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Tetrodotoxin levels in lab-reared Rough-Skinned Newts (*Taricha granulosa*) after 3 years and comparison to wild-caught juveniles☆

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ABSTRACT

The origin and biogenesis of tetrodotoxin (TTX) is one of the most interesting and perplexing questions remaining for TTX researchers. Newts can possess extreme quantities of TTX and are one of the most well-studied of all TTX-bearing organisms, yet seemingly conflicting results between studies on closely related species continues to generate debate. In this study, eggs from 12 female newts (*Taricha granulosa*) were reared in captivity and the metamorphosed juveniles were fed a TTX-free diet for 3 years. Using a non-lethal sampling technique, we collected skin samples from each individual each year. Wild-caught juveniles from the same population were also sampled for TTX. In lab-reared juveniles, mass increased rapidly, and after only 2 years individuals approached adult body mass. TTX levels increased slowly during the first two years and then jumped considerably in year three when fed a diet free of TTX. However, wild-caught juvenile newts of unknown age were more toxic than their lab-reared counterparts. These results, coupled with additional data on the long-term production and synthesis of TTX in adult newts suggest that TTX is unlikely to come through dietary acquisition, but rather newts may be able to synthesize their own toxin or acquire it from symbiotic bacteria.

Author contributions

Brian G. Gall: Conceptualization, Methodology, Data curation, analyses, writing, Visualization, reviewing, editing. Amber N. Stokes: Methodology, Validation, Data curation. Edmund D. Brodie III.: Conceptualization, Funding acquisition. Edmund D. Brodie, Jr.: Conceptualization, Supervision, Funding acquisition.

1. Introduction

Tetrodotoxin (TTX) is a potent non-proteinaceous neurotoxin that inhibits the propagation of action potentials in nerve and muscle cells causing rapid asphyxiation and death (Mosher et al., 1964; Narahashi et al., 1967). While the structure of TTX has been known for almost 60 years (Woodward, 1964), debate continues regarding the origin of this toxin, even in well-studied species. Tetrodotoxin has been documented in species occupying freshwater, brackish, marine, and terrestrial environments (Jal and Khora, 2015) including phylogenetically unrelated species such as bacteria, protists, ribbonworms, flatworms, annelids, numerous mollusks, horseshoe crabs, an insect, sea stars, fish, amphibians, and garter snakes (Hanifin, 2010; Jal and Khora, 2015; Lorentz et al., 2016; Miyazawa and Noguchi, 2001; Stokes et al., 2014; Strand et al., 2016; Williams, 2010). Part of the difficulty in evaluating the origin of TTX may be due to the existence of multiple evolutionary pathways in which these various organisms have likely come to acquire the toxin.

Cumulative evidence on a diversity of species indicates three potential routes by which organisms acquire TTX. First, numerous bacteria have been reported to produce TTX (Jal and Khora, 2015). From these bacteria (or other TTX producing organisms) TTX may be acquired by higher organisms via accumulation through the food chain. For example, pufferfish fed a diet containing TTX accumulate the toxin in the liver which may then be shunted to other organs (Kono et al., 2008; Kudo et al., 2017). Itoi et al. (2018) conducted a thorough investigation

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on the relationship between pufferfish and TTX-containing flatworms. The authors demonstrated that pufferfish at all developmental stages eat these flatworms, and this directly contributes to the toxicity of pufferfish at all stages of their life cycle. Second, TTX-producing bacteria may also reside symbiotically in metazoans, thus providing a continuous supply of TTX that would not depend on dietary acquisition. Tetrodotoxin has been detected in bacteria cultures collected from the skin and ovaries of pufferfish (Yang et al., 2010; Yotsu et al., 1987), and may be responsible for an increase in TTX toxicity in paralarval blue-ringed octopuses (Williams et al., 2010). While Lehman et al. (2004) failed to find bacterial DNA in Rough-Skinned Newts (*Taricha granulosa*), a recently conducted study by Vaelli et al. (2020) isolated TTX-producing bacteria from the skin of toxic newts. Third, some species may be capable of producing tetrodotoxin endogenously. This hypothesis is supported by a number of observational and life-history studies in Rough-Skinned Newts. For example, newts reared long-term in captivity under a TTX-free diet maintain or increase their toxicity (Gall et al., 2012; Hanifin et al., 2002) and are even capable of regenerating TTX after it is released via electrical stimulation (Cardall et al., 2004). In addition, toxic newts that are "milked" of their toxin via mild electrical stimulation and maintained on an antibiotic regimen for a full year also regenerate TTX (E. Brodie III., unpublished data). Recently, Mailho--Fontana et al. (2019) discovered that TTX concentration in newts is correlated with the volume of a particular cell type found in the granular glands, indicating a relationship between toxicity and the morphological structure of the granule. Most convincingly however is the recent discovery of two new TTX analogs, two bicyclic guanidinium shunt products for TTX, and a proposed biosynthetic pathway for TTX production in terrestrial newts (Kudo et al. 2020, 2021). Of course, these hypotheses may not be mutually exclusive, and it is also possible that some species require a combination of mechanisms such as dietary TTX precursors which are then modified endogenously or by bacteria to produce TTX.

