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Tetrodotoxin levels of the rough-skin newt, *Taricha granulosa*, increase in long-term captivity

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Abstract

We investigated the persistence of the neurotoxin tetrodotoxin (TTX) in individual captive newts (*Taricha granulosa*) from the Willamette Valley of Oregon using a non-lethal sampling technique. We found that the TTX levels of newts held in the laboratory for 1 yr increased. TTX stereoisomer-analog profiles were not affected by captive husbandry. Levels of TTX were high in newts from our study population and we observed substantial within population variation in quantitative levels of TTX. Females possessed more TTX than males, but the response of TTX levels to captivity did not differ between females and males. The stability of TTX toxicity in newts is consistent with other amphibian species where TTX is present and may indicate that exogenous factors play a less important role in TTX toxicity of newts than previously thought.

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

Despite decades of study, the biogenesis and biosynthesis of tetrodotoxin (TTX) is still poorly understood. In marine taxa the best-supported hypothesis is that TTX is produced by symbiotic bacteria (Noguchi et al., 1986; Yotsu et al., 1987; Yasumoto and Yotsu-Yamashita, 1996), but this model of TTX biogenesis may not be appropriate for amphibian taxa which posses TTX such as the newt *Taricha granulosa*. TTX producing bacteria have not been isolated from any of the amphibian species that possess TTX. Additionally, Daly (1995) has argued that the presence of multiple TTX analogs in single species of the frog genus *Atelopus* (e.g. zetekitoxin found solely in *Atelopus zeteki* and chiriquitoxin found solely in *Atelopus chiriquiensis*) may indicate that the origin of TTX in amphibians is different from marine taxa.

The biogenesis of TTX in *T. granulosa* is of particular interest because of the importance of TTX in the coevolutionary interaction between *T. granulosa* and a snake predator, *Thamnophis sirtalis* (Brodie and Brodie, 1990, 1999; Hanifin et al., 1999). While low levels of TTX are lethal to most potential predators of newts (Brodie, 1968) snakes of the genus *Thamnophis* (*T. sirtalis* in particular) have evolved a resistance to TTX, which allows them to eat highly toxic newts (Brodie and Brodie, 1990, 1991, 1999; Hanifin et al., 1999). TTX resistance in snakes has been shown to possess a heritable genetic basis (Brodie and Brodie, 1999), but the genetic basis of TTX toxicity in newts is as yet unknown.

Few studies have attempted to explore the biogenesis and biosynthesis of TTX in newts (Wakely et al., 1966; Shimizu and Kobayashi, 1983), and in these studies the persistence of TTX in captive populations was not the central question. Wakely et al. (1966) were primarily interested in the distribution of TTX in newt organs, but found that *Taricha torosa* kept in their laboratory for 1 yr had TTX levels similar to the levels of newly captured animals. Shimizu and Kobayashi (1983) were primarily interested in measuring active TTX synthesis in *T. granulosa*. However, they report that overall TTX levels of newts decreased as a correlate of reduced body weight in their captive animals. This correlation between reduced body weight and reduced toxin production has also been shown in ambystomatid salamanders (Williams and Larsen, 1986), and in *Salamndra salamandra* (Phisalix-Picot, 1900), but toxins secreted by

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those salamanders are structurally dissimilar to TTX and are thought to derive from energy storage molecules (Habermehl, 1981; Hamning et al., 2000).

As part of an ongoing study of TTX toxicity in *T. granulosa*, we tested the persistence of TTX toxicity in captive newts using a novel, non-lethal sampling technique. We developed this non-destructive sampling technique to allow us to repeatedly sample the same individuals. Because preliminary data on newts indicated that variation in TTX levels can be remarkably high within some populations of newts, we examined the lability of TTX in captive newts by tracking the change in TTX levels of individual newts over a 1 yr period rather than measuring changes in the means of groups of newts, as has been done in previous studies (Wakely et al., 1966; Shimizu and Kobayashi, 1983). Additionally, we examined the stereoisomer profiles of individual newts to see if these profiles changed in captivity.

1. Methods

We examined 29 adult (23 male, 6 female) *T. granulosa* from a single population (Soap Creek Ponds) located in the Willamette Valley of Oregon (Benton County). These animals were collected in October, 2000, and sampled 4 days, 6 months, and 1 yr after capture. Animals were held at 14 °C in individual 10 gal aquaria filled with 6 cm of filtered tap water. Animals were kept under a 14:10 h light dark cycle and fed a diet of earthworms, tubifex worms, and crickets offered weekly. We recorded total length, snout-vent length, and weight at each sampling period. Tissue samples were stored at −80 °C.

