Journal of Chemical Ecology, Vol. 31, No. 2, February 2005 (© 2005)
DOI: 10.1007/s10886-005-1345-x

PARALLEL ARMS RACES BETWEEN GARTER SNAKES AND NEWTS INVOLVING TETRODOTOXIN AS THE PHENOTYPIC INTERFACE OF COEVOLUTION

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(Received February 23, 2004; accepted October 21, 2004)

Abstract—Parallel "arms races" involving the same or similar phenotypic interfaces allow inference about selective forces driving coevolution, as well as the importance of phylogenetic and phenotypic constraints in coevolution. Here, we report the existence of apparent parallel arms races between species pairs of garter snakes and their toxic newt prey that indicate independent evolutionary origins of a key phenotype in the interface. In at least one area of sympatry, the aquatic garter snake, *Thamnophis couchii*, has evolved elevated resistance to the neurotoxin tetrodotoxin (TTX), present in the newt *Taricha torosa*. Previous studies have shown that a distantly related garter snake, *Thamnophis sirtalis*, has coevolved with another newt species that possesses TTX, *Taricha granulosa*. Patterns of within population variation and phenotypic tradeoffs between TTX resistance and sprint speed suggest that the mechanism of resistance is similar in both species of snake, yet phylogenetic evidence indicates the independent origins of elevated resistance to TTX.

Key Words—Coevolution, parallel evolution, resistance, *Taricha*, tetrodotoxin, TTX, *Thamnophis*, toxicity.

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INTRODUCTION

Both the fitness consequences that drive an "arms race" and the traits that evolve as a result depend on the phenotypic interface of coevolution. In other words, the phenotypic traits that mediate the interaction between ecological partners serve as both agents and targets of selection (Brodie and Brodie, 1999b; Brodie and Ridenhour, 2003). In chemically mediated interactions, the phenotypic interface may revolve around a toxin. If the toxin is severe enough to drive an arms race between predator and prey, we would expect to see similar coevolutionary patterns in multiple predator–prey pairs wherein prey possesses this toxin. This scenario, of course, requires that the usual prerequisites for coevolution are met, including the genetic ability of each species to respond to reciprocal selection and the occurrence of ecological interactions that allow the traits to generate selection (Berenbaum and Zangerl, 1992). The existence of such "parallel arms races" would be evidence that the phenotypic interface in question is a driving force behind the patterns of trait covariation observed. Although parallel arms races might be observable as a single prey species with different successful predators in different parts of its range, the strongest case for the evolutionary significance of the phenotypic interface would be independent species pairs of coevolving predators and prey.

Parallel arms races might involve identical traits on both sides of the phenotypic interface or on only one. If two prey species share a common deadly toxin, their respective predators might have responded by evolving the same means of circumventing the toxin, or they might have evolved different mechanisms of exploitation. If both a chemical and mechanism of resistance to the chemical are similar in parallel arms races, this suggests that constraints of some type are involved in determining the evolutionary response to selection in the parallel systems. Some of the best examples of constrained parallel evolution come from the phylogenetically diverse but mechanistically similar adaptations of insects to insecticides (Mallet, 1989; McKenzie and Batterham, 1994). Conversely, other cases of insecticide resistance involve traits as varied as behavioral, enzymatic, and physiological defenses to the same classes of chemicals, indicating that evolutionary responses need not always play out in similar dimensions (Mallet, 1989; Denholm et al., 1999). Analogous possibilities exist for the other side of the interaction as well, such that different prey species might repeatedly evolve similar defenses with respect to a single predator, or evolve unique defenses with respect to a common exploitative trait of its predators. Characterization of the existence and nature of parallel arms races is a first step in understanding the generality of the ecological context related to any phenotypic interface of coevolution.