To complicate the understanding of TTX production, studies on closely related species of newts have yielded mixed results. For example, the Japanese Fire-Bellied Newt (*Cynops pyrrhogaster*) fed a diet containing TTX convert approximately 40% of that toxin into their body and viscera (Kudo et al., 2017), indicating dietary acquisition of TTX may be possible. In addition, a long-term study on this same newt found that endogenous production of TTX is unlikely (Kudo et al., 2015; Mebs and Yotsu-Yamashita, 2021). When reared in captivity from eggs, these newts slowly lose their maternally derived TTX, and at metamorphosis are non-toxic (Kudo et al., 2015). When these juveniles were reared in the lab on a TTX-free diet for a year after hatching, no measurable TTX was detected (Kudo et al., 2015). Yet, when reared with their parents, larvae and juveniles possessed TTX (Mebs and Yotsu-Yamashita, 2021). The slow decline in maternally-provided TTX demonstrated in these studies is very similar to that which occurs in Rough-Skinned Newts (Gall et al., 2011). Yet, TTX levels of lab-reared Rough-Skinned Newts are unknown beyond metamorphosis. We investigated changes in toxicity of Rough-Skinned Newts raised from eggs in the lab. Juvenile newts were reared on a TTX-free diet and their toxicity was assessed yearly for three years. In addition, we sampled TTX from wild-caught juvenile newts from this same population and compare them to lab-reared juveniles.

2. Materials and methods

Twelve gravid female newts were collected by hand from a series of manmade ponds near Corvallis OR (Benton CO) 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 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. Eggs were collected and larvae were reared according to Gall et al. (2011). Briefly, eggs were transferred into 9 cm diameter plastic dishes with 50 mL filtered water until hatching. Upon hatching, larvae were transferred to 5.7 L plastic containers and fed *Daphnia pulex* and blackworms (*Lumbriculus variegatus*) ad libitum. Newts, eggs, and larvae were rinsed with filtered water at each transfer and/or water change. After two weeks, larvae were transferred into 37L tanks filled with 30L of water and dry grass from the edges of a pond near Logan, UT. Blackworms were added to these tanks twice weekly.

Larvae initiated metamorphosis between November 2010 and November 2011. Containers were monitored weekly for newts initiating metamorphosis, and when identified, groups of metamorphs were transferred to a 5-L container. The container was positioned at an angle creating a small pool at one end which sloped upward into a terrestrial area containing damp sphagnum moss. Once in the terrestrial portion of the container, groups of 2–14 newts were transferred into 5 L containers containing damp sphagnum. Metamorphs were fed fruit flies (*Drosophila melanogaster*) and blackworms three times per week for the first 6 months, followed by blackworms only until the completion of the study (year 3). The containers were cleaned, and sphagnum moss was replaced bi-weekly. Individual newts from a single container were given a unique number and toe clipped accordingly to monitor changes in toxicity of individuals over time. Individuals were re-clipped every 3 months. As the newts grew, groups of juveniles from the same metamorphic cohort were moved into larger containers as needed.

