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TETRODOTOXIN RESISTANCE IN GARTER SNAKES: AN EVOLUTIONARY RESPONSE OF PREDATORS TO DANGEROUS PREY

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Abstract.—The use of the “arms race” analogy as a conceptualization of evolutionary predator-prey interactions has been criticized because of the lack of evidence that predators can and do adapt to increased antipredator ability of prey. We present evidence that the garter snake *Thamnophis sirtalis* has evolved resistance to tetrodotoxin (TTX) in response to the toxicity of the newt *Taricha granulosa* on which the snake feeds. A bioassay (locomotor performance before and after injection of TTX) was used to obtain repeated measures of resistance for individual snakes. We studied interpopulation and interspecific variation by comparing resistance in *Thamnophis sirtalis* from populations occurring sympatrically and allopatrically with *Taricha granulosa*, and in *Thamnophis ordinoides* (which does not feed on the newt) occurring sympatrically with *Taricha granulosa*. We also examined intrapopulation variation in TTX resistance using snakes from a population known to feed on *Taricha granulosa*. Resistance differed significantly among individuals and litters; repeatability and heritability estimates of the assay were significantly different from zero, demonstrating the potential for response to selection. The population of *Thamnophis sirtalis* that occurs with *Taricha granulosa* exhibited levels of resistance much greater than either of the other groups. These results suggest that the predator-prey arms race analogy may be applicable to this system.

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The “arms race” analogy recently has come under attack as a conceptualization of evolutionary predator-prey interactions on the grounds that predators may not respond evolutionarily to antipredator adaptations by prey. Some authors argue that predators are under weaker selection to respond to advances by prey than vice versa, the so-called “life-dinner principle” (Dawkins and Krebs, 1979; Vermeij, 1982; Sih, 1984). Theoretical work indicates that in most scenarios prey must respond to predator advances with increased antipredator adaptation, but the reciprocal response of predators to prey is never necessitated and is possible only in some cases (Abrams, 1986). Inequalities in the potential for coevolution also suggest that predators may not be held in lock-step coevolution with their prey (Slobodkin, 1974; Bakker, 1983; see also Lenski and Levin, 1985, on bacteria-virulent phage interactions). Bakker (1983) points to an “adaptive gap” between predators and ungulates in the fossil record as evidence that “extinct predators were not

evolving fast enough to track their prey closely.”

Empirical studies that invoke the arms race analogy suffer from a bias toward explaining adaptation by the prey. Elaborate antipredator mechanisms are typically attributed to the arms race between predator and prey, but reciprocal adaptation by the predator is rarely invoked. Some paleontological studies indirectly support an arms race process, but generally fail to provide evidence of coevolutionary response by predators (reviewed in Vermeij, 1983; Stanley et al., 1983; Steneck, 1983). There are indications that herbivores evolve in response to plant defenses (Berenbaum, 1983), but there is no evidence of a pair of species in a highly coupled arms race (Futuyma, 1983; Jermy, 1984).

To evaluate the arms race view of predator-prey evolution, we must determine if predators can and do adapt to increased antipredator ability in prey lineages. An arms race is most likely to occur between a predator and a dangerous prey because the pred-

ator will be under stronger selection to respond to prey adaptations (Vermeij, 1982, 1983). An inefficient predator in such a confrontation risks losing not only its dinner but also its life, thus rendering the life-dinner principle inapplicable.

We examined the predator-prey association between the garter snake *Thamnophis sirtalis* and the newt *Taricha granulosa* to evaluate the potential for evolutionary response by a predator to the antipredator adaptations of a dangerous prey species. *Taricha granulosa* has high concentrations of the neurotoxin tetrodotoxin in its skin as an antipredator mechanism (Mosher et al., 1964; Wakely et al., 1966; Brodie et al., 1974). Interspecific and interpopulation variation in tetrodotoxin resistance was examined comparing forms of *Thamnophis* that feed on *Taricha granulosa* with those that do not. Intrapopulation variation in resistance also was inspected using *Thamnophis sirtalis* from a population known to eat *Taricha granulosa*.

