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Toxicon 43 (2004) 243–249

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A predictive model to estimate total skin tetrodotoxin in the newt *Taricha granulosa*

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Received 21 May 2003; revised 21 November 2003; accepted 26 November 2003

Abstract

We developed a predictive model to estimate the total amount of tetrodotoxin (TTX) in the skin of individual newts (*Taricha granulosa*) based on measures of the amount of TTX present in dorsal skin. We found that regions of skin on a newt could be reliably differentiated using granular gland density and that patterns of variation in granular gland density matched intra-individual variation in TTX levels. Tetrodotoxin is uniformly distributed in dorsal skin and TTX levels in dorsal skin are strongly predictive of TTX levels in other regions of skin on a newt. Our model is both accurate and precise and includes the effect of body size through surface area, variation in granular gland density, as well as positional variation in toxicity apparently associated with variation in granular gland density. This technique allows us to detect patterns that would be unclear if only dorsal skin toxicity or total skin toxicity based on extracts from an entire animal were used and demonstrates that the levels of TTX present in some populations of *T. granulosa* are remarkably high.

Keywords: Tetrodotoxin; Coevolution; Predator–prey; *Taricha granulosa*; Salamandridae; Caudata

1. Introduction

Tetrodotoxin (TTX) in the skin of the rough-skin newt, *Taricha granulosa*, is an important chemical defense against predators (Brodie, 1968), and a critical trait in the coevolutionary arms-race between *T. granulosa* and a TTX resistant snake predator, *Thamnophis sirtalis* (Brodie and Brodie, 1990, 1991, 1999; Brodie et al., 2002). Dorsal skin toxicity of individual adult *T. granulosa* varies considerably within and among populations (Hanifin et al., 1999, 2003), and these individual differences in toxicity may have important ramifications in regard to selection by *T. sirtalis* (Williams et al., 2003). However, measures of TTX in a small region of skin (e.g. dorsal skin toxicity) may not be the relevant phenotypic trait upon which selection acts. Although dorsal skin toxicity provides a convenient means for comparing relative toxicity within and among populations of *T. granulosa*, other measures of toxicity (e.g. whole animal toxicity) may be as or more significant in the ecological interaction between predator and prey. The newt's primary predator, *T. sirtalis*, swallows its prey whole. As a result, the amount of TTX a snake ingests or is exposed to should be more closely related to the total amount of TTX in the entire skin of a newt rather than the amount of TTX present per unit weight or area of dorsal skin. The total amount of TTX present in the skin of a newt results from a complex interaction of multiple factors. Size may be important because of the relationship between size and skin surface area, but understanding the contribution that differences in size make to total skin TTX requires a model or measure that can asses the effect of size independently of total skin toxicity.

We undertook this study to estimate the total amount of TTX present in the skin of a given newt from a measure of dorsal skin toxicity (defined as the amount of TTX present). Estimates of total skin TTX in newts based on dorsal skin toxicity can provide a more detailed understanding of TTX toxicity in newts because they incorporate variation in

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0041-0101/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2003.11.025

toxicity that results from anatomical or biochemical differences in skin composition as well as the effect of differences in individual newt size. Because TTX is not uniformly distributed in the skin of a newt (Brodie et al., 1974), we needed to assess the variation present in TTX levels in different positions on the surface of a newt as well as determine what, if any, anatomical structures could be used to reliably define qualitatively different regions on the surface of a newt. Although the origin of TTX in newts is still unclear, the TTX present in newts is stored in and secreted by granular skin glands (Toledo and Jared, 1995; Tsuruda et al., 2002; Mahmud et al. 2003). Therefore, we used granular skin gland density as the primary anatomical indicator of variation in skin structure and, thus, toxicity. We examined and quantified granular gland density to differentiate between regions of skin on newts and to calculate the proportion of each of these identifiably different regions. We then measured the amount of TTX present in these regions and used these measures of toxicity to assess the relationship between dorsal toxicity and other regions of skin where gland density was measurably different. Using these data, we developed a predictive model for whole animal toxicity based on measures of dorsal skin toxicity that integrated size, and the relationship between dorsal toxicity and the toxicity of other skin regions. These estimates of total toxicity were used to assess the relative importance of size in total skin TTX. In addition, we compared patterns of variation in dorsal skin toxicity and total skin TTX to assess the degree of congruence between these two measures of toxicity. Finally, we tested our predictive model by measuring the dorsal skin and whole skin TTX levels of a randomly selected series of animals from our study population and comparing the predicted total skin TTX levels (derived from dorsal skin TTX levels) with actual measured total skin TTX levels in those animals.

