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RESISTANCE OF NEONATES AND FIELD-COLLECTED GARTER SNAKES (*Thamnophis spp.*) TO TETRODOTOXIN

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Abstract—Prior studies of tetrodotoxin (TTX) resistance in garter snakes (*Thamnophis spp.*) have used laboratory-reared neonates as subjects, but the use of field-caught individuals would reduce cost and effort. We compared estimates of TTX resistance in field-caught and laboratory-born garter snakes. We found that a mass-adjusted dose of TTX administered to field-caught garter snakes produces an estimate of a population 50% dose that is comparable and unbiased with respect to those previously reported using laboratory-born neonates. Dose-response curves estimated for three field-caught populations closely matched the curves estimated from neonate data. The method was tested using populations with levels of TTX resistance ranging between approximately 5–90 mass-adjusted mouse units for their respective 50% doses. The technique of using field-caught snakes as test subjects provides larger genetically independent data sets that are more easily obtained. Our results indicate that changes in mass during development parallel ontogenetic shifts in TTX resistance.

Key Words—Tetrodotoxin, resistance, dose-response curve, mass adjustment, *Thamnophis sirtalis*, *Thamnophis couchii*.

INTRODUCTION

The study of drug resistance and toxicity is important to disciplines that range from biomedical research to agriculture. Given this broad range, it is not surprising that bioassays measuring resistance or toxicity are usually tailored to the system and question of interest. Many newer techniques have arisen as improved alternatives to prior methods. The improvement may come in the form of reduced cost, higher

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throughput, reduced effort, or alternatives to laboratory animal usage (see Raposo et al., 1995; Plasencia and Banttari, 1997; Spielmann et al., 1999; Sousa and Poiares da Silva, 1999; Olmstead et al., 2001; Valentin-Severin et al., 2002, for examples). Although there are many ways to measure resistance or toxicity, two categories of assays are particularly prevalent. The first method, referred to as the 50% lethal dose or LD_{50}, measures the dose that is lethal to 50% of the test organisms (Kadir et al., 1999; Maa and Liao, 2000; Schmuck et al., 2001). The second, called the 50% inhibition concentration or IC_{50}, measures the dose at which a biological function (e.g., growth or binding rate) is inhibited by 50% (Sousa and Poiares da Silva, 1999; Lee and Adams, 2000; Ismail et al., 2002). The use of measures such as the LD_{50} or IC_{50} provides a simple way to compare the potency of a toxin or resistance across toxins or organisms. However, LD_{50} and IC_{50} only measure the *average* response of individuals within a population and are not useful for assessing individual variation. The estimation of *individual* differences in trait values is fundamental to the study of evolution.

The coevolutionary interaction between the common garter snake *Thamnophis sirtalis* and the rough-skinned newt *Taricha granulosa* revolves around tetrodotoxin (TTX) toxicity and resistance. *Taricha granulosa* (and other newts of the genus *Taricha* found in western North America) possess TTX as a potent chemical defense (Brodie, 1968). In an apparent coevolutionary arms race, the common garter snake *Thamnophis sirtalis* has evolved TTX resistance in parallel with the toxicity level of *Taricha granulosa* (Brodie et al., 2002). By measuring the ratio of unimpaired crawl speed to TTX impaired crawl speed of neonate garter snakes, Brodie and Brodie (1990) developed an assay that estimates individual variation in resistance. The geographic mosaic theory of coevolution (Thompson, 1994) predicts that due to evolutionary forces other than selection, populations within the larger metapopulation will have different evolutionary endpoints (i.e., there will be variation among populations for trait values). Therefore, Brodie et al. (2002) developed a population resistance measure similar to an IC_{50} to allow for easy comparison among groups; the so-called 50% TTX dose is obtained by regression techniques utilizing the data from the individual assay of Brodie and Brodie (1990). In combination, the methods of Brodie and Brodie (1990) and Brodie et al. (2002) provide a technique that yields both the individual measures of TTX resistance necessary to address evolutionary questions and also a measure similar to an IC_{50} that is useful for comparison across groups.

