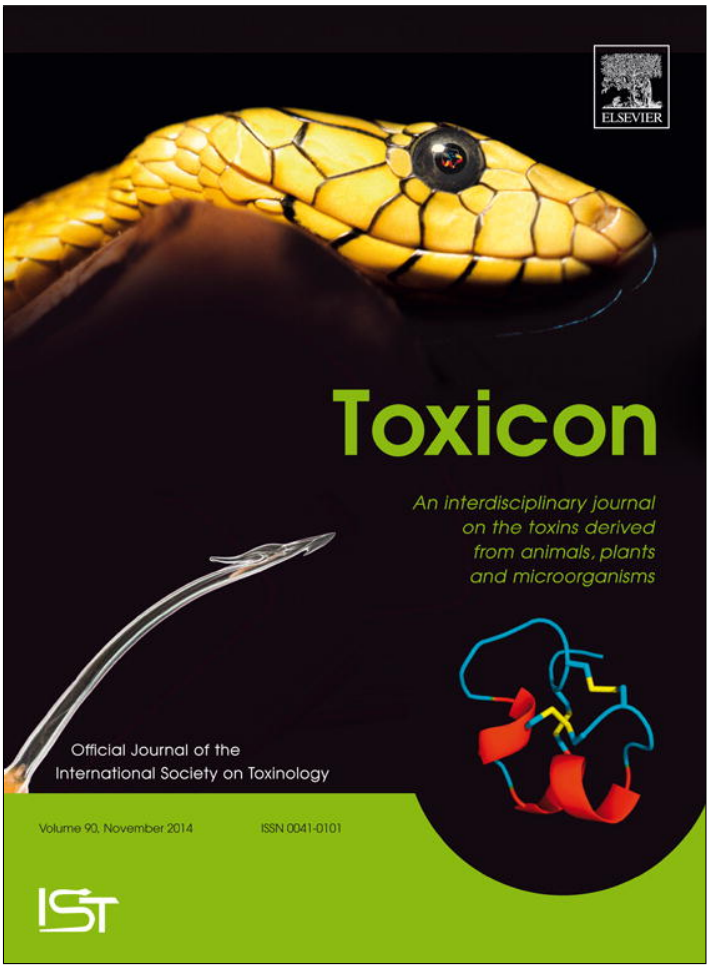

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Tetrodotoxin concentrations within a clutch and across embryonic development in eggs of the rough-skinned newts (*Taricha granulosa*)

Brian G. Gall [a, *], Amber N. Stokes [b], Jory J. Pett [c], Kari L. Spivey [a], Susannah S. French [c], Edmund D. Brodie III [d], Edmund D. Brodie, Jr. [c]

[a] Department of Biology, Hanover College, P. O. Box 108, Hanover, IN, 47243, USA
[b] Department of Biology, California State University, 9001 Stockdale Highway, Bakersfield, CA, 93311, USA
[c] Department of Biology, Utah State University, 5305 Old Main HL, Logan, UT, 84322, USA
[d] Mountain Lake Biological Station and Department of Biology, University of Virginia, P.O. Box 400328, Charlottesville, VA, 22904, USA

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ABSTRACT

Tetrodotoxin is an enigmatic neurotoxin that is found in a wide-variety of organisms. Unfortunately, tetrodotoxin (TTX) toxicity across life-history stages is poorly understood in most organisms. Rough-skinned newts (*Taricha granulosa*) possess the greatest known quantities of TTX of any organism and numerous studies have begun to elucidate these patterns in this species. We conducted a series of studies to answer the following questions: (1) do eggs from a single female's clutch vary in toxicity? (2) does TTX concentration change during embryonic development? and (3) does the jelly coat from newt eggs possess TTX? We found that the amount of TTX in newt eggs depended on the relative "position" of the egg within a clutch; eggs deposited at the beginning of the clutch had substantially more TTX than those at the end. During development egg toxicity remained consistent until hatching. The jelly coat contained small quantities of TTX, but these were not correlated with the toxicity of the embryo. These results clarify several long-held interpretations about embryo toxicity and continue to elucidate the life-history patterns of tetrodotoxin toxicity in this amphibian.