2. Materials and methods

2.1. Specimens

We examined the toxicity of 26 animals collected from the central Willamette Valley, Oregon (Benton County). These animals were euthanized and frozen at −80 °C. A subset of six of these animals was used to examine variation in dorsal skin toxicity as well as positional variation in TTX levels. An additional subset of six animals (chosen at random) was used to test our predictive model.

2.2. Tissue sampling

2.2.1. Variation in dorsal skin TTX levels of Taricha granulosa

We sampled skin with a 5 mm diameter human skin biopsy punch (Acupunch™ Acuderm, Inc.). The use of a biopsy punch allows for a high degree of accuracy in estimating levels of TTX per unit area as well as minimizing the amount of subdermal tissue in our samples. We removed six punches from the dorsal surface of each of six animals. The position of these punches was designed to sample as much of the dorsal surface as possible without sampling from regions of the newt where gland density was visibly decreased. Punches were removed in pairs one on either side of the vertebral column. The first of these pairs was removed posterior to the scapula, the second at the midpoint between the scapula and the pelvic girdle; the last pair was taken anterior to the pelvic girdle.

2.2.2. Positional variation in TTX levels within individual Taricha granulosa

Examination of newt skin using backlighting and a dissecting microscope indicated that even though granular gland density decreases gradually (rather than discretely) in the transition from dorsum to vent we could still use granular gland density to identify three distinct regions (‘dorsal’, ‘ventral’, and ‘lateral’) on a newt’s skin. We quantified the relative proportions of these three distinct regions on a typical newt by carefully examining the transition zones between dorsal, ventral, and lateral skin in the midbody of four animals. We considered the tail as an extension of the main body of the newt when we calculated the relative proportions of dorsal, ventral, and lateral skin on a newt because variation in glandular density in the tail mirrored the midbody. We quantified positional variation in granular gland density by counting the granular skin glands present in 4 mm^2 sections of skin taken from the dorsal, ventral, and lateral regions of three animals. These skin sections were removed using a human biopsy skin punch and fixed in either 70% ethanol or formalin and then examined with a dissecting microscope.

We quantified the TTX levels of these three regions using the same methodology as the dorsal variability experiment. Because of the high repeatability of our assay we chose to measure dorsal skin toxicity with either one ($n = 8$) or two ($n = 6$) skin punches taken from the midbody. Ventral skin toxicity measures were based on a single punch taken from the central midline of the vent. Lateral skin toxicity measures were based on either one ($n = 14$) or two ($n = 6$) skin punch(s) taken from the midpoint between the pelvic and pectoral girdle midway between the dorsum and vent. The six animals from the dorsal skin variability study were also included in this experiment. Dorsal skin toxicity estimates for these animals were calculated as the mean of all six dorsal subsamples taken in the first experiment and additional punches from the vent and lateral surface were taken as described above.

2.2.3. Model testing

We tested our predictive model by measuring both dorsal skin TTX levels and the total amount of TTX present in the skin of each of six randomly selected animals that had not been used in the previous experiments. We sampled dorsal

skin TTX with the same methodology as the dorsal variability and positional variation experiments. We used a single 5 mm diam punch removed from the center of the dorsum for TTX analysis. After removing the punch the animal was refrozen at − 80 °C and all of the skin removed using a scalpel and forceps. Care was taken during this procedure to keep the samples frozen to reduce any possible loss of TTX from the skin due to handling.