THE SYSTEM

Tetrodotoxin (TTX) is one of the most potent neurotoxins known and is found in a variety of taxa including amphibians, fishes, molluscs, echinoderms, platyhelminths (reviewed in Kodama et al., 1985; Daly et al., 1987), and bacteria (Narita et al., 1987; Noguchi et al., 1986, 1987). Recent evidence suggests that the seemingly phylogenetically independent origination of TTX may be caused by production of the compound by a symbiotic gut bacteria in some species (Narita et al., 1987; Noguchi et al., 1986, 1987). This does not appear to be the case for amphibians (Mosher et al., 1964; Wakely et al., 1966; Daly et al., 1987), which are believed to synthesize TTX "for the primary function of defense" (Daly et al., 1984).

All amphibians have skin secretions, many of which contain toxins (Duellman and Trueb, 1986). The antipredator effects of these skin secretions in salamanders have been well documented (reviewed in Brodie, 1983). Newts of the family Salamandridae are especially toxic and, among these, members of the genus *Taricha* from the Pacific coast of the United States and Canada are by far the most potent (Wakely et al., 1966; Brodie et al., 1974). *Taricha* are lethal to a

wide range of potential predators (Brodie, 1968), and their toxicity results almost entirely from high concentrations of TTX in the skin (Mosher et al., 1964; Wakely et al., 1966; Brodie et al., 1974). However, *Taricha granulosa* is the only toxic salamander with a known resistant predator, the garter snake *Thamnophis sirtalis* (Brodie, 1968).

The garter snake *Thamnophis sirtalis* is an amphibian specialist in most of its range (Wright and Wright, 1957; Fitch, 1965) and feeds on *Taricha granulosa* where they are sympatric (Nussbaum et al., 1983). These snakes have even been observed aggregating around and feeding in pit-traps containing *Taricha granulosa* in the field (Brodie, 1968). Along the Oregon coast, *Taricha granulosa* is sympatric with two species of garter snakes, *Thamnophis sirtalis* and *Thamnophis ordinoides*. *Thamnophis ordinoides* is a slug and worm specialist that occasionally may take small amphibians, but has never been reported to eat *Taricha* (Fitch, 1941; Gregory, 1978).

Our laboratory observations indicate that, although sympatric *Thamnophis sirtalis* are not completely resistant to *Taricha granulosa*, neither do they normally die after eating a newt. Of 11 adult snakes that freely ate adult *Taricha granulosa* in the laboratory, 9 showed no apparent ill effects, but 2 exhibited classic symptoms of TTX poisoning—loss of muscle coordination and righting reflex. Both affected snakes recovered, one after 1.5 hours and the other after at least 7 hours. In addition, an adult snake encountered basking in the field and later found to contain a freshly eaten adult newt did not attempt to move until touched and even then was unable to crawl, instead adopting a defensive posture. This variation in effect might be caused by differences in toxicity of the newts or in resistance of the snakes.

METHODS

The Bioassay

We developed an assay for TTX resistance based on the negative effect of TTX on locomotor performance. This estimate of resistance is biologically appropriate because TTX is a neurotoxin and therefore affects muscle control. Locomotor perfor-

mance is an ecologically relevant trait to use in the assay because of its importance in prey capture and escape from predators.

We used intraperitoneal injections rather than force-feeding to introduce TTX into experimental snakes because a non-lethal amount could be administered and dosages could be controlled and replicated, thus allowing repeated measures of resistance within individuals. The injection methodology also allowed us to examine physiological resistance without the confounding effects of absorption from the digestive system and of food in the stomach on locomotor performance. Skin extracts from *Taricha granulosa* produce the same symptoms when injected as when ingested and the toxin is not neutralized digestively (Brodie, 1968).

Sprint speed of neonate garter snakes was estimated on a 2-m racetrack (9 cm wide) lined with astroturf at $25.5^{\circ} \pm 0.5^{\circ}\text{C}$. Snakes were stimulated to crawl by tapping them on the tail with a cotton swab, and times were taken with a stopwatch at 1-m intervals. The fastest 1-m time in each trial was taken as an estimate of sprint speed (after Bennett, 1980; Huey et al., 1981; Garland, 1988; Brodie, 1989).

