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Recovery of Garter Snakes (*Thamnophis sirtalis*) from the Effects of Tetrodotoxin

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km S Totontepec; 14169 UTA OAX, Sierra Mixe, 0.8 km S Totontepec; 14170 UTA OAX, Sierra Mixe, 0.8 km S Totontepec.

*Toluca conica*: 2898 MZFC GRO, Chilpancingo, Omiltemi Salida E del pueblo; 2899 MZFC GRO, Chilpancingo, Omiltemi 2 km E-SE; 2900 MZFC GRO, Chilpancingo, Omiltemi on trail to Las Joyas 500 m NW; 2901 MZFC GRO, Chilpancingo, Omiltemi Barranca de Potrerillos; 2902 MZFC GRO, Chilpancingo, Omiltemi 2 km E.

*Toluca lileata acuta*: 3258, 3258-3, 3258-4, 3258-6, 3258-7 MZFC PUE, Chapulco, 4 km E.

*Toluca lileata acuta* × *Toluca lineata lineata*: 0840 MZFC HGO, Tejocotal approx 500 m NE of town.

*Toluca lineata lineata*: 3216 MZFC PUE, town of Amozoc; 3217-18 MZFC PUE, Chignahuapan, Puente rojo 0.5-1 km W; 3534 MZFC PUE, Chignahuapan, Chignahuapan 10 km S.

*Toluca lineata varians*: 7108 MZFC MEX, Atlacomulco km 21 carr. Toluca-Atlacomulco; 5739 MZFC PUE, Tehuacan, 8 km E Chapulco.

*Toluca lineata wetmorei*: 11453-54 MZFC OAX, Cerro de Yucunino; 11455-57 MZFC OAX, Llano de Gualupe.

*Toluca megalodon*: 6557 MZFC OAX, Sierra de Juárez, km 148 carr. 185 Oaxaca-Tuxtepec; 8301 MZFC OAX, Sierra de Juárez, La Cumbre carr. Oaxaca-Tuxtepec.

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## Recovery of Garter Snakes (*Thamnophis sirtalis*) from the Effects of Tetrodotoxin

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The arms race analogy is a popular view of evolutionary interactions between predators and prey but one whose generality is questionable because of the paucity of empirical studies in natural systems. Predators and prey are expected to experience asymmetrical selection from ecological interactions, leading some authors to question whether predators are under direct selection to respond to evolutionary advances by prey. The consequences of interactions are generally less severe for predators than for prey (the "lifer-dinner principle"; Dawkins and Krebs, 1979) and even severe consequences may not be predictable ("dodging the bullet"; Brodie and Brodie, 1999a). Consequently, evolutionary arms races between predators and prey are most likely to occur when prey are dangerous and, therefore, exert strong selection on predators (Brodie and Brodie, 1999a).

Strong selection by prey on predators is expected when prey are toxic or otherwise dangerous (Brodie and Brodie, 1999a). For toxic prey, selection may result

from the immediate effects of toxins (i.e., injury or death) or from indirect effects that result from the action of the toxin such as temporary immobility, alterations to physiology or metabolism, or reduced organismal performance. Because evolutionary response of predators to prey might result in any predator adaptation that ameliorates the fitness deficits associated with prey toxins, predator resistance might include behavioral avoidance of toxic prey, reduced susceptibility to a toxin directly, or reduced duration of the effects of a toxin, or some combination thereof.

From a microevolutionary perspective, one of the best documented predator-prey systems includes the newt *Taricha granulosa* and its predator, the garter snake *Thamnophis sirtalis* in the Pacific Northwest of North America (Brodie and Brodie, 1990, 1991, 1999b,a). Newts of the genus *Taricha* possess tetrodotoxin (TTX; Mosher et al., 1964; Wakely et al., 1966; Brodie, 1968; Brodie et al., 1974; Daly et al., 1987), an extremely potent neurotoxin that acts as a Na<sup>+</sup> channel blocker. Although all three species of the genus *Taricha* possess this toxin, *Taricha granulosa* is many times more toxic than any other species. The only predator known to forage on newts and resist the effects of this toxin is *Thamnophis sirtalis*.