The TTX resistance bioassay (Brodie and Brodie, 1990) used neonate garter snakes as test subjects. The use of neonates is advantageous for several reasons. First, little is known about the ontogenetic change in TTX resistance levels in *T. sirtalis* other than that TTX resistance cannot be altered through exposure (Ridenhour et al., 1999). Using neonates provides a set developmental time point at which testing may occur and be compared across populations (approximately 5-days postbirth). Second, the use of neonates provides a distribution of resistance

within the population prior to selective influences. Third, garter snakes' litters can be large; one gravid female can provide multiple data points. The use of neonates, however, has problems. Capture of gravid female *T. sirtalis* can be difficult due to the reduced number of potential animals in the target population; capture of gravid females from small populations is particularly difficult. Once gravid females are caught, they must be returned to the laboratory and cared for. Husbandry of gravid females is expensive, time-consuming, and may produce no offspring in the end. Finally, although litters produce larger sample sizes, they are not genetically independent data points and, thus, overestimate the confidence placed in the resistance distribution.

We tested an alternative method of measuring TTX resistance in garter snakes by comparing population estimates of resistance in field-caught *T. sirtalis* and *T. couchii* to neonate assays from the same populations. The use of field-caught garter snakes obviates the problems associated with the capture and husbandry of gravid females and, given that collected individuals are not littermates or from highly inbred populations, provides genetically independent resistance measures. Field-caught animals, both male and female, were captured from populations with known levels of neonate resistance and assayed for resistance by using a mass-adjusted TTX dose. The estimated 50% dose from the field-caught snakes was then compared to the estimated 50% dose, using neonate snakes.

METHODS AND MATERIALS

Resistance of neonate garter snakes was estimated in two species. Gravid female *T. sirtalis* were collected from two different populations: Bear Ridge, Humboldt Co., CA ("Bear Ridge") and Adair, Benton Co., OR ("Benton"). Gravid female *T. couchii* were collected from Cold Springs, Tulare Co., CA ("Cold Springs"). Gravid females were housed individually in $25 \times 50 \times 30$ cm aquaria placed on heat-tape to generate a thermal gradient; each aquarium contained a sphagnum-filled hide box and a water bowl. Females were fed fish once per week. The animal chamber was held on a 12L:12D cycle at $26 \pm 1^{\circ}$C. Females were checked multiple times per day for the presence of neonates in their aquaria. Neonate snakes were housed separately in plastic tubs (15 cm diam. $\times$ 10.5 cm tall) and assayed for resistance starting approximately 3–5 days postparturition. Neonates were given water on a daily basis; on trial days, they were given water posttesting.

From each of the three populations, captured animals that were not gravid females were returned to the laboratory for resistance tests ("field-caught"). Field-caught animals were housed individually and kept in conditions identical to those of the gravid females. Field-caught individuals ranged from young-of-the-year ($\sim$4–7 g) to full-grown adults ($\sim$50 g). Assays for resistance on field-caught individuals were not performed at a predetermined time interval after entering captivity

(i.e., the time interval from capture to trial varied among individuals). All field-caught animals were considered to be in good condition prior to use in the experiment.

Using the methodology of Brodie and Brodie (1990), snakes were assayed for resistance to TTX. An adjustable electronic racetrack, 4 m in length, was used to measure crawl speeds. Because the field-caught snakes were larger than neonates, the racetrack was configured differently for running neonate and field-caught animals. For field-caught animals, the track was set to 20 cm in width; for neonates, it was set at 11 cm. Crawl speed for both categories was measured over the central 2 m of the track, with electronic sensors every 0.5 m (thus providing four half-meter speeds); the fastest 0.5 m segment was used as the maximum crawl speed of the snake. Animals were stimulated to crawl down the length of the track by tapping their tail with a finger. The baseline crawl speed for an animal was calculated as the average crawl speed from two separate uninjected time trials performed on the same day approximately 4 hrs apart. An artificial turf substrate was used to aid in crawling. All trials were performed at $26 \pm 1^{\circ}$C.