The phenotypic interface of the interaction between the garter snake *Thamnophis sirtalis* and the newt *Taricha granulosa* is the neurotoxin tetrodotoxin

(TTX). Tetrodotoxin binds to voltage-gated sodium channels in nerves and muscles, thereby blocking action potential propagation. Because TTX has high binding affinity for most sodium channel types in most species (Hille, 1992; Narahashi, 2001), it has broad and extreme toxicity. TTX is found in a wide variety of taxa (Miyazawa and Noguchi, 2001) including all species of newts in the genus *Taricha* (Brodie et al., 1974; Yotsu et al., 1990; Yotsu-Yamashita, 2001). Levels of TTX in *Taricha granulosa* can be extremely high, making the newts lethal prey to almost all potential predators (Brodie, 1968; Hanifin et al., 1999). The common garter snake, *Thamnophis sirtalis*, appears to have entered such an arms race. Some populations of *T. sirtalis* that are sympatric with toxic *T. granulosa* have evolved physiological resistance to TTX, and levels of resistance generally covary with toxicity of newts across a broad geographic range (Brodie et al., 2002). Phylogenetic comparisons suggest that the entire genus *Thamnophis* has slightly elevated resistance to TTX, predisposing this group to engagement at a phenotypic interface involving TTX (Motychak et al., 1999). Despite this predisposition to and apparent evolutionary lability of TTX resistance, *Thamnophis sirtalis* is the only species known to be apparently evolving with toxic newts. The apparent lack of parallel arms races involving TTX and resistant snake predators is paradoxical with predictions of the importance of dangerous prey to predator–prey coevolution (Brodie and Brodie, 1999b).

Following the observations of predation in the wild, we investigated TTX resistance and toxicity in sympatric populations of a second species pair of garter snakes (*T. couchii*) and newts (*T. torosa*) from California. Our results indicate that not only has this species of garter snake evolved elevated resistance to TTX present in local newts, but also that similar patterns of costs to TTX resistance exist, suggesting similar mechanisms of resistance. The phylogenetic relationships of *T. couchii* and *T. sirtalis* (de Queiroz et al., 2002) indicate independent origins of elevated TTX resistance in these two species. The similarity of both predator and prey sides of the phenotypic interface in these apparent parallel arms races indicates not only that TTX is a potent driving force behind coevolution in these taxa, but also that some form of evolutionary or physiological constraint has led to parallel phenotypic evolution in the predatory traits mediating this coevolution.

METHODS AND MATERIALS

Population Samples. Adult female garter snakes (*Thamnophis couchii*) and both juvenile and adult newts (*Taricha torosa*) were collected from Cold Springs Creek, nearby Tyler Creek, and small adjacent ponds, from the Greenhorn Mountains in the Sierra Nevada Range, Tulare County, CA, USA. Subjects were collected (18–20 May and 12–14 June 2001) and will be deposited at the California Academy of Sciences.

Snake Resistance. Tetrodotoxin resistance data were collected on 68 neonate snakes born in the laboratory to six wild-caught females 4 August-5 September, 2001. Females were housed individually in 10 gallon glass aquaria with a thermal gradient (24–30°C) and a 14:10 L:D photoperiod. Females had constant access to water and were fed farm-raised mollies (*Poecillia* sp.) weekly. After parturition, neonates were measured for mass, snout-vent length (SVL), and total length, housed individually in plastic tubs (15 cm diam by 10.5 cm tall), and watered once daily.

Resistance to TTX was scored by using a bioassay of whole organism performance (Brodie and Brodie, 1990; Brodie et al., 2002). At 3–5 d after birth, each individual was raced for 2 m on a 4-m by 0.1-m racetrack with a substrate of indoor/outdoor carpet and equipped with infrared sensors to electronically time sprint speed over 0.5-m intervals. Each neonate was tested twice on one day (3–4 hr apart) to determine "baseline speed." The maximum 0.5-m speed in each trial was taken as a measure of maximum sprint speed.

The following day (20–21 hr after the last speed trial) each neonate was given an intra-peritoneal injection of a known dose [see below) of TTX [crystalline $3\times$ in citric acid–sodium citrate buffer (Sigma) diluted in amphibian ringer solution]. Thirty min after injection snakes were tested on the racetrack to determine "postinjection speed." Forty-eight hr later, snakes were again tested, up to three times total per snake. Control injections of physiological saline have no effect on snake performance (Brodie and Brodie, 1990). "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, while those unaffected by a dose of TTX crawl 100% of their baseline speed.