Tetrodotoxin was assessed 1-, 2-, and 3-years post metamorphosis in each individual. Skin punches were collected on the specific date corresponding with the initiation of metamorphosis for each group of individuals, and not on the same date for all samples. In addition, skin samples were collected from wild-caught juvenile newts in March 2011 and 2012. Wild-caught juveniles (N = 16) were collected on rainy nights from two roads that border the same breeding site from which gravid females were collected. Individual newts were anesthetized via immersion in 1% tricaine methanesulfonate (MS222) buffered to a pH of 7 and a 3 mm piece of dorsal skin was removed from each individual using a human skin biopsy punch (Acupunche Acuderm, Inc.) (Hanifin et al., 2004). Sample collection was alternated between the right and left sides of the body such that two years separated samples in the same general region on each lab-reared individual. Samples were immediately frozen (−80 C) in individual microcentrifuge tubes until TTX extraction and quantification; dorsal skin samples from wild-caught juvenile newts were collected and prepared in the same manner as described above. Extraction of skin punches was performed according to Hanifin et al. (2002), and quantification was executed 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 calculate 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 [22]. All plates were read at 405 nm.

The TTX present in each skin punch was then used to estimate total TTX and TTX/mg body mass, as per Hanifin et al. (2004). These data were treated as a randomized complete block design with the mean of animals within each tray in each year as the response variable. A linear mixed model fit by REML with a one-factor treatment (Year Punched) in which animals were nested within trays and repeated measurements were made on animals over time was used for the analysis. Only animals with data for all three years were included in this analysis (N = 52 newts from 12 trays); 6 animals died over the course of the study. Total TTX and TTX concentration were log-transformed to meet assumptions of normality and homogeneity of variance. Analyses were conducted in SAS v9.4.

3. Results

After reaching metamorphosis, juvenile newts acclimated to artificial conditions and grew quickly. After only 2 years, many individuals approached or exceeded the mass (~10g) of breeding adult newts collected in the wild (Fig. 1). While the age of wild-caught juvenile newts is unknown, the masses overlap with those of lab-reared newts (Fig. 1), and based on the minimal overlap between clusters of individuals we suspect that they encompass at least 4 unique pre-breeding age cohorts.

A linear mixed model detected a highly significant effect of year on the total TTX present in lab-reared newts ($F_{[2,22]} = 24.9$, $P < 0.0001$). In this case, TTX increased approximately 6-fold between metamorphosis (mean ± SE; 0.444 ± 0.062 μg (see Gall et al., 2011)) and year one (2.7 ± 0.67 μg), remained relatively unchanged through year two (1.5 ± 0.39 μg), and increased ten-fold in year three (15 ± 3.9 μg; Fig. 2). Tukey post-hoc comparisons found that year 3 was significantly higher than year 1 and year 2 ($P \leq 0.0001$), but the total TTX present in lab-reared newts 1 and 2 years after metamorphosis were not significantly different ($P = 0.25$). Similarly, TTX concentration in lab-reared newts was also significantly different across the three years of this study ($F_{[2,22]} = 16.7$, $P < 0.0001$), with newts in year two (mean ± SE; 0.225 ± 0.059 μg TTX/g body mass) having the lowest TTX concentration and TTX concentration being highest in year 3 (1.84 ± 0.49 μg TTX/g body mass; Fig. 2; Tukey post-hoc comparisons all $P \leq 0.01$).

Wild-caught juvenile newts had substantially higher total TTX (mean ± SE; 222 ± 240 μg) and TTX concentrations (48.43 ± 6.8 μg TTX/g) relative to newts reared in the lab for three years (Fig. 3). While comparing these values is difficult due to the unknown age of wild-caught juveniles, wild-caught juveniles that are likely 2–3 years post-metamorphosis (based on apparent breaks in mass between groups) have, on average, approximately 10 times more total TTX than lab-reared newts of the same age (Fig. 3).

4. Discussion

After three years on a TTX-free diet under laboratory conditions, juvenile Rough-Skinned Newts possessed 34x more total TTX than at metamorphosis. Tetrodotoxin increased during the first year, remained relatively unchanged through year two, and finally increased again during the third year. While these data do not exclude the possibility of accumulating TTX via dietary sources, when combined with previous data on adult newts (Cardall et al., 2004; Gall et al., 2012; Hanifin et al., 2002), dietary acquisition of tetrodotoxin seems unlikely to be the primary mechanism of toxicity in Rough-Skinned Newts. In this case, either symbiotic bacteria, or endogenous production of TTX by the newts themselves, is the likely mechanism by which TTX is acquired.