1. Introduction

Tetrodotoxin (TTX) is one of the most toxic, well studied, and yet mysterious natural products found in nature. This neurotoxin is a non-proteinaceous, guanidinium ion that binds to voltage-gated sodium channels in nerves and skeletal muscles (Mosher et al., 1964; Narahashi et al., 1967). This action prevents the propagation of action potentials which has made it an integral natural toxin in the study of neurophysiology (Moczydlowski, 2013). Unfortunately, this action also results in asphyxiation and death (Kao, 1966; Moczydlowski, 2013), and TTX has been documented to be lethal to a wide variety of organisms (Brodie, 1968), including humans (Noguchi and Arakawa, 2008; Noguchi and Ebesu, 2001).

Despite the toxicity of tetrodotoxin, it is found in a great diversity of aquatic and terrestrial organisms, including, bacteria, invertebrates, and vertebrates (see review in Chau et al., 2011). For example, small quantities of TTX are produced by multiple species of marine bacteria including *Alteromonas*, *Lysinibacillus*, and *Vibrio* (Lee et al., 2000; Pratheepa and Vasconcelos, 2013; Simidu et al., 1987; Wang et al., 2010). In eukaryotes, TTX has been identified in tissues from marine organisms including dinoflagellates, flatworms, a sea slug, crabs, a starfish, and an octopus (Chau et al., 2011; Simidu et al., 1987). In vertebrates, TTX is phylogenetically restricted to amphibians and fish. The

* Corresponding author. Tel.: +1 812 866 7249.
E-mail address: gall@hanover.edu (B.G. Gall).

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toxin is named for the family Tetraodontidae (which includes the pufferfishes), members of which contain large quantities of TTX in the skin, liver, and ovaries. The consumption of traditional Japanese fugu (pufferfish liver) accounts for many of the human intoxication events (Noguchi and Arakawa, 2008). In amphibians, TTX is prevalent in several distantly related frog species as well as many newts in the family Salamandridae.

Despite our knowledge of the diverse taxa that possess this toxin, its source and synthesis remain unclear. In marine organisms, the presence of TTX producing bacteria has led some authors to hypothesize that the toxin is produced by symbiotic bacteria and is co-opted by the host for other functions (e.g. defense) (Simidu et al., 1987; Wang et al., 2010). The life-history patterns of TTX variation are most well-documented in terrestrial newts, and details from this system indicate that the bacterial production hypothesis is less supported for this group. For example, adult newts possess extreme quantities of TTX in the skin and ovaries, and a single newt can contain as much as 29 mg of TTX or enough to kill as many as 58 people (Stokes et al., 2015). These quantities are between 600 and 5500 times higher than quantities derived from bacteria in culture (Wang et al., 2010, and references herein) and would require between 309 and 1103 L of culturing medium to produce (see Supplementary material). Lehman et al. (2004) analyzed newt tissues for 16S rRNA genes of bacteria and found its presence only in the intestines, which contain little TTX. Bacterial DNA was absent from the skin, ovaries, and liver which contain large quantities of TTX. In addition, TTX toxicity increases in newts reared in the lab over time, is deposited in the eggs from captive female newts for multiple years, and can be rapidly regenerated when released by electrical stimulation (Cardall et al., 2004; Gall et al., 2012a; Hanifin et al., 2002).