Garter snakes were assessed for intoxicated crawl speed 30 min after an i.p. injection. Individual snakes were repeatedly injected to test resistance at different doses, but no snake was injected more than five times *in toto*. Snakes were not injected on consecutive days to ensure the elimination of residual TTX effects (Brodie and Brodie, 1990) and to reduce the effect of fatigue. Resistance was measured as the ratio of crawl speed before injection to crawl speed after injection of TTX. For example, a resistance score of one indicates that a snake was unaffected by a given dose of TTX, while a score of 0.25 (i.e., 25% resistant) indicates that the snake's crawl speed was reduced by 75% at that dose.

The effective dosages were calculated in the following manner. A variable volume (up to 0.5 ml) of TTX solution of known concentration (mg TTX/ml amphibian Ringer solution) was administered via intraperitoneal injection. The absolute dose (mg TTX) was then transformed to a mass-adjusted mouse unit (MAMU). A mouse unit is the amount of TTX (0.0002857 mg) required to kill a 20-g mouse in 10 min. Mass-adjusted mouse units were calculated using different methods for field-caught and neonate snakes. For neonates, MAMU dose was calculated as

$$\text{dose}_{\text{n}} = \frac{\text{dose mg TTX}}{\bar{m}\,\text{g}} \times \frac{20\,\text{g}}{0.0002857\,\text{mg TTX}}$$

where $\bar{m}$ is the average mass of a neonate snake in the population of origin. The MAMU dose for field-caught animals was calculated as

$$\text{dose}_{\text{f}} = \frac{\text{dose mg TTX}}{m\,\text{g}} \times \frac{20\,\text{g}}{0.0002857\,\text{mg TTX}}$$

where m is the mass of the individual snake tested. The only difference between the two methods of calculating the dose is the use of the population mean mass, $\bar{m}$, in neonates vs. individual mass, m. The use of $\bar{m}$ also implies that neonates

were given the identical absolute amount of TTX (mg TTX) at a given MAMU dose. In contrast, field-caught individuals received variable absolute amounts of TTX for a given MAMU dose. Both measures adjust dose relative to mass and interpret this adjustment in the biologically relevant mouse unit. Because of the relatively low variance in mass at birth in a population of garter snakes, using $\bar{m}$ is effectively similar to using m in the equation but captures the expectation for an average neonate in the population.

The doses administered to snakes from the three populations varied. The use of different doses between populations was necessary due to the variability in population resistance levels. For example, the doses given to Bear Ridge snakes would have little to no effect on snakes from Benton or Cold Springs. The approximate range of doses given to field-caught animals was 5–100 MAMU for Benton, 2–17 MAMU for Bear Ridge, and 8–206 MAMU for Cold Springs. The approximate range of doses given to neonates was 12–253 MAMU for Benton, 3–19 MAMU for Bear Ridge, and 19–97 MAMU for Cold Springs. Doses were chosen for field-caught individuals on the basis of the 50% doses found in neonates (Brodie et al., 2002), and to be roughly equivalent to the doses given to neonates. The estimates for the 50% doses were 34.1 MAMU for Benton and 6.6 MAMU for Bear Ridge; no estimate has been published for Cold Springs neonates.

