Secretion and regeneration of tetrodotoxin in the rough-skin newt (*Taricha granulosa*)

Brian L. Cardall[a], Edmund D. Brodie Jr.[a], Edmund D. Brodie III[b], Charles T. Hanifin[a],*

[a]*Department of Biology, Utah State University, 5305 Old Main Hill, Logan, UT 84322-5305, USA*
[b]*Department of Biology, Indiana University, Bloomington, IN 47405-3700, USA*

Abstract

Rough-skin newts (*Taricha granulosa*) released tetrodotoxin (TTX) in their skin secretions in response to mild electric stimulation. This release resulted in a large (21% to almost 90% of the pre-stimulation levels) reduction in the amount of TTX present in the dorsal skin of individual newts. Over the next 9 months newts significantly regenerated the levels of TTX in their skin. These data, in combination with previously published results, are consistent with the hypothesis that these newts produce their own TTX.
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1. Introduction

Some genera of newts in the family Salamandridae (e.g. *Taricha* and *Cynops*) possess the neurotoxin tetrodotoxin (TTX) in their skin (Mosher et al., 1964; Wakely et al., 1966; Brodie et al., 1974; Yotsu and Yasumoto, 1990). TTX toxicity in these newts is thought to play an important ecological role as an antipredator defense (Brodie, 1983; Daly, 1995). The importance of TTX as an antipredator trait has been well documented in one species (*Taricha granulosa*) where TTX is the critical trait governing the predator–prey arms-race between *T. granulosa* and its snake predator (*Thamnophis sirtailis*) (Brodie, 1968; Brodie and Brodie, 1990; Brodie et al., 2002). TTX toxicity in newts (both *Cynops* and *Taricha*) is associated with granular skin glands (Tsuruda et al., 2002; Hanifin et al., 2002). This association is not surprising given that amphibian granular skin glands are thought to primarily function in the production and secretion of noxious or toxic substances used as an antipredator defense (Daly, 1995; Toledo and Jared, 1995). In addition, granular skin glands have an important role in toxin production and storage in other salamanders (Toledo and Jared, 1995; Williams and Larsen, 1986).

TTX is of particular interest because of its presence in a large number of relatively unrelated taxa (reviewed in Miyazawa and Noguchi, 2001). The traditional model of TTX production in 'higher' taxa is that it results from symbiosis with TTX producing bacteria (Noguchi et al., 1986; Yotsu et al., 1987; Yasumoto and Yotsu-Yamashita, 1996; reviewed in Miyazawa and Noguchi, 2001), but recent work (Shimizu, 2002; Hanifin et al., 2004; Lehman et al., 2004) has questioned this model of TTX toxicity in newts. Lehman et al. (2004) demonstrated that bacteria are not present in newt skin glands nor are bacteria present, with the exception of the gut, in other tissues of the body where TTX is found. Although bacteria are present in the intestines of newts (Lehman et al., 2004), Wakely et al. (1966) found that TTX levels in viscera were 200 times lower than that of skin in *T. torosa*. The presence of specialized structures associated with TTX storage and secretion in newts

* Corresponding author. Tel.: +1 435 797 2450; fax: +1 435 797 1575.

E-mail address: chanifin@biology.usu.edu (C.T. Hanifin).

(i.e. skin granular glands) that do not contain bacteria (Lehman et al., 2004) raises the possibility that newts may produce their own TTX.

Secretion of TTX in *Cynops pyrrhogaster* in response to handling has already been demonstrated (Tsuruda et al., 2002). We undertook this study to assess the presence of TTX in the glandular secretions of *T. granulosa* and to determine if TTX stores in the dorsal skin can be regenerated after secretion. We quantified the reduction in dorsal skin TTX levels of individual newts after electrically stimulating the skin in order to assess the amount of TTX secreted. We then monitored the TTX levels of our study animals over a 9-month period to determine if regeneration of TTX occurred.

2. Materials and methods

We used 36 individual *T. granulosa* (29 males, 7 females) collected from a single population in the Willamette Valley of Oregon, Benton County. Newts were housed individually in 38-l aquaria with 6 cm of filtered tap water at a temperature of 19 °C and a photoperiod of 14L: 10D; they were fed earthworms and crickets.

2.1. Tissue sampling

We measured changes in individual newt TTX levels over time with a non-lethal sampling technique developed in our laboratory (Hanifin et al., 2002). We anesthetized our study animals in a 1% tricaine solution, rinsed them with filtered tap water, and removed a small (5 mm diameter) circle of skin with a human skin-biopsy punch (Acu-Punch™, Acuderm, Inc.). Care was taken to ensure only skin and the thin layer of connective tissue between the skin and dorsal muscle was removed. Samples 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 different parts of the dorsum show little within–individual variation (Hanifin et al., 2004). We did not sample skin from other regions (e.g. vent) of our study animals because TTX levels of other regions of the skin are much lower than dorsal skin (Hanifin et al., 2004) and because skin punches taken from the ventral and lateral surfaces can easily rupture the body wall and cause mortality. Skin punches sampled at different time intervals did not physically overlap; tissue samples were stored at −80 °C.