Within populations, resistance to TTX varies among neonate snakes and has a heritable basis (Brodie and Brodie, 1990). Resistance is not affected by either short-term or long-term exposure to TTX (Brodie and Brodie, 1990; Ridenhour et al., 1999), hence, environmental effects are unlikely to explain familial differences. Among populations, the levels of both newt toxicity and garter snake resistance are variable but roughly matched (Brodie and Brodie, 1990, 1991, 1999a), suggesting a geographic mosaic of coevolutionary outcomes (Thompson, 1994, 1999a,b). Garter snake populations allopatric with newts are not resistant to TTX, whereas sympatric populations are resistant (Brodie and Brodie, 1990). Island populations of newts from British Columbia lack TTX (Hanifin et al., 1999), and garter snakes from these populations are nonresistant (Brodie and Brodie, 1991). Other garter snakes that coexist with *Taricha* are susceptible to TTX (Brodie, 1968; Brodie and Brodie, 1990; Motychak et al., 1999), supporting the view that resistance to TTX is an adaptation by a predator to its toxic prey.

Past studies of TTX resistance have used a bioassay based on locomotor performance to assess variation (Brodie and Brodie, 1990, 1991, 1999b; Ridenhour et al., 1999). The bioassay examines an individual snake's reduction in crawl speed 30 min after an injection of TTX. The bioassay is ecologically relevant because reduced locomotor performance is expected to impair the ability of snakes to escape predators or thermoregulate. The length of time that a snake is impaired from a given dose of TTX is unknown and may further contribute to the selective scenario in the interaction between newts and snakes. In this paper, we explore the time to recovery for individual snakes from a population resistant to TTX. We further examine the correlation between resistance as measured by reduction in locomotor performance after 30 min and the relative recovery at longer intervals.

The subjects for this experiment were neonate *T. sirtalis*, born during the summer of 2000 in captivity to wild-caught females from Benton County, Oregon.

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Gravid females were collected in June 2000, housed individually in  $25 \times 50 \times 30$  cm terraria, and fed fish or newts once a week until parturition. Fifty-five neonates from four litters were used in the experiment.

The effects of exposure to TTX were tested with a whole organism performance bioassay developed by Brodie and Brodie (1990). Each individual snake was raced at  $26^\circ\text{C}$  ( $\pm 1^\circ$ ) on a 2-m linear racetrack, lined with artificial turf, and equipped with infrared sensors at 0.5-m intervals to electronically time sprint speed. The maximum 0.5-m speed in a given trial was taken as the sprint speed score. Individuals were first measured for sprint speed on the third day of life. Trials were repeated after four hours, and the mean of these two trials was taken as an individual's "Baseline Speed." Repeatability of this assay of Baseline Speed in previous studies of *T. sirtalis* from Benton County and other localities ranges from 0.5–0.7 (Brodie and Brodie, 1990, 1999b; unpubl. data). Twenty-four hours after the last sprint test, neonates were given intraperitoneal injections of known quantities of TTX. Thirty minutes after injection, snakes were tested again to obtain a measure of "Postinjection Speed." "Resistance" was scored as the percentage of an individual's baseline speed crawled after injection (Postinjection Speed/Baseline Speed). Individuals that are greatly impaired by TTX crawl only a small proportion of their normal speed, whereas those that are unaffected by an injection of TTX crawl 100% of their baseline speed. Control injections of physiological saline have no effect on snake performance (Brodie and Brodie, 1990), and TTX resistance is not affected by repeated exposure (Brodie and Brodie, 1990; Ridenhour et al., 1999). Compared to ingested TTX, interperitoneal injections of TTX have the same qualitative effect on *T. sirtalis* (Brodie, 1968) but require approximately  $40\times$  less TTX for a comparable quantitative reduction in locomotor performance (Williams et al., pers. comm.). The sprint speed bioassay of resistance is highly repeatable (0.5–0.7; Brodie and Brodie 1990, 1999b; unpubl. data) and enables us to assess individual differences in susceptibility to TTX.

