Tetrodotoxin levels in larval and metamorphosed newts (*Taricha granulosa*) and palatability to predatory dragonflies

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ABSTRACT

Some populations of the newt *Taricha granulosa* possess extremely high concentrations of the neurotoxin tetrodotoxin (TTX). Tetrodotoxin is present in adult newts and their eggs, but has been assumed to be absent from the larval stage. We tested larval and meta-morphosed juveniles for the presence of TTX and evaluated the palatability of these developmental stages to predatory dragonfly nymphs. All developmental stages retained substantial quantities of TTX and almost all individuals were unpalatable to dragonfly nymphs. Tetrodotoxin quantity varied greatly among individuals. When adjusted for mass, TTX concentrations declined steadily through metamorphosis. Several juveniles were palatable to dragonflies and these individuals had significantly lower TTX levels than unpalatable juveniles. These results suggest that despite previous assumptions, substantial quantities of TTX, originally deposited in the embryo, are retained by the developing larvae and metamorphosed juveniles and this quantity is enough to make them unpalatable to some potential predators.

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1. Introduction

The neurotoxin tetrodotoxin (TTX) is found in an enormous range of taxa including marine bacteria, ribbon worms, flatworms, mollusks, and puffer fishes as well as several species of terrestrial amphibians (reviewed by Hanifin, 2010; Miyazawa and Noguchi, 2001; Williams, 2010). Tetrodotoxin is an extremely powerful toxin, binding to sodium channels in nerve and skeletal muscle thereby blocking the propagation of action potentials resulting in asphyxiation and death (Kao, 1966; Narahashi et al., 1967). The ecological function of TTX is likely diverse, and TTX may function in both intra-specific (Matsumura, 1995; Zimmer et al., 2006) and inter-specific (Hwang et al., 2004; Ito et al., 2006) communication,

prey immobilization and capture (Ritson-Williams et al., 2006), or as an antipredator mechanism (Brodie, 1968; Williams et al., 2010).

The role of TTX in deterring predation has been well documented in adult rough-skinned newts (*Taricha granulosa*). Newts may contain extremely high concentrations of TTX in the skin (Hanifin et al., 1999), and cutaneous extracts are lethal to almost all potential predators (Brodie, 1968). Moreover, newts with higher toxicity are more likely to survive encounters with potential predators, thus conferring a fitness advantage to toxicity (Williams et al., 2010).

The presence of TTX and its role in other life-history stages including eggs, larvae, and metamorphosed juveniles is less clear. Substantial quantities of TTX are present in the ovaries and ova of adult females, as well as recently deposited eggs (Hanifin et al., 2003; Wakely et al., 1966). Maternal toxicity is highly correlated with egg toxicity and there is little variation in toxicity within a clutch (Hanifin et al., 2003). Due to their sedentary nature, embryos are

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vulnerable to predation (Orians and Janzen, 1974), and the TTX present in newt eggs may limit the scope of predators capable of consuming them (Daly et al., 1987; Twitty, 1966; Gall et al., in press). Twitty and Johnson (1934) and Twitty (1937) conducted the only experiments investigating the toxicity of late stage embryos and larvae. When *Taricha torosa* embryos were grafted to *Ambystoma tigrinum* the host became paralyzed. The paralysis persisted until the yolk was absorbed (shortly after hatching), whereupon mobility was regained. Extracts from macerated embryos (developmental stages: neurula to active swimming) were also injected into larval *Ambystoma* yielding similar results. Compared to embryonic extract, the length of paralysis was reduced when *Ambystoma* was injected with extract from larvae that had recently started feeding, and injection of extract from developmentally advanced larvae yielded no locomotor impairment.

These experiments led to the conclusion that the toxin is primarily located in the yolk, and prevailing views assume that newt larvae retain no toxin. Nevertheless, specific toxicity levels of larvae and juveniles have not been measured. Moreover, these developmental stages have not been presented to potential predators to determine whether toxicity (if present) functions to deter predation. We reared *Taricha* from egg deposition to metamorphosis in the laboratory and tested the palatability of four developmental stages to predatory dragonflies (*Anax junius*). We also screened each developmental stage for the presence of TTX using a Competitive Inhibition Enzymatic Immunoassay (CIEIA).