Despite the advancements with regard to our understanding of ontogenetic changes in toxicity in newts, several major questions remain regarding the toxicity of newts at various life-history stages. In addition to the adults, female newts deposit substantial quantities of TTX in their eggs (Mosher et al., 1964), and although we know the toxicity of recently deposited eggs (Gall et al., 2012a; Hanifin et al., 2003), it is unknown whether the eggs within a single clutch possess similar quantities of toxin. Many species can adjust the amount of maternally apportioned compounds within their eggs (Williams, 2012), and this process could have important implications for the fitness of female newts (Mousseau and Fox, 1998). Second, it is unknown how TTX toxicity changes from egg deposition to hatching. Recent studies have hinted that egg toxicity may remain constant throughout embryo development (Gall et al., 2011), yet historical reports also suggest that toxicity may decrease slowly (Twitty, 1937; Twitty and Johnson, 1934). Answering this question will clarify the ontogenetic changes that occur during this early life-history stage. We set out to fill gaps in our knowledge of TTX toxicity in the life-history of newts by answering these questions. Specifically, we conducted three studies to quantify (1) the amount of TTX in eggs across a single clutch, (2) the change in TTX during embryonic development, and (3) the amount of TTX in the embryo versus the jelly coat of newt eggs.

2. Materials and methods

2.1. Animal collection and maintenance

Gravid female newts (*Taricha granulosa*) were collected in March 2012 and 2013 from Soap Creek ponds in the central Willamette Valley, OR. Newts were immediately transported to Utah State University where they were housed in 5.7-L containers with 2 L of filtered tap water. Each female was housed in an environmental chamber at 21 °C and injected with 15 μl LHRH (de-Gly10, [d-His(Bzl) 6]-Luteinizing Hormone Releasing Hormone Ethylamide; Sigma #L2761) to stimulate egg deposition. Each female was provided with a small clump of polyester fiber to serve as an oviposition site. This substrate is readily accepted as an oviposition site by newts. Females were not fed until the completion of egg deposition.

2.2. Experiment 1 – does TTX level differ between eggs within a single clutch?

To determine whether TTX levels differ between eggs within a single clutch, each of four females was monitored frequently throughout deposition such that small batches of eggs (10–50) were removed and the "position" of each of these batches within the clutch was known. Females were monitored for the beginning of egg deposition at which point the first 10 eggs were removed, weighed to the nearest 0.001 g, and frozen at −80 °C. After these initial eggs were collected, females were permitted to deposit a larger batch of eggs (generally between 50 and 100 eggs) which were removed from the fiber, counted, and used in experiment 2 (see below). After this larger batch of eggs had been removed, the following 10 eggs that were deposited were removed, weighed, and frozen at −80 °C. This process was repeated until each female had deposited all of its eggs and we had a sequence of eggs at four different positions from the beginning to the end of the clutch (beginning, first third, second third, end).

For TTX analysis, 3 eggs from each female and time-period throughout the clutch laying period were randomly selected and analyzed for total TTX and TTX concentration (see below for TTX extraction and quantification). Because each female laid a different number of eggs, the sampling periods within a clutch are not the same for each female.

The relationship between total TTX (or TTX concentration) and lay order (i.e. position within the clutch) was assessed using a random coefficients model with random intercepts, slopes and covariance using a generalized linear mixed model assuming a gamma distribution for the response. Computations were made using Laplace estimation in the GLIMMIX procedure in SAS/STAT 12.3 in the SAS System for Windows Release 9.4.

2.3. Experiment 2 – does TTX level change during embryonic development?

To assess whether TTX quantity changes during embryonic development, we collected the first 100 eggs from each female (excluding the 10 used in experiment 1), and placed them individually in 5 ml cups with 4 ml filtered tap

water and maintained them at 21 °C. These 100 eggs were generally laid in less than 24 h, although one female required 48 h to deposit a sufficient number of eggs for the experiment. Immediately after placing these eggs in cups, ten eggs from each female were randomly selected, removed from the cups, and their developmental stage was recorded using an Olympus stereo microscope with an ocular micrometer according to Harrison (1969). Each egg was then weighed to the nearest 0.001 g and frozen at −80 °C for later TTX analysis. This process was repeated at the following days post deposition: day 6 (developmental stages 19–23), day 10 (developmental stages 36–38), and day 18 (developmental stages 40–43); for purposes of analysis, each egg was assigned to one of four developmental stages associated with each of the time-points listed above (stage 4, 20, 37, 43h; h = hatched). All embryos had hatched by day 18, therefore TTX measurements on day 18 samples do not include the egg jelly (see below for analysis of TTX in egg jelly).