Trials were started three to six days after birth and were conducted twice a day (four hours apart) for the first three days (a total of six trials). The first two trials were discarded as "training trials" (Bennett, 1980) and the last four trials used as estimates of "baseline speed."

On the fourth day of testing, each snake received a 0.1 ml intraperitoneal injection of TTX or saline. Purified TTX in citrate buffer, manufactured by Sankyo, was diluted with physiological saline to produce the reported dosages. Thirty minutes after being injected, snakes were tested for sprint speed ("post-injection speed"). In cases where the same individual received multiple injections, trials were conducted 48 hr apart. Post-injection speeds were compared to baseline speed for each individual to determine the effect of TTX on locomotor performance. This assay of resistance has the advantage of preserving individual differences in locomotor behavior.

Neonate garter snakes used in all parts of this study were laboratory born from preg-

nant snakes captured in the field one to two months prior to parturition. Adult females were fed as much as they would eat twice weekly during captivity; *Thamnophis sirtalis* were offered goldfish, whereas *Thamnophis ordinoides* were offered earthworms. All neonates were housed individually from birth with water always available but were not fed until after the last trial. Individual masses were recorded following the first baseline speed trial.

Interpopulation and Interspecific Variation in Tetrodotoxin Resistance

To compare patterns of TTX resistance among garter snakes from races that eat *Taricha granulosa* and those that do not, we assayed resistance in neonates from three groups: (1) sympatric *Thamnophis sirtalis* from Benton Co., Oregon (42 individuals from 5 litters), a population that co-occurs with *Taricha granulosa*; (2) allopatric *Thamnophis sirtalis* from Bear Lake Co., Idaho (13 individuals from 1 litter), a population that does not co-occur with *Taricha granulosa*; and (3) sympatric *Thamnophis ordinoides* from Benton Co., Oregon (125 individuals from 10 litters).

Resistance was assayed at a number of different concentrations of TTX. Not all groups received all concentrations because of obvious differences in susceptibility. Each individual received only one injection of TTX. Injections of an equal amount of saline were given to some subjects as controls.

Resistance was calculated as the ratio of post-injection speed (1 trial) to mean baseline speed (average of four trials). For illustration, dosages also were reported in mass-adjusted mouse units to account for size differences among the snakes. One mass-adjusted mouse unit equals the amount of TTX needed to kill 1g of mouse in 10 minutes multiplied by the mean mass of each snake population. This is based on the amount of TTX needed to kill a 20-g mouse in 10 minutes.

Intrapopulation Variation in Tetrodotoxin Resistance

To examine patterns of TTX resistance within a population of *Thamnophis sirtalis* sympatric with *Taricha granulosa*, we assayed resistance in 328 neonates from 23

litters of snakes from Tenmile Creek, Lane Co., Oregon. Three repeated measures of post-injection speed were recorded for each snake using a dosage of 0.001 mg of TTX.

To avoid the statistical problems associated with analyzing ratios (Sokal and Rohlf, 1981; Packard and Boardman, 1987), the residuals from a regression of post-injection speed on mean baseline speed (=“residual resistance”) were used instead of the analogous ratio in all statistical analyses. A mean residual resistance for each individual was obtained by regressing mean post-injection speed on mean baseline speed. The three repeated measures of post-injection speed also were separately regressed on mean baseline speed to provide three repeated measures of residual resistance for each individual.

We statistically removed individual mass effects and maternal effects related to the condition of the female by regressing mean residual resistance on female mass and individual mass and using the resultant residuals (=“mass adjusted resistance”) in further analyses (c.f. Garland, 1988; Brodie, 1989). This analysis also was performed for each of the three repeated measures of residual resistance to provide multiple estimates of mass adjusted resistance for each individual.

