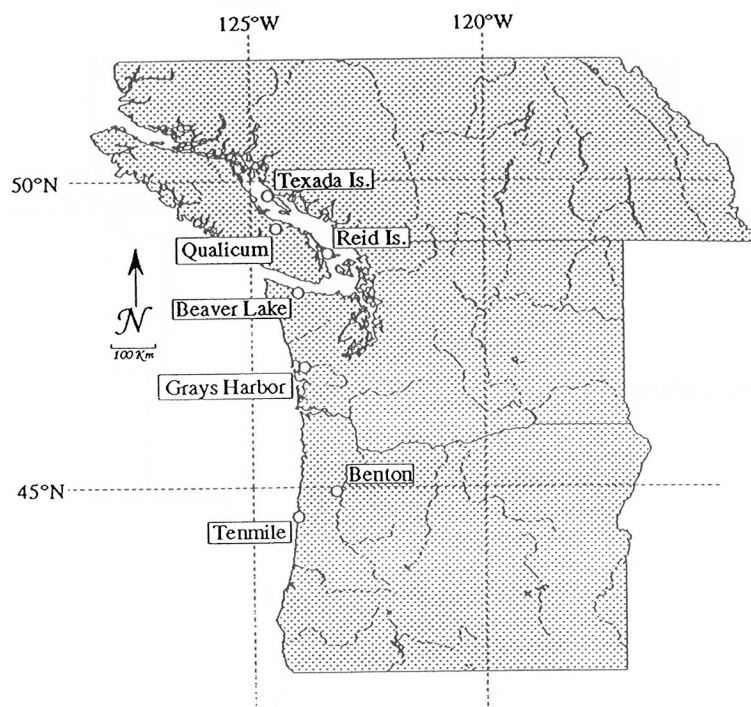


FIG. 1. Map of populations surveyed.

TTX levels. Therefore, frozen and preserved samples for any given population were pooled in this study.

Toxin Extraction. Extracts from each sample were prepared from dorsal skin that had either been removed from subjects and immediately frozen at -80°C (specimens from 1996 or 1997) or removed from subjects just prior to toxin extraction (preserved specimens from 1989). Each tissue sample was weighed and cut into small pieces, and enough extraction buffer (0.1 M aqueous acetic acid) was added to bring the final concentration of the sample to 0.05 g skin/ml of buffer. Samples were heated in a boiling water bath for 5 min and 1 ml of the resultant extract spun at 10,000 rpm for 10 min. Following this first spin, 0.5 ml of the supernatant was spun at 10,000 rpm for 10 min in 0.5-ml Millipore centrifuge filter tubes (Ultrafree-MC, 10,000 NMWL filter unit). Aliquots of 10 μl of this final filtrate were used for analysis. Repeatability of the extraction protocol was measured with two independent skin extractions on individuals from Tenmile ($N = 5$). Each of these extractions was analyzed as an independent sample, and the repeatability of the extraction was calculated using a one-way

ANOVA (Becker, 1992). The repeatability for this extraction protocol and toxin analysis was high ($r = 0.95$), indicating that the extraction protocol was not a significant source of variance.

TTX Assay. The levels of TTX and TTX analogs present in newts were quantified by fluorometric HPLC (Yasumoto and Michishita, 1985; Yotsu et al., 1989). Separation of analogs was performed on a Develosil ODS-5 column (0.46×25 cm, Nomura Chemical, Seto, Japan) with a 50 mM ammonium acetate and 30 mM ammonium heptafluorobutyrate buffer (pH 5.0) containing 3% acetonitrile run at a flow rate of 0.5 ml/min. The eluate was mixed with an aqueous 4 N NaOH solution from another pump (1.2 ml/min), and passed through a stainless steel tube (0.5 mm \times 10 m) heated at 105°C to derive analogs to fluorophore. After cooling in a 30-cm water jacket, the fluorescent derivatives were detected by a Jasco FP110 fluoromonitor (Jasco Co. Ltd., Tokyo, Japan). The excitation wavelength of the detector was set at 365 nm and emission wavelength set at 510 nm. The peak areas were measured and integrated with a Hitachi Chromato-Integrator D-2500.