A population-level dose response curve was calculated from individual neonate responses to five levels of TTX injections (0.5, 1, 2, 5, and 10 μg) using the linear regression $y' = \alpha + \beta x'$, with the transforms $y' = \ln((1/y) - 1)$, where y is resistance as a percentage of baseline crawl speed, and $x' = \ln(x)$, where x is the dose of TTX in mass-adjusted mouse units ("MAMU") (for further details of analysis, see Ridenhour et al., 2004). From this regression model, we estimated the "50% dose," defined as the amount of TTX required to reduce the average snake to 50% of its baseline speed. Because TTX resistance is related to body size within and among some populations of *Thamnophis sirtalis* (Brodie and Brodie, 1990, 1999a; Ridenhour et al., 2004), and to compare levels of TTX resistance to populations of *Thamnophis sirtalis* (Brodie et al., 2002), we transformed doses using a population-level mass-adjusted measure. A dose in MAMUs was calculated by dividing a given dose of TTX by the mean neonate mass of the population (as measured after the final baseline speed trial), then dividing by the amount of TTX sufficient to kill 1 g of mouse in 10 min (Brown and Mosher, 1963); 1 "mouse unit" = 0.0143 μg of TTX. One MAMU is, therefore, one mouse

unit of TTX per gram of snake. Neonate garter snakes ($N = 56$) were tested at one common dose (0.005 mg TTX) to determine whether families differed with respect to average resistance at this dose; resistance scores were analyzed using a one-way ANOVA in JMP v 5.01 (JMP, 1989–2002).

Phenotypic tradeoffs between locomotor performance and TTX resistance have been detected in populations of *Thamnophis sirtalis* (Brodie and Brodie, 1999a). To investigate the presence of similar tradeoffs in *T. couchii*, we examined the slope of a regression of postinjection speed on baseline speed. If TTX affects all individuals equally, then the slope of the regression will be one, and the effect of TTX is purely additive and reflected in the intercept. If, however, the effect of TTX is related to the speed of an individual, then the slope should differ from one: a slope <1 indicates a tradeoff wherein the fastest individuals have low resistance, while a slope >1 indicates that faster individuals have greater resistance. Regression analyses were performed in JMP v 5.01 (JMP, 1989–2002). Because both variables are measured with error, reduced major axis (RMA) regression was also examined. Results of RMA converge quantitatively with Model I regression as the error ratio exceeds 2, and so only Model I results are reported.

Newt Toxicity. Newts were brought to the laboratory (Utah State University), weighed, SVL measured, and frozen at -80°C within 5 d of field collection. Individual tissue samples from each subject were taken from the dorsal surface between the pelvic and pectoral girdle. This region of skin has a uniform distribution of skin glands, and TTX levels from the dorsum show little within individual variation (Hanifin et al., 2004). We removed a small (5 mm diam) circle of skin with a human skin-biopsy punch (Acu-Punch^{TM}, Acuderm Inc.) for toxin analysis. Only skin and the thin layer of connective tissue between the skin and dorsal muscle was removed.

Toxin was extracted from each skin sample by grinding a single tissue plug (0.19 cm^2) with 800 μl extraction solution (0.1 M aqueous acetic acid). Samples were shaken, heated, and spun following procedures described previously (Hanifin et al., 1999, 2004). The levels of TTX were quantified by fluorometric HPLC following the protocol of (Yasumoto and Michishita, 1985; Hanifin et al., 1999). Data acquisition and chromatographic analysis were performed with System Gold software (version 8.1, Beckman Inc.). Peak area concentration curves were calculated with standards prepared from commercial TTX (Sigma).

We estimated whole newt toxicity by using the relationship of dorsal skin toxicity (from skin punches) to whole animal toxicity described by Hanifin et al. (2004). The relationships between TTX concentration and newt size SVL, and between whole newt toxicity and newt SVL were estimated with regression using JMP v5.01 (JMP, 1989–2002). Graphical comparisons of whole newt toxicity and snake resistance at the population-level were made using these whole newt toxicity measures and a projection of average snake resistance as a function of body size.

To determine how much toxin would reduce the performance of the average snake in a population by a given amount, we used the relationship of injected to oral doses of TTX (Williams et al., 2001) and interpolation of the population average MAMU dose from the population curve (described above or from Brodie et al., 2002 for Benton Co., OR *T. sirtalis*) yielding the equation: mg TTX = [(Mouse Unit × oral/injected dose) × snake mass] × resistance in MAMU. Curves were estimated for 15% (near immobility) and 50% baseline performance for the Cold Springs Creek population of *T. couchii* and sympatric *T. torosa*, and for the Benton Co., OR population of *T. sirtalis* and sympatric *T. granulose*. Data for *T. sirtalis* and *T. granulosa* were taken from Brodie et al. (2002) and Hanifin et al. (1999, 2002).