2.3. Toxin extraction

Toxin extraction was performed as per Hanifin et al. (2002). Each biopsy punch was ground in a 1 ml glass tissue grinder (Kontes Duall 20) with 800 μl extraction solution (0.1 M Acetic Acid). These samples were then vortexed and heated in a boiling water bath for 5 min. After heating, the samples were cooled in an ice-water bath and spun at 13,000 rpm for 20 min. Following this first centrifuge spin, 0.5 ml of the supernatant was removed and spun at 13,000 rpm for 20 min in 0.5 ml Millipore centrifuge filter tubes (Ultrafree-MC, 10,000 NMWL filter units). The final filtered product was then used for TTX analysis.

The TTX present in our whole skin samples was extracted by grinding the skin in a large volume tissue homogenizer in either 40 or 50 ml of 0.1 M Acetic Acid. After grinding the extracts were spun at 10,000 rpm for 30 min. A 500 ml aliquot of the resultant supernatant was the filtered as per our standard extraction protocol (see above).

2.4. TTX assay

The levels of TTX were quantified by fluorometric HPLC (Yasumoto and Michishita, 1985; Yotsu et al., 1989; Hanifin et al., 2002). We used protocols modified from both Yotsu et al. (1989) and Hanifin et al. (2002). Separation of TTX and TTX analogs was performed on either a Synergi 4μ Hydro-RP 80A (0.46×25 cm^2, Phenomenex, USA) reverse-phase column or a Develosil RP-Aqueous (0.46×25 cm^2 or 0.46×15 cm^2, Phenomenex, USA) reverse-phase c30 column. The mobile phase consisted of either a 50 mM ammonium acetate and 60 mM ammonium heptafluorobutyrate buffer (pH 5.0) containing 1% acetonitrile or a 25 mM ammonium acetate and 35 mM ammonium heptafluorobutyrate buffer (pH 5.0) containing 1% acetonitrile. We used either a Beckman 126 or a Shimadzu LC-10AD-vp pump system coupled with a secondary pump for the 5N NaOH derivitization reaction. Fluorescent derivative were detected with either a Jasco Fp-1520 or Rainin Dynamax Fl-2 fluorescence detector. The excitation wavelengths of the detectors were set at 365 nm and the emission wavelengths were set at 510 nm. Peak area concentration curves were calculated with standards prepared from commercial TTX (Calbiochem).

2.5. Total skin surface area calculations

We calculated total skin surface area using the formula for skin surface area in *Taricha granulosa* from Whitford and Hutchison (1967):

$$\log S = 0.945 + 0.719 \log W$$

where S is the surface area (in cm^2) and W is the mass (in g).

2.6. Analysis and statistics

We analyzed variation in dorsal skin toxicity with a mixed-model repeated measures analysis of variance (ANOVA) with individual newts as our random factor and dorsal region as our treatment variable (PROC MIXED SAS/STAT, version 8.1, SAS Institute). Repeatability of the dorsal skin toxicity measure was calculated as per Becker (1992) using all six dorsal punches as independent estimates of dorsal toxicity. Because PROC MIXED directly estimates $\partial^2 W$ and $\partial^2 E$ we used these parameters to calculate repeatability rather than MS_E and MS_W. Variation in TTX levels between different regions within individuals was analyzed with a mixed-model analysis of variance (ANOVA) with newt as the random variable and body region as the treatment variable (PROC MIXED SAS/STAT, version 8.1, SAS Institute). We used Tukey's adjusted least-square-means test to examine post hoc, pair-wise comparisons between all regions. Linear regression (Statview, version 4.02) was used to test the relationship between dorsal skin and both ventral and lateral skin. Because an animal with no TTX in its dorsal skin is unlikely to posses TTX in other regions of the skin, we assumed an intercept of zero in these regressions. The slope coefficients from these analyses were used to predict toxicity of both lateral and vent skin. We tested the accuracy of our total skin TTX equation by substituting measured ventral and lateral skin toxicities into the equation for each animal and recalculating total skin TTX with these values. These recalculated values were then compared to the total skin TTX values produced by our equation with a paired t-test (Statview, version 4.02).

We tested for sexual dimorphism in mass, dorsal toxicity, and total skin toxicity with a one-way analysis of variance (ANOVA) with sex as the treatment factor (Statview, version 4.02). We used linear regression to test for a relationship between mass and total skin toxicity (Statview, version 4.02). Because males and females differed in mass, this analysis was done separately for males and females.

