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A RESISTANT PREDATOR AND ITS TOXIC PREY: PERSISTENCE OF NEWT TOXIN LEADS TO POISONOUS (NOT VENOMOUS) SNAKES

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Abstract—The Common Garter Snake (*Thamnophis sirtalis*) preys upon the Rough-skinned Newt (*Taricha granulosa*), which contains the neurotoxin tetrodotoxin (TTX) in the skin. TTX is toxic, large quantities are present in a newt, and highly resistant snakes have the ability to ingest multiple newts; subsequently snakes harbor significant amounts of active toxin in their own tissues after consuming a newt. Snakes harbor TTX in the liver for 1 mo or more after consuming just one newt, and at least 7 wk after consuming a diet of newts. Three weeks after eating one newt, snakes contained an average of 42 μg of TTX in the liver. This amount could severely incapacitate or kill avian predators, and mammalian predators may be negatively affected as well.

Key Words—*Taricha granulosa*, *Thamnophis sirtalis*, toxicity, resistance, chemical defense, tetrodotoxin, aposematism, coevolution.

INTRODUCTION

Amphibians are characterized by epidermal granular glands that secrete substances with primarily defensive functions (Duellman and Trueb, 1986), some of which are lethal to predators. Yet many snakes have evolved resistance to toxins that kill other predators, such as *Heterodon* and *Xenodon* that eat toads of the genus *Bufo*

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(see Smith and White, 1955), *Liophis* that eat harlequin frogs (*Atelopus sp.*), and poison dart frogs *(Dendrobates* and *Phyllobates*; Myers et al., 1978), and Central American colubrids (*Thamnophis fulvus, Coniophanes fissidens, Pliocercus elapoides*, and *Rhadinaea* spp.) that eat the plethodontid salamander *Bolitoglossa rostrata* (see Brodie and Ducey, 1991). Similarly, the Common Garter Snake (*Thamnophis sirtalis*) preys upon the Rough-skinned Newt (*Taricha granulosa*).

The Rough-skinned Newt contains the powerful neurotoxin tetrodotoxin (TTX) in the skin (Mosher et al., 1964; Hanifin et al., 1999), which blocks voltage-gated sodium channels in nerve and muscle tissue, thereby inhibiting action potentials (Narahasi et al., 1967). TTX is extremely potent; the oral LD_{50} for a mouse is 334 μg/kg (Mosher et al., 1964; Kawasaki et al., 1973). In other words, only 6–7 μg of orally administered TTX will kill 50% of 20 g mice. *T. granulosa* are variable in toxicity throughout their geographic range and can vary greatly within a population as well (Hanifin et al., 1999, 2002). However, in one toxic population (Soap Creek in the Willamette Valley, Benton Co., Oregon), adult newts contain from 0.6 to 13.0 mg of TTX in their skin (Hanifin et al., 2004), an amount of toxin far in excess of that necessary to kill almost any potential predator of *T. granulosa* (Brodie, 1968).

Nonetheless, *T. sirtalis* consume *T. granulosa* on a regular basis (e.g., Brodie, 1968). Resistance to the toxin occurs in certain populations of *T. sirtalis* that prey upon the newt and have apparently coevolved with them (e.g., Brodie and Brodie, 1990, 1999a,b; Geffeney et al., 2002). The outcomes of coevolutionary interactions may vary over the geographic range in species interactions because of gene flow or differences in local selection (Thompson, 2000). Although some locations exhibit newts with little or no toxicity and correspondingly low to nonresistant snakes, other populations of snakes and newts have been caught in an escalation of reciprocal selection on toxicity and resistance that has led to extremely toxic newts and correspondingly highly resistant snakes—hotspots of snake–newt coevolution (Brodie et al., 2002). Benton Co., in the Willamette Valley, Oregon, is one such locality. A captive snake from the Benton Co., Oregon, population consumed eight adult newts in a 2-wk period (Brodie, 1968). Thus, an individual garter snake may ingest phenomenal amounts of toxin and survive.

Because of the extreme toxicity of TTX, the large quantities present in a newt, and the ability of highly resistant snakes to ingest multiple newts, the possibility arises that snakes may harbor significant amounts of active toxin in their own tissues after consuming a newt. Dendrobatid and mantelline frogs acquire toxicity from dietary precursors (Daly et al., 1997; Saporito et al., 2003). A genus of Natricine snake (*Rhabdophis*, closely related to *Thamnophis*) encompasses several species that appear to sequester toad (*Bufo*) toxins, which are then secreted from nuchal glands in the neck region (Akizawa et al., 1985). The snakes exhibit unique antipredator behavior that exposes predators to the glandular secretion (Mori et al., 1996).

Passive or active containment, sequestration, or failure to flush TTX from the system could generate elevated concentrations of the toxin in the tissues of *T. sirtalis*. The possibility arises that snakes may exploit the toxic newts and receive protection, *via* the chemical defense of TTX, from their own predators. Here, we investigate the location and quantities of TTX accumulation in Common Garter Snakes (*T. sirtalis*) after ingestion of Rough-skinned Newts (*T. granulosa*), factors that influence the accumulation of TTX in the snakes, and possible consequences of that accumulation for snake predators.