After the initial skin punch, we allowed the wound to heal for 1 month. The newts were then anesthetized again, as above, and glandular secretions from the animals were collected with the use of a bipolar stimulating electrode (Astro-med, Inc.) powered by a Grass SD-9 stimulator set at 5.4 V, 10 pps, with a pulse duration of 14 ms. We tested the possibility that our stimulus was destroying TTX present in glands (rather than forcing secretion of TTX) by collecting secretions with a cotton swab. These swabs were checked for the presence of TTX by HPLC, but because we did not attempt to collect all of the secretion of a given newt we did not quantify levels of TTX present in the swabs. One day after stimulation, another skin punch was taken from each individual to determine whether toxicity was reduced after the skin glands had been stimulated to secrete. We tested for TTX regeneration by taking additional skin punches at 9 months post-stimulation from all animals. In addition, we divided our study animals into sub-groups that were sampled at 1-month intervals to assess rates of regeneration. All of the animals in each of these groups were sampled at least once during the 9 months between stimulation and the end of our study. Both of these samples (i.e. the 9-month estimate and the intermediate sample) were used in our analysis of regeneration.

2.2. Toxin extraction

Extracts from each skin sample were prepared by grinding the tissue in a 1 ml glass tissue grinder (Kontes Duall 20) with 800 μl of our extraction solution (0.1 M aqueous acetic acid). Samples were 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 spin, 0.5 ml of the supernatant was spun at 13,000 rpm for 20 min in 0.5 ml Millipore centrifuge filter tubes (Ultrafree-MC, 10,000 NMWL filter units). Aliquots of 20 μl of the supernatant were used for analysis. This extraction procedure is highly repeatable ($r = 0.95$) (Hanifin et al., 2004) and is not a significant source of variance in our analysis.

2.3. TTX assay

Levels of TTX in our samples were quantified by fluorometric HPLC (Yasumoto and Michishita, 1985; Yotsu et al., 1989; Hanifin et al., 1999), using a protocol modified from Yotsu et al. (1989). Separation of TTX and TTX analogs was performed on a Synergi 4 μ Hydro-RP 80A (0.46×25 cm, Phenomenex, USA) reverse-phase column with a 50 mM ammonium acetate and 60 mM ammonium heptafluorobutyrate buffer (pH 5.0) containing 1% aceto-nitrile run at a flow rate of 0.5 ml/min with a Beckman 126 pump system. The eluate was mixed with an aqueous 5 N NaOH solution from pump B of the Beckman system (1.0 ml/min) and passed through a Pickering CRX 400 post column reactor (1 ml reaction loop) heated to 115 °C in order to derive analogs to fluorophore. After cooling in a 2 cm water jacket, the fluorescent derivatives were detected by a Jasco FP-1520 fluoromonitor. The excitation wave-length of the detector was set at 365 nm and the emission wavelength at 510 nm. Data acquisition and chromato-graphic analysis were performed with System Gold software (version 8.1, Beckman, Inc.). Peak area concentration curves were calculated with standards prepared from

commercial TTX (Calbiochem or Sigma). Levels of 4-*epi* TTX and 4,9-anhydroTTX were not quantified because they were present at very low levels relative to TTX.

2.4. Analysis and statistics

At the time of the final skin punch, our sample consisted of 31 individuals (5 females, and 26 males). Four individuals died and one was removed from the study because of a sampling error. Because of the possibility that the response of an individual newt to stimulation might be proportional rather than absolute (i.e. the amount of reduction in dorsal skin TTX might be a proportion of total skin TTX rather than an absolute amount) we used a regression analysis to examine TTX secretion in response to stimulation. We used the regression slope coefficients from this analysis to estimate the population average of the percentage of total initial TTX that each animal secreted in response to stimulation. Additionally, we calculated each individual reduction (the ratio of post- versus

pre-stimulation skin TTX concentration values for each individual, subtracted from one) in order to qualitatively assess variation in secretion within the population.

We analyzed regeneration of TTX in our animals with a random coefficients model using Proc Mixed in SAS (version 9.0, SAS institute). The regression line describing changes in TTX over time (months post-stimulation) was calculated for each animal. This line included the TTX measure taken between the post-stimulation value and the final value as well as the final TTX measure. We used the means from the distributions of the slope and intercept coefficients derived from this analysis of individual regeneration to assess TTX recovery as a population level phenomenon.