Data analysis was conducted by using SAS (version 8.0, SAS Institute, Inc.). To characterize the resistance level of neonate and field-caught animals, a 50% dose was calculated using curvilinear regression on natural-log-transformed dosages to provide a simple estimate of a population-wide 50% dose (Brodie et al., 2002). The curvilinear regression was performed by utilizing the linear regression $y' = \alpha + \beta x'$, where $y' = \ln(1/y - 1)$ and $x' = \ln(x)$; y is TTX resistance, x is TTX dose, and α and β are the estimated parameters. Data adjustment was performed in a manner slightly different from the method described in Brodie et al. (2002); data points that were greater than or equal to one were treated as 0.999, and those that were zero were treated as 0.001. The estimated 50% dose of field-caught animals was then compared to that of laboratory-born neonates to determine differences. Because the estimated 50% doses are ratios of two parameters, bootstrapping was done to create a sample t-distribution for the difference of two 50% estimates. The test statistic was chosen to test the hypotheses

H_0: $|\tau_n - \tau_f - \delta| > 0$ or H_1: $|\tau_n - \tau_f - \delta| = 0$, where τ_n and τ_f are the estimated 50% dose for neonates and field-caught animals, respectively. Because we sought to test equivalence of methods, H_1 tests the hypothesis of equality (i.e., a reverse test). A standard statistical test would only show that τ_n and τ_f were not equal and not truly test that the parameters were equal (the case in which we are interested). With the test we performed, the parameter δ estimates the observed difference between the two 50% estimates and produces equality in our hypothesis. Using a data set created from 1000 bootstrap samples, values of δ were found that satisfied H_1 at the $P < 0.05$ level (one-tailed), thus producing a range of the observed difference between the neonate and field-caught 50% doses.

TABLE 1. REGRESSION RESULTS FOR FIELD AND LABORATORY (NEONATE) GROUPS FROM THREE DIFFERENT POPULATIONS OF *Thamnophis.*

Population	N	N_i	α	P_α	β	P_β
Benton						
Field	104	22	−7.35	<0.001	2.10	<0.001
Laboratory	416	361	−3.93	<0.001	1.11	<0.001
Bear Ridge						
Field	32	9	−3.26	0.0087	1.16	0.0044
Laboratory	51	23	−4.55	<0.001	2.22	<0.001
Cold Springs						
Field	59	14	−6.97	<0.001	1.51	<0.001
Laboratory	125	56	−6.91	<0.001	1.55	<0.001

Note. N is the number of injections given to the group and used in the analysis; N_i is the number of individuals actually used; α and β are the intercept and slope of the curvilinear regression respectively given with their significance level (P).

RESULTS

Curvilinear regression for the field and laboratory groups from each population was significant at the $\alpha = 0.05$ level (Table 1). Bear Ridge had the lowest estimated 50% doses for both neonate and field-caught groups (6.78 and 6.03 MAMU, respectively), followed by Benton (33.65 and 31.84 MAMU), and then Cold Springs (86.47 and 99.27 MAMU). The same pattern was observed for δ, with the smallest absolute values calculated for Bear Ridge and the largest for Cold Springs (Table 2). The level of matching between field-caught and laboratory estimates can be visualized by constructing the paired dose-response curves with their respective 50% TTX doses for all three populations (Figure 1).

TABLE 2. ESTIMATES FOR GROUP 50% TTX DOSES AND THE DIFFERENCE BETWEEN THE ESTIMATES FOR THE LABORATORY (NEONATE) AND FIELD ANIMALS

	50% Dose (MAMU)		$\delta(P < 0.05)$	
Population	Field (τ_f)	Laboratory (τ_n)	Minimum	Maximum
Bear Ridge	6.03 (3.87, 9.17)	6.78 (5.58, 8.20)	0.65	0.84
Benton	31.84 (25.16, 40.23)	33.65 (30.42, 40.39)	1.62	2.00
Cold Springs	99.27 (67.97, 144.78)	86.47 (70.33, 106.26)	−11.84	−13.75

Note. Group 50% doses (τ_f and τ_n) are given with their 95% confidence interval in parentheses. The difference between groups (δ) is given as the minimum/maximum value for the difference based on bootstrapping analysis.