For TTX analysis, 3 eggs from each female and developmental stage were randomly selected and analyzed for total TTX and TTX concentration (see below for TTX extraction and quantification). To determine if TTX levels change during embryonic development, we compared the total TTX and TTX concentration between the four developmental stages using a randomized complete block design with developmental stage as a fixed-effect factor and female treated as a random factor. Post-hoc comparisons were conducted using the REGWQ procedure. Assessments of distributional assumptions were based on graphical analysis of residuals; all assumptions appeared to be adequately met for all response variables. All analyses were performed using PROC GLM in SAS/STAT software version 9.1 (SAS Institute, Inc., Cary, NC).

2.4. Experiment 3 – how much TTX is present in the jelly coat of newt eggs?

To assess whether there is TTX present in the jelly coat of newt eggs, we collected three recently deposited eggs from three female newts and used iris scissors to carefully cut the jelly and remove the intact embryo. The embryo was weighed to the nearest 0.001 mg using a Mettler Toledo (Switzerland) AB265-S/FACT digital balance with a draft shield and transferred to a microcentrifuge tube. The jelly was then rinsed in DI water (to remove any remaining residue from the embryo), weighed, and transferred to a microcentrifuge tube. Due to their extremely delicate nature, we did not rinse the embryos prior to weighing and freezing. All samples were immediately frozen at −80 °C for later TTX analysis (see below). Linear regression was used to compare the amount of TTX in the embryo with the amount of TTX in the jelly.

2.5. Tetrodotoxin quantification

Frozen egg samples were extracted for analysis using previously described techniques (Hanifin et al., 2002). Tetrodotoxin was quantified using a Competitive Inhibition Enzymatic Immunoassay (CIEIA) as in Stokes et al. (2012). 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 estimate the TTX concentration using a 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., 2012). All samples were diluted 1:2, 1:4, 1:8, 1:16, or 1:32 in Bovine Serum Albumin (BSA) to assure they were within the linear range of the standard curve. All plates were read at 405 nm. The average coefficient of variation on each plate was between 5.04 and 11.09%.

3. Results

3.1. Do TTX levels differ between eggs within a single clutch?

The total amount of TTX deposited in the eggs of female newts declined from the beginning to the end of the clutch ($F_{[1,3]} = 8.47$, $P = 0.062$, Fig 1). Similarly, there was a negative relationship between TTX concentration and the lay order within the clutch ($F_{[1,3]} = 7.09$, $P = 0.076$, Fig 2). For each female, mean egg toxicity declined by 88%, 84%,