METHODS AND MATERIALS

Common Garter Snakes (*T. sirtalis*) were fed Rough-skinned Newts (*T. granulosa*), and the fate of the toxin within the snakes was determined. These animals were collected from Benton Co., Oregon, where newts are highly toxic and snakes comparably resistant (Brodie et al., 2002). Snakes were housed in 38-l aquaria, given access to a thermal gradient with an ambient temperature of 25°C, and exposed to a 12:12 L/D cycle. Previous work showed that the majority of toxin exposure to snakes consuming amphibians occurs in the upper gastrointestinal (GI) track (Brodie and Tumbarello, 1978; Williams et al., 2002), and that exposure time (defined as the length of time a newt occupied the upper GI track) influenced the intoxication of a snake (Williams et al., 2003). Thus, snakes were randomly offered live newts for feeding trials.

Feeding Trials and Tissue Collection. Newt snout–vent length (SVL), total length (TL), and mass were recorded immediately before snake-feeding; snake SVL, TL, and mass were taken after the feeding trial. For those snakes that consumed newts, mass was estimated by subtracting the mass of the newt. Snakes were euthanized with 0.5–1.5 ml of 10% chlorotone, or 0.05–0.25 ml Beauthanasia-D, depending on snake mass. In concordance with tissues examined in rats and mice after administration of TTX (Ogura, 1958; Kao, 1966), tissue samples of liver, kidney, heart, skeletal muscle, blood, and musk were collected from each snake. Tissues were immediately frozen at −80°C after collection.

Snakes were assigned to four groups. Group A ($N = 9$) from Benton, Co., OR and Clatsop Co., OR was housed in the lab as above and fed only fish weekly for over 1 year. Snakes in Clatsop Co., OR also exhibit high resistance to TTX, though less so on average than those from Benton Co., OR (Brodie et al., 2002). Group A was offered a diet of newts (two newts/wk for 5 wk). After 5 wk, snakes were returned to a diet of fish. Snakes were sacrificed weekly, and tissues were collected to examine TTX concentrations. Sampling in Group A was expanded to six snakes during the fourth week after the last newt was consumed in order to evaluate variation in toxicity. Group A was sampled before a nonlethal assay for newt toxicity was developed, thus, the quantity of toxin ingested by these snakes is unknown. By

monitoring the decay of TTX in Group A, we were able to evaluate the assumption that 9 mo was enough time for TTX to be purged from wild-caught treatment snakes (Group D below). *T. sirtalis* from Bear Lake Co., Idaho were allopatric with newts, and served as a negative control (Group B; $N = 2$). Snakes in Group C ($N = 6$) from Benton Co., OR had been kept in the lab for 12 mo and fed only fish. They were screened for signs of the toxin in their liver as an additional negative control.

Treatment snakes (Group D; $N = 15$) were fed a diet of fish weekly for at least 9 mo, enabling us to assume that they were TTX-free before treatment. Group D was fed one newt each then returned to a diet of fish. Tissues were collected on a weekly basis by sacrificing snakes; tissues were collected from five snakes 1 wk after newt consumption, four snakes after 2 wk, four snakes after 3 wk, and four snakes after 4 wk. For snakes in Group D only, the mass of the liver, two kidneys combined, and heart were recorded during tissue collection. A skin sample was collected from each newt given to treatment snakes (Group D) in order to later determine newt toxicity.

Before snake-feeding trials, each newt was anesthetized with 3% Tricaine, and a skin-biopsy punch (Acu-Punch™, Acuderm Inc.) was used to extricate a 5-mm diam circle of mid-dorsal skin as per Hanifin et al. (2002). After a 24-hr recovery period, newts were offered to snakes. The quantity of TTX in the skin sample was assayed in the same manner as TTX in snake tissues as described below.

One snake from the Benton Co., OR, population was encountered in the field on 14 October 2000 after consuming an adult female newt containing eggs. Hanifin et al. (2003) found that the quantity of TTX per clutch of eggs was as much as 1–3 μg, while females contained 0.046–0.487 mg TTX/cm^2 skin. Females were also found to be slightly more toxic than males (Hanifin et al., 2002). The newt ingested by the wild-caught snake was palpated out upon capture in the field; we estimated the newt was consumed 1–2 d previously. The skin was digested, however, the body form was mostly intact, as were the eggs. This opportunity provided an empirical test of TTX retention in the wild. Four days later on 18 October 2000 the snake was euthanized and tissues were collected.