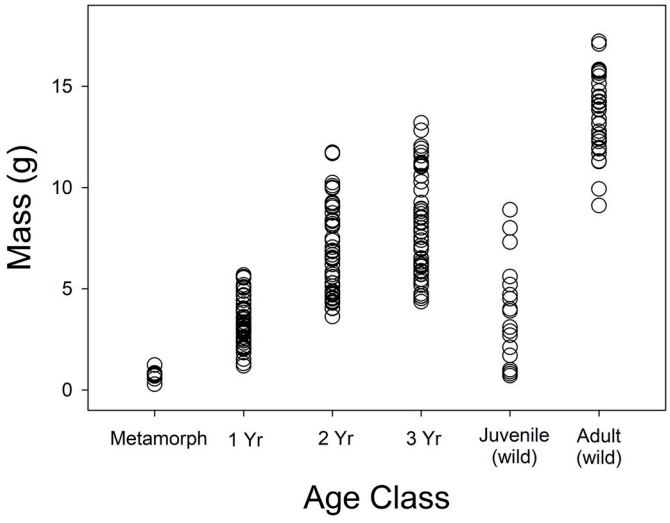

Fig. 1. Comparison of Rough-Skinned Newt (*Taricha granulosa*) mass (g) from individuals in various age classes. Open circles represent data from individual animals. Metamorph, 1 year old (1 Yr), 2 year old (2 Yr), and 3 year old (3 Yr) were reared in captivity from eggs on a TTX-free diet and with no exposure to adult newts. Juvenile (wild) and Adult (wild) newts were sampled from the same population in which the lab-reared individuals descended.

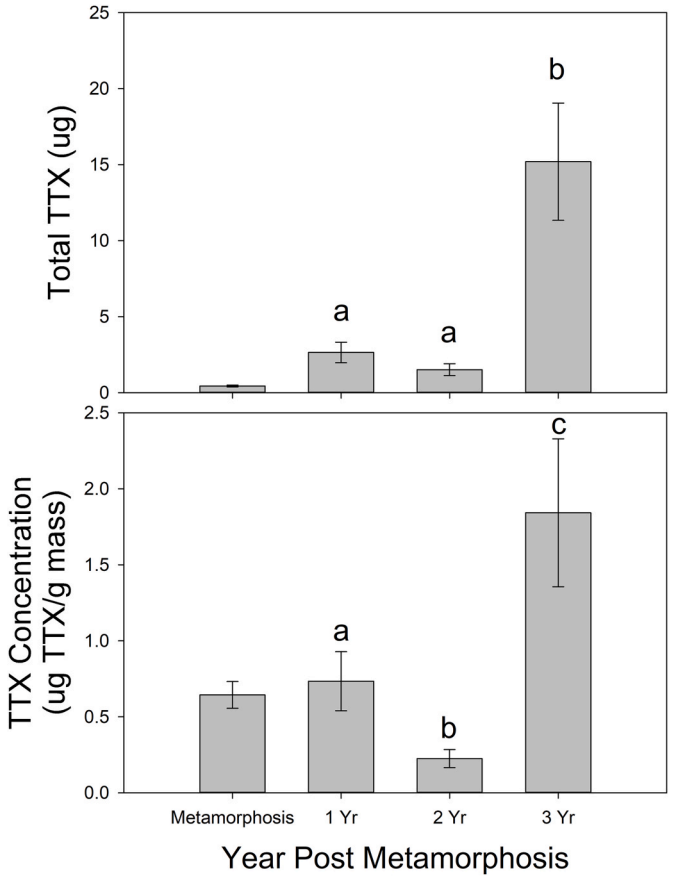

Fig. 2. Mean (±SE) total TTX (μg) present (top) and TTX concentration (bottom) in Rough-Skinned Newts reared from eggs in the lab on a TTX-free diet for three years. Tetrodotoxin values for recently metamorphosed newts are provided for reference from Gall et al. (2011) and were not included in the analysis. Different letters indicate significant differences in total TTX and TTX concentration between years (Tukey post-hoc comparisons, all $P \leq 0.01$).

