

Repeated Injections of TTX Do Not Affect TTX Resistance or Growth in the Garter Snake *Thamnophis sirtalis*

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The garter snake *Thamnophis sirtalis* is a known predator of the toxic newt *Taricha granulosa*. Resistance to the primary toxin of the newt, tetrodotoxin (TTX), varies among individuals and has a genetic basis. We investigated the consequences of long-term exposure to this toxin in an experiment designed to determine (1) whether increased tolerance to tetrodotoxin occurs following repeated injections of tetrodotoxin in *T. sirtalis* and (2) whether repeated exposure to tetrodotoxin has an effect on the growth of juvenile snakes. We measured resistance to tetrodotoxin for each individual based on reduction in crawl speed. Pairs of neonatal snakes were chosen based on similar resistance and size and randomly split into two groups, control and experimental. Each group received intraperitoneal injections every two weeks of either saline (control) or tetrodotoxin (experimental) for six months. Mass data were collected five times during the year. Length data were collected twice during the year. After one year, resistance was again measured and compared with neonate resistance. Analyses showed that repeated injections of tetrodotoxin had no effect on resistance or growth.

A great variety of predators and herbivores feed on toxic prey. The repeated exposure to toxins may produce positive or negative side effects, even in organisms that have evolved the ability to exploit such prey. Repeated exposure to a toxic food item sometimes increases the ability of predators to detoxify the poison. For example, the tobacco hornworm *Manduca sexta* shows signs of increased nicotine detoxification after being fed nicotine (Snyder and Glendinning, 1996), and the fall armyworm *Spodoptera frugiperda* demonstrates increased tolerance toward indol 3-carbinol postingestion (Glendinning and Slansky, 1995). Alternatively, repeated exposure and the costs of detoxification may lead to the retardation of growth and development. The ruffed grouse *Bonasa umbellus* preferentially forages on quaking aspen buds with low levels of coniferyl benzoate to avoid the damaging energy expenditure associated with metabolizing the toxin (Guglielmo et al., 1996). The southern armyworm *Spodoptera eridania* suffers reduced growth from energy expenditures associated with the breakdown of dietary nicotine (Cresswell et al., 1992). However, such effects are not universal. Repeated exposure may have no effect as in the case of parsnip webworm *Depressaria pastinacella*, which does not suffer retarded growth after exposure to xanthotoxin (Berenbaum and Zangerl, 1994).

Amphibians have evolved a great diversity of toxic skin secretions for defense (Brodie, 1983; Daly et al., 1987) but are still eaten by many snake predators (Duellman and Trueb, 1994; Greene, 1997). It has even been suggested that

"all species of amphibians with skin secretions toxic to some predators coexist with a snake resistant to their secretions" (Brodie et al., 1991). The best studied system of a snake evolving resistance to a toxic amphibian involves the garter snake *Thamnophis sirtalis* that feeds on newts of the genus *Taricha* (Brodie and Brodie, 1990, 1991).

Newts of the genus *Taricha* possess the neurotoxin tetrodotoxin (Mosher et al., 1964), which effectively protects *Taricha* against all potential predators except the garter snake *Thamnophis sirtalis* (Brodie, 1968; Brodie and Brodie, 1990). *Thamnophis sirtalis* exhibits heritable resistance to tetrodotoxin (TTX) that varies among populations, among litters of the same population and even among littermates (Brodie and Brodie, 1990). Although the underlying genetic basis to resistance is clear, it is unclear what consequences may accompany this trait.

To better understand variation in TTX resistance and the consequences of feeding on newts, we conducted an experiment to investigate the effects of repeated exposure to TTX on *Thamnophis sirtalis*. From this experiment, we hoped to determine whether repeated exposure to tetrodotoxin altered TTX resistance levels of snakes and whether the growth of *Thamnophis sirtalis* neonates was affected by repeated exposure to TTX.

MATERIALS AND METHODS

The subjects for this experiment were neonate *T. sirtalis*, born during the summer of 1996

in captivity to wild-caught females from Warrenton, Clatsop County, Oregon. Females were collected in June 1996, housed individually in 25 × 50 × 30 cm aquaria, and fed fish once a week until parturition. In total, 30 neonates from 11 different litters were selected for the experiment. Neonates were housed in pairs in 25 × 50 × 30 cm aquaria. Individuals were marked with PIT tags for identification.

Following the methodology of Brodie and Brodie (1990), snakes were timed to determine base speed under normal conditions and speed while impaired by tetrodotoxin. All snakes were timed one-half hour after injection. Times were measured with the aid of electronic racetracks. To time the snakes, an individual was placed within the racetrack and forced to crawl down the track by tapping its tail. Different tracks were used for neonate and yearling time trials. Neonates were tested on a track 3 m in length and 11 cm wide; yearlings were tested on a track 4 m in length and 20 cm wide. Both tracks were equipped with built-in infrared sensors every 0.5 m of a 2-m central segment. The tracks were thereby divided into four timed 0.5-m segments with two end segments of equal length for starting and stopping the snake (1 m and 0.5 m for the 4-m and 3-m tracks, respectively). An Astroturf substrate was provided for traction. Each of the four 0.5-m segments of the tracks yielded one-half meter speeds (time/0.5 m); the fastest speed of the four was used to reduce behavioral inconsistency. All trials were conducted at a temperature of 26 ± 1 C.