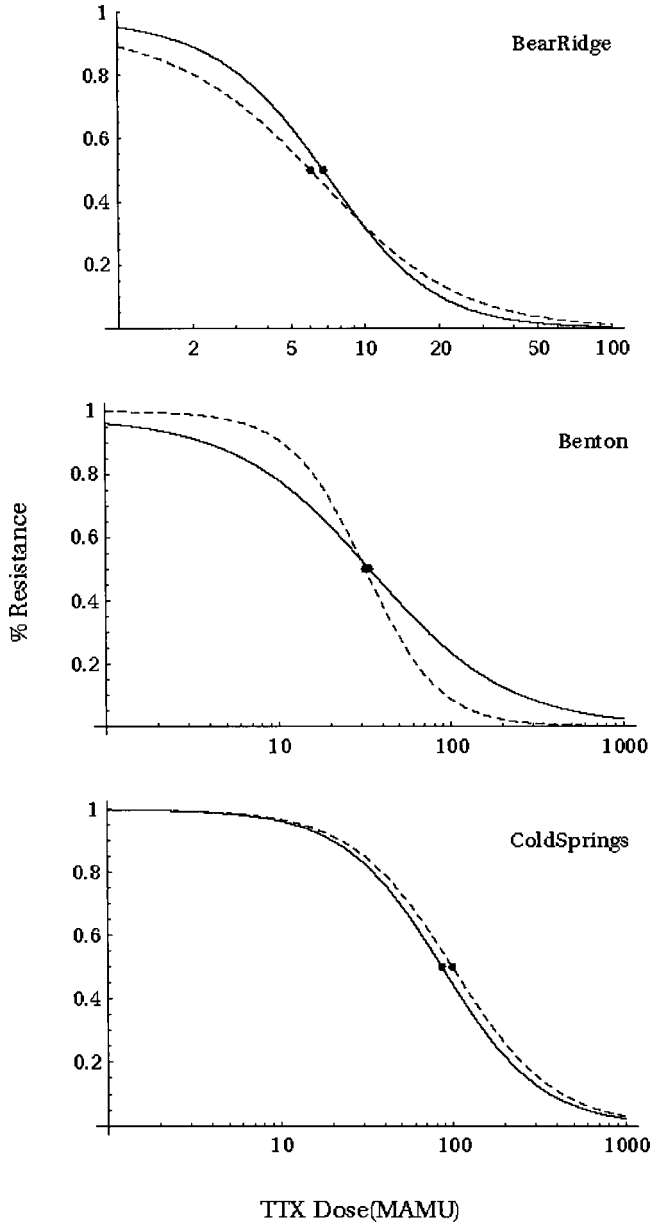

FIG. 1. Population dose-response curves for TTX resistance in neonate (—) and field-caught (- - -) garter snakes. The • indicates the estimated 50% dose for the group. The dose-response curves were estimated using curvilinear regression techniques (see text).

DISCUSSION

The use of field-caught snakes provides a comparable alternative to laboratory-reared neonates for bioassays of TTX resistance in the two species of *Thamnophis* tested. Field-caught and neonate estimates obtained from the populations from Bear Ridge, Benton (both *T. sirtalis*) and Cold Springs (*T. couchii*) produced nearly identical dose-response curves (Figure 1). More importantly for quantitative comparisons, the estimated 50% doses for both populations are similar (Table 2). For Benton, the estimated 50% doses are less than 6% different ($\delta \max/\tau_n$). For Bear Ridge and Cold Springs, the estimated 50% doses are approximately 13% different and 16% different, respectively. The increased precision of the Benton sample is probably due to the large sample size. These populations provide evidence that using field-caught garter snakes for TTX bioassays provides data similar to assays performed on laboratory-born neonate *Thamnophis*.

By comparing the values of δ, it appears there is no bias in the difference between the field and laboratory estimates. Benton and Bear Ridge both exhibit positive values of δ (though both are relatively small). Cold Springs on the other hand exhibited a negative deviation in field and laboratory estimates. The Cold Springs estimate does, however, exhibit the largest deviation of all three populations; this larger value is most likely due to the nature of the analysis (discussed later) rather than a systematic bias. More observations are needed to show conclusively that the estimation of a population 50% dose from field-caught individuals is unbiased.