These results are in stark contrast to data on another newt, *Cynops pyrrhogaster*. Like *Taricha* (Gall et al., 2011), *Cynops* reared from eggs slowly lose their maternally derived toxicity during larval development (Kudo et al., 2015). However, it is at metamorphosis that differences in toxicity and toxin acquisition begin to appear. While Fire-Bellied Newts are non-toxic at metamorphosis (Kudo et al., 2015), Rough-Skinned Newts retain measurable quantities of TTX which can be enough to repel potential predators (Gall et al., 2011, 2014). When Fire-Bellied Newts were reared in the lab for a year, TTX was not detected (Kudo et al., 2015). Yet captive newts in our study did increase in toxicity during this same time frame on a TTX-free diet. Such markedly different results are surprising given the TTX-bearing phenotype occurs within the Modern Newt clade of the family Salamandridae; with few exceptions tetrodotoxin presence among animals appears phylogenetically haphazard (Hanifin, 2010; Jal and Khora, 2015; Lorentz et al., 2016). These newts have a series of mutations in the sodium-channel proteins that confer TTX resistance (Hanifin and Gilly, 2015; Gendreau et al., 2021). While some of these mutations' pre-date the origin of tetrodotoxic newts, others co-occur with the ancestor of all newts and during the subsequent divergence of the clade (Hanifin and Gilly, 2015; Kudo

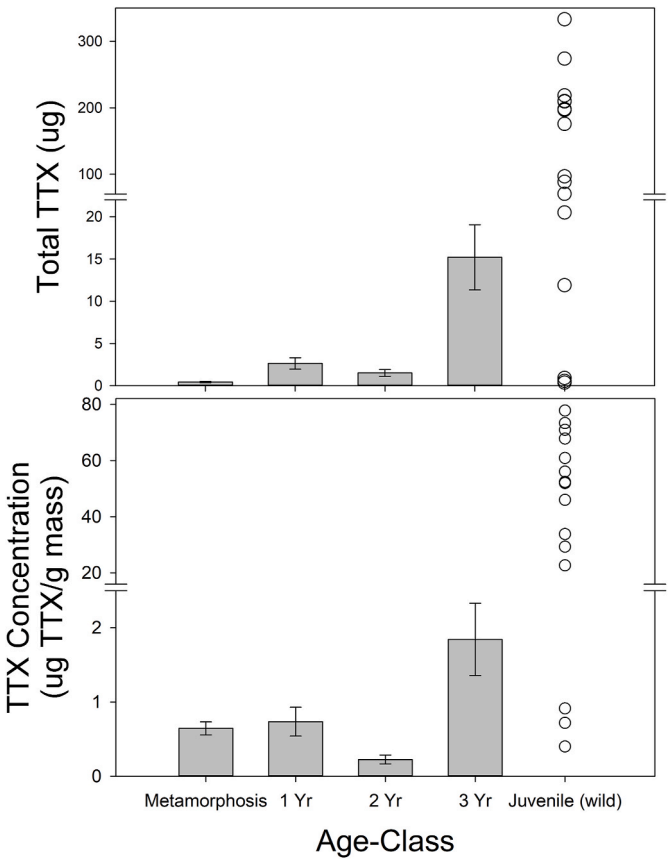

Fig. 3. Mean (±SE) total TTX (μg) (top) and TTX concentration (bottom) in Rough-Skinned Newts reared from eggs in the lab on a TTX-free diet for three years compared to toxicity of juvenile newts collected in the wild (open circles). Tetrodotoxin values for recently metamorphosed newts are provided for reference from Gall et al. (2011). Line breaks and data from the most toxic 4 wild-caught juvenile newts were excluded to enhance visual acuity of the lab-reared means.

et al., 2017; Gendreau et al., 2021). While the most basal Salamandrids (True salamanders & Primitive newts) do not possess TTX, the Modern newt genera do possess the TTX-bearing phenotype (Hanifin and Gilly, 2015; Kudo et al., 2017; Yotsu-Yamashita et al., 2017) indicating a phylogenetic component to both TTX resistance and presence and making different results surprising. Regardless, discretion should be taken when making direct comparisons between these species. Our study site includes the most toxic known population of Rough-Skinned Newts, with adults possessing as much as 30 mg of TTX and individual eggs containing 1–4 μg TTX (Hanifin et al., 2003; Gall et al. 2012, 2014). Fire-Bellied Newts are substantially less toxic with average TTX concentration of 36 μg TTX per gram in adults and a sample of 16 pooled eggs containing only 1 μg (Mebs and Yotsu-Yamashita, 2021).