Standards and Analysis. Standard solutions of TTX, 4-epi-TTX, and 4,9-anhydro-TTX were isolated from pooled eggs of *Fugu poecilonotus* and *F. pardalis* (Nakamura and Yasumoto, 1985). The 6-epi-TTX standard was isolated from the newt *Cynops ensicauda* (Yasumoto et al., 1988). The purity of these compounds was checked by a combination of HPLC, thin-layer chromatography (TLC), ^1H NMR spectroscopy, and mass spectrometry (Yotsu et al., 1990). Tetrodotoxin was quantified by weighing. Because of the difficulty of weighing small amounts of hydroscopic TTX isomers, quantification was made by ^1H NMR spectroscopy with TTX as the standard (Yotsu-Yamashita, et al., unpublished data). Because the HPLC analyzer is 20 times more sensitive to 6-epi-TTX than to TTX (Figure 2) (Yasumoto and Michishita, 1985; Yotsu et al., 1989), a standard solution of 6-epi-TTX was prepared separately from the standard mixture of TTX, 4,9-anhydro-TTX, and 4-epi-TTX. This difference in sensitivity also means that direct comparison of peak area or height for different analogs (Figure 2) is not an accurate method of gauging the relative amounts of each analog. Therefore, the amount of each analog was calculated based on the standard curve for each analog. This relationship is linear (Yasumoto and Michishita, 1985; Yotsu et al., 1989) within the ranges of TTX and 6-epi-TTX of our samples and could be used to quantify the amount of TTX and 6-epi-TTX present in our samples. The lower limit of detection of this system is $4.5\text{ }\mu\text{g}$ of TTX per gram of skin and $0.23\text{ }\mu\text{g}$ of 6-epi-TTX per gram of skin. The analyzer was calibrated by injecting the TTX and 6-epi-TTX standards before and after assaying each sample. All samples were assayed twice and the mean of those measures used for analysis.

Statistical Analysis. Because our raw data were heteroscedastic and had variances proportional to group means, we used a square root transformation on

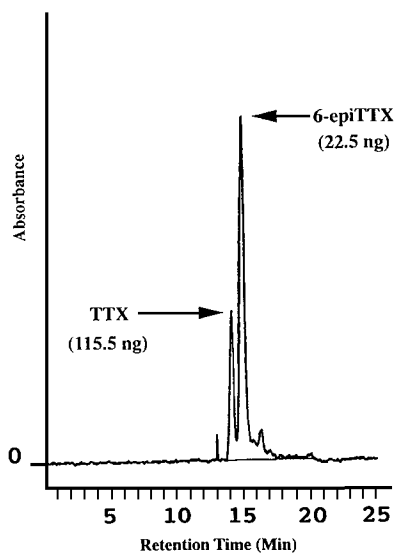


FIG. 2. HPLC chromatogram of TTX (115.5 ng) and 6-epi-TTX (22.5 ng) standards showing the differential sensitivity of the analyzer. The area under each peak is linearly related to the quantity of that compound. Note that the scale of this figure is different from that of other figures and cannot be used for direct comparison of TTX and TTX analog concentrations.

our data (Zar, 1984). These transformed data were used for all analyses. Mean levels of TTX and 6-epi-TTX for populations that possessed these compounds were compared by one-way ANOVA. Post-hoc pairwise comparisons of all population means were done by using the Student-Newman-Keuls test.

RESULTS

We found TTX in newts from four of seven sites (Table 1, Figure 3). Animals from Oregon (Tenmile, Benton County) and Washington (Grays Harbor, Beaver Lake) possessed TTX, while no individual from either Vancouver Island (Qualicum) or islands adjacent to Vancouver Island (Reid Island, Texada Island) possessed TTX (Table 1, Figure 3). In both populations from Oregon and one population from Washington (Grays Harbor), all of the individuals had TTX. In the second population from Washington (Beaver Lake) only two of four individuals had TTX.