Extraction of Toxin. TTX was extracted from the tissues by modifying the procedure of Hanifin et al. (1999). Two replicates of 125 mg of snake tissue per ml of 0.050 N acetic acid were mixed in a 1 ml Dounce glass homogenizer (Wheaton, USA). Newt tissues were similarly extracted for a final concentration of 10 mg/ml. Extracts were immersed in boiling water for 5 min and cooled in an ice bath before centrifugation at 13,000 rpm for 15 min. The supernatant was collected and filtered though 5,000 NMWL (snakes), or 10,000 NMWL (newts) 0.5 ml Ultrafree-MC Millipore tubes in the centrifuge at 13,000 rpm for 20 min; filtrates were stored at -80°C.

Detection and Quantification of Toxin. Twenty microliters aliquots of sample extracts were quantified on a reverse phase high performance liquid chromatography (HPLC) system by modifying the procedures of Yotsu et al. (1989), and Hanifin et al. (1999). Modification of the HPLC parameters, particularly with respect to the mobile phase, resulted in appropriate phase shifts of elutants such that we were able to quantify accurately TTX in snake tissues. TTX from newt tissues was eluted with an isocratic gradient of 3.0% (2.0% for snakes) by volume acetonitrile, 0.010% (0.013% for snakes) by weight heptafluorobutyric acid, and 0.049 N (0.010 N for snakes) acetic acid. The pH of this mobile phase was adjusted with 50% NH_4OH to pH 5.0 for newts and pH 6.0 for snakes. Analytes were separated with a C18 reverse phase column, Develosil ODS-UG-5 (250 $\times$ 4.6 mm Nomura Chemical, Japan) for newts and Synergi 4 μ Hydro-RP 80A (250 $\times$ 4.6 mm, Phenomenex, USA) for snakes. A flow rate of 0.5 ml per min was produced by a Shimadzu LC-10ADVP pump. The post-column reaction was induced with 5 N sodium hydroxide in a Pickering CRX 400 post-column reactor at 130°C with a flow rate of 0.9 ml per min from a Beckman Model 110A pump. Products were cooled in a water jacket before detection by a Dynamax fluorescence detector Model FL-2 with excitation wavelength set at 365 nm and emission wavelength at 510 nm. An HP 3390A integrator was used to record the chromatographs and calculate peak areas.

TTX peaks in tissue samples were identified and quantified by comparison with a standard of 50 ng TTX (Calbiochem) in 20 μl of 0.050 N acetic acid. Identification of TTX in snake tissues was also evaluated by spiking snake tissues with alleged TTX peaks with TTX standard to elucidate possible co-elution discrepancies. The samples were also compared with TTX-free tissues of two snakes from Bear Lake Co., ID where *T. sirtalis* are not resistant to TTX and *Taricha* are absent. A standard curve for TTX was created between 10 and 500 ng; however, quantification was less accurate at low concentrations in control snake tissues spiked with TTX. Thus, control tissue samples from Bear Lake snakes were spiked with 10, 25, 50, and 160 ng to determine sensitivity of quantification in actual snake tissues. Detector response was linear, accurate, and precise (within 1–10%) at all TTX concentrations in newt tissues and at 50 ng and above in snake tissues. Thus, all snake tissue samples with detectable TTX, but less than 50 ng, were spiked with 47 ng TTX. A snake liver with over 50 ng TTX was spiked with 165 ng TTX as a check on the spiking procedure; quantification in this case was accurate within one SE.

Analysis. For Group A, fed a diet of newts, simple linear regression of the log TTX concentration in the liver vs. time was used to evaluate the assumption that 9 mo was sufficient to purge snakes with a large TTX load. Because the data set did not include points where the concentration of TTX in liver was zero, a 95% confidence interval (CI) for the predicted time that TTX concentration decreased to zero was extrapolated.

Data from treatment snakes (Group D) were analyzed by multiple regression to evaluate the effects of newt toxicity and time (since newt consumption) on snake toxicity. Total newt toxicity was estimated using a formula that incorporates surface area, ratios of general toxin distribution in newts, and the concentration of toxin in dorsal skin by area (Hanifin et al., 2004). Total snake toxicity was conservatively estimated as the concentration of TTX in the liver of each snake multiplied by the mass of the liver. Data were transformed to better approximate normal distributions as follows: log (time), log (newt toxicity + 1), and log (snake toxicity$^{0.8}$ + 1).

In order to put the persistence of TTX in snake livers in context, we estimated the rate of elimination of the toxin based on first order kinetics. For a first order process, the rate of elimination is proportional to the amount of chemical in the body. We assumed a 1st order process because TTX is water soluble and expected to rapidly equilibrate between tissues. Also, a plot of the log concentration vs. time appears to yield a straight line. Based on these assumptions, the decay of TTX over time should follow a one compartment open model. In this case, the decay can be represented by this equation:

$$\log(C/D) = \log C_0 - (k_{\text{el}} \cdot t)/2.303$$

(modified from Medinsky and Klaassen, 1996)

where C represents the concentration of TTX in a snake's liver, D is the dose that an individual snake received (total newt toxicity/mass snake), C_0 represents the y-intercept, t is time, and k_{el} is the first order elimination rate constant. Once the rate of elimination (k_{el}) was estimated, the half-life of TTX in the liver was estimated with the following relationship: $t_{1/2} = 0.693/\, k_{\text{el}}$ (Medinsky and Klaassen, 1996). All data analyses were performed using SAS software, Version 8 of the SAS System for Windows (SAS, 1999).