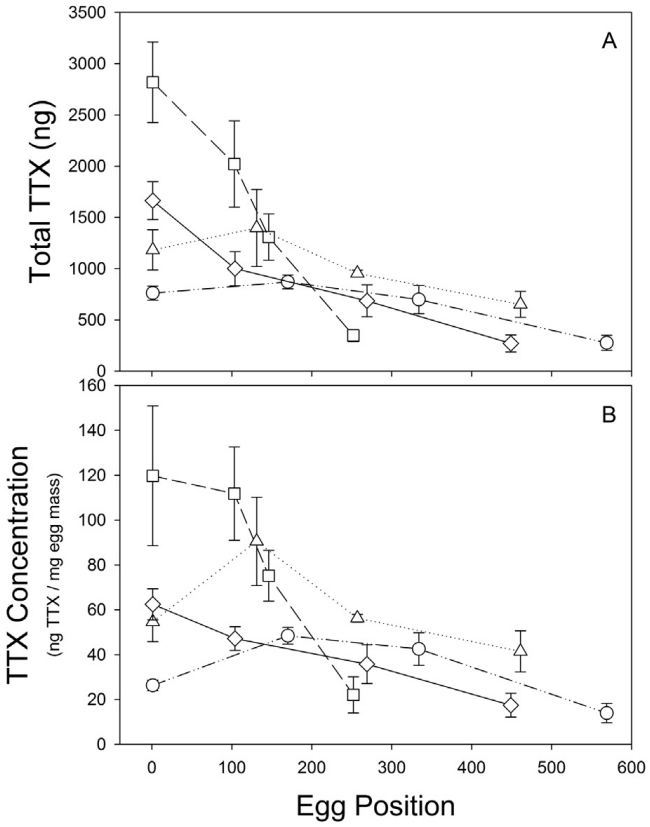

Fig. 1. Mean (±SE) total nanograms of TTX (A) or TTX concentration (B) present in rough-skinned newt embryos at different relative positions within a females clutch. There is a significant decline in toxicity from the first to last eggs within a clutch (Total TTX: $F_{[1,3]} = 8.47$, $P = 0.062$; TTX conc: $F_{[1,3]} = 7.09$, $P = 0.076$). Different symbols (circle, triangle, diamond, and square) represent eggs from four different females; matching symbols in Figs. 1 and 2 correspond to the same female. Lines are present to highlight trends within females.

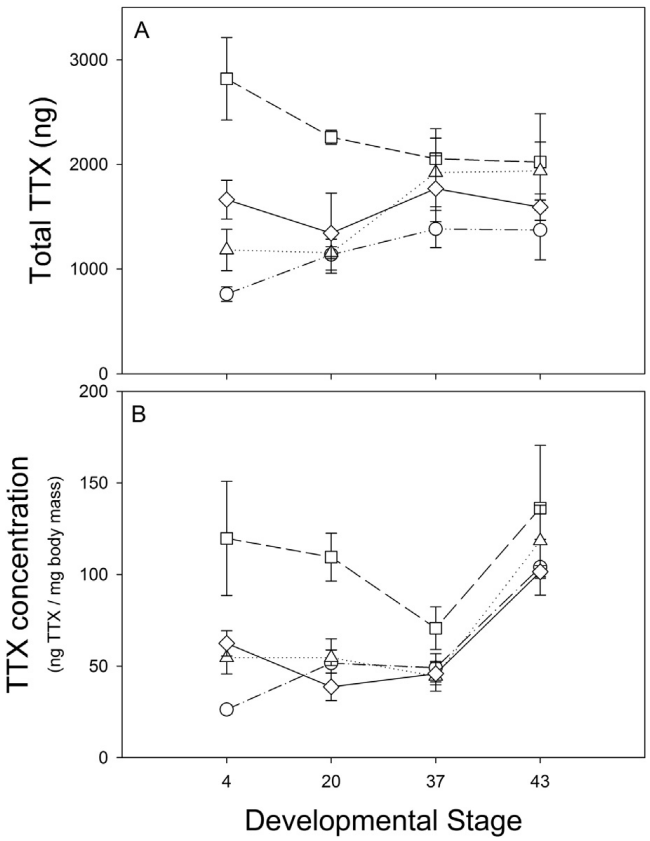

Fig. 2. Mean (±SE) total nanograms of TTX (A) or TTX concentration (B) present in rough-skinned newt embryos from one of four developmental stages. There was no change in total TTX across developmental stages ($F_{[6,41]} = 0.93$, $P = 0.44$), but there was a significant difference in TTX concentration across development due to the loss of the jelly and fluids after hatching ($F_{[6,41]} = 13.58$, $P < 0.0001$). Different symbols (circle, triangle, diamond, and square) represent eggs from four different females; matching symbols in Figs. 1 and 2 correspond to the same female. Developmental stages were assigned according to Harrison (1969). Lines are present to highlight trends within females.

64%, and 45% from the first to the last eggs within their clutch.

3.2. Does TTX level change during embryonic development?

The total amount of tetrodotoxin in newt eggs did not significantly change during embryonic development ($F_{[6,41]} = 0.93$, $P = 0.44$, Fig 2A). The concentration (ng TTX per mg egg) of TTX in newt eggs did differ across embryonic developmental stages ($F_{[6,41]} = 13.58$, $P < 0.0001$, Fig 2B). In this case, the concentration of TTX was significantly higher immediately after hatching (developmental stage 43), whereas there was no difference in TTX concentration between the first three developmental stages (Fig 2B). The total TTX and TTX concentration present in eggs did however differ between females (Total TTX: $F_{[6,41]} = 10.76$, $P < 0.0001$; TTX concentration: $F_{[6,41]} = 9.79$, $P < 0.0001$; Fig. 2A,B).