1.1. Tissue sampling

We developed a non-lethal technique for skin sampling that allowed us to measure changes in individual newt toxicity. We anesthetized our study animals in a 1% tricaine solution, rinsed them with filtered tap water and then removed a small (5 mm diameter) circle of skin with a human skin-biopsy punch (Acu-Punch™, Acuderm Inc.). 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, unpublished data). Skin punches sampled at 6 months and 1 yr did not physically overlap punches from previous times, but were taken from areas close to earlier punches to minimize any variance associated with skin sampling.

1.2. 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 extraction buffer (0.1 M aqueous acetic acid). Samples were vortexed and then 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 were used for analysis. This extraction procedure is highly repeatable ($r = 0.95$) (Hanifin et al., 1999) and is not a significant source of variance in our analysis.

1.3. TTX assay

The levels of TTX were quantified by fluorometric HPLC (Yasumoto and Michishita, 1985; Yotsu et al., 1989; Hanifin et al., 1999). We used a protocol modified from Yotsu et al. (1989). Separation of TTX and TTX analogs was performed on a Synergi 4μ Hydro-RP 80A (0.46×25 cm^2, Phenomenex, USA) reverse-phase column with a 50 mM ammonium acetate and 60 mM ammonium heptafluorobutyrate buffer (pH 5.0) containing 1% acetonitrile run at a flow rate of 0.5 ml/min with a Beckman 126 pump system. The eluate was mixed with an aqueous 5N 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 wavelength of the detector was set at 365 nm and the emission wavelength at 510 nm. Data acquisition as well as all chromatographic analysis was performed with System Gold software (version 8.1, Beckman, Inc.). Peak area concentration curves were calculated with standards prepared from commercial TTX (Calbiochem).

1.4. Analysis and statistics

We measured TTX levels from the 21 animals that survived the entire year of captivity. Of these 21 individuals, two were excluded from the final analysis because their tubes leaked during extraction. Thus, our final analysis was based on three samples from each of 19 individuals (15 males, 4 females).

The effect of captivity on TTX levels was tested using a mixed-model, repeated measures analysis of variance (ANOVA). Because preliminary data indicated that males and females might possess different levels of TTX, we included gender in our model. We used a two-factor (time and gender) mixed-model ANOVA assuming a symmetrical model of covariance structure for covariance estimates between individual time measures (PROC MIXED SAS/STAT, version. 8.1, SAS Institute). Tukey's adjusted least-square-means test was used to assess the differences between the sample groups. A similar analysis was performed on weight.

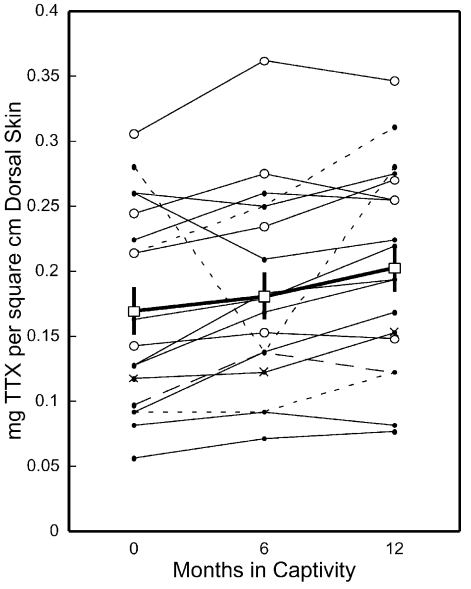

Fig. 1. TTX levels of individual newts (*T. granulosa*) showing an increase in toxicity over 1 yr in captivity. Mean TTX levels (open squares with standard error bars and heavy line) also increased over the 1 yr period of captivity. Females are open dots and males are solid dots; one male is denoted with an X symbol because two individuals had identical levels. Dashed lines are used to clarify individuals where two or more individual newts had the same level of TTX.