RESULTS

Snake Resistance. An adult *T. couchii* (50 g, 480 mm SVL-CAS 212868) was observed (J. V. Vindum and C. R. F., personal observation) swallowing a large juvenile *T. torosa* (1.85 g, 41 mm SVL-CAS 212869) in the wild at the Cold Springs Creek locality on 7 June, 2000. When discovered, the newt was visibly covered with secretion and was swallowed head first as far as its forelimbs. Upon collection, the snake disgorged the newt and both animals appeared unharmed for 3 d, after which they were preserved as voucher specimens.

At testing, the mean mass of neonate *Thamnophis couchii* ($N = 68$) was 3.7 ± 0.07 (SE) g, mean SVL was $195 + 1.3$ (SE) mm, and mean total length was 258 ± 1.7 (SE) mm. The average litter size was 11.2 and ranged from 7 to 15.

Neonate *Thamnophis couchii* from this population exhibit high levels of resistance to TTX. The population resistance curve was characterized by the regression: $y' = -6.91 + 1.55x'$. This relationship yielded an estimated 50% dose of 86.5 MAMU (95% CI 70.3–106.3 MAMU) for the population (Figure 1). Significant family level variation in resistance to 0.005 mg of TTX was detected (ANOVA: $F_{4,50} = 11.85$, $P < 0.001$).

Tradeoffs between resistance and locomotor performance were detected at the phenotypic level (Figure 2). The regression of postinjection speed on baseline speed was postinjection speed = 0.106 + 0.185 [baseline speed]. The slope of this regression was less than 1 ($t = -4.30$, df = 1, $P < 0.001$), indicating that slower snakes were relatively more resistant than faster snakes. An insufficient number of families prevented analysis of the genetic tradeoffs.

Newt Toxicity. Tetrodotoxin was detected in each of the newts collected from the Cold Springs Creek locality. Adult newts (>65 mm SVL; $N = 8$) had an average concentration of 0.065 mg TTX/cm^2 dorsal skin (range 0.028–0.133); juvenile newts (<50 mm SVL; $N = 11$) had an average concentration of 0.009 mg TTX/cm^2 dorsal skin (range 0.001–0.026). The concentration of

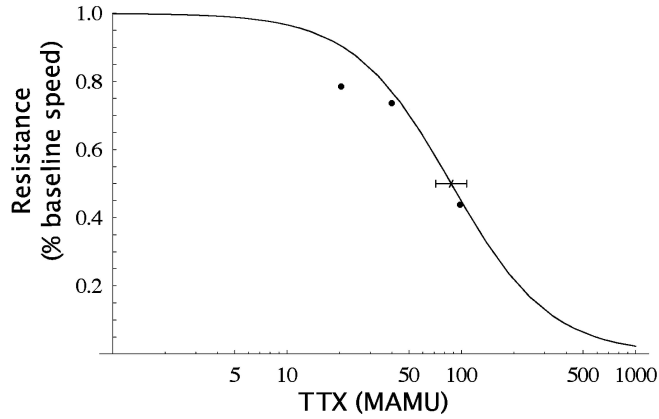

FIG. 1. Tetrodotoxin resistance in *Thamnophis couchii* from Cold Springs Creek, CA. The dose–response curve for neonate *T. couchii* was estimated over a range of TTX doses using curvilinear regression. The 50% dose (with 95% CI) is shown as a bar at 86.5 MAMU of TTX. Symbols indicate average response at each tested dose.