3. Results

Electrical stimulation produced a visible secretion in newts that contained TTX. All 15 of the cotton swabs

Table 1
Levels of TTX in *T. granulosa* before and after electrical stimulation as well as 9-months post stimulation

| Sex	Mass (g)	Initial TTX (mg/cm² skin)	Post-stimulus TTX (mg/cm² skin)	TTX secreted (percentage of initial TTX)	Final TTX (mg/cm² skin)	Final TTX/ initial TTX
m	16.0	0.0861	0.0677	21	0.1082	1.26
f	11.1	0.0797	0.0349	56	0.0849	1.07
m	15.1	0.2372	0.0561	76	0.2051	0.86
m	21.0	0.1663	0.0917	45	0.1374	0.83
m	17.9	0.1715	0.0337	80	0.1274	0.74
m	13.3	0.0657	0.02	70	0.0485	0.74
m	16.8	0.1779	0.0885	50	0.1122	0.63
m	13.5	0.2692	0.0473	82	0.1679	0.62
m	18.4	0.0377	0.0256	32	0.0224	0.59
m	18.8	0.1066	0.0337	68	0.0633	0.59
m	16.3	0.1767	0.0381	78	0.0994	0.56
f	9.4	0.3305	0.1462	56	0.1815	0.55
f	12.2	0.1462	0.0284	81	0.0769	0.53
f	15.1	0.2396	0.0821	66	0.1094	0.46
m	13.0	0.1655	0.0361	78	0.0713	0.43
m	14.7	0.1639	0.0381	77	0.0661	0.40
m	16.0	0.2003	0.0653	67	0.0745	0.37
m	16.8	0.0897	0.0156	83	0.0308	0.34
m	16.0	0.1398	0.0813	42	0.0453	0.32
m	21.5	0.0601	0.028	53	0.0192	0.32
m	16.0	0.1879	0.0321	83	0.0597	0.32
m	18.8	0.1258	0.0333	74	0.0397	0.32
m	19.4	0.1058	0.0184	83	0.0333	0.31
f	11.0	0.3049	0.0673	78	0.0721	0.24
m	15.8	0.1567	0.0381	76	0.0369	0.24
m	15.2	0.2288	0.0697	70	0.0501	0.22
m	11.6	0.1763	0.0373	79	0.0385	0.22
m	14.1	0.2368	0.0481	80	0.0465	0.20
m	16.1	0.1278	0.0349	73	0.0164	0.13
m	15.4	0.2252	0.024	89	0.016	0.07
m	13.9	0.3229	0.0777	76	0.01298	0.04
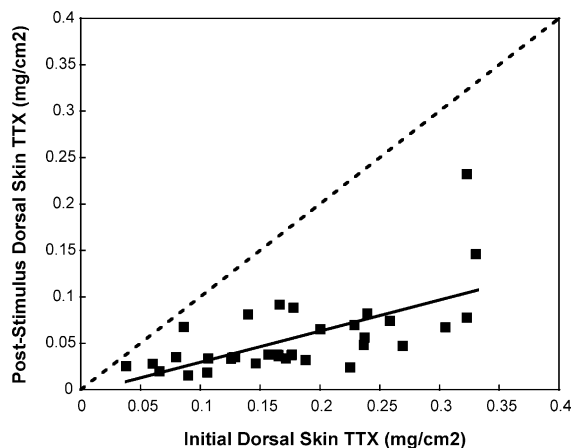
Fig. 1. Tetrodotoxin released from *T. granulosa* in skin secretion in response to mild electrical stimulation. Each individual newt's TTX level before and after stimulation is plotted. The slope of the solid line (Post-Stimulation TTX = Initial TTX × 0.335–0.003881) is the mean of the distribution of slope estimates derived from regression model used to assess TTX secretion. The dashed line represents the relationship between pre- and post-stimulation TTX if no reduction in toxicity occurred as a result of electrical stimulation (i.e. a slope of 1) and indicates that all animals in our study secreted some TTX in response to electrical stimulation.

used to collect secretions post-stimulation contained TTX and the stereoisomer profile of TTX in the swabs matched that of dorsal skin. Individuals in our study secreted an average of 66.5% (measured as a reduction in individual TTX) of their dorsal skin TTX when stimulated (Table 1). There was, however, variation in the amount of toxin secreted. The animal most affected by electrical stimulation secreted almost 90% of its initial TTX, while the least affected animal secreted 21% of its initial TTX (Table 1). Electrical stimulation of the dorsum resulted in a significant reduction in dorsal skin toxicity ($F_{1,31} = 20.793$, $p < 0.0001$, $r^2 = 0.40$) (Fig. 1). The regression line describing dorsal skin TTX reduction in response to electrical stimulation for all animals combined was: Post-Stimulation TTX = Initial TTX × 0.335–0.003881. The slope of this line, $b = 0.335$ (SE = 0.73), was significantly different than zero ($t = 4.56$, $p < 0.0001$) and 1 ($t = -8.83$, $p < 0.0001$) indicating that the electrical stimulation resulted in a reduction of dorsal skin TTX and that this reduction was proportional rather than absolute. The intercept of this line was not significantly different from zero ($t = -0.271$, $p = 0.79$).