2. Results

The amount of TTX present in the skin of individual newts increased over a 1 yr period of captivity ($F_{2,34} = 4.25$, $p = 0.0225$) (Fig. 1), with a 20.7% increase in the mean TTX of all animals from capture (0.169 ± 0.02 mg TTX/cm^2 skin) to 1 yr (0.204 ± 0.02 mg TTX/cm^2 skin) (Table 1). Of the 19 animals included in the final analysis, 16 (84%) had TTX levels that increased over the 1 yr period in which they were held in the laboratory (Fig. 1). One animal lost toxicity (but maintained 86% of its initial toxicity), while two animals had 1 yr TTX levels that were equal to their initial TTX levels (Fig. 1). A comparison of the means for each time period shows a monotonic upward trend over the entire period of captivity (Table 1). Post-hoc comparisons indicated that significant differences in mean TTX levels exist between the initial capture and 1 yr samples ($t_{34} = -2.90$, $p = 0.0172$). Females were more toxic than males ($F_{1,34} = 4.23$, $p = 0.0475$) (Table 1), but the effect of captivity on TTX levels was not different for females and males ($F_{2,34} = 1.0$, $p = 0.393$). The range of TTX values for individual newts at capture from Soap Creek Ponds was almost a full order of magnitude (0.056 mg TTX/cm^2 skin for the least toxic animal to 0.306 mg TTX/cm^2 skin for the most toxic).

There was no effect of captivity on the stereoisomer

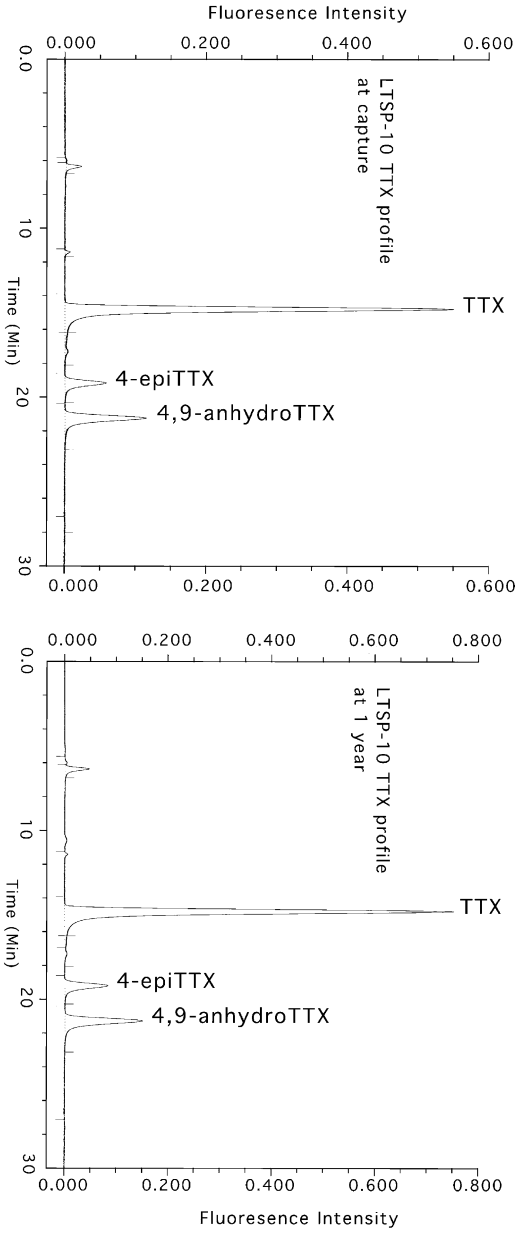

Fig. 2. HPLC–FLD chromatograms of a single typical individual newt (*T. granulosa*) from Soap Creek Ponds in the Willamette Valley of Oregon showing no differences between its stereoisomer-analog profile at capture (left) and after 1 yr in captivity (right). Note that all TTX variants present at capture are present in the 1 yr sample and that no new variants are present at 1 yr sample.

profile of individual newts. No new stereoisomers or analogs of TTX were present in tissues taken from either the 6 months or 1 yr samples, and all analogs present in the initial sample were present in both the 6 months and 1 yr samples (Fig. 2). While we did not quantify levels of TTX-analogs

Table 1
Mean (±SE) TTX values in mg TTX/cm^2 skin for captive *T. granulosa* split by gender and time in captivity

Time	All animals	Females	Males
Capture	0.169 ± 0.02	0.224 ± 0.03	0.153 ± 0.02
6 months	0.178 ± 0.02	0.255 ± 0.04	0.163 ± 0.02
1 yr	0.204 ± 0.02	0.255 ± 0.04	0.188 ± 0.02

other than TTX, a qualitative assessment of chromatograms indicated that ratios between TTX and TTX-analogs for individual animals did not change over time (Fig. 2).

The weight of our study animals was not affected by captivity ($F_{2,34} = 1.45$, $p = 0.249$). Males weighed more than females ($F_{2,34} = 13.11$, $p = 0.0009$), but the response to captivity of male and females was not different ($F_{2,34} = 2.01$, $p = 0.15$).