RESULTS

Because of their larger size, female snakes from the population in Benton Co., OR, were more often able to consume adult newts. Only one male snake consumed a small recently metamorphosed newt during preliminary trials. After garter snakes consumed toxic newts, peaks in the HPLC chromatographs of tissue extracts appeared to correspond to the newt toxin, TTX. The alleged TTX in snake tissues co-eluted with the TTX standard, further verifying the identity of the compound. Snakes from Bear Lake Co., ID, (Group B), which are allopatric to newts, showed no signs of TTX in any tissues, including liver (Figure 1). Snakes from Benton Co., OR, (Group C) fed exclusively a diet of fish for 9 mo also lacked TTX in their livers. During preliminary investigations, screening of a snake 1 hr after consuming a newt revealed TTX in the kidney, liver, skeletal muscle, blood,

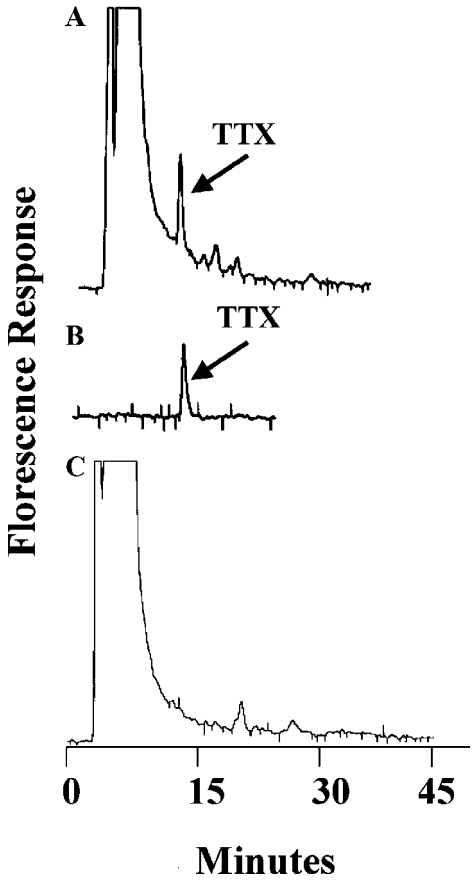

FIG. 1. Chromatographs of (A) a snake liver containing tetrodotoxin (TTX) 1 wk after consuming a newt, (B) commercial TTX standard, and (C) control snake liver from a snake population allopatric to newts.

and heart (by decreasing concentration). In snakes that had consumed a diet of newts (Group A), TTX was present in the liver (up to 7 wk, the last sampling period) and kidney (up to 3 wk), but not in skeletal muscle, cardiac muscle, or blood by 1 wk after newt consumption. One week after consumption of a single newt, TTX was not detectable in kidney, skeletal muscle, cardiac muscle, or blood of treatment snakes (Group D), but was detectable in the liver up to the final sampling period of 4 wk. The liver of the wild-caught snake found with the half-digested newt contained approximately 108 μg of TTX. TTX could not be identified in snake musk with this method because of numerous peaks that eluted

at or near the same time as the toxin. However, the size of peaks in this region indicated that little or no TTX was present in snake musk following newt ingestion. A summary of the location of TTX in snake tissues, number of newts eaten, time elapsed since the last newt eaten, and concentration of TTX in the liver for all snakes is given in Table 1.

Variation between replicate snake tissue extracts fell within the detector variation of 1–10%. Variation between replicate newt tissue extracts was slightly higher at 1–13%, likely due to the small size of tissue samples and sensitivity of the scale used to measure the sample mass. Newts fed to treatment snakes (Group D) ranged in toxicity from 0 to 8.34 mg TTX per newt (mean $\pm$ SD = 3.00 ± 2.19 mg). Tissues collected from one treatment snake (Group D) 4 wk after consuming one newt were excluded from all analyses because this newt contained no TTX. Although TTX was absent from the toxin profile of this newt, other TTX isomers were present.

Snakes in Group A (offered a diet of newts) consumed between four and eight newts in the 5-wk period. Simple linear regression of the log TTX concentration in the liver vs. time for snakes in Group A predicted that our assumption of 9 mo was sufficient time to purge these wild-caught snakes of TTX (Figure 2; df = 7; $F = 7.92$; $P = 0.03$; $r^2 = 0.57$; $y = -0.02x + 1.88$). The regression predicted a loss of toxin in the livers at 62 d with a 95% CI of 24–100 d, approximately one third of the time snakes were held in the lab before being fed newts. Because of the log conversion, a snake with a TTX concentration of zero was excluded, resulting in a more conservative estimate for time required to purge TTX. Because TTX was detected only in the liver of treatment snakes (Group D) 1 wk after consuming a single newt and was not present in other tissues after this time, total snake toxicity was conservatively estimated as the concentration of toxin in the liver multiplied by the mass of the liver. Analyses were then focused on the TTX in the liver of snakes.