We examined the effects of two different doses of TTX (Sankyo, Lot B19481) mixed in 0.1 ml of amphibian Ringer solution (Carolina Biological Supply Co.). All individuals were injected from the same stock solution of a given dose. The doses were selected because they were known to reduce individual crawl speed in snakes from the Benton County, Oregon population to an average of approximately 54% (at 0.0001 mg; the 50% dose) and 17% (at 0.0005 mg) of Baseline Speed 30 min after injection (Brodie and Brodie, 1999a). Within population variation in neonate mass is uncorrelated with resistance in *T. sirtalis* populations that are resistant to TTX (Brodie and Brodie, 1990, 1999b); thus, no attempt to adjust doses to size differences among individuals was made. At the 0.0005 mg dose, subjects were retested at 90 min, 180 min and 360 min after injection, whereas individuals were only retested at 90 min at the 0.0001 mg dose. At this lower dose, virtually every individual had fully recovered after 90 min.

To determine whether individuals with higher resistance also recovered from the effects of TTX more quickly, nonparametric correlations (Spearman's  $\rho$ ,  $\rho_s$ ) were estimated between the percent base crawl

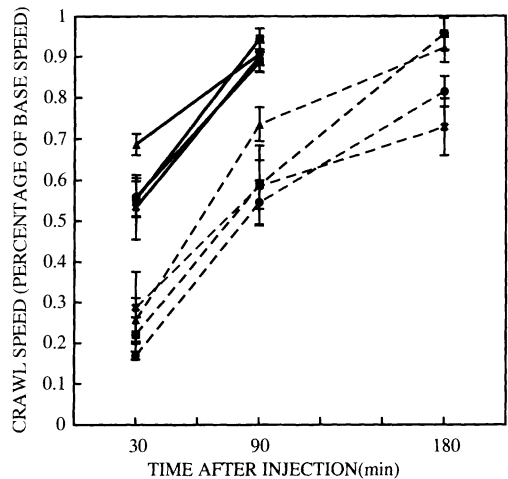


FIG. 1. Percent Baseline Speed as a function of time after injection of TTX. Responses to 0.0001 mg are indicated by solid lines, and responses to 0.0005 mg are indicated by dashed lines. Each line represents a family mean ( $\pm$  SE), with families represented by the same symbol at each dose.

speed at 30 min (resistance) and the percent baseline speed at 90 min and 180 min. Because of potential autocorrelations between these measures (i.e., low values of % baseline speed at later times could not occur in individuals with high initial resistance), six individuals with extremely high initial resistance ( $>$  mean + 2 SD) to the 0.0005 dose were removed from all analyses. Removal of these individuals from the analyses reduced the skew in the distribution of resistance, minimizing potential autocorrelations. This censure did not alter the qualitative results of the analyses.

Neonate *T. sirtalis* in this sample ( $N = 55$ ) were reduced to an average of  $57.7 \pm 2.6$  (SE)% and  $21.0 \pm 1.9\%$  of their Baseline Speeds at 0.0001 and 0.0005 mg of TTX, respectively. At the 50% dose the population average had recovered almost fully ( $90.1 \pm 1.2\%$  of Baseline Speed) 90 min after injection (Fig. 1), and every individual had recovered to 69% of Baseline Speed or greater. At the higher dose recovery was slower, with the population average reaching only  $59.8 \pm 3.2\%$  after 90 min. After 180 min, individuals had recovered to an average of  $84.4 \pm 1.9\%$ , and only two individuals had not recovered to at least 60% of Baseline Speed. The subset of 16 individuals that had not recovered to at least 80% at 180 min was tested again at 360 min, and had reached an average of  $79.5 \pm 2.2\%$  at this time. Only two individuals of this subset had not recovered to at least 71% after 360 min.