Significant differences in TTX levels were found among newt populations with TTX ($F = 28.889$, $df = 3$, $P = 0.0001$). Pairwise comparisons indicated that significant differences existed between all population pairs, with the exception

TABLE 1. COMPARISON OF LEVELS OF TTX AND 6-epi-TTX OF 7 POPULATIONS OF *Taricha granulosa* FROM THE PACIFIC NORTHWEST

Population	Sample size (N)	TTX (mg/g skin, mean \pm SE)	6-epi-TTX (mg/g skin, mean \pm SE)
Benton County, OR	5	1.024 \pm 0.142	0
Tenmile, OR	5	1.752 \pm 0.110	0.236 \pm 0.027
Grays Harbor, WA	4	0.143 \pm 0.031	0.271 \pm 0.125
Beaver Lake, WA	4	0.007 \pm 0.004	0
Qualicum, BC	1	0	0
Texada Island, BC	3	0	0
Reid Island, BC	4	0	0

of Beaver Lake–Grays Harbor (Table 2). Mean levels of TTX were highest in populations from Oregon and lower in Washington (Tables 1 and 2). Because 220 ng of TTX kills one mouse [ddy, male, 15–20 g body weight (Fuhrman, 1986)], on average 1 g of skin from individuals from Oregon contained enough TTX to kill between 5000 (Benton County) and 9000 (Tenmile) mice, while 1 g of skin from individuals from Grays Harbor had enough TTX to kill 650 mice. Beaver Lake, the mainland population closest to Vancouver Island, had the lowest mean level of TTX (Tables 1 and 2), but 1 g of skin of an average individual from this population still had enough TTX to kill 32 mice. We found at least some intrapopulation variation in TTX levels in all populations from Oregon and Washington (Figure 4).

Newts from only two populations (Tenmile and Grays Harbor) possessed the C-6 stereoisomer of TTX, 6-epi-TTX (Table 1, Figure 3), and there was no significant difference between the mean levels of 6-epi-TTX of these populations ($F = 0.277$, $df = 1$, $P = 0.61$). In these samples, all individuals possessed 6-epi-TTX. No newts from either Benton County or Beaver Lake had 6-epi-TTX (Figure 3). All newts from Benton County and Tenmile possessed 4-epi-TTX (Figure 3), but concentrations of this analog were too low to quantify accurately. Other TTX analogs, including 4,9-anhydro-TTX and 4,9-anhydro-6-epi-TTX, were present in all individuals from populations in Oregon and Washington (Figure 3), but these compounds are unlikely to be important in the antipredator ecology of *Taricha* because of their low toxicity relative to TTX and 6-epi-TTX (Yasumoto and Yotsu-Yamashita, 1996). These anhydro isomers of TTX (i.e., 4,9-anhydro-TTX and 4,9-anhydro-6-epi-TTX) have been shown to have one-fiftieth the binding affinity of TTX in frog skeletal muscle and squid axon Na^+ channels (Kao and Walker, 1982; Kao and Yasumoto, 1985; Yang et al., 1992). Although relative toxicity of these anhydro analogs in snakes is unknown, these analogs are unlikely to possess dramatically different toxicity.