We tested the congruence of predicted whole TTX levels with measured TTX levels with a paired t-test (Statview version 4.02). We also tested the accuracy and robustness of our predictive model by regressing measured whole skin TTX levels on predicted skin TTX levels (Statview, 4.02)

3. Results

3.1. Dorsal skin variability

Estimates of toxicity from dorsal skin did not vary significantly among different dorsal samples within a given animal ($F_{5,25} = 0.81, p = 0.55$). Repeatability of dorsal skin toxicity estimates across the body was high ($R = 0.95$).

3.2. Whole animal variation and positional variation in toxicity

Granular gland density was much greater in dorsal skin than lateral or ventral skin, with the lowest granular gland density found in ventral skin (Table 1). Our examination of granular gland density indicated that the proportions of the three regions of skin in a typical newt were: dorsal skin = 30% of total skin area, lateral skin = 25% of total skin area, and ventral skin = 45% of total skin area.

The TTX toxicities of the three areas assayed were significantly different ($F_{2,37} = 9.25$, $p = 0.0006$). Dorsal skin was the most toxic followed by lateral skin and then ventral skin. Post hoc comparisons indicated that the toxicity of both dorsal ($t = 5.84$, $p < 0.0001$) and lateral ($t = 3.99, p = 0.0003$) skin was significantly different from ventral skin, but that the toxicity of lateral skin was not significantly different than dorsal ($t = 1.34$, $p = 0.19$). However, in 17 of the 20 animals assayed lateral skin toxicity was lower than dorsal skin. Dorsal skin toxicity was a strong predictor of both lateral skin toxicity ($F_{1,19} = 26.207$, $p < 0.0001$, $r^2 = 0.56$) and ventral skin toxicity ($F_{1,18} = 134.007, p < 0.0001, r^2 = 0.88$). With the intercept constrained at zero, the regression line describing the relationship between dorsal skin toxicity and ventral skin toxicity was: Ventral TTX (mg/cm^2 skin) = 0.208 (dorsal TTX (mg/cm^2 skin)). The 95% CI for the slope was 0.170–0.246. The regression line describing the relationship between dorsal skin toxicity and lateral skin toxicity was: Lateral TTX (mg/cm^2 skin) = 0.502 (dorsal TTX (mg/cm^2 skin)). The 95% CI for the slope was 0.297–0.708.

We used the slope estimates from the two regression lines to predict the toxicity of ventral and lateral skin in newts where these values were unknown. We combined estimates of ventral and lateral skin toxicity with estimates of total skin surface area (S) based on mass (Whitford and Hutchison, 1967) and our estimates of the relative proportion of each skin region (e.g. dorsal = 0.3 of total skin surface area, ventral = 0.45 of total skin surface area, and lateral = 0.25 of total skin surface area) to estimate the total amount of TTX present in the skin of a single animal as:

$$\text{total skin TTX} = \text{dorsal skin TTX} + \text{ventral skin TTX} + \text{lateral skin TTX}$$

and

$$\text{ventral TTX} = 0.208\ (\text{dorsal TTX})$$

$$\text{lateral TTX} = 0.502\ (\text{dorsal TTX})$$

$$\text{skin surface area } (S) = 0.3(S)\ \text{`dorsal area'} + 0.45(S)\ \text{`ventral area'} + 0.25(S)\ \text{`lateral area'}$$

then

$$\text{total skin TTX} = ((0.3(S))(\text{dorsal TTX}) + ((0.45(S))(0.208(\text{dorsal TTX})) + ((0.25(S))(0.502(\text{dorsal TTX})))$$

Total skin TTX estimated solely from dorsal skin toxicity (i.e. predicting ventral skin and lateral skin toxicity from dorsal skin toxicity) was not significantly different from total toxicity estimates that used measured values of ventral skin and lateral skin toxicity (df = 18, $t = -1.148$, $p = 0.27$, Table 2). Total skin TTX ranged from a low of 0.62 mg TTX to a high of 10.63 mg TTX (Fig. 1). Variation in dorsal skin TTX was comparable with a low of 0.17 mg TTX/cm^2 and a high of 0.428 mg TTX/cm^2.