2. Material and methods

2.1. Animal collection

Twelve gravid female newts were collected by hand from a series of manmade ponds near Corvallis OR in March 2010. Newts were transported to Utah State University where they were housed in 5.7-L containers with 2 L tap water filtered by reverse osmosis (henceforth: filtered water) in an environmental chamber at 6 °C. Females were transferred to a separate environmental chamber at 17 °C and injected with 2 μl/g LHRH (de-Gly10, [d-His(Bzl)6]-Luteinizing Hormone Releasing Hormone Ethylamide; Sigma #12761) to stimulate egg deposition. Some species of *Taricha* (e.g. *Taricha torosa*) attach clusters of eggs to stones or vegetation, however, *T. granulosa* deposit eggs singly on aquatic vegetation over several weeks in nature (Petranka, 1998). Eggs were collected 72 h after the initiation of deposition and maintained in 9 cm diameter plastic dishes with 50 ml filtered water until hatching. Upon hatching, larvae were transferred to 5.7 L plastic containers with 2 L filtered water. Larvae were maintained in these containers without food until the yolk was completely absorbed (3–5 days). Larvae were then fed *Daphnia pulex* ad libitum for one month. After this period, clutches were transferred to 38-L aquaria filled with 35 L of pond water, detritus, and aquatic vegetation (*Myriophyllum spicatum*); this mixture contained abundant quantities of *Daphnia* and other microorganisms. The tank mixture was supplemented with blackworms (Order: Lumbriculida; *Lumbriculus variegatus*)

weekly. Blackworms were obtained from a commercial supplier (Aquatic Foods, Fresno CA). Immediately prior to testing 15 week old larvae (see below), the tanks were drained and the larvae were transferred to 5.7 L containers with 2 L filtered water to monitor development. Larvae were fed blackworms ad libitum until the initiation of metamorphosis (see below), whereupon they were transferred to a 1.2 L container filled with damp sphagnum moss and fed fruit flies (*Drosophila melanogaster*) until testing.

Dragonfly nymphs (*Anax junius*) are major predators of anuran and caudate larvae (Buskirk, 1988; Storfer and White, 2004; Yurewicz, 2004). We used dragonflies from a population (near Preston ID) that does not co-occur with newts to ensure predators had no prior experience with newt larvae; sympatric dragonfly nymphs may learn to avoid newt larvae if they are unpalatable. Nymphs were maintained individually in 275 ml glass bowls with 200 ml filtered water and a small stone (perch site) in an environmental chamber at 17 °C. Dragonflies were fed blackworms ad libitum.

2.2. Testing

We tested the palatability of larvae (three age classes) and recently metamorphosed juvenile *T. granulosa* to predatory dragonfly nymphs (*A. junius*). (1) First stage larvae (hatchlings: 0 weeks old) hatched two days prior to testing at developmental stage 39–41 (Harrison, 1969) and were tested on 3 June 2010. (2) Second stage larvae (4 weeks old) hatched on 27 to 30 April 2010 and were tested at developmental stage 46 (Harrison, 1969) on 27 May 2010. (3) Third stage larvae had fully formed front and back limbs, fully developed digits, and were tested 15 weeks after hatching (hatched: 27 to 30 April 2010; tested: 14 August 2010). (4) Metamorphosed juveniles were identified based on a reduction of the gills, change from smooth larval skin to granulated adult skin, and development of orange pigmentation on the venter. Because larvae initiated metamorphosis at different times, small groups of metamorphs were tested in November and December 2010 (roughly 28 weeks old, hatched on 27 to 30 April 2010). Juveniles were tested exactly 25 days after the initiation of metamorphosis. Half the individuals in each group were used to test palatability while the other half served as controls.

Each trial consisted of two 20 min periods during which a nymph was provided either a single blackworm (control) or a newt. Each period was separated by 40 min and the order of testing (control or newt) was randomly determined before the start of each trial. Twenty dragonfly nymphs were randomly chosen from a pool of individuals and fed three blackworms three days prior to testing to standardize hunger level between individual nymphs. The dragonfly holding containers were cleaned prior to each test period. Individual newts within a treatment (i.e. developmental stage) were collected from a minimum of five families. A prey (control or newt) was introduced to a bowl with a pipette and we recorded whether the prey was seized, rejected or consumed, and injured, uninjured, or dead. Trials were terminated after 20 min if the nymph did not consume the prey, or after a prey was seized and either rejected or consumed. Seizure usually occurred

within the first minute. Dragonfly nymphs and newt larvae and metamorphosed juveniles were never retested.