We used a partial regression model to examine the effects of total newt toxicity and time on total snake toxicity (Figure 3). There was no interaction between total newt toxicity and time (df = 14; $t = 2.73$; $P = 0.16$), thus, the interaction term was dropped from the model. As expected, total newt toxicity affected the amount of toxin per snake (df = 14; $t = 3.17$; $P = 0.01$). Time was less influential on snake toxicity (df = 14; $t = -1.93$; $P = 0.08$); however, the trend of decreasing toxin concentration over time is evident (Figure 3). For Group D (treatment snakes), the regression of the log of the concentration of TTX in snake liver divided by dose vs. time is shown in Figure 4 (df = 14; $F = 3.97$; $P = 0.07$; $r^2 = 0.234$; $y = -0.03x + 2.0$). Using this regression, the first order elimination rate constant, k_{el} , was calculated at 14.0 d^{-1}. Consequently, an estimate of the half life of TTX in snake liver based on first order kinetics is 9.7 d. After 7 1/2 lives, 99.2% of a chemical is eliminated (Medinsky and Klaassen, 1996), corresponding to 73 d after consumption of a single newt in garter snakes.

TABLE 1. SUMMARY OF ALL SNAKES AND NEWTS, SAMPLING REGIMES, TOXICITIES, AND PRESENCE/ABSENCE OF TETRODOTOXIN (TTX) IN SNAKE TISSUES

Group designation for snake (see text)	Number of newts eaten	Total newt toxicity (mg TTX/newt)	Time since last newt consumption (days)	Tissues in which TTX was detectable (+/−)					Concentration of TTX in snake liver (μg TTX/g liver)	Total snake toxicity (μg TTX)
				Liver	Kidney	Heart	Skeletal Muscle	Blood		
N/A	1	NE	1 hr	+	+	+	+	+	NE	NE
N/A	1	NE	6 d?, field caught	+	NE	NE	NE	NE	17.6	108.0
A	5	NE	8	+	+	−	−	−	48.6	NE
A	4	NE	14	+	+	−	−	−	75.2	NE
A	8	NE	28	+	NE	NE	NE	NE	12.1	NE
A	3	NE	28	+	NE	NE	NE	NE	8.8	NE
A	7	NE	28	+	NE	NE	NE	NE	14.2	NE
A	6	NE	28	+	NE	NE	NE	NE	22.8	NE
A	8	NE	28	+	NE	NE	NE	NE	31.2	NE
A	4	NE	28	−	NE	NE	NE	NE	0.0	NE
A	7	NE	49	+	NE	NE	NE	NE	10.4	NE
B	0	N/A	N/A	−	−	−	−	−	0	0
B	0	N/A	N/A	−	−	−	−	−	0	0
C	0	N/A	N/A, housed in lab 1 year	−	NE	NE	NE	NE	0	0
C	0	N/A	N/A, housed in lab 1 year	−	NE	NE	NE	NE	0	0
C	0	N/A	N/A, housed in lab 1 year	−	NE	NE	NE	NE	0	0
C	0	N/A	N/A, housed in lab 1 year	−	NE	NE	NE	NE	0	0
C	0	N/A	N/A, housed in lab 1 year	−	NE	NE	NE	NE	0	0
C	0	N/A	N/A, housed in lab 1 year	−	NE	NE	NE	NE	0	0
D	1	0	N/A	NE	NE	NE	NE	NE	NE	NE
D	1	2.94	7	+	−	−	−	−	10.6	40.4
D	1	3.09	7	+	−	−	−	−	6.4	21.0
D	1	2.47	7	+	−	−	−	−	25.0	84.3

TABLE 1. CONTINUED

Group designation for snake (see text)	Number of newts eaten	Total newt toxicity (mg TTX/newt)	Time since last newt consumption (days)	Tissues in which TTX was detectable (+/−)					Concentration of TTX in snake liver (μg liver)	Total snake toxicity (μg TTX)
				Liver	Kidney	Heart	Skeletal Muscle	Blood		
D	1	1.53	7	+	−	−	−	−	4.7	20.1
D	1	4.07	7	+	−	−	−	−	19.8	62.1
D	1	2.92	14	+	NE	NE	NE	NE	3.8	12.9
D	1	8.34	14	+	NE	NE	NE	NE	12.3	62.7
D	1	0.59	14	+	NE	NE	NE	NE	2.0	9.2
D	1	1.78	14	+	NE	NE	NE	NE	4.2	15.5
D	1	1.12	21	+	NE	NE	NE	NE	11.5	35.0
D	1	5.88	21	+	NE	NE	NE	NE	10.9	48.7
D	1	4.19	21	+	NE	NE	NE	NE	8.5	35.9
D	1	2.60	21	+	NE	NE	NE	NE	20.1	47.4
D	1	1.03	28	−	NE	NE	NE	NE	0.0	0.0
D	1	5.45	30	+	NE	NE	NE	NE	9.9	32.8

Note. NE = Not examined, N/A = Not applicable.