Because the technique of estimating 50% doses utilizes a linear regression performed on appropriately transformed variables, sampling regime plays an important role in obtaining accurate results. In order to obtain good regression results, sufficient variation in the x-variable and appropriate sample sizes are needed. As an example of the importance of sampling regime, data that were collected from Benton field-caught snakes prior to the current study were analyzed (not shown). This group of snakes ($N = 31$) was given doses that ranged only between 23 and 31 MAMUs (as compared to the 5–100 MAMUs given to the Benton field-caught snakes for this study). Because of the lack of variation in dose, a non-significant regression result was obtained ($P_\alpha = 0.1962$, $P_\beta = 0.2060$). The 50% dose estimated for this group was 26.85 MAMU (22.23, 32.40). This estimate, therefore, had δ values that were more than three times as large as those estimated for the current study ($6.69 < \delta < 6.91$). Without a proper sampling scheme to produce a significant curvilinear regression, it is impossible to estimate accurately the population dose-response curve (Figure 2). Future assays for TTX resistance in garter snakes should employ a sampling regime over a broad range of doses to provide sufficient variation in the x-variable. Ideally the distribution of doses should be uniform about the 50% dose, and the tails of the distribution should encompass doses that produce resistance measures greater than 80% and doses that produce

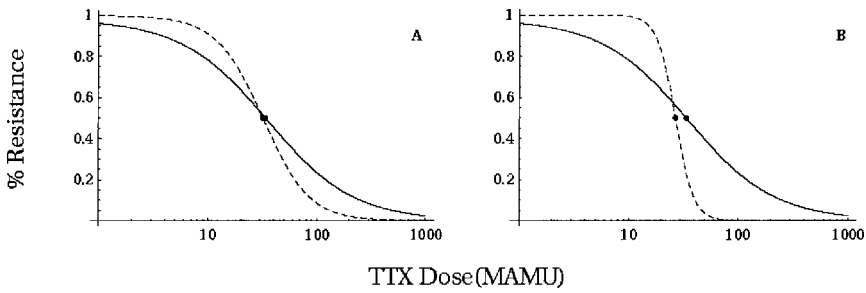

FIG. 2. A graphical comparison of field-caught animals from Benton, OR. **A** and **B** show the estimated 50% dose and dose-response curves for the field-caught animals (- - -) used for this study and a prior sample respectively. Estimates using the data collected for this study (**A**) were done using a proper sampling regime and more closely match the neonate estimates (——). The prior sample (**B**) was tested over a restricted range of doses near the known 50% dose.

resistance measures of less than 20% (higher doses may damage animals). Multiple doses either above the 80% level or below the 20% level should be avoided, as they reduce the accuracy and provide no new information. If such "repetitive" doses exist, it is suggested that they be dropped from the analysis.

Sample size also plays a critical role in achieving good results using this technique. The number of injections used in the curvilinear regression is inflated over the number of actual individuals used for the study (Table 1). However, the use of field-caught animals alleviates some of the sample-size issues that occur when using neonates. Field-caught animals, assuming littermates are not caught, provide genetically independent data points, while multiple littermates are typically used in neonate analyses. For example, 125 total injections were given to 56 neonates for the estimation of the Cold Springs data, but the 56 neonates came from 5 litters. Brodie and Brodie (1991) have demonstrated a genetic basis to TTX resistance in *T. sirtalis*. The neonate estimate for Cold Springs may, thus, represent a smaller, more biased, sample than the 14 field-caught individuals used for the analysis. The 50% dose estimate for field-caught snakes from Cold Springs may actually lie closer to the population mean, though fewer injections (59) were used in the analysis.