To ensure injuries incurred during the predation event were not due to handling, we tested the survival of each newt compared to control larvae that were treated the same except no predator was present in the bowls. The tested and control larvae were transferred to glass bowls and the number of uninjured, injured, and dead larvae was recorded after 48 h. Each larva was removed from the bowl and nudged with the pipette. If the larva responded immediately by swimming in a general linear path it was classified as uninjured. Injured larvae often had difficulty swimming or swam in convoluted patterns. We verified the health of metamorphs by placing them on their back and recording the time to right themselves (<5.0 s, classified as uninjured). Of 78 control larvae 76 were uninjured and two died; this variable did not affect the results and these data were removed from the analysis. At the termination of the experiment all unpalatable larvae were frozen at −80 °C for TTX analysis. Larvae that were seized and partially consumed were removed from the predator after 1 h and frozen at −80 °C to quantify TTX so the toxicity of palatable larvae could be compared to larvae that were rejected.

2.3. TTX analysis

Tetrodotoxin was quantified using a Competitive Inhibition Enzymatic Immunoassay (CIEIA) as in Stokes et al., submitted for publication. This assay is highly specific and works by binding anti-TTX monoclonal antibodies to TTX. In the absence of TTX or in low concentrations of TTX, the antibodies bind to the conjugate on the plate allowing secondary antibodies to also bind to the plate, resulting in a high absorbance reading. This value is then used to calculate the TTX concentration using the linear standard curve. The assay is able to detect TTX at a minimum concentration of 10 ng/mL, and has a linear range of 10–500 ng/mL (Stokes et al., submitted for publication). Anti-TTX antibody concentrations for all plates were 0.391 μg/mL. All samples were diluted 1:2, 1:4, 1:8, or 1:16 depending on the total TTX concentration, to assure they were within range of the standard curve. Blanks (no antibody), negative controls (blackworms), and a standard curve (100, 75, 50, 25, 10 ng) were included on each plate. All plates were read at 405 nm. The average coefficient of variation for each plate was between 4.55 and 6.55%.

2.4. Statistical analysis

We compared the total and mass corrected (ng TTX/mg body mass, log transformed to meet assumptions of normality) amount of TTX (ng) in individuals from each developmental stage with ANOVAs followed by Holm-Sidak multiple comparisons. Finally, we compared the concentration of TTX in unpalatable and palatable metamorphosed juveniles with a t-test.

We determined whether the frequency of palatable and unpalatable individuals the same for the four developmental stages using a contingency table and a chi-square test. The survival data were analyzed by combining the number of injured and dead larvae into a single category (wounded) and comparing the frequency of uninjured and wounded larvae in the four developmental stages with a contingency table and a chi-square test.

3. Results

Tetrodotoxin levels in unpalatable larvae and juveniles ranged between 229 and 2354 ng TTX per individual or 0.463–3344 ng TTX/mg body mass, with hatchlings exhibiting the highest concentration and toxicity generally decreasing with increasing developmental stage (Table 1). Palatable juveniles contained 296 ± 103 ng TTX or 0.33 ± 0.08 ng TTX/mg body mass (Table 1). There were significant differences in the total amount (ng) of TTX from the different developmental stages (df = 3, $F = 5.02$, $P < 0.008$, Fig. 1), with toxicity greatest in hatchlings, declining at 4 weeks old, and remaining relatively constant for the remainder of development. There was also a significant difference between developmental stages in the concentration of TTX (ng TTX/mg body mass) (df = 3, $F = 65.1$, $P < 0.001$, Fig. 1), whereby TTX concentration decreased after hatching and was the lowest after metamorphosis (Fig. 1). Palatable metamorphs had significantly lower TTX concentrations relative to unpalatable metamorphs (df = 11, $t = 2.83$, $P = 0.016$, Fig. 2, Table 1). The negative control (blackworms) contained no TTX and was not different from the BSA control.

The frequency of palatable and unpalatable individuals was not the same for the four developmental stages (df = 3, $\chi^2 = 10.22$, $P < 0.025$) whereby the palatability of newts generally increased with developmental stage (Table 2). Hatchling and four week old larvae were completely unpalatable, whereas one 15 week old larva was palatable and four juveniles were consumed (Table 2).

The frequency of uninjured and wounded individuals was not the same for the four developmental stages (df = 3, $\chi^2 = 16.89$, $P < 0.001$). Younger larvae were more likely to be wounded after being seized by predatory dragonflies than older larvae (Table 2).

Table 1
The quantity (total and mass corrected) of TTX present in unpalatable larvae and juveniles from four developmental stages and palatable (palat) 28 week old juveniles. Conc.: concentration. Values are ± SE.