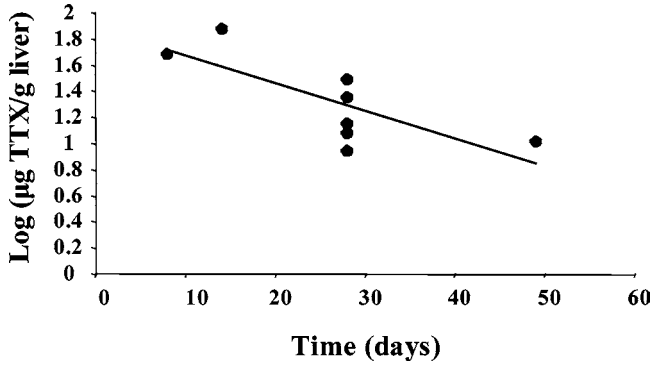

FIG. 2. Logarithmic regression of tetrodotoxin concentration in the liver of snakes fed a diet of newts (Group A) vs. time since last newt consumed. Expanded sampling at 4 wk illustrates variation in snake toxicity.

DISCUSSION

Individuals of *T. sirtalis* from the population in the Willamette Valley, Benton Co., OR, are capable of ingesting massive amounts of the potent neurotoxin TTX. The toxin lingered in snake livers for at least 7 wk and kidneys up to 3 wk after snakes were fed a diet of newts. The presence of TTX in the liver and kidney

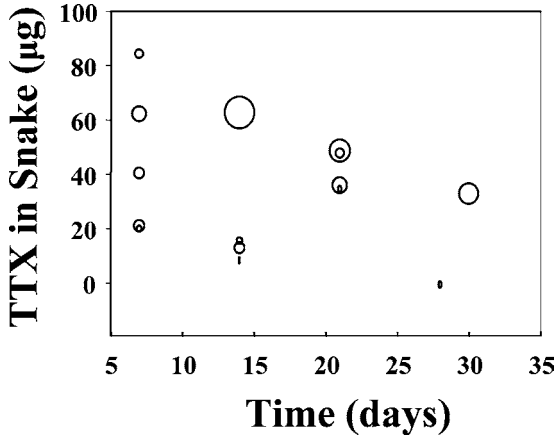

FIG. 3. Relationship of total snake toxicity with time after consumption of one newt (Group D). Bubble area varies in proportion to individual newt toxicity such that larger bubble areas correspond to higher newt toxicity.

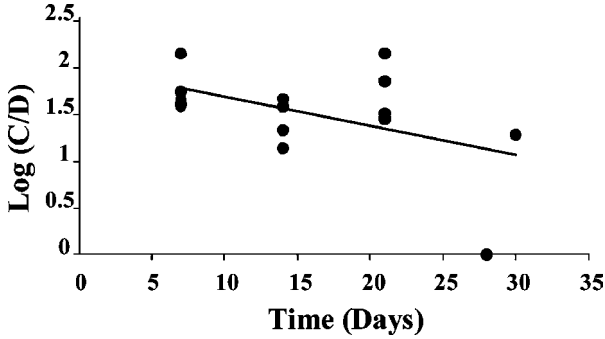

FIG. 4. The regression of the log of the concentration of TTX in snake liver (ng TTX/μl extract) divided by dose (newt toxicity per mass snake in mg TTX/g snake) vs. time (days) for Group D (treatment snakes).

and the absence thereof in other tissues is not surprising. The liver is primarily responsible for detoxification in the body, and Ogura (1958) found that after a subcutaneous injection, large amounts of TTX were present in the kidney of rats, pending elimination. In snakes that consumed only one newt, TTX was present in the liver in appreciable quantities after 1 mo. As expected, snake toxicity depended on the amount of toxin ingested (total newt toxicity). The decrease of toxin concentration over time is also evident, though weak (Figure 3). The shallow slope of toxin elimination over time indicates that TTX is being eliminated at a fairly slow rate.

The estimate of the half-life of TTX in snake liver based on first order kinetics is 9.7 d. The half-life of TTX in rat liver is only 3–4 hr (Ogura, 1958). After 7 1/2 lives, 99.2% of a chemical is eliminated (Medinsky and Klaassen, 1996), thus, rats functionally have no TTX left in the liver after, at most, 28 hr, compared with 73 d for *T. sirtalis* after the consumption of one newt. The persistence of saxitoxin (STX) for 2 mo in the hepatopancreas of lobsters (Cembella and Desbiens, 1994) suggests that there may be differences in longevity of these toxins resulting from divergent physiological characteristics in endotherms and ectotherms. Another possibility is that livers of snakes may contain some kind of molecule that binds TTX. The liver of a species of puffer fish contained a high molecular weight component that released TTX upon digestion with RNase (Kodama et al., 1983). Nagashima et al. (2003) found that puffer fish liver, as opposed to three other fish, differentially accumulated TTX when incubated in a TTX solution and retained the toxin even after incubation in a TTX free solution for an additional 48 hr. TTX is concentrated in the liver of snakes, removed from the sites of action of the toxin. Thus, snakes may not need to expel TTX before they can function normally, resulting in the persistence in livers for several weeks.