Neonates were tested using the following protocol. Individual snakes were tested twice, three to five days postparturition to determine their average base speed. Individuals were raced twice more one and three days after the initial trials; in these trials, each neonate was injected with 0.0005 mg TTX (Sankyo, Lot W029H) in 0.1 ml of amphibian Ringer solution (Carolina Biological Supply Company). All injections for the experiment were administered intraperitoneally. Interperitoneal injections of TTX have the same qualitative effect on *T. sirtalis* as ingestion of TTX (Brodie, 1968). However, more tetrodotoxin is required in oral doses to achieve the same quantitative reduction in locomotor performance (B. Williams, unpubl.).

The ratios of the postinjection speeds to the base speed were averaged to determine the index of resistance to TTX for each individual. Therefore, if a snake suffered no impairment from tetrodotoxin injection, its index of resistance would be 100%. If a snake crawled half as fast postinjection as normally, its resistance would be 50%. Preliminary analysis showed that

resistance data followed a nearly normal distribution; therefore, the use of ratios in statistical analyses is valid.

After initial testing, pairs of snakes from the same litter were chosen that demonstrated similar resistance to tetrodotoxin (less than 16% different) and were of similar size (less than 1 g different). One member of each pair was assigned randomly to the control or the experimental group. During the ensuing year, both groups received injections every two weeks for 26 weeks. A dose of 0.001 mg TTX in 0.1 ml amphibian Ringer solution was administered to the experimental group on 11 November and 25 November 1996. Between 9 December 1996 and 12 May 1997, the experimental group received doses of 0.002 mg TTX in 0.2 ml amphibian Ringer solution every two weeks. The control group was given equal volumes (with respect to the experimental group) of amphibian Ringer solution for all injections. In total, 14 injections were administered to each individual.

We were primarily concerned with the long-term effects of repeated exposure to tetrodotoxin on resistance to TTX and growth. On 25 September 1997, more than one year postparturition and more than three months after injections ceased, individual resistance was assayed again to examine the effect of repeated doses of TTX. This assay was originally conducted in June 1997, but the TTX solution used was found to be of an improper salinity and the data were not analyzed. All subjects were tested again in September. No apparent effects of the June assay were detected. Base speed was determined for each of the 30 yearlings as before. Both the control and experimental groups received injections of a mass-adjusted dose of TTX calculated as

$$dose_{adj} = \frac{dose_{org} \times g_c}{2g_b},$$

where g_b and g_c represent birth mass and mass after one year, respectively, and $dose_{org}$ and $dose_{adj}$ represent original and adjusted dosages, respectively. Resistance was once again calculated and compared to the neonate results using two-way ANOVA procedures, with year and treatment group as main effects. Using these procedures, we compared neonate score for the same individual and to the paired control individual to eliminate the effect of year.

Mass, snout-vent length (SVL), and total length (TL) were measured five times during the year (at birth, 2 November 1996, 15 January 1997, 20 May 1997, and 25 September 1997) to examine the effects of TTX on growth. The dif-

TABLE 1. SUMMARY STATISTICS FOR BOTH CONTROL AND EXPERIMENTAL (TTX) GROUPS OF *Thamnophis sirtalis* IN ALL MEASURES GIVEN AS MEAN \pm STANDARD ERROR. *n* for each group was 15.

	Control	Experimental
Resistance (%)		
Neonate	52.52 \pm 4.09	55.86 \pm 4.87
Yearling	44.84 \pm 2.93	48.49 \pm 3.35
Net Growth (mm)		
Snout-vent length	316 \pm 9.6	312 \pm 10.5
Total length	418 \pm 10.3	418 \pm 11.0
Mass (g)		
Birth	2.97 \pm 0.09	2.86 \pm 0.12
2 Nov. 1996	7.33 \pm 0.37	7.38 \pm 0.38
15 Jan. 1997	16.95 \pm 0.62	16.72 \pm 0.94
20 May 1997	34.97 \pm 1.88	36.57 \pm 2.32
25 Sept. 1997	40.58 \pm 1.61	43.55 \pm 3.05

ference between the 25 September 1997 mass and birth mass was calculated for each individual snake as a measure of growth. Mean growth in mass was compared between treatments with a standard *t*-test. The difference between the 25 September 1997 length and birth length also was calculated for each individual snake as a measure of growth. Mean growth in length again was compared using a standard *t*-test.

RESULTS

Table 1 contains summary statistics for all groups. Repeated injections of tetrodotoxin had no effect on resistance. Three different tests were performed to check the interactions between treatment (control vs experimental) and year ($F < 0.001$, $P = 0.965$, $df = 14$), pair and treatment ($F = 2.12$, $P = 0.168$, $df = 14$), and pair and year ($F = 5.85$, $P = 0.030$, $df = 14$). The only *F*-test of significance ($P = 0.030$) was between pair and year, which indicates a shift in pairwise resistance between 1996 and 1997. More important, the *F*-test for treatment and year clearly indicates that the mean of both groups changed identically between 1996 and 1997 ($F < 0.001$, Fig. 1).