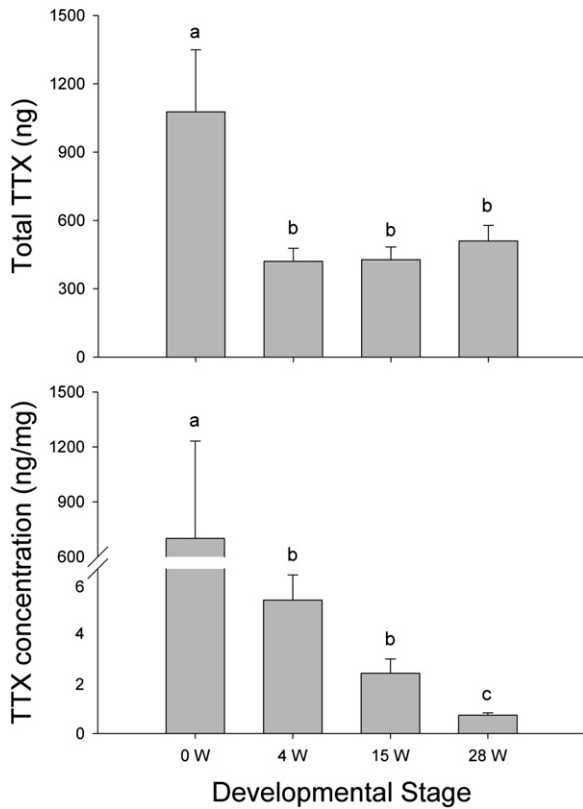| Treatment	N	mass ± SE (mg)	Total TTX (ng)	Range	TTX conc. (ng/mg)	Range
0 Week	6	5.9 ± 1.3	1076.3 ± 272.5	488–2354	700.02 ± 530.9	55.18–3344.36
4 Week	6	77.6 ± 4.3	420.3 ± 57.5	259–580	5.67 ± 1.1	3.24–9.88
15 Week	6	203.0 ± 41.1	427.5 ± 55.9	229–518	2.56 ± 0.6	1.14–5.17
28 Week	9	681.8 ± 71.5	509.7 ± 68.5	316–929	0.78 ± 0.1	0.46–1.24
28 Week (Palat)	4	836.5 ± 130.2	296.0 ± 103.4	90–583	0.33 ± 0.08	0.12–0.48

Fig. 1. (top) Mean (±SE) total amount of TTX (ng) in newt larvae from four developmental stages. (bottom) Mass corrected (mean (±SE) ng TTX/mg body mass) TTX in newt larvae from four developmental stages. 0 W = Larvae that had recently hatched, 4 W = Four week old larvae, 15 W = fifteen week old larvae, 28 W = Larvae that had recently completed metamorphosis. Different letters indicate significant differences between treatments ($P < 0.015$).
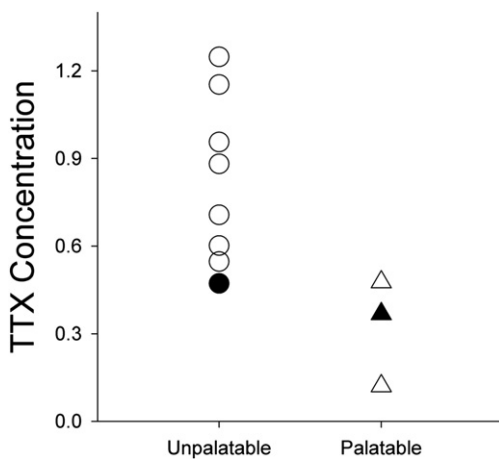

Fig. 2. TTX concentration (ng TTX/mg body mass) present in unpalatable and palatable individual juvenile newts (approx. 28 Weeks post hatching; exactly 25 days post metamorphosis). Palatable juveniles had significantly lower TTX concentrations relative to unpalatable juveniles (df = 11, $t = 2.83$, $P = 0.016$). Circle: unpalatable juvenile newts. Triangle: palatable juvenile newts. Filled symbols indicate two overlapping values.

4. Discussion

T. granulosa larvae and metamorphosed juveniles possess substantial quantities of TTX and most are unpalatable to predatory dragonfly nymphs. Female newts deposit large quantities of TTX in their eggs prior to deposition (Hanifin et al., 2003), and it was previously unknown how toxicity changed ontogenetically. Recently deposited eggs may contain as much as 2767 ng of TTX per egg (Hanifin et al., 2003), and early experiments suggested toxicity decreased with increasing development, ultimately resulting in nontoxic larvae (Twitty, 1937; Twitty and Johnson, 1934). Our study indicates toxicity does decline slightly during development (mean egg toxicity = 1528 ng (Hanifin et al., 2003); mean hatchling toxicity = 1072 ng), however, larvae remain extremely toxic at hatching and all developmental stages, including metamorphosed juveniles, possess substantial quantities of TTX. The total toxicity of a single larva at hatching is 1076 ± 272 ng of TTX. Total individual toxicity declines between hatching and four weeks old (420 ± 57 ng), and then remains stable through metamorphosis. These results contradict previous assumptions about the ontogenetic change of toxicity in *Taricha* and indicate some TTX is retained at least through metamorphosis.