Although there was considerable individual variation in the rate and amount of TTX recovery in our study animals (Table 1), newts in our study regenerated dorsal skin TTX over the 9-month sampling period ($F_{1,30} = 7.52$, $p = 0.0102$). Individual regression lines describing TTX recovery varied considerably and included eight animals whose recovery rate was not significantly different from zero, but the regression line describing average regeneration (i.e. the average of all individual regression lines) for the population was TTX = Time × 0.0035 + 0.04735 with a slope that was significantly different from zero ($t = 2.74$, $p = 0.0102$). The 95% confidence interval of the slope (0.000895–0.006121) was positive and did not include zero. Only 2 of the 30 animals fully regenerated TTX to pre-stimulation levels and 8 of our 30 animals did not appear to regenerate any TTX (Table 1). Regeneration (or failed regeneration) of TTX did not appear to be associated with the sex of the study animal (Table 1). Stereoisomer profiles of newt TTX did not change over time or as a result of stimulation.

4. Discussion

We demonstrate here that *Taricha granulosa* secreted high levels of TTX from glands in the skin. *Taricha granulosa* appears to be capable of secreting remarkable levels of TTX. The presence of TTX in our swabs indicates that TTX is being secreted when then skin is stimulated rather than being destroyed. Because our measures of post-stimulation skin toxicity and pre-stimulation skin toxicity are more accurate than our measures of TTX levels in our cotton swabs we used those measures to assess secretion of TTX in our animals. Given the results presented here (e.g. newts may be capable of secreting up to 90% of their TTX) and the high levels of TTX present in newts from this locality (from 1 to 13 mg of total skin TTX (from Hanifin et al., 2004)) newts from our study sample may be capable of secreting up to 12 mg of TTX.

T. granulosa appeared to be capable of regenerating TTX toxicity over a 9-month period in captivity. Although we considered the hypothesis that newts in our study did not regenerate TTX but instead replenished dorsal skin TTX from other regions of the body (e.g. internal organs, or other regions of skin) we feel this is unlikely. Previous work (Wakley et al., 1966; Hanifin et al., 2004) indicated that only low levels of TTX are present in tissues other than dorsal skin and ovarian tissue making these tissues an unlikely source of TTX for newts. Because TTX regeneration appeared to be independent of sex, we also discount the possibility that the increases in dorsal skin TTX post-stimulation are a result of TTX replenishment from ovarian tissue. There was, however, considerable variation in the rate and amount of TTX regenerated by individual newts and the factors involved in TTX regeneration in captive newts are still unclear.

Tsuruda et al. (2002) in a study of the Japanese newt *Cynops pyrrhogaster* demonstrated that TTX, absent in larval newts, increased in juveniles coincident with development of the granular skin glands and was concentrated in these glands in adult newts. *T. granulosa* also lack TTX as larvae (Twitty, 1937; Hanifin, unpublished data) and have low levels as juveniles (Hanifin, unpublished data). Granular skin glands are associated with toxin production by other

salamanders, and skin glands are also associated with toxins in a variety of other amphibians (Toledo and Jared, 1995). Palatability of amphibians decreases as the granular skin glands develop and produce toxins during metamorphosis (Garton and Mushinsky, 1979; Formanowicz and Brodie, 1982).

Symbiotic bacteria may not be the source of TTX in *T. granulosa*; examination of the tissues containing the highest levels of TTX (skin, liver, and ovaries; Wakeley et al., 1966) determined the absence of bacteria (Lehman et al., 2004). Additionally, Shimizu (2002) has questioned the bacterial origin of TTX in newts because of the disparity in the low levels of TTX produced by bacterial cultures and the high levels present in *Taricha* and *Cynops*. Our results are consistent with the hypothesis that newts are producing TTX in captivity. These results are also congruent with previous studies demonstrating the correlation of granular skin gland density and levels of TTX in *T. granulosa* (Hanifin et al., 2004), the association between TTX and skin glands in *Cynops* (Tsuruda et al., 2002), and the increase of TTX levels of long-term captive newts (Hanifin et al., 2002). Taken together, this work indicates that a reevaluation of TTX production in salamanders may be appropriate.

Acknowledgements

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