Given these stark differences in TTX production, we perceive two hypotheses for the origin of TTX in these newts. First, as in some marine systems, newts may not produce TTX themselves, but rather depend on the presence of symbiotic bacterial species. This is supported by resent research on Rough-Skinned Newts whereby population-level differences in the presence of bacterial species was discovered between toxic and non-toxic populations of newts (Vaelli et al., 2020). In this case, numerous bacterial genera were isolated and cultured from the skin of toxic newts, some of which were found to produce TTX (Vaelli et al., 2020). These data are particularly interesting when coupled with recent research on metamorphic Fire-Bellied Newts. When reared with their parents, juvenile Fire-Bellied Newts possess TTX, yet when reared apart they are non-toxic (Mebs and Yotsu-Yamashita, 2021); although the sample size of Mebs and Yotsu-Yamashita (2021) is exceedingly small and most juveniles were reared for less than half of the duration of our study. Together these studies suggest that either 1) some type of natural bacterial acquisition and subsequent sequestration by the host newt may be responsible for TTX toxicity, or 2) a chemical signal produced by the adult newts induces an endogenous TTX pathway in the offspring. If TTX is acquired symbiotically, the results presented here, combined with those of Mebs and Yotsu-Yamashita (2021), indicates direct vertical transmission of TTX-producing bacteria from females to their eggs is unlikely.

The second hypothesis for TTX production in newts is that the necessary biosynthetic pathway for TTX production is present in the newt genome itself (e.g. endogenous TTX production). Larvae in our study were reared in benign laboratory conditions in the absence of any wild-caught newts and without any natural products from a field-site in which newts are present. While bacteria may be capable of producing large quantities of TTX under natural conditions, TTX-producing bacterial isolates have never been found that produce ecologically relevant levels of TTX in the lab (Vaelli et al., 2020). When combined with the rapid increase in TTX in lab-reared Rough-Skinned Newts and the rapid regeneration of large quantities of TTX in adult newts in the lab (Cardall et al., 2004), it seems unlikely that these large volumes could be sequestered strictly from symbiotic bacteria residing in or on these newts. Until recently, the endogenous origin hypothesis was supported largely by observational studies (such as this one) on the ontogeny of TTX in various species, and differentiation between competing hypotheses was impossible without a proposed mechanism for the synthesis or sequestration of TTX. Kudo et al. (2020) recently described new TTX analogs and proposed a biosynthetic and metabolic pathway for TTX production in terrestrial newts. This hypothesis provides a functional template for endogenous TTX production in terrestrial species (Kudo et al., 2020). When combined with the discovery of two novel bicyclic intermediates in the proposed pathway (Kudo et al., 2021), these data support an endogenous pathway as the mechanism for the production of TTX and its analogs in terrestrial TTX-producers, including the Fire-Bellied Newt and Rough-Skinned Newt (Kudo et al. 2020, 2021).

While it is difficult to compare the toxicity of lab-reared newts to wild juvenile newts of unknown age, some general comparisons can be made. First, the sizes of wild-caught juvenile newts suggest at least 3 or 4 discrete cohorts, and previous research has suggested that 4–8 years are necessary for juveniles to reach sexual maturity (Chandler, 1918; Twitty, 1961). While the pattern of TTX toxicity (total and concentration) in the smallest of these wild juveniles overlaps with our lab reared newts, most wild juveniles were considerably more toxic. Any number of factors could explain this discrepancy between mass, age, and toxicity. For example, lab-reared newts were essentially fed a single food item for the entirety of their life. While free of TTX, nutritional diversity may be necessary to produce the large quantities of TTX seen in wild juveniles. Alternatively, the difference in toxicity between lab and wild juveniles may support the hypothesis that symbiotic bacteria produce TTX and that lab-reared newts simply lack the bacterial diversity or abundance to produce these quantities. Finally, the abundance of food and subsequent rapid rate of growth, coupled with the relative “safety” of the lab environment, may have facilitated the lower overall TTX levels in lab-reared newts. The production of TTX may incur a considerable energetic investment that must be balanced with other life-history traits such as growth. Such trade-offs involving toxin production and growth/reproduction exist in plants, invertebrates, and vertebrates, including other amphibians (Glynn et al., 2007; Agrawal et al., 2010; Leong and Pawlik 2010; Blennerhassett et al., 2019; Harris and Jenner 2019). For example, a study in Cane toads (*Rhinella marina*) found a negative correlation between the amount of stored toxin and the size of the liver and gonads suggesting a tradeoff between the production of defensive chemicals and both energy metabolism and reproduction (Blennerhassett et al., 2019). Experimental manipulations of toxin stores also led to