When adjusted for body mass, TTX continues to decline through metamorphosis, despite no change in total toxicity. Due to their small size, hatchlings remain extremely toxic relative to eggs. However, by four weeks old, total toxicity decreased and body mass increased resulting in a 100 fold decrease in the amount of TTX (per mg body mass) relative to hatchlings.

Egg toxicity is correlated with maternal toxicity (Hanifin et al., 2003), and the TTX present in larval and metamorphosed juveniles is likely residual from the quantity initially deposited in the eggs. At our study site, TTX quantity is highly variable among individual adults (Hanifin et al., 1999, 2003). Because larvae for the current study were collected from at least five different clutches, it is likely initial clutch variability is driving some of the variation in larval and juvenile toxicity that we observed. This large amount of variation suggests the quantity of TTX in larval and juvenile newts may be correlated to the initial investment of TTX in the embryo. If female toxicity is correlated with all ontogenetic changes in toxicity, a selective advantage would likely be conferred to all age groups if derived from a highly toxic lineage.

Taricha larvae are unpalatable to one of the most pervasive predators of amphibian larvae. For TTX to function as an antipredator mechanism, individuals with greater toxicity must be more likely to survive a predatory encounter. Williams et al. (2010) directly tested the role of TTX toxicity on the survival probability of encounters between adult newts and toxin resistant garter snakes. The authors found that newts that survived the encounters with predatory snakes had higher TTX concentrations than newts that were eaten, thereby directly testing the efficacy of TTX as an antipredator mechanism. In this study, unpalatable juveniles exhibited higher toxicity than palatable individuals suggesting TTX toxicity also is directly related to probability of survival for larval newts.

Table 2
Number and survival of newt larvae from four developmental stages presented to predatory dragonfly nymphs. H = Hatchling, M = Metamorph.

Developmental stage	N	Seized	Rejected	Uninjured	Injured	Dead	Consumed
0 Week	20	19	19	9	6	5	0
4 Week	20	19	19	16	1	3	0
15 Week	20	15	14	17	1	1	1
28 Week	18	17	13	13	0	0	4

Tetrodotoxin is primarily located in the skin of adult newts (Mosher et al., 1964; Wakely et al., 1966; Brodie et al., 1974), and in the yolk of embryos (Twitty, 1937). The location of the toxin in larval newts is unknown; however, rejected larvae and juveniles were struck on all regions of the body (tail, limbs, head, torso). Dragonflies slowly consume pieces of their prey (rather than whole). The greater palatability of older larvae is likely due to a reduction in TTX per unit mass in larger individuals as well as greater sensitivity to TTX concentration rather than total quantity in predatory dragonflies.

Although toxicity makes newt larvae unpalatable to potential predators, this antipredator mechanism functions after the prey has been seized and when the risk of injury is high (Endler, 1986; Brodie et al., 1991). Tetrodotoxin toxicity cannot confer a selective advantage if the prey still dies from injuries received during the attack. Dragonfly nymphs possess palpal lobes on the labium that terminate in movable grasping hooks. These hooks function to pierce and subjugate prey (Corbet, 1999). Larvae in early developmental stages (especially hatchlings) are vulnerable to injury or death from these predatory attacks. However, numerous individuals did survive these encounters, thus conferring a selective advantage to unpalatability. The predators used in this study had no prior experience with newts, requiring each individual to sample and reject the prey. Odonates are capable of learning (e.g. Chivers et al., 1996), and may be able to learn to avoid dangerous prey (O'Donnell, 1996). If dragonfly nymphs learn to avoid chemical or visual cues associated with newt larvae, the effective advantage of unpalatability increases, especially for those individuals that survive the initial encounter.

Despite previous assumptions, *Taricha* larvae and most metamorphosed juveniles were unpalatable to predatory dragonfly nymphs. All age classes retained TTX, yet palatable juveniles possessed reduced levels of TTX relative to their unpalatable cohorts. A wide range of organisms possess tetrodotoxin, and understanding the ontogenetic changes in toxicity and the role TTX plays in the various life-history stages is paramount to determining the function of TTX for these organisms.

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