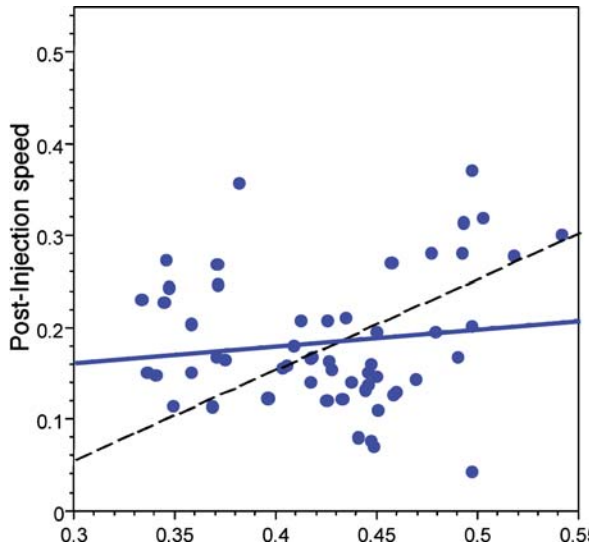

FIG. 2. Costs of tetrodotoxin resistance in *Thamnophis couchii*. Regression of postinjection speed on baseline speed (m/s) reveals a slope (solid line) significantly less than 1 (dashed line), indicating that slower snakes are relatively more resistant than faster snakes. Regression based on data from neonates snakes ($N = 56$) tested at 95.4 MAMU of TTX.

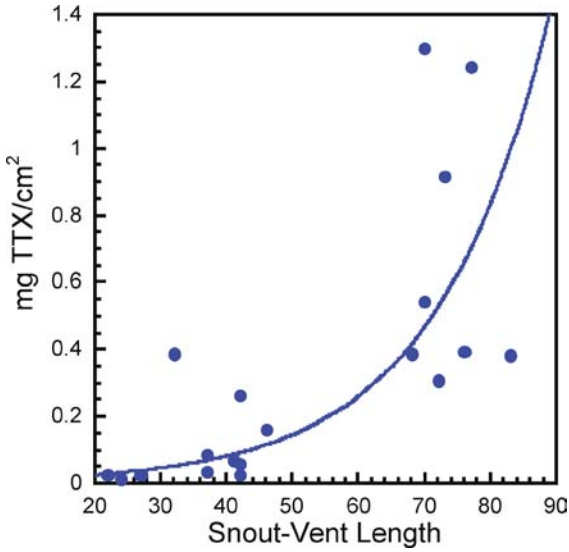

FIG. 3. Tetrodotoxin levels of *Taricha torosa* as a function of SVL (mm). The concentration (by area) of TTX in dorsal skin increases exponentially with SVL. This relationship appears to be driven primarily by differences between juvenile (<50 mm) and adult (>65 mm) newts.

TTX in dorsal skin increased with size of newt, but varied greatly among individuals [Figure 3; $\ln(\text{TTX/cm}^2)$ skin $= -7.596 + 0.642$ [SVL], $F_{1,17} = 54.34$, $P < 0.001$]. Whole animal estimates of TTX per individual indicated the average juvenile newt possessed 0.081 mg TTX in its skin, whereas the average adult newt possessed 2.41 mg of TTX. Whole animal TTX also increased with body size [TTX/individual $= -7.197 + 1.072$ [SVL]; $F_{1,17} = 145.77$, $P < 0.001$]. The effect of size on both skin concentration of TTX and total body TTX appeared to be primarily due to difference between newly metamorphosed and adult newts.

DISCUSSION

Thamnophis couchii from the Cold Springs Creek region of California have evolved high levels of resistance to tetrodotoxin (TTX). Previous studies of *T. couchii* and congeners from other parts of western North America have not detected TTX resistance in this group, even where these snakes are sympatric with newts of the genus *Taricha* (Motychak et al., 1999). As is the case for TTX resistance in another species of garter snake, *T. sirtalis*, among-family variation in level of resistance is apparent. Furthermore, tradeoffs between resistance and

locomotor performance similar to those detected in populations of *T. couchii* are observed in *T. sirtalis* (Brodie and Brodie, 1999a). The presence of such a cost suggests that similar physiological mechanisms might underlie TTX resistance in both species of snakes. Relatively high levels of TTX are present in sympatric *T. torosa*, though the quantity of the toxin varies among individuals and increases dramatically with size after metamorphosis (Figure 3). Field observations of an adult *T. couchii* eating a newt in the wild indicate at least some frequency of ecological interaction between these species.