At the higher dose, resistance at 30 min was positively correlated with both percent Baseline Speed at 90 min ( $\rho_s = 0.470$ ,  $P < 0.0003$ ) and 180 min ( $\rho_s = 0.389$ ,  $P < 0.0033$ ). Resistance and recovery at 90 min were not related at the lower dose ( $\rho_s = 0.074$ , ns).

*Thamnophis sirtalis* recover from the effects of injected TTX surprisingly quickly, but time to recovery depends on the dose and initial effect (Fig. 1). At the 50% dose, virtually all individuals were crawling within 10–15% of full speed after only 90 min (recovery may have been even quicker, but we did not assay

recovery at earlier times). Even at higher doses that virtually immobilized some individuals, the majority of the sample was near full performance after 180 min. Only two individuals had not reached 80% of full capacity after 6 h. The relationship between these recovery times and the actual impairment experienced by snakes that ingest newts is unclear for several reasons. The effects of ingested TTX are quantitatively different than injected TTX (Williams et al., in press). Furthermore, substantial variation exists among individual newts in toxin level (Hanifin et al., 1999) and among individual snakes in resistance (Brodie and Brodie, 1990, 1999a); thus, duration of effect of TTX is likely to be a function of individual predators and prey. We have observed adult *T. sirtalis* to be noticeably impaired for up to seven hours after eating an adult newt in both field and laboratory situations (Brodie and Brodie, 1990).

Impaired locomotor function may represent several selective consequences (Brodie and Brodie, 1990). After eating a meal, snakes must thermoregulate to aid in digestion (Stevenson et al., 1985); hence, a snake that has eaten a newt is likely to be both exposed and impaired immediately following a predation event. As a result, the effects of TTX toxicity may reduce survival in two distinct ways. First, an exposed and debilitated snake is at higher risk of predation. The greater and longer the snake is impaired, the greater the risk. Second, a snake that becomes immobilized for even a short period of time may be unable to avoid lethal temperatures as the thermal environment changes (Peterson, 1987). The longer the duration of impairment from TTX, the greater is the risk from either source of selection.

Time to recovery is related to resistance, as previously measured by reduction in locomotor performance but only at higher doses. The same relationship may hold at lower doses, but the rate of recovery was sufficiently rapid in our experiment that only minimal variation in recovery at 90 min was present at the dose resulting in a 50% loss of ability. The relationship between time to recovery and resistance suggests that individual *T. sirtalis* have a general ability to withstand the effects of TTX in both the degree of initial impairment and duration of impairment. Because the mechanism of TTX resistance in *T. sirtalis* is not yet known, it is unclear whether the physiological bases of resistance and recovery are related. Likewise, it is unclear whether the ecological costs that covary with resistance (Brodie and Brodie, 1999b) also covary with recovery time.

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### Microhabitat Use and Orientation to Water Flow Direction by Tadpoles of the Leptodactylid Frog *Thoropa miliaris* in Southeastern Brazil

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Frog larvae develop in a wide array of microhabitats including permanent and nonpermanent ponds, rivers, lakes, tank bromeliads, and other water reservoirs (Duellman and Trueb, 1994). A unique form of development and microhabitat specialization occurs in tadpoles of leptodactylid frogs of the genus *Thoropa*: tadpoles live in the film of water on rock surfaces at the wet borders of waterfalls in rain-forest areas, and in rocky fields of mountain ranges of southeastern Brazil (Bokermann, 1965; Caramaschi and Sazima 1984; Cocroft and Heyer, 1988). The tadpoles of this genus have an elongated, dorsoventrally compressed body, reduced fins, an expanded and flattened abdomen with an adherent ventral disk, and a long muscular tail, which, together with the labium, are used for movement and adhesion to the substrate (Bokermann, 1965; Wassersug and Heyer, 1983).