The importance of large sample size increases in tandem with resistance levels. The spread of the estimated difference exhibits a heteroscedastic pattern, with small variance at low 50% doses and large variance at large 50% doses. This pattern results from using natural log transformed doses prior to regression. The estimates for Cold Springs, Benton, and Bear Ridge are exemplary of this behavior. The regression estimates for the 50% dose for the field and laboratory groups from all three populations showed almost identical levels of difference after

analysis (approximately 0.05–0.14). However, upon reversing the transform of x (MAMU) to $\ln(x)$ (ln MAMU), the values of δ for Cold Springs are approximately six times as large as those for Benton, and almost 12 times as large as those for Bear Ridge (Table 2); though δ for Benton is larger than δ for Bear Ridge, Benton estimates actually showed the least deviation prior to the reverse transform. This discrepancy is because the 50% dose of Cold Springs (86.47 MAMU) is larger than that of Benton (33.65 MAMU) and much larger than that of Bear Ridge (6.78 MAMU). This behavior implies that larger sample sizes and better sampling regimes are critical to produce accurate estimates for populations that are more resistant to TTX.

The use of multiple injections on one individual for the analysis introduces the issue of pseudoreplication. Because the injections outnumbered the individuals, the degrees of freedom for the analysis are exaggerated in a standard regression. The same type of analysis can be performed using a mixed linear model, where individuals represent a random effect (i.e., individuals have different dose-response curves but are chosen at random from the population). The effect of using a mixed linear model is a reduction in the degrees of freedom associated with the estimation of the parameters, but the variance due to individual differences is removed producing better (i.e., lower variance) parameters. For populations where this type of analysis has been performed, the effect on the estimation of the 50% dose is negligible. For example, for the field-caught Bear Ridge animals, the degrees of freedom dropped from 31 to 22, the 50% dose only changed from 6.03–6.07 MAMU, and the variance of the 50% dose estimate decreased. The use of a mixed linear model is advantageous for proper parameter estimation but makes little difference in the estimation of the 50% dose. We have chosen to use linear regression in this paper because prior work used linear regression techniques (cf. Brodie et al., 2002).

The deviations observed between field-caught and neonate estimates could be due to natural selection but are more likely the result of statistical inaccuracy. The broad-sense heritability, an upper-bound to narrow-sense heritability, of TTX resistance for a population of *T. sirtalis* near Benton was estimated to be 0.715 ± 0.162 (Brodie and Brodie, 1990). In combination, the observed deviations and the potentially high heritability of resistance would imply rapidly shifting TTX resistance levels in some populations. The larger negative deviation observed at Cold Springs could be due to a cost of resistance (Brodie and Brodie, 1999), but such a drastic drop in resistance levels from birth to adulthood makes this explanation unlikely. Ideally, the method of using field-caught garter snakes could lead to producing measures of selection through "cross-sectional" studies (cf. Lande and Arnold, 1983). Being able to perform such a study would be of great benefit because the use of catch-and-release studies for studying selection in *Thamnophis* is problematic.

We achieved equivalent estimates of TTX resistance in both field-caught and laboratory animals by using a mass-adjusted dose. Because of the correlation

between mass and resistance, we gain insight into developmental changes in resistance as snakes mature by looking at the change in mass through ontogeny. This pattern may be further extended to muscular development because the mechanism for TTX resistance is thought to be altered sodium channel morphology in muscle tissue (Geffeney et al., 2002). Selection for TTX resistance *within* a population may favor either faster size development or larger overall size, depending on the timing of selection and assuming that the mass-resistance correlation is not purely phenotypic. The positive covariance of mass and resistance has not been examined across populations.

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REFERENCES

BRODIE, E. D. JR. 1968. Invesigations on the skin toxin of the adult rough-skinned newt, *Taricha granulosa*. *Copeia* 1968:307–313.

BRODIE, E. D., III and BRODIE, E. D., JR. 1990. Tetrodotoxin resistance in garter snakes: An evolutionary response of predators to dangerous prey. *Evolution* 44:651–659.

BRODIE, E. D., III and BRODIE, E. D., JR. 1991. Evolutionary response of predators to dangerous prey: Reduction of toxicity of newts and resistance of garter snakes in island populations. *Evolution* 45:221–224.