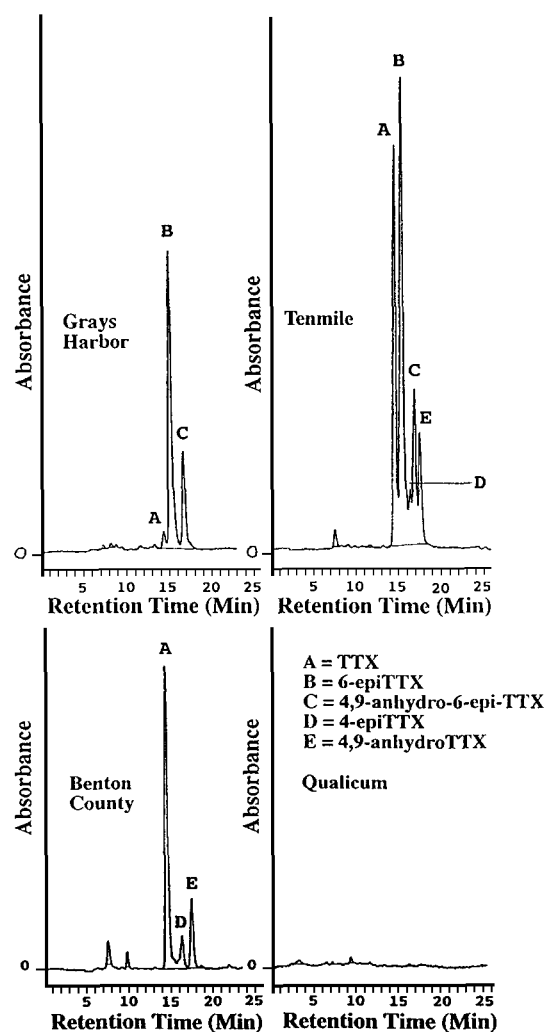


FIG. 3. HPLC chromatograms of representative individuals from four different populations showing differences in the amount of TTX present as well as differences in the types of analogs present. Newts from Qualicum, Vancouver Island, British Columbia, lack TTX and TTX analogs. Newts from Grays Harbor, Washington, and Tenmile, Oregon, show the presence of 6-epi-TTX, which is lacking in newts from Benton County, Oregon. Peak area for each compound is linearly related to its quantity. It is important to note that our analyzer is 20 times more sensitive to 6-epi-TTX than to TTX; thus, direct comparison of TTX and 6-epi-TTX peak area is not an accurate method of gauging the relative abundance of those compounds.

TABLE 2. PAIRWISE POST-HOC COMPARISONS OF MEAN TTX LEVELS FOR ALL POPULATIONS^a

Population	Compared with	Critical difference $\sqrt{(\text{TTX ng/g skin})}$		Observed Difference $\sqrt{(\text{TTX ng/g skin})}$
Beaver Lake	Grays Harbor	7.06	n.s.	5.5
	Benton County	8.62	<i>b</i>	19.6
	Tenmile	9.58	<i>b</i>	27.2
Grays Harbor	Benton County	7.06	<i>b</i>	14.0
	Tenmile	8.62	<i>b</i>	21.6
Benton County	Tenmile	7.06	<i>b</i>	7.6

^aStudent-Newman-Keuls test was used. Tests were performed on square root transformations of ng/g measurements.

^bSignificance levels of $P < 0.05$.

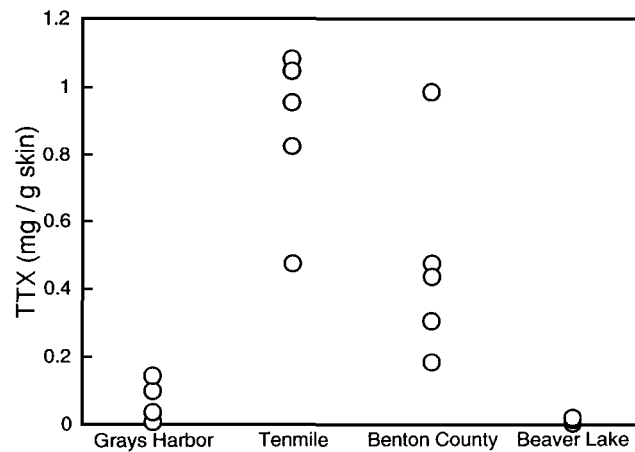


FIG. 4. Graph of individual values (mg TTX/g skin) of tetrodotoxin for all individuals from populations where TTX was present. Both interpopulation and intrapopulation variation is present, although small sample sizes make precise evaluation of intrapopulation variation in TTX levels difficult.

DISCUSSION

Quantifying the variation in TTX levels of *Taricha granulosa* is a requisite step in evaluating whether coevolution occurs between the newt and its garter snake predator. Previous studies of this system have focused on variation within and among populations of the predator (Brodie and Brodie, 1990, 1991). The results presented here document a pattern of variation within and among newt

populations that is both necessary for, and consistent with, the occurrence of chemically mediated coevolution between predator and prey.

If TTX is the central trait in a coevolutionary interaction between *Taricha granulosa* and *Thamnophis sirtalis*, then we should see a pattern of matched exploitative ability and defense among populations of the two species (Brodie and Brodie, 1991, 1999; Thompson, 1994; Berenbaum and Zangerl, 1998). Our analysis of TTX levels confirms an explicit link between the chemical ecology of prey and predator species and reveals some unexpected patterns of geographic variation. Newts from Vancouver Island (and surrounding islands) possess no TTX and are sympatric with snakes that lack TTX resistance (Brodie and Brodie, 1991). Where snakes are known to be highly resistant to TTX (Brodie and Brodie, 1990), newts possess high levels of TTX (Benton County, Tenmile). Geographically intermediate (Washington) populations of newts have low, but highly variable, levels of TTX.