Separate one-way analyses of variance were used to test for trial and individual differences in baseline speed and post-injection speed and for individual and litter differences in mass adjusted resistance. Repeatabilities and heritabilities (based on full-sib correlations) and associated standard errors were estimated using formulae in Becker (1984) and Falconer (1981). These estimates were judged significant (at $P <$

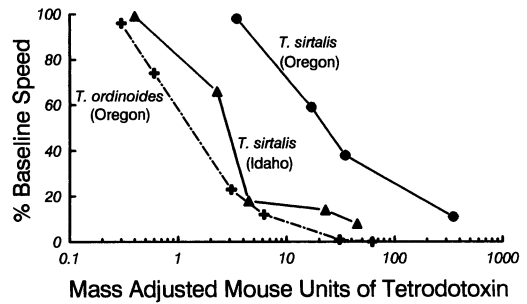


FIG. 1. Mean resistance (in % baseline speed) as a function of mass-adjusted mouse units of TTX (shown on a \log_{10} scale) for each of three garter snake (*Thamnophis* sp.) populations.

0.01) if 99% confidence intervals excluded zero. All other significance was judged at $P < 0.05$. Statistical analyses were performed using PC-SAS version 6 (SAS Institute, 1985).

RESULTS

Interpopulation and Interspecific Variation in Tetrodotoxin Resistance

The assay for resistance to TTX based on sprint speed revealed marked differences between sympatric *Thamnophis sirtalis* and allopatric *Thamnophis sirtalis* and between sympatric *Thamnophis sirtalis* and sympatric *Thamnophis ordinoides* (Table 1), even when dosages were adjusted to account for differences in mean mass of the three groups (Fig. 1). At each level of TTX tested on all three groups, sympatric *Thamnophis sirtalis* showed much less reduction in speed than each of the other populations. A concentration of TTX (0.00015 mg) that had virtually no effect on sympatric *Thamnophis sirtalis* reduced the sprint speed of each of the other groups to less than 20% of base-

TABLE 1. Mean resistance (in % baseline speed) \pm standard error at different dosages of TTX for each of three snake populations. Sample sizes are in parentheses.

Dose mg TTX	Population		
	Sympatric <i>T. sirtalis</i>	Allopatric <i>T. sirtalis</i>	Sympatric <i>T. ordinoides</i>
0.015	11 \pm 3.7 (14)	—	—
0.0015	37 \pm 7.0 (15)	9 \pm 6.6 (2)	0 (3)
0.00075	59 \pm 7.7 (7)	13 \pm 1.0 (2)	1 \pm 0.9 (19)
0.00015	97 \pm 3.9 (3)	18 \pm 2.5 (3)	12 \pm 1.3 (25)
0.000075	—	64 \pm 5.5 (3)	30 \pm 4.3 (3)
0.000015	—	94 \pm 11.7 (3)	72 \pm 3.5 (25)
0.0000075	—	—	96 \pm 4.0 (8)
Saline	101 \pm 3.0 (30)	122 \pm 2.3 (5)	105 \pm 2.4 (37)

TABLE 2. Results from analyses of variance to determine individual and litter effects in baseline speed, post-injection speed and mass-adjusted resistance for sympatric *Thamnophis sirtalis* (***) = $P < 0.0001$).

	Individual effect			Litter effect		
	F	d.f.	P	F	d.f.	P
Baseline speed	5.55	327, 984	***	—	—	
Post-injection speed	5.17	327, 656	***	—	—	
Mass-adjusted resistance	4.05	327, 656	***	8.90	22, 305	***

line. An amount of TTX (0.0015 mg) sufficient to slow sympatric *Thamnophis sirtalis* to 38% of baseline speed rendered sympatric *Thamnophis ordinoides* unable to crawl; the latter subjects did not recover for more than eight hours. The injection control (saline) had no effect on locomotor performance in any group (Table 1).

Intrapopulation Variation in *Thamnophis sirtalis*

The four repeated measures of baseline speed did not differ significantly ($F_{3, 1308} = 1.84$, $P > 0.05$). Post-injection speed was significantly different among trials ($F_{2, 981} = 6.39$, $P < 0.01$). The first (mean = 25.4 cm/s) and second (mean = 24.0 cm/s) post-injection speeds were different (Bonferroni T test: d.f. = 981, $T > 2.40$, $P < 0.05$) but neither was different from the third (mean = 24.7 cm/s). Mean speed did not consistently increase or decrease over these three trials, so the difference does not constitute a meaningful order effect.