Despite the relationship between mass and surface area, mass was not correlated with total skin TTX for either males ($F_{1,9} = 0.97$, $p = 0.35$, $r^2 = 0.097$) or females ($F_{1,9} = 0.0025$, $p = 096$, $r^2 = 0.0004$) (Fig. 1). Even though males, on average, weighed considerably more than females ($x_{\text{males}} = 17.82$ g, $x_{\text{females}} = 9.94$ g, $F_{1,18} = 182.29$, $p < 0.0001$) females possessed more total skin TTX ($F_{1,18} = 10.76$, $p = 0.0042$) as well as higher levels of dorsal skin TTX ($F_{1,18} = 18.40, p = 0.0004$) than males (Fig. 1).

3.3. Predictive model testing

Our final predictive model was both accurate and precise (Fig. 2). Actual measured levels of whole skin TTX were not different than predicted levels ($t = -1.295$, $p = 0.25$) (Table 3). The regression line describing the relationship between predicted skin TTX levels and measured skin TTX levels (Measured Skin TTX = Predicted Skin TTX × 1.1175 − 0.7) was significant ($F_{1,4} = 69.15$, $p = 0.0011$, $r^2 = 0.95$). The 95% CI for the slope was 0.783–1.567. The intercept of the line was not significantly different

Table 1
Granular skin gland density in glands/mm^2 for three *Taricha granulosa* (one male, and two females) split by region of the skin

	Number of granular glands/mm^2		
	Ventral	Lateral	Dorsal
Male	1	5.75	12.25
Female	1.5	3	16
Female	1	6.25	12.25

Table 2
Total skin TTX estimates for all study animals

Sex	Mass (g)	Total skin area (cm^2)	Ventral skin TTX (mg TTX/cm^2)	Lateral skin TTX (mg TTX/cm^2)	Dorsal skin TTX (mg TTX/cm^2)	Total skin TTX (mg) (all measures)	Total skin TTX (mg) (from dorsal Skin TTX)
F	7.81	38.62	0.080	0.054	0.245	4.75	4.91
F	7.90	38.94	0.024	0.089	0.173	3.31	3.50
F	9.15	43.28	0.017	0.045	0.105	2.17	2.35
F	9.42	44.19	0.081	0.165	0.427	9.08	9.78
F	10.42	47.52	0.038	0.087	0.139	3.84	3.44
F	10.50	47.78	0.061	0.212	0.428	9.98	10.63
F	10.71	48.46	0.073	0.330	0.268	9.47	6.74
F	11.18	49.98	0.055	0.298	0.221	8.27	5.73
F	12.38	53.79	0.005	0.036	0.058	1.54	1.63
M	16.00	64.68	0.010	0.030	0.060	1.94	2.01
M	16.18	65.20	0.001	0.021	0.031	0.96	1.04
M	17.10	67.85	0.013	0.040	0.079	2.68	2.77
M	17.57	69.18	0.006	0.022	0.029	1.15	1.03
M	17.75	69.69	0.004	0.007	0.017	0.59	0.62
M	17.93	70.20	0.017	0.029	0.047	2.03	1.71
M	18.07	70.59	0.003	0.009	0.021	0.69	0.76
M	18.08	70.62	na	0.056	0.050	na	1.82
M	18.80	72.63	0.005	0.015	0.022	0.88	0.81
M	18.80	72.63	0.064	0.110	0.150	7.37	5.64
M	19.67	75.03	0.002	0.026	0.074	2.22	2.90

from zero ($t = -0.64$, $p = 0.56$). The measured dorsal skin TTX levels of the six animals used to test our model were comparable to other animals from this study population as was their mass (Table 3). Whole skin TTX levels (both predicted and measured) were also comparable to levels of TTX predicted in our other samples (Tables 2 and 3).