Compared to known populations of *T. sirtalis*, the Cold Springs Creek population of *T. couchii* has relatively high resistance to TTX; this population would rank in the second highest category with only four populations of *T. sirtalis* exceeding its average 50% level resistance of 86.5 MAMU (Brodie et al., 2002). Interpopulational comparisons of snake resistance and newt toxicity in the *T. sirtalis* and *T. granulosa* interaction suggest fairly close phenotypic matching (Brodie et al., 2002). Projections of relative toxicity and resistance for a range of body sizes (Figure 4) suggest that adult *T. couchii* would be able to ingest most adult newts in the population without experiencing a complete loss of locomotor function. By contrast, adult *T. sirtalis* from the Benton County population in Oregon (one of the more resistant populations of snakes) are expected to frequently encounter newts toxic enough to fully immobilize them (Figure 4). Thus, the Cold Springs population of *T. couchii* appears to be more resistant relative to the toxicity of its sympatric prey than most studied populations of *T. sirtalis*.

Elevated resistance to TTX is a derived character state in the genus *Thamnophis* previously known only in *T. sirtalis*, despite investigations of other species of garter snakes that prey on amphibians and co-occur with *Taricha*. *Thamnophis sirtalis* and *T. couchii* are distantly related within the genus; *T. sirtalis* is a member of the sister group (with ribbon snakes) to all other garter snakes, whereas the aquatic garter snake species group that includes *T. couchii* is relatively derived (de Queiroz et al., 2002). The elevated resistance of Cold Springs Creek *T. couchii* likely represents an independent origin of TTX resistance within the genus, though other alternatives such as introgression of resistance alleles through hybridization currently cannot be ruled out. Species relationships among newts of the genus *Taricha* are less well known (Tan and Wake, 1995), but the presence of TTX in all three members of the genus, combined with the presence of TTX in *Notophthalmus* (the sister genus to *Taricha*) and other salamandrids (e.g., *Cynops* spp.) suggests that some level of TTX toxicity is ancestral in the group (Brodie et al., 1974; Yotsu et al., 1990; Yotsu-Yamashita, 2001). Previously examined populations of *T. torosa* from the Sierra Nevada of California possess TTX at levels well below those observed at the Cold Springs Creek locality (C. Hanifin, unpublished data). Although the source of TTX toxicity in newts is unclear, recent work (Hanifin et al., 2002; Shimizu, 2002; Cardall et al., 2004; Lehman et al., 2004) provides evidence that newts may be more active in the biosynthesis of their

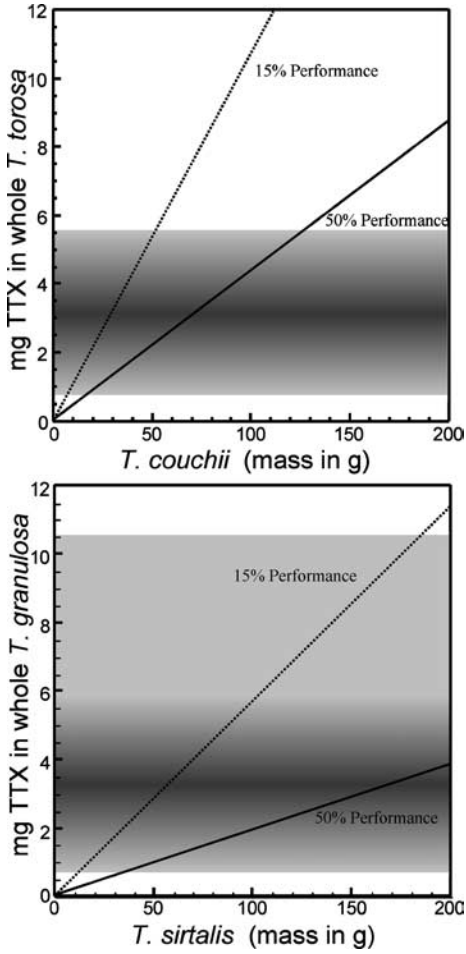

FIG. 4. Comparisons of prey toxicity and predator resistance from two localities. The average resistance for snakes as a function of body size is shown predicting the amount of ingested toxin that would reduce a snake to 15% (near immobility; dotted line) and 50% (solid line) baseline performance. On the vertical axis, the total skin toxicity of adult newts is shown, with the range of observed values shaded and the mean shown as a darker band. For a given size snake, the predicted effect of ingesting a newt of given toxicity can be estimated by finding the intersection of performance lines and newt toxicity. (A) Most adult *T. couchii* from the Cold Springs Creek, CA population (approximately 60–200 g) can ingest the average adult newt without suffering a performance reduction of 50%. (B) Few if any adult *T. sirtalis* (approximately 40–200 gm) from Benton Co., OR could ingest the average adult newt with less than a 50% reduction, and most would be expected to suffer much greater reductions of performance.