Survivorship of our experimental group was high. Over 70% (21) of the initial sample of 29 animals survived the entire year. Animals healed quickly following tissue removal (i.e. complete wound closure within 2 weeks). The majority (7 of 8) of the deaths in our lab-held animals occurred rapidly and appeared to be correlated with poor body condition at capture. None of the eight animals that died did so during or immediately after skin tissue was removed.

3. Discussion

Our results indicate that high levels of TTX in newts are not only maintained over a period of 1 yr in captivity, but that these levels increase significantly over this time period (Table 1, Fig. 1). The levels of TTX of newts from Soap Creek Ponds are remarkably high. The mean level of TTX for all animals at capture (0.169 ± 0.02 mg TTX/cm^2 skin or approximately 3.3 mg of TTX/g skin) is over three times greater than that previously reported from this population (1.02 ± 0.14 mg of TTX/g skin) (Hanifin et al., 1999), but this earlier value was based on a small sample size ($n = 5$). Our values are approximately 20 times greater than those previously reported for *T. granulosa* in Yotsu et al. (1990), and 50 times greater than those reported for *T. torosa* in Wakely et al. (1966). Newts measured here possess almost 1000 times more TTX in their skin than atelopid frogs (8.4 μg/g skin; Daly et al., 1994).

While we did not specifically manipulate the diet of our study animals to test the correlation between body weight and TTX levels, the fact that TTX levels increased in our captive animals while body weight remained stable may indicate that this factor is not strongly correlated with TTX levels.

Earlier studies of TTX persistence in newts reported either no loss of toxicity over a period of 1 yr (Wakely et al., 1966), or a reduction of TTX levels as a correlate of body mass (Shimizu and Kobayashi, 1983). Both of these studies were, however, problematic. The earliest of these studies (Wakely et al., 1966) presented no data supporting their conclusion that TTX levels of newts are stable over a 1 yr period. In the later study (Shimizu and Kobayashi, 1983), the authors claimed that newts kept for 167 days lost TTX as a function of reduced body weight, but their TTX extraction and measurement methodology did not allow them to accurately measure differences between fresh-caught and captive groups or test the relationship between individual weight and individual toxicity. They estimated toxicity by combining large numbers of animals ($n = 273$ for one group and $n = 179$ for another) and then extracting and measuring TTX from these pooled extracts. TTX levels were calculated as the total amount of TTX in these extracts divided by the total weight of newts included in each extract. Thus, their estimates of toxicity were not a result of measurements of individual newts. Unfortunately, no attempt was made to ensure that animals grouped together came from the same populations or were collected simultaneously. Evidence reported here, and earlier work (Brodie and Brodie, 1991; Hanifin et al., 1999) demonstrates significant variation in TTX levels within and among populations of newts. This pre-existing variation among newt populations could easily explain the differences reported by Shimizu and Kobayashi (1983). Additionally, their experimental design did not allow them to test the relationship between weight and TTX. Our results provide the first conclusive evidence that *T. granulosa* is capable of maintaining high levels of TTX toxicity in captivity.

The persistence of TTX in other taxa where it is present is poorly studied. No studies of TTX lability in wild caught pufferfish have been performed, but good evidence exists that captive reared fish possess either no TTX or very low levels (Matsumura, 1996). Wild caught *Atelopus oxyrhynchus* maintained a high level of TTX after 3.5 yr in captivity (Yotsu-Yamashita et al., 1992), but captive reared *Atelopus* lacked TTX (Daly et al., 1997). The lability of TTX in other amphibian species where TTX is present is unknown.

It is unclear how our observed increase of newt toxicity in captivity relates to the biogenesis of TTX in newts. One possibility is that TTX already present in other tissues of the newt is moved to the skin of our study animals. Our results neither support nor disprove this hypothesis, but the distribution of TTX in newt tissues makes this hypothesis unlikely. In *T. torosa* males, the skin contains four times the TTX found in blood, ten times the TTX in muscle, and over 800 times the TTX found in liver or the testes (Wakely et al., 1966). In females, only the ovaries contain TTX levels comparable to skin levels (Wakely et al., 1966). This distribution of TTX means that it is unlikely that other tissues in the body could act as a source for TTX in the skin.

The fact that newts are capable of increasing skin toxicity over a 1 yr period could also mean that they are actively synthesizing TTX during this period. If newts are synthesizing

TTX, our results indicate that they are unlikely to require a complex dietary precursor. We made no attempt to feed newts a diet similar to that which they would experience in the wild, and the food items we offered (earthworms, tubifex worms, and crickets) are dissimilar to food items wild newts would likely eat.

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References

Brodie Jr., E.D., 1968. Investigations on the skin toxin of the adult rough-skinned Newt, *Taricha granulosa*. Copeia 2, 307–313.