3.3. How much TTX is present in the jelly coat of newt eggs?

Four out of nine eggs had detectable quantities of TTX present in the jelly coat. These concentrations ranged from 11.8 ng to 28 ng of TTX; the jelly from 5 newt eggs had toxin levels below the detectable limit of the assay (<10 ng TTX). Large quantities of TTX were present in the embryos of each egg, which ranged between 1791 ng and 4945 ng of TTX. There was no relationship between jelly toxicity and egg toxicity ($F_{[1,7]} = 0.09$, $R^2 = 0.01$, $P = 0.77$).

4. Discussion

Two historical conclusions have been drawn regarding the tetrodotoxin toxicity of early developmental stages in *Taricha*. The first was based on evidence from the well-known embryologist Victor Twitty and his work with *Taricha* embryos (Twitty, 1961). In experiments conducted in the 1930's, *Ambystoma* embryos were grafted to *Taricha* embryos and the former became paralyzed (Twitty, 1937, 1966; Twitty and Johnson, 1934). The paralysis persisted until shortly before the *Taricha* embryos started feeding or the yolk had been absorbed. This paralysis (and later recovery) led subsequent researchers to conclude that tetrodotoxin was present during early developmental stages but was slowly metabolized or lost as the embryo approached hatching. The data presented here show that tetrodotoxin concentrations in rough-skinned newt embryos do not decline during embryo development. Shortly after egg deposition (Harrison stage 4), embryos contained between 650 and 3400 ng of TTX, which is within the established range for eggs of the rough-skinned newt (Gall et al., 2012a; Hanifin et al., 2003). Although there was substantial variation among females in the pattern of toxicity across development (possibly due to the small number of eggs assayed), the total amount of TTX remained constant and was not metabolized or lost by the developing embryo. In a similar manner, TTX concentration remained constant for the first three embryo stages. Tetrodotoxin concentrations did, however, increase sharply after hatching which is not due to an actual change in the total quantity of TTX, but rather to the loss of the jelly coat and the fluids that surround the embryo, which dramatically decreased the mass of the sample to roughly 50% of pre-hatching values.

The second interpretation was that female newts provision all of their eggs with similar quantities of tetrodotoxin. Previous analyses suggested tremendous variation in toxicity among females but very little variation within a single clutch (Hanifin et al., 2003). This conclusion was based on relatively small samples (maximum of 10 eggs from one clutch), all of which were collected at the beginning of the deposition period. The results presented here indicate that TTX provisioning is indeed different for eggs at different points in the laying order. Eggs deposited early in the lay order were provisioned with the greatest quantities of TTX whereas those deposited at the end contained between 12% and 55% of the TTX in the earlier eggs. These results are surprising given that TTX is believed to be present primarily in the yolk (Twitty and Johnson, 1934) and its deposition appears to be independent of the size of the embryo (Hanifin et al., 2003).

Unfortunately, characterizing the proximate mechanism for differences in TTX within a clutch is difficult because it is unknown where or how TTX is produced (Hanifin, 2010).

Regardless, one possible explanation may be that TTX is a limited resource and female newts provision their eggs until the available TTX becomes depleted. Differential allocation of maternally derived resources is a common occurrence in many oviparous organisms. For example, female canaries (*Serinus canaria*) allocate different quantities of testosterone in their eggs such that the concentration of hormones increases with the order of egg deposition (Schwabl, 1993). Similarly, birds may differentially allocate lipids (yolk) to their eggs such that early laid eggs are larger and have a competitive advantage, or late eggs are larger thereby equalizing the competitive advantage across the clutch (Hadfield et al., 2013; Howe, 1976). Alternatively, some eggs may simply have more time to absorb TTX if egg toxicity is a passive by-product of the physiology of newt reproductive cycles; this may explain why TTX toxicity is not correlated with the volume of the embryo (Hanifin et al., 2003). For example, Miller and Robbins (1954) examined the histological changes in the ovaries of a closely related newt (*Taricha torosa*) and found that deposition of yolk, the likely site of TTX, occurs slowly over 5–6 months prior to ovulation. Moreover, this process was completed only shortly before ovulation, indicating that some embryos may have the potential to absorb different quantities of TTX depending on their position within the clutch; it is unclear which eggs (first or last) complete yolk deposition immediately prior to ovulation.