TTX than previously thought and that variation in newt toxicity within and among populations of newts may have a genetic basis. Taken together, these ecological and phylogenetic observations suggest that the predator–prey interaction between *T. couchii* and *T. torosa* in the southern Sierra Nevada of California has evolved in parallel to the interaction between *T. sirtalis* and *T. granulosa* in other parts of western North America.

Parallel "arms races" among distinct species pairs of TTX resistant garter snakes and toxic newts reveal both evolutionary lability and phylogenetic bias. The results presented herein show that resistance to TTX has evolved in at least two unrelated species groups in the genus *Thamnophis*, as well as at least twice within one species (*T. sirtalis*; Brodie et al., 2002; Geffeney et al., 2002). This pattern of diversity suggests considerable flexibility for a fundamental neurophysiological feature that is conserved across most vertebrates, the structure of voltage-gated sodium channels in skeletal muscle (Geffeney et al., 2002). Conversely, all known occurrences of elevated resistance to TTX by predators occur in just this single genus of snakes. Such restricted phylogenetic distribution of evolutionary response to dangerous prey is consistent with some form of historical bias, possibly related to the ancestral occurrence of low level resistance to TTX observed in the genus *Thamnophis* (Motychak et al., 1999). Similarly, TTX has been found throughout the genus *Taricha*, as well as in other salamandrids (Mosher et al., 1964; Brodie et al., 1974; Yasumoto et al., 1988), but not in other groups of urodeles. This restricted pattern of TTX distribution is difficult to interpret, however, because the mechanism of production of TTX in these species is still unclear.

The selective importance of deadly toxicity, and TTX in particular, as a feature driving predator–prey arms races is emphasized by the discovery of these parallel coevolutionary systems. The fact that independently derived predator lineages have evolved similar phenotypic responses to prey toxicity further suggests that there are phenotypic constraints to the array of evolutionary counteradaptations available to combat TTX toxicity. Lineage-specific constraints might also contribute to the phenotypic similarity in response, since the predator species in question belong to the same clade. Similar parallel evolution is known from other sorts of biological systems involving deadly toxins, most notably the evolution of insecticide resistance. Many phylogenetically diverse groups of insects have evolved resistance to human-introduced toxins through strikingly similar physiological and genetic mechanisms. In many cases, the parallelism of these adaptations can be attributed to the specific actions of insecticides designed to target fundamental metabolic or neurological pathways. Resistance to such toxins often is achieved by common changes to binding sites or enzymes targeted by the toxins (Mallet, 1989; McKenzie and Batterham, 1994; Ffrench-Constant et al., 2000). Parallelism in these cases is thought to result because there are limited ways to disable the toxins. A similar phenomenon might underlie the parallelism in predator resistance to TTX. Tetrodotoxin is known to bind to sodium

channels in nerves and muscles, thereby blocking action potentials. Resistance to TTX in one species of garter snake (*T. sirtalis*), as well as in other organisms that utilize TTX as a defense (pufferfish, newts), involves changes in the binding affinity of the sodium channel to TTX (Kao and Fuhrman, 1967; Kidokoro et al., 1974; Yotsu-Yamashita et al., 2000; Geffeney et al., 2002). If *T. couchii* shares a similar mechanism as suggested by the observed patterns of variation in and tradeoffs with resistance, then predator–prey arms races involving deadly prey may be under the same sort of metabolic control constraints as insect–insecticide systems.

Acknowledgments—We thank J. V. Vindum and M. S. Koo of the California Academy of Sciences for assistance in the field, M. Pfrender for comments on the paper, and B. Ridenhour for help with analysis and Figure 1. Specimens were collected with permission from the California Department of Fish and Game (scientific collecting permit 803045-04 to CRF). The use of vertebrate subjects was approved by the Utah State University IACUC (protocols 712, 693). This work was supported by NSF grants DEB-9903829, DEB-0316004 to EDB III and DEB-99004070, DEB-0315172 to EDB Jr.

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