Dramatic population differences in TTX levels of newts may occur on a fine geographic scale. Populations at Benton County and Tenmile are separated by about 70 km, yet significant differences existed in mean TTX levels in these two populations. In addition, these populations differ substantially in the presence of some TTX analogs. Snakes at these two sites differ slightly in their average resistance to TTX as well (Brodie and Brodie, 1999). Newts and snakes from Tenmile exhibit somewhat higher levels of TTX and TTX resistance, respectively. The pattern of geographic variation in both defensive and exploitative abilities is consistent with the geographic mosaic perspective of coevolution that predicts coevolution will occur at the population scale and may result in different outcomes in different localities (Thompson, 1994).

Individual, heritable variation in trait values must be present at both sides of the phenotypic interface for coevolution to occur within any single population. These criteria have been demonstrated for TTX resistance in *Thamnophis sirtalis* (Brodie and Brodie, 1990), but have not been previously explored in *Taricha granulosa*. While our experimental design does not allow us to speculate on the inheritance of TTX levels in newts, we do observe individual differences in TTX levels that selection might act upon. Individual variation ranged from quantitative differences in TTX concentrations within most populations, to discrete differences in the population at Beaver Lake, where two of the four individuals sampled completely lacked TTX and TTX analogs.

Conclusions about the interpopulation and intrapopulation variability of newt TTX levels are limited by the small sample sizes of our study. Additional sampling at one or more populations may change quantitative estimates of mean toxicity or variation within some populations. However, significant population differences in toxicity do exist. Additionally, our estimate of mean TTX levels of newts from the Benton County population closely matched previously published estimates (Brodie et al., 1974). We view our data as preliminary evidence

supporting a rough pattern of coevolution between these two species rather than a definitive assessment of patterns of geographic variation in the TTX levels of *Taricha granulosa*.

The genetic control of TTX production in taxa that possess the toxin is poorly understood. Tetrodotoxin in pufferfish is thought to originate from bacteria (Yotsu et al., 1987; Yasumoto and Yotsu-Yamashita, 1996, but see Matsumura, 1995), but this condition has not been shown in newts. Toads of the genus *Atelopus* possess TTX in the wild, but do not produce TTX when raised in captivity (Daly et al., 1997) indicating that at least some aspect of their TTX production is affected by environmental factors. The existence of newts that either possess TTX or lack TTX from a single population (Beaver Lake) may indicate that TTX production is not entirely environmentally determined. If newts are producing their own TTX, a number of factors, including diet, ontogeny, and season, are likely to affect the toxicity of individual newts (Shimizu and Kobayashi, 1983; Daly et al., 1997).

Finally, our results suggest that 6-epi-TTX is the only TTX analog of possible ecological importance in interactions between newts and snakes. While we found multiple TTX analogs in our populations (including 6-epi-TTX, 4,9-anhydro-6-epi-TTX, 4-epi-TTX, and 4,9-anhydro-TTX), only 6-epi-TTX and 4-epi-TTX are likely to be sufficiently toxic (Yasumoto and Yotsu-Yamashita, 1996) to be biologically significant. Although data on the relative toxicity of TTX analogs are based on data from squid axon and frog skeletal muscle (Kao and Walker, 1982; Kao and Yasumoto, 1985; Yang et al., 1992), the action of TTX and the structure of the TTX binding site in Na⁺ channels makes it unlikely that either 4,9-anhydro-6-epi-TTX or 4,9-anhydro-TTX possess much toxicity in garter snakes. Of the analogs with high toxicity (6-epi-TTX and 4-epi-TTX), only 6-epi-TTX was present at concentrations that are likely to generate toxic effects. On average, individual newts from both Tenmile and Grays Harbor possessed enough 6-epi-TTX to kill 500 mice, indicating that it could play an important role in the antipredator arsenal of newts from these populations. As with TTX, interpopulational variation in the presence or absence of 6-epi-TTX was detected, but no variation was seen in the mean levels between 6-epi-TTX-bearing populations. Why newts from some populations possess other TTX analogs but do not possess 6-epi-TTX remains to be explained, as does the homogeneity of mean 6-epi-TTX levels in populations where it is present. Answers to questions about TTX analog variability may shed light not only on the coevolutionary interaction between *Taricha granulosa* and *Thamnophis sirtalis* but also on the biosynthesis of TTX.

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