reduced growth rates in both captive and wild toads (Blennerhassett et al., 2019). This is particularly interesting given that amphibians have also been documented to differentially allocate defensive chemicals based on numerous ecological conditions, including relative predation risk (Benard and Fordyce 2003; Uveges et al., 2017; Bókony et al., 2019), as well as the finding that another closely related species of newt, *Taricha torosa*, can induce TTX defenses in response to simulated predation risk (Buciarelli et al., 2017). Furthermore, newt larvae reared in the lab and under semi-natural conditions differed in TTX and body condition, suggesting they may be capable of differentially allocating chemical defenses based on environmental stimuli (Buciarelli et al., 2017). The finding of such inducible defenses in *Taricha* suggest that the abundance of food, combined with the relative safety of the lab environment, may have led to the prioritization of energy allocated to growth and minimized allocation to defense.

Tetrodotoxin production in animals, and especially in newts, is one of the most enigmatic and contested questions still posed by those interested in natural toxins. While we cannot rule out all competing hypotheses, the production and increase in TTX by lab-reared newts over several years indicates that it is unlikely that newts acquire TTX via the food chain. These results can be interpreted to support both of the remaining hypotheses of endogenous toxin production and bacterial TTX production and sequestration by newts. However, when combined with the recent proposal of a biosynthetic pathway and the associated discovery of TTX intermediates within this pathway (Kudo et al. 2020, 2021), it seems plausible that at least some terrestrial species may be capable of producing TTX endogenously.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Agrawal, A.A., Conner, J.K., Rasmann, S., 2010. Tradeoffs and negative correlations in evolutionary ecology. In: Bell, M.A., Futuyma, D.J., Eanes, W.F., Levinton, J.S. (Eds.), Evolution since Darwin: the First 150 Years. Sinauer Assocates, Sunderland, MA, pp. 243–268.

Benard, M.F., Fordyce, J.A., 2003. Are induced defenses costly? Consequences of predator-induced defenses in western toads, *Bufo boreas*. Ecology 84, 68–78.

Blennerhassett, R.A., Bell-Anderson, K., Shine, R., Brown, G.P., 2019. The cost of chemical defence: the impact of toxin depletion on growth and behaviour of cane toads (*Rhinella marina*). Proc Royal Soc B 286, 20190867.

Bókony, V., Üveges, B., Verebélyi, V., Ujhegyi, N., 2019. Toads phenotypically adjust their chemical defences to anthropogenic habitat change. Sci. Rep. 9, 1–8.

Buciarelli, G.M., Shaffer, H.B., Green, D.B., Kats, L.B., 2017. An amphibian chemical defense phenotype is inducible across life history stages. Scientific Reports 7, 1–8.

Cardall, B.L., Brodie , Jr., B.D., Brodie III., E.D., Hanifin, C.T., et al., 2004. Secretion and regeneration of tetrodotoxin in the rough-skin newt (Taricha granulosa). Toxicon 44, 933–938.

Chandler, A.C., 1918. The western newt or water-dog (*Notophthalmus torosus*) - a natural enemy of mosquitoes. Oregon Agricult Coll Exp Stat Bull 15, 1–24.

Gall, B.G., Stokes, A.N., French, S.S., Brodie III., E.D., Brodie Jr., E.D., 2012. Female newts (*Taricha granulosa*) produce tetrodotoxin laden eggs after long term captivity. Toxicon 60, 1057–1062.

Gall, B.G., Stokes, A.N., French, S.S., Schlepphorst, E.A., Brodie, E.D., Brodie, E.D., 2011. Tetrodotoxin levels in larval and metamorphosed newts (*Taricha granulosa*) and palatability to predatory dragonflies. Toxicon 57, 978–983.

Gall, B.G., Stokes, A.N., Pett, J.J., Spivey, K.L., French, S.S., Brodie III., E.D., Brodie Jr., E.D., 2014. Tetrodotoxin concentrations within a clutch and across embryonic development in eggs of the Rough-Skinned Newts (*Taricha granulosa*). Toxicon 90, 249–254.