Brodie III, E.D., Brodie Jr., E.D., 1990. Tetrodotoxin resistance in garter snakes: an evolutionary response to dangerous prey. Evolution 44, 651–659.

Brodie III, E.D., Brodie Jr., E.D., 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 III, E.D., Brodie Jr., E.D., 1999. Predator–prey arms races. Bioscience 49, 557–568.

Daly, J.W., 1995. The chemistry of poisons in amphibian skin. In: Esiner, T., Meinwald, J. (Eds.). The Chemistry of Biotic Interaction. National Academy of Science, Washington, DC, pp. 17–28.

Daly, J.W., Gusovsky, F., Meyers, C.W., Yotsu-Yamashita, M., Yasumoto, T., 1994. First occurrence of tetrodotoxin in a dendrobatid frog (*Colostethus inguinalis*), with further reports for the bufonid genus *Atelopus*. Toxicon 32, 279–285.

Daly, J.W., Padgett, W.L., Saunders, R.L., Cover Jr., J.F., 1997. Absence of tetrodotoxins in a captive-raised riparian frog *Atelopus varius*. Toxicon 35, 705–709.

Habermehl, G.G., 1981. Venomous Animals and their Toxins. Springer, Berlin.

Hamning, V.K., Yanites, H.L., Peterson, N.L., 2000. Characterization of adhesive and neurotoxic components in skin granular gland secretions of *Ambystoma* tigrinum. Copeia 2000, 856–859.

Hanifin, C.T., Yotsu-Yamashita, M., Yasumoto, T., Brodie III, E.D., Brodie Jr., E.D., 1999. Toxicity of dangerous prey: variation of tetrodotoxin levels within and among populations of the newt *Taricha granulosa*. J. Chem. Ecol. 25, 2161–2175.

Matsumura, K., 1996. Tetrodotoxin concentrations in cultured puffer fish, *Fugu rubripes*. J. Agric. Food Chem. 44, 1–2.

Noguchi, T., Jeon, J.-K., Arakawa, O., Sugita, H., Deguchi, Y., Shida, Y., Hashimoto, K., 1986. Occurrence of tetrodotoxin and anhydrotetrodotoxin in *Vibrio* sp. isolated from the intestines of a xanthid crab, *Atergatis floridus*. J. Biochem. 99, 311–314.

Phisalix-Picot, M., 1900. Recherches embryologiques histologiques et physiologiques les glands a venin de la slamandre terrestre. Schleicher Frères, Paris.

Shimizu, Y., Kobayashi, M., 1983. Apparent lack of tetrodotoxin biosynthesis in captured *Taricha torosa* and *Taricha granulosa*. Chem. Pharm. Bull. 31, 3625–3631.

Wakely, J.F., Fuhrman, G., Fuhrman, F.A., Fischer, H.G., Mosher, H.S., 1966. The occurrence of tetrodotoxin (tarichatoxin) in amphibia and the distribution of the toxin in the organs of newts (*Taricha*). Toxicon 3, 195–203.

Wiliams, T.A., Larsen Jr., J.H., 1986. New function for the granular skin glands of the eastern long-toed salamander, *Ambystoma macrodactylum columbianum*. J. Exp. Zool. 239, 329–333.

Yasumoto, T., Michishita, T., 1985. Fluorometric determination of tetrodotoxin by high performance liquid chromatography. Agric. Biol. Chem. 49, 3077–3080.

Yasumoto, T., Yotsu-Yamashita, M., 1996. Chemical and etiological studies on tetrodotoxin and its analogs. J. Toxicol.-Toxin Rev. 15, 81–90.

Yotsu, M., Yamazaki, T., Meguro, Y., Endo, A., Murata, M., Naoki, H., Yasumoto, T., 1987. Production of tetrodotoxin and its derivatives by *Pseudomonas* sp. isolated from the skin of a pufferfish. Toxicon 25, 225–228.

Yotsu, M., Endo, A., Yasumoto, T., 1989. An improved tetrodotoxin analyzer. Agric. Biol. Chem. 53, 893–895.

Yotsu, M., Iorizzi, M., Yasumoto, T., 1990. Distribution of tetrodotoxin, 6-epiTetrodotoxin, and 11-deoxytetrodotoxin in newts. Toxicon 28, 238–241.

Yotsu-Yamashita, M., Mebs, D., Yasumoto, T., 1992. Tetrodotoxin and its analogues in extracts from the toad *Atelopus oxyrhynchus* (family: Bufonidae). Toxicon 30, 1489–1492.