4. Discussion

Our model for estimating total skin TTX levels from dorsal skin TTX levels is highly robust and indicates that we can reliably estimate the total amount of TTX present in the skin of a single animal from a small sample of dorsal

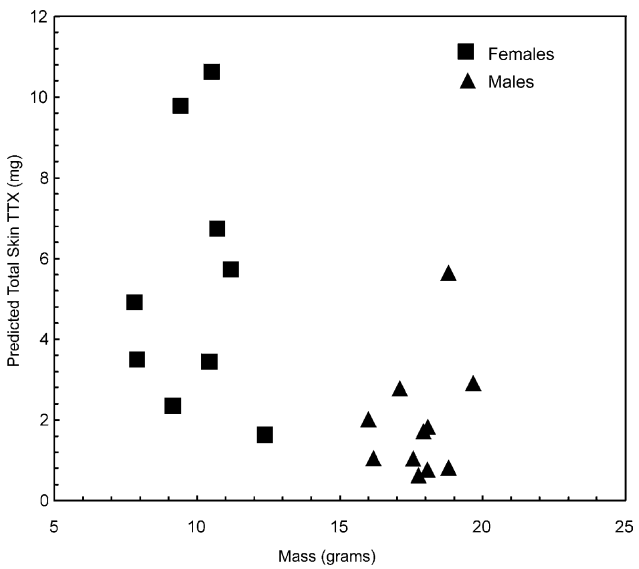

Fig. 1. Total skin TTX (mg) plotted against mass for males (circles) and females (squares). Mass is not a good predictor of total toxicity for either males ($F_{1,9} = 0.97$, $p = 0.35$, $r^2 = 0.097$) or females ($F_{1,9} = 0.0025$, $p = 096$, $r^2 = 0.0004$).

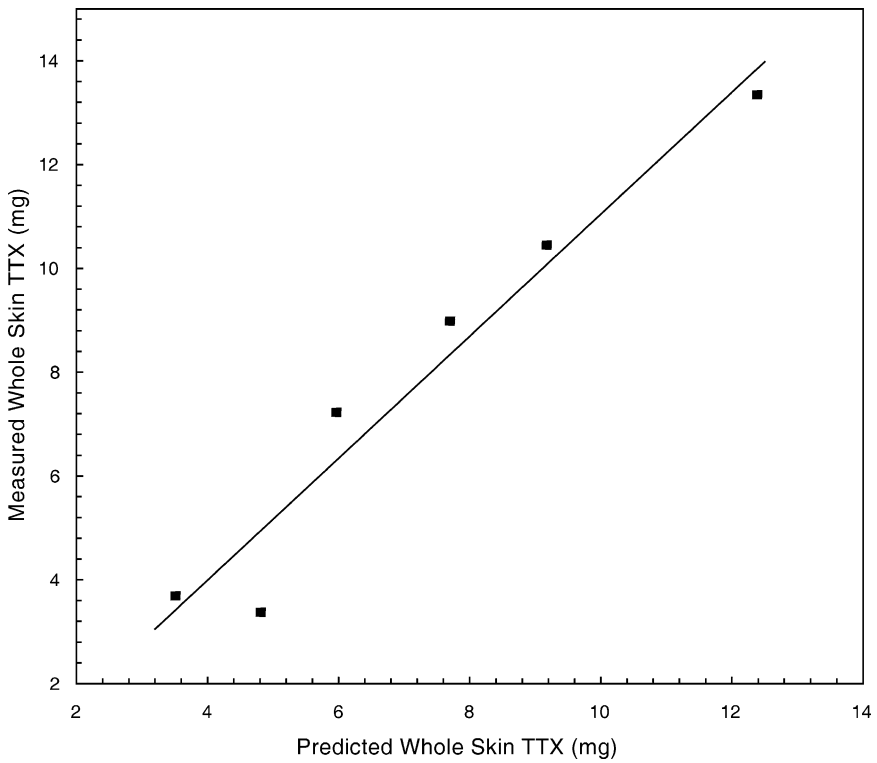

Fig. 2. Total measured whole skin TTX plotted against predicted whole skin TTX for a subset of six individuals chosen at random from the study population. Predicted and measured values do not differ significantly and our predictive model (Measured Skin TTX = Predicted Skin TTX × 1.1175 − 0.7) is highly robust ($F_{1,4} = 69.15$, $p = 0.0011$, $r^2 = 0.95$).

skin. All of the regression parameters (e.g. slope ≈ 1, high r^2, and intercept ≈ 0) indicate that our predicted total skin TTX values are in close agreement with actual total skin TTX values. The close congruence of our predicted and measured TTX levels (Table 3) as well as the similarity of estimates based solely on dorsal skin with estimates based on directly measured lateral and ventral skin toxicities (Table 2) indicates that the our assessment of the various subcomponents of our predictive model (e.g. the relationship between dorsal skin toxicity and both ventral and lateral skin toxicity, estimates of total skin surface area, and the relationship between gland structure and toxicity) are also robust.