This persistence has potential consequences for predators of the snake. Although many snakes (e.g., vipers) are venomous (differentiated by an active delivery of toxin, as in biting or stinging), *Rhabdophis* is the only documented (Akizawa et al., 1985) poisonous snake (passive delivery of toxin, usually by ingestion). Known garter snake predators include herons (*Ardea, Butorides*; Hancock and Kushlan, 1984), ravens and crows (*Corvus*; Shine et al., 2001), hawks (*Buteo*; Fitch, 1965; Richardson et al., 2001), raccoons (*Procyon*), minks (*Mustela*), foxes (*Vulpes*), and badgers (*Taxidea*; Fitch, 1965; and see Rossman et al., 1996). American Bitterns (*Botaurus*; Rapp, 1954; Hancock and Kushlan, 1984) are known to eat *Thamnophis* up to 2 ft in length. Susceptibility to TTX of a variety of taxa has been explored by several investigators (Ishihara, 1918; Kao, 1966; Brodie, 1968). Unfortunately, the variability in newt toxicity between and within populations was not documented until three decades later (Hanifin et al., 1999). Additionally, the standard measurement of TTX quantity is based on mouse units—the reaction of mice to an intraperitoneal injection of toxin extract (Kawabata, 1978). How much purified TTX a mouse unit represents varied between early studies (see Kao, 1966). Hence, the exact amount of TTX administered in these previous studies testing toxin susceptibility is unknown. Yet, predicting the qualitative effect of consuming snake livers on some predators is still possible.

Three weeks after consuming just one newt, the mean quantity of toxin in snake livers was 42 μg ($N = 4$). Birds (common snake predators) are more susceptible to TTX than mammals and reptiles (Ishihara, 1918; Kao, 1966; Brodie, 1968). Several documented instances of predation by birds on *Taricha* yielded no survivors (see Mobley and Stidham, 2000). Although an estimate for a minimum lethal oral dose for birds does not exist, there was some precedent to assume that such a dose could be predicted (Williams et al., 2002) from the minimum lethal dose of subcutaneous injection of TTX for a pigeon (2.0 μg/kg; Ishihara, 1918; Kao, 1966). In mice, approximately 23 times the minimum lethal subcutaneous dose was required to produce a minimum lethal oral dose of TTX (Kawasaki et al., 1973). An estimate of the minimum lethal oral dose estimate for a pigeon is then $23 \times 2.0 = 46$ μg/kg. Even with the inherent variability of such an estimate, the extreme susceptibility of birds to TTX is apparent.

The amount of TTX in snake livers 3 wk after ingesting one newt averaged 42 μg. One may infer that avian predators of garter snakes such as Northern Harriers (420 g), Red-tailed hawks (1080 g), American Bitterns (700 g), and American Crows (450 g; Sibley, 2000) would be severely affected—if not killed—by consuming a snake weeks after the snake had consumed a single newt. Less severe effects of this dose on mammals would be expected. Ten genera of mammals were found to be similarly susceptible to TTX as mice by weight (Brodie, 1968); the oral LD_{50} of TTX for a mouse is 334 μg/kg (Mosher et al., 1964; Kawasaki et al., 1973). Mammalian predators of *T. sirtalis* range in average size from 1–12 kg (Nowak, 1991), thus, a TTX dose of the order of 42 μg would not

likely be lethal. However, because TTX may also cause an emetic response and the onset of symptoms is extremely rapid (Kao, 1966; Brodie, 1968), mammalian predators may be negatively affected at this dose.

These comparisons have been made with the apparent toxicity of snakes based on the consumption of a single newt. A snake that ate seven newts during a 5-wk period possessed considerable amounts of TTX in the liver 7 wk after this snake had been switched to a diet of fish—about 60 μg. The number of newts a snake may eat is unknown; however, one snake from Benton Co., OR, consumed eight adult newts in 2 wk in the lab (Brodie, 1968). The snake discovered in October with a half-digested newt in its stomach contained approximately 108 μg TTX. Tissues were collected from this snake 4 d after the newt had been palpated out of the stomach. Had the snake been able to completely digest the newt, the concentration of TTX in the liver may have been higher. Regardless, the occurrence of large quantities of TTX in wild snakes was verified.