A regression of residual resistance on both female mass and individual mass showed a significant slope effect of both variables ($F_{2, 325} = 17.09$, $P < 0.0001$, $P_{\text{mass}} < 0.0001$, $P_{\text{fmass}} < 0.02$). For this reason, resistance was adjusted for mass effects by using the residuals of this regression in all subsequent analyses (=“mass adjusted resistance”).

Baseline speed, post-injection speed and mass adjusted resistance differed signifi-

cantly among individuals (Table 2). Repeatabilities estimated by intraclass correlation for each of these variables also were significant (Table 3).

Significant litter differences in mass-adjusted resistance were revealed through analysis of variance (Table 2). Broad-sense heritability of this trait as estimated through full-sib correlation also was significantly different from zero (Table 3).

DISCUSSION

Variation in TTX Resistance

Thamnophis sirtalis from populations that occur with *Taricha granulosa* were much more resistant to TTX than were conspecifics that occur outside the range of the newt. Another snake (*Thamnophis ordinoides*) that occurs with *Taricha granulosa* in the Pacific Northwest showed levels of resistance roughly similar to allopatric *Thamnophis sirtalis*. These facts suggest that TTX resistance is not a property of the genus *Thamnophis* or even of the species *Thamnophis sirtalis* at large, but rather has arisen only in populations of *Thamnophis sirtalis* that feed on *Taricha granulosa*.

Variation in TTX resistance also was observed within a population of *Thamnophis sirtalis* that, on average, exhibited high levels of resistance. Some of this variation can be explained by the direct relationship between size and resistance, but even after resistance

TABLE 3. Repeatabilities and broad-sense heritabilities of baseline speed, post-injection speed and mass-adjusted resistance for 328 neonates from 23 families of sympatric *Thamnophis sirtalis*. See text for estimation techniques and tests (** = $P < 0.01$).

	Repeatability			Heritability		
	R	SE	P	h ²	SE	P
Baseline speed	0.532	0.028	**	—	—	
Post-injection speed	0.581	0.025	**	—	—	
Mass-adjusted resistance	0.504	0.032	**	0.715	0.162	**

was adjusted for differences in mass, individual variation was apparent. Repeatability estimates indicated that individual differences were consistent.

Resistance to TTX was not attributable to an acquired tolerance. No consistent order effect was found during the three post-injection trials, indicating that individual resistance did not improve during the study.

Differences in TTX resistance were detected among litters, suggesting a genetic basis for resistance in this population. An estimate of heritability also suggested a significant amount of additive genetic variation for the trait. This estimate is based on a full-sib correlation and as such includes fractions of dominance variance and variance caused by common family environments. The contribution of the latter is probably minimal because individuals were separated at birth and some maternal effects related to female condition during pregnancy were removed statistically (Garland, 1988). However, because dominance variation may affect the estimate, the heritability reported here should not be considered a precise measure of additive genetic variance.

Evolutionary Response by a Predator to Dangerous Prey

The potential for *Thamnophis sirtalis* to respond evolutionarily to increased toxicity in *Taricha granulosa* is established by two lines of evidence: (1) individual differences for TTX resistance within a population of *Thamnophis sirtalis* and (2) an additive genetic component to this variation. These are the two necessary conditions for a trait to respond to natural selection (Lande and Arnold, 1983). Any form of selection applied to a trait with these properties will result in adaptive evolution.

Brodie (1968) found that virtually all potential predators of *Taricha granulosa* were killed either when injected with small doses of skin homogenate or force fed newt pieces. Anecdotal literature accounts (Mosher et al., 1964) and our own observations also indicate that ingestion of *Taricha granulosa* has lethal consequences for most animals, including garter snakes from outside the range of *Taricha granulosa* and humans (Bradley

and Klika, 1981). In predators that are not resistant to TTX, this would result in strong selection for either avoidance of the newt or resistance to the toxin.