BRODIE, E. D., III and BRODIE, E. D., JR. 1999. Costs of exploiting poisonous prey: Evolutionary trade-offs in a predator-prey arms race. *Evolution* 53:626–631.

BRODIE, E. D., JR., RIDENHOUR, B. J., and BRODIE, E. D., III. 2002. The evolutionary response of predators to dangerous prey: Hotspots and coldspots in the geographic mosaic of coevolution between garter snakes and newts. *Evolution* 50:2067–2082.

GEFFENEY, S., BRODIE, E. D., JR., RUBEN, P. C., and BRODIE, E. D., III. 2002. Physiological mechanisms of adaptation in a predator-prey arms race: Tetrodotoxin-resistant sodium channels in garter snakes. *Science* 297:1336–1339.

ISMAIL, M., PHANG, S. M., TONG, S. L., and BROWN, M. T. 2002. A modified toxicity testing method using tropical marine microalgae. *Environ. Monit. Assess.* 75:145–154.

KADIR, H. B. A., PAYNE, C. C., CROOK, N. E., FENLON, J. S., and WINSTANLEY, D. 1999. The comparative susceptibility of the diamondback moth *Plutella xylostella* and some other major lepidopteran pests of brassica crops to a range of baculoviruses *Biocontrol Sci. Technol.* 9:421–433.

LANDE, R. and ARNOLD, S. J. 1983. The measurement of selection on correlated characters. *Evolution* 37:1210–1226.

LEE, D. and ADAMS, M. E. 2000. Sodium channels in central neurons of the tobacco budworm, *Heliothis virescens*: Basic properties and modification by scorpion toxins. *J. Insect Physiol.* 46:499–508.

MAA, W. C. J. and LIAO, S. C. 2000. Culture-dependent variation in esterase isozymes and malathion susceptibility of the diamondback moth, *Plutella xylostella* 1. *Zool. Stud.* 39:375–386.

OLMSTEAD, J. W., LANG, G. A., and GROVE, G. G. 2001. Assessment of severity of powdery mildew infection of sweet cherry leaves by digital image analysis. *Hortscience* 36:107–111.

PLASENCIA, J. and BANTTARI, E. E. 1997. Comparison between a culture plate method an an immunoassay to evaluate vascular colonization of potato by *Verticillium dahliae. Plant Dis.* 81:53–56.

RAPOSO, R., COLGAN, R., DELCAN, J., and MELGAREJO, P. 1995. Application of an automated quantitative method to determine fungicide resistance in *Botrytis cinerea. Plant Dis.* 79:294–296.

RIDENHOUR, B. J., BRODIE, E. D., JR., and BRODIE, E. D., III. 1999. Repeated injections of TTX do not affect TTX resistance or growth in the garter snake *Thamnophis sirtalis. Copeia* 1999:531–535.

SCHMUCK, R., SCHONING, R., STORK, A., and SCHRAMEL, O. 2001. Risk posed to honeybees (*Apis mellifera* 1. Hymenoptera) by an imidacloprid seed dressing of sunflowers. *Pest Manag. Sci.* 57:225–238.

SOUSA, M. C. and POIARES DA SILVA, J. 1999. A new method for assessing metronidazole susceptibility of *Giardia lamblia* trophozoites. *Antimicrob. Agents Chemother.* 43:2939–2942.

SPIELMANN, H., GENSCHOW, E., LIEBSCH, M., and HALLE, W. 1999. Determination of the starting dose for acute oral toxicity (ld50) testing in the up and down procedure (udp) from cytotoxicity data. *ATLA—Altern. Lab. Anim.* 27:957–966.

THOMPSON, J. N. 1994. The Coevolutionary Process. University of Chicago Press, Chicago, IL.

VALENTIN-SEVERIN, I., LAIGNELET, L., LAUGUENOT, J. C., and CHAGNON, M. C. 2002. Uridine uptake inhibition assay: An automated micromethod for the screening of cytotoxicity. *Toxicology* 171:207–213.