The literature, combined with our data, suggests that snakes may harbor TTX in the approximate range, or just below a lethal dose for certain predators. Shine et al. (2001) found that crows were the main predators of *T. sirtalis* emerging from dens in Manitoba (outside the geographic range of *T. granulosa*). Remarkably, the crows excised the garter snake livers preferentially (an easily located, highly nutritional source in snakes), and consumed several in quick succession. Notably, live snakes were observed with ventral scars that have been attributed to partial liver removal by attacks from bird predators (M. Pfrender and R. Mason, pers. comm.).

If a predator became sick after ingesting a toxic snake liver but survived, it might learn to avoid these snakes. The ability of birds to acquire aversions to aposematically colored prey has been demonstrated repeatedly (Nicolaus et al., 1983; Roper and Wistow, 1986), even after just one exposure to noxious food items (Brower et al., 1970). The possibility of predators learning to avoid poisonous garter snakes may be accentuated (Fisher, 1930) by the fact that TTX is fast-acting, may cause an emetic response (Kao, 1966; Brodie, 1968), and garter snakes may exhibit defensive displays towards predators including open mouth strikes and flattening of the body to appear larger (Arnold and Bennett, 1984). Snakes in Manitoba reveal their distinctive red lateral coloration during the body flattening displays in response to a crow model (Shine et al., 2000). *T. sirtalis* from Benton, Co., OR, have far more brilliant red coloration, not only in their distinct lateral bars, but also on their head, which contrasts with their black background. Contrasting prey coloration accelerates predator learning (Schuler and Hesse, 1985; Sillén-Tullberg, 1985). Avian predators are visually oriented, discriminate objects based on color (Cuthill and Bennett, 1993; Hunt et al., 1997), and their foraging behavior can be influenced by variation in color and light (Maddocks et al., 2001). The contrasting colored rings of venomous coral snakes (*Micrurus, Micruroides*) and their sympatric nonvenomous kingsnake and milk snake mimics (*Lampropeltis*)

are aposematic to avian predators (Brodie and Janzen, 1995; Pfennig et al., 2001). Additionally, Hensel and Brodie (1976) found that Blue Jays (*Cyanocitta cirstata*), Common grackles (*Quiscalus quiscula*), and Brown Thrashers (*Toxostoma rufum*) recognized not only red on black warning coloration, but minute variations in form of distasteful salamander prey.

A combination of cues, such as chemosensory (Terrick et al., 1995), combined with aposematic colors may accelerate predator learning. Garter snakes often void their cloaca when threatened by predators, and the odor of the musk they secrete is distinctive. Musk, as an additional cue, may facilitate predator learning.

Additionally, Roper and Redston (1987) found that the rate of acquisition and duration of learned avoidance for predators may be increased by the frequency of exposure to aversive-tasting prey. The probability of encountering a toxic snake does not necessarily equate with the frequency of toxic snakes in the population. Brodie and Brodie (1999a) have shown that highly resistant individuals of *T. sirtalis* have reduced crawl speed in comparison with non-resistant individuals from the same population. Therefore, the ability of resistant snakes to escape from their predators may be reduced even before consuming a newt. As resistance increases, snakes are more likely to successfully consume a newt (Williams et al., 2003). After consuming a newt, the mobility of a snake is further impaired by the effects of TTX for up to several hours (Brodie and Brodie, 1990; Williams et al., 2003). During this time, snakes are especially vulnerable to predators. Finally, size matters—female snakes, which are larger than males, are more often able to consume adult newts. The combination of lethargy due to intoxication, bright coloration, visual sensitivity of birds, and larger size would render poisonous snakes more conspicuous. Conspicuousness increases the rate of consumption of a prey item, at least initially (Gittleman et al., 1980; Gittleman and Harvey, 1980). A higher probability of encountering toxic prey results in predators experiencing a higher proportion of unpalatable prey, which may accelerate predator learning. The higher proportion of toxic prey encountered also increases the possibility of an automimetic complex occurring within a population (Brower et al., 1970). Additionally, as the probability of prey entering the perceptual field of a predator increases, selective pressure on antipredator mechanisms, including toxicity, increases (Brodie et al., 1991). Because toxic snakes should be more frequently encountered than their innocuous counterparts, the evaluation of the role of snake toxicity as an antipredator mechanism requires consideration.

The occurrence of TTX in livers of garter snakes in such large quantities effectively renders some snakes of this population poisonous to potential predators. How poisonous depends on individual variation in newt toxicity and consequent variation in toxicity of snakes as well as specific and individual differences in predator susceptibility to TTX, which remains to be tested empirically. Whether toxicity in snakes constitutes chemical protection, or otherwise benefits the snakes is unknown and depends on the probability of predators encountering a poisonous

snake and the predators' ability to learn to avoid them. However, individual snakes that consume even a single newt are subsequently poisonous at least 3–4 wk later. The persistence of foreign toxins in an organism may be the necessary first step for evolution of the sequestration of toxin for consequent protection. The possibility that some populations of garter snakes may employ TTX obtained from their newt prey as an antipredator mechanism adds yet another dimension to the multifaceted coevolutionary interactions between *T. granulosa* and *T. sirtalis*.

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