Ingestion of *Taricha granulosa*, though not directly lethal, could have adverse effects on survivorship even for a predator as resistant as sympatric *Thamnophis sirtalis*, which typically right themselves slowly and are unable to crawl for several hours after consuming a newt. Furthermore, snakes must thermoregulate after a meal to aid in digestion (Stevenson et al., 1985), so an individual that has eaten a newt is likely to be both exposed and immobilized for some time after ingestion. This could decrease survivorship in two ways: (1) an exposed, debilitated snake is at high risk of predation and (2) a basking snake that becomes non-ambulatory for even a short time risks being unable to avoid lethal temperatures as the thermal environment changes (Peterson, 1987). Thus, ingestion of *Taricha granulosa* may result in selection for increased resistance, even among predators that are only partially susceptible.

The lack of TTX resistance in allopatric populations of *Thamnophis sirtalis* suggests that conspecifics sympatric with newts evolved TTX resistance in response to newt toxicity. The existence of heritable variation for resistance in the face of the present directional selection for the trait must result in continuing adaptive evolution. Because this selection is a product of *Taricha granulosa* toxicity, such evolution constitutes an evolutionary response by a predator to the antipredator mechanisms of a dangerous prey.

The Arms Race Analogy

The arms race analogy describes antagonistic reciprocal coevolution of predator and prey, not simply an evolutionary response by one species to the other. For the *Thamnophis sirtalis*-*Taricha granulosa* system to constitute an arms race, *Taricha granulosa* must also be evolving greater toxicity in response to the increased resistance of its predator. Although we present no data directly demonstrating reciprocal evolution between *Thamnophis sirtalis* and *Taricha granulosa*, we can examine observed pat-

terns of toxicity and resistance to evaluate the potential applicability of the arms race analogy to this system.

Genetic complementarity (Lenski and Levin, 1985) is a necessary (but not sufficient) condition for an arms race. Without additive genetic variation for complementary traits in both predator and prey, reciprocal coevolution cannot occur. The data presented here establish this condition in the predator, but the basis of the observed individual variation in toxicity of *Taricha granulosa* (Wakely et al., 1966; Brodie et al., 1974) is unknown.

The predator and prey must also exert selection on one another with respect to the complementary traits for an arms race to develop. If *Taricha granulosa* is an important food source for *Thamnophis sirtalis*, then the newt's toxicity creates strong selection for resistance in the predator. However, it is difficult to explain the evolution of prey toxicity through individual selection by predation (Fisher, 1958). If a prey kills its predator but loses its own life in the process, toxicity is not selectively advantageous. Brodie (1968) reports a *Taricha granulosa* that was eaten by a bullfrog later emerging unscathed from the dead frog's mouth, and we have removed live newts from non-resistant garter snakes that died up to 85 min. after ingestion. *Taricha granulosa* also have been observed to crawl out of the mouths of *Thamnophis couchi* that became immobilized after ingesting the newts (S. J. Arnold, *pers. comm.*). This phenomenon would allow the evolution of toxicity through individual selection on newts and would be a particularly effective mechanism against snake predators that swallow their prey whole. Thus, resistance in *Thamnophis sirtalis* may create a selective pressure to increase toxicity in *Taricha granulosa*.

Taricha granulosa does not occur outside the range of *Thamnophis sirtalis* so it is impossible to compare populations with and without resistant predators. However, it is known that the genus *Taricha* is 100 times more toxic to mice than are other genera of newts that have been tested (Brodie et al., 1974; Nowak and Brodie, 1978; Brodie et al., 1984). None of these other genera are known to have resistant predators, implying

that newts in general may be more toxic in the presence of a resistant predator.

Finally, the level of resistance in *Thamnophis sirtalis* and toxicity in *Taricha granulosa* appear to be roughly matched. *Taricha granulosa* are not lethal to *Thamnophis sirtalis*, but neither is the predator completely resistant. Some individual *Thamnophis sirtalis* from resistant populations show no ill effects after eating a newt, others are debilitated for several hours. If an arms race is taking place between predator and prey, one would not expect either species to be fully capable of exploiting or repelling the other.

Although we have established that predators can and do respond evolutionarily to antipredator adaptations of dangerous prey, our data are not sufficient to determine whether or not *Thamnophis sirtalis* and *Taricha granulosa* are involved in an evolutionary arms race. Indirect evidence suggests that this is the case, but information on intrapopulation variation in toxicity and the selective advantage of increased toxicity in *Taricha granulosa* are required before this can be stated with confidence.

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