Growth was unaffected by repeated exposure to tetrodotoxin (Table 1). The growth in mass was not significantly different between treatments ($t = 1.025$, $df = 14$, $P = 0.375$). Neither growth in SVL nor TL were significantly different between treatments ($t = 1.028$, $df = 14$, $P = 0.321$; $t < 0.001$, $df = 14$, $P > 0.999$, respectively).

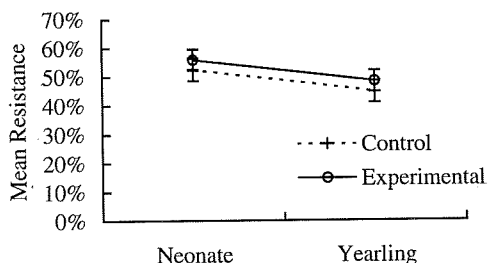


Fig. 1. Resistance in experimental and control groups of *Thamnophis sirtalis* from neonates to yearlings. The shift in resistance between 1996 and 1997 was paralleled in each group ($F < 0.001$). The reduction in resistance between years resulted from the use of an arbitrary mass adjusted dose (see text).

DISCUSSION

Despite the dramatic effects of tetrodotoxin on locomotor performance, repeated exposure to TTX had no effect on growth or resistance to TTX in *T. sirtalis*. We conclude that increased resistance to tetrodotoxin in *T. sirtalis* cannot be induced through repeated exposure to the toxin. The strongest support for this interpretation comes from the lack of any treatment by year interaction, which indicates that there is no difference in resistance between our control and experimental group means. A shift in mean resistance between the 1996 trials and the 1997 trials occurred largely due to experimental design. The dose given to the yearlings in 1997 was one-half of a full mass-adjusted dose. Because no formula for the change in resistance with respect to change in mass and age is yet known, an approximation was used. The use of a one-half mass-adjusted dose resulted in speeds similar to those recorded for neonates in 1996. Though the use of a nonidentical dose in 1997 caused a shift in the net mean for the two groups, its use did not affect the statistical outcome. In fact, any dose could have been used as long as it did not prevent the snakes from crawling or was so small that no impairment occurred. The use of a one-half mass-adjusted dose avoided these two problems. The fact that tetrodotoxin injections were given two days apart during the assays did not influence results. Previous research (Brodie and Brodie, 1990) showed that the injection of TTX had no effect on resistance over this time span.

Tetrodotoxin did not affect growth in the test snakes. The two test groups followed a nearly identical growth curve. We see that, through both the lag and exponential phases of growth, both groups' mean mass remained the same. Though a formal ANCOVA was not performed

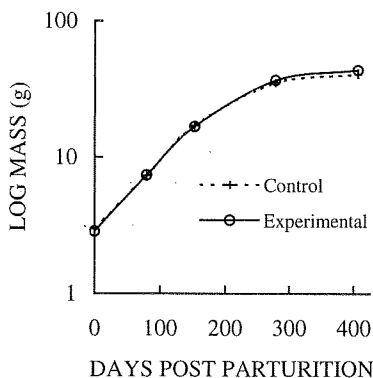


Fig. 2. Plot of mean treatment group mass in *Thamnophis sirtalis* based on days postparturition. Both groups of subjects exhibited identical growth patterns throughout the experiment with respect to mass (above) and TL (see also Table 1).

with mass and time and covariates, it is obvious that the relationship between groups never changed over time (Fig. 2).

The *T. sirtalis* and *Taricha* interaction differs from that of most predators and their toxic food items. Other systems, such as the hornworm and armyworm, use digestive enzymes to break down toxins (Glendinning and Slansky, 1995; Snyder and Glendinning, 1996). The concentrations of such enzymes can be altered within an individual based on exposure. However, alteration by an individual of its enzymatic levels may adversely affect its growth, as in the case of *S. eridania* (Cresswell et al., 1992). Tetrodotoxin, however, is a neurotoxin that blocks Na⁺ channels in nervous and muscle tissues. Therefore, the most likely source of resistance in *T. sirtalis* comes from Na⁺ channels that have a lower affinity for TTX binding (Brodie and Brodie, in press).

The fact that repeated exposure to TTX does not change individual resistance implies that there is little interaction between the environment and the genotype influencing resistance. Therefore, the variation observed among neonate snakes should be characteristic of genetic variation found within natural populations. In addition, the lack of environmental effect suggests that the resistance of *T. sirtalis* to TTX is under strong genetic control. Because direct exposure to TTX has no effect on resistance in *T. sirtalis*, we expect there to be no effect of maternal exposure to TTX. Furthermore, the lack of effect of TTX on growth eliminates one possible cost of exploiting toxic prey for *T. sirtalis*.

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