Although there may be proximate mechanisms for the differences in TTX across a clutch, we propose an evolutionary explanation for this variation. Rough-skinned newts lay eggs singly over weeks or months in the late spring and early summer (Petranka, 1998). Because the introduction of eggs occurs slowly over time, any potential predator that samples the eggs at the beginning of the cycle may learn to avoid this resource entirely. By investing some minimum threshold of TTX in their embryos, females may be able to protect an entire clutch of eggs, yet also preserve TTX for self-defense or future reproductive events. A similar scenario was demonstrated by Brodie and Formanowicz (1987), who showed that despite being palatable, toad tadpoles at intermediate developmental stages derive protection from predators because both early stage tadpoles and late-stage metamorphosed juveniles are unpalatable. Predators, including dragonfly naiads, giant water bugs, and eastern newts, quickly learn to avoid the unpalatable stages due the presence of toxic bufodienolides. The predator's generalization of avoidance to all toad life-history stages incidentally provides protection despite the absence of toxins. Given the large quantity of TTX that would be deposited in an entire clutch if all eggs contained similar quantities of TTX (2 mg as estimated by Gall et al., 2012a, b), this strategy could have a large effect on the amount of TTX that is available for personal defense by females or future offspring. Given the decline in TTX across a clutch demonstrated here, we estimate 0.53 mg of TTX is invested in an entire clutch by a single female.

Together with the study of Gall et al. (2011), these data provide a clear picture of the ontogenetic changes in TTX in early newt developmental stages. After migrating to a pond and mating, a single female will deposit eggs with varying toxicities depending on their lay order (this study). The most toxic eggs from the initial part of the female's clutch continue developing and ultimately hatch into larvae. These embryos and newly hatched larvae remain as toxic as when they were initially deposited (this study; Gall et al., 2011). As the larvae from this female continue developing, they lose all traces of their yolk and begin feeding on their own (approximately 4 weeks post-hatching). At this point, total TTX declines to approximately 400 ng in each larva (Gall et al., 2011), which is still sufficient to protect the larvae from dragonfly nymphs (Gall et al., 2011). This initial decrease likely accounts for the regained mobility observed in *Ambystoma* by Twitty (Twitty, 1937; Twitty and Johnson, 1934). After the decrease in TTX toxicity, newt larvae continue developing and by 28-weeks begin to undergo metamorphosis. These newly metamorphosed juveniles still retain 400–500 ng of TTX which also provides protection from aquatic predators (Gall et al., 2011).

This study has clarified two major components regarding the ontogenetic and within-clutch changes in TTX toxicity that occur in rough-skinned newts. When combined with previous studies (e.g. Cardall et al., 2004; Gall et al., 2012a, 2011; Hanifin et al., 2002, 2003), these data provide the clearest picture of the origin and within-individual changes in toxicity of any TTX-bearing organism.

Ethical statement

This manuscript "Tetrodotoxin concentrations within a clutch and across embryonic development in the eggs of rough-skinned newts (*Taricha granulosa*)" represents original research that is not being for considered for publication in another journal or publication. All methodologies have been sufficiently described to permit replication by readers. All previously published work cited in the manuscript has been fully acknowledged, and all authors have contributed substantially to the manuscript and approved the final submission. All procedures were approved and performed under Utah State University's animal care and use committee permit #1008R.

Acknowledgments

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Conflict of interest

The authors declare that there are no conflicts of interest and all financial support has been fully acknowledged.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2014.08.060.

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