*Thoropa miliaris* (Spix, 1824) occurs near freshwater bodies along the Atlantic rain forest of the states of Rio de Janeiro, São Paulo, Minas Gerais, and Espírito Santo in southeastern Brazil (Cocroft and Heyer, 1988) and also on rocky marine shores (Abe and Bicudo, 1991). Information available on microhabitat use by tadpoles of this species is restricted to general notes reporting the use of a very shallow film of current water on rock surfaces at the side of waterfalls with varying slopes, including vertical. These reports suggest that the tadpoles tend to remain with the head oriented against the water flow when adhered to the rock surface (Bokermann, 1965; Abe and Bicudo, 1991). However, there is no quantitative description of microhabitat use by this species. In this study, we analyze microhabitat use by *T. miliaris* tadpoles to address the following questions. Do tadpoles orient randomly or selectively in relation to water flow? Do tadpoles tend to remain at the shallowest sites or use the available film of water indiscriminately? Do tadpoles occupy some slopes available on the waterfall surface more than others? Does water temperature where tadpoles occur differ from that of the main drainage of the waterfalls nearby?

We studied microhabitat use by *T. miliaris* tadpoles along the sides of a waterfall with wet granite substrate. The waterfall is located in the Barra Grande River at a place locally called Mãe D'água Dam (23°10'92.3"S, 44°12'04.4"W) at Ilha Grande, Rio de Janeiro State, south-eastern Brazil, at an altitude of approximately 90 m above sea level. The study was carried out in November and December 1998.

Study sites were delineated using a 1 × 1 m wooden frame internally subdivided with cotton strings in 25 subplots of 20 × 20 cm. These subplots were considered sampling units. We systematically sampled all the wet rocky area at both sides of the waterfall. We considered this area the small drainage compared to the main drainage of the waterfall. At each subplot we checked for the presence of tadpoles of *T. miliaris*. Although the water volume and current at the main drainage prevented us using the same methodology, we also checked along the main drainage for the presence of tadpoles of *T. miliaris*. Whenever a tadpole was found within a subplot, we placed a toothpick to indicate its original position and orientation relative to the water flow direction. We included only tadpoles that had not been disturbed by our presence. Because tadpoles of *T. miliaris* are found in very shallow water, sometimes it was not possible to determine visually the direction of the water flow. In such cases, we dropped a pinch of powder on the water beside the tadpole to identify the direction of the water flow. We defined the direction of water flow as zero degree (0°) on a hypothetical circle, and we recorded to the nearest 1° the tadpole's head orientation, relative to the water flow direction, using a protractor. To randomize the possible influence of sunlight on tadpole compass orientation, we recorded observations throughout the day, from early morning to late afternoon. We also recorded, at the center of each 20 × 20 cm subplot, the slope of the rock (using a clinometer, to the nearest 1°) and the depth of the water film. For depth measurements, we put a dried toothpick perpendicularly to the wet rock surface and removed it immediately after it had touched the rock surface; we then measured the length of the wet column on the toothpick using a digital caliper, to the nearest 0.1 mm. The water depth in cells containing tadpoles was measured immediately adjacent to the original position of the tadpole. We estimated tadpole density by counting the number of tadpoles found in each square meter of wet rock surface.

The relationship between tadpole body orientation and the direction of water flow was tested using statistics for circular distributions. A mean vector direction (the mean vector with length "r") expresses the dispersion of points around the mean angle ("α") of orientations around the circle (Batschelet, 1965). The mean vector length (0 < r < 1) varies according to the concentration of data around the mean angle, where r = 1 indicates that all points are concentrated in the same direction (Batschelet, 1965; Gandolfi and Rocha, 1998). We also tested for differences between the depth of the water where tadpoles occurred and the depth of water in subplots where no tadpole occurred, using one-way analysis of variance (ANOVA; Zar, 1999). To evaluate whether tadpoles of *T. miliaris* preferred particular substrate slopes, we compared the frequency distribution of slopes used by tadpoles with the frequency distribution of slopes from all subplots. For this comparison, we used the Kolmogorov-Smirnov test for two groups (Zar, 1999).

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