These estimates of total toxicity allow us to assess the relative importance of dorsal skin toxicity and size for this population. Dorsal skin toxicity appears to be the most important factor for whole animal toxicity. Although the size of an individual newt contributes to total toxicity because of the relationship between mass and surface area this factor appears to be less important than that of dorsal skin toxicity. In this population, dorsal skin TTX appears to be a reliable estimate of phenotypic toxicity (i.e. the trait

Table 3
Mass, and TTX levels (dorsal, predicted whole skin, and measured whole skin) of animals used to test the predictive model

Mass (g)	Total skin surface area (cm^2)	Dorsal skin TTX (mg/ cm^2)	Predicted whole skin TTX (mg)	Measured whole skin TTX (mg)
7.10	36.06	0.257	4.82	3.38
7.58	37.80	0.180	3.52	3.68
8.17	39.89	0.599	12.39	13.32
10.04	46.26	0.250	5.98	7.21
10.1	46.46	0.381	9.19	10.44
11.41	50.72	0.293	7.71	8.97

acted upon by selection). The factors that lead to the dramatic levels of variation in toxicity measured in this population (Table 2) are still unclear, but our study indicates that differences in individual size do not have a dramatic effect on this variation in toxicity.

Total skin TTX varies considerably within this population of newts with a 20-fold difference between the least toxic animal and the most toxic. Variation in dorsal skin toxicity is even greater with a 30-fold difference between the least (0.017 mg TTX/cm^2 skin) and the most toxic (0.599 mg TTX/cm^2 skin) animals. This level of variation in dorsal skin is comparable to previously reported toxicities for this population (Hanifin et al., 1999, 2003). The total amount of TTX present in the skin of newts from this population is remarkable. The 13.32 mg of TTX present in the most toxic of our study animals is enough to kill over 50,000 mice and even the least toxic animal (0.62 mg) contains enough TTX to kill over 2800 mice (1 MU, or 220 ng, is the amount of TTX required to kill a 20 g mouse, Matsumura, 1996) (Table 2).

Tetrodotoxin is uniformly distributed in dorsal skin and dorsal skin is the most toxic skin on a newt. The high repeatability of our extraction protocol (as demonstrated by the dorsal skin variability experiment) indicates that we can reliably measure dorsal skin TTX levels. Ventral skin and lateral skin contain less TTX than dorsal skin, and this decrease in toxicity appears to be associated with a lower density of granular glands in the skin. The association between TTX levels and granular gland density in different areas of newt skin is congruent with other studies demonstrating an association between TTX and skin glands in TTX bearing salamanders (Toledo and Jared, 1995; Tsuruda et al., 2002; Mohmud et al., 2003). This association indicates that glands in the skin of newts are likely to be important in the production and/or sequestration of TTX in this species (and other TTX bearing salamanders), and suggests that newts may be capable of producing their own TTX.

Acknowledgements

We thank S. Durham for statistical advice and assistance and S. Geffeney, T. Gardner, and two anonymous reviewers for comments and aid in preparing this manuscript. Jon Hanifin provided technical assistance with tissue sampling. We also thank S. Boyes of Oregon State University for permission and assistance in collecting newts from Soap Creek Ponds and D. Dewald for the use of additional laboratory equipment. These studies was supported by the National Science Foundation under grant DEB -9904070 to EDB Jr. and grant DEB-9903829 to EDB III. The Utah State University IACUC panel approved animal care, as well as our tissue sampling protocol. Voucher specimens have been deposited in The University of Texas at Arlington Collection of Vertebrates.

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