Gendreau, K.L., Hornsby, A.D., Hague, M.T.J., McGlothlin, J.W., 2021. Gene conversion facilitates the adaptive evolution of self-resistance in highly toxic newts. Mol. Biol. Evol. 38, 4077–4094.

Glynn, C., Herms, D.A., Orians, C.M., Hansen, R.C., Larsson, S., 2007. Testing the growth–differentiation balance hypothesis: dynamic responses of willows to nutrient availability. New Phytol. 176, 623–634.

Hanifin, C.T., 2010. The chemical and evolutionary ecology of tetrodotoxin (TTX) toxicity in terrestrial vertebrates. Mar. Drugs 8, 577–593.

Hanifin, C.T., Brodie III., E.D., Brodie Jr., E.D., 2002. Tetrodotoxin levels of the rough-skin newt, *Taricha granulosa*, increase in long-term captivity. Toxicon 40, 1149–1153.

Hanifin, C.T., Brodie III., E.D., Brodie Jr., E.D., 2003. Tetrodotoxin Levels in Eggs of the Rough-Skinned Newt, *Taricha granulosa*, Are Correlated with Female Toxicity.

Hanifin, C.T., Brodie III., E.D., Brodie Jr., E.D., 2004. A predictive model to estimate total skin tetrodotoxin in the newt *Taricha granulosa*. Toxicon 43, 243–249.

Hanifin, C.T., Gilly, W.F., 2015. Evolutionary history of a complex adaptation: tetrodotoxin resistance in salamanders. Evolution 69, 232–244.

Harris, R.J., Jenner, R.A., 2019. Evolutionary ecology of fish venom: adaptations and consequences of evolving a venom system. Toxins 11, 60.

Itoi, S., Ueda, H., Yamada, R., Takei, M., Sato, T., Oshikiri, S., Wajima, Y., Ogata, R., Oyama, H., Shitto, T., Okuhara, K., Tsunashima, T., Sawayama, E., Sugita, H., 2018. Including planocerid flatworms in the diet effectively toxifies the pufferfish, *Takifugu niphobles*. Sci. Rep. 8, 12302.

Jal, S., Khora, S.S., 2015. An overview on the origin and production of tetrodotoxin, a potent neurotoxin. J. Appl. Microbiol. 119, 907–916.

Kono, M., Matsui, T., Furukawa, K., Yotsu-Yamashita, M., Yamamori, K., 2008. Accumulation of tetrodotoxin and 4,9-anhydrotetrodotoxin in cultured juvenile kusafugu *Fugu niphobles* by dietary administration of natural toxic komonfugu *Fugu poecilonotus* liver. Toxicon 51, 1269–1273.

Kudo, Y., Chiba, C., Konoki, K., Cho, Y., Yotsu-Yamashita, M., 2015. Confirmation of the absence of tetrodotoxin and its analogues in the juveniles of the Japanese Fire-Bellied Newt, *Cynops pyrrhogaster*, captive-reared from eggs in the laboratory using HILIC-LC-MS. Toxicon 101, 101–105.

Kudo, Y., Chiba, C., Konoki, K., Cho, Y., Yotsu-Yamashita, M., 2017. Dietary administration of tetrodotoxin and its putative biosynthetic intermediates to the captive-reared non-toxic Japanese Fire-Bellied Newt, *Cynops pyrrhogaster*. Toxicon 137, 78–82.

Kudo, Y., Hanifin, C.T., Kotaki, Y., Yotsu-Yamashita, M., 2020. Structures of N-Hydroxy-Type tetrodotoxin analogues and bicyclic guanidinium compounds found in toxic newts. J. Nat. Prod. 83, 2706–2717.

Kudo, Y., Hanifin, C.T., Yotsu-Yamashita, M., 2021. Identification of tricyclic guanidino compounds from the tetrodotoxin-bearing newt *Taricha granulosa*. Org. Lett. 23, 3513–3517.

Lehman, E.M., Brodie Jr., E.D., Brodie III., E.D., 2004. No evidence for an endosymbiotic bacterial origin of tetrodotoxin in the newt *Taricha granulosa*. Toxicon 44, 243–249.

Leong, W., Pawlik, J.R., 2010. Evidence of a resource trade-off between growth and chemical defenses among Caribbean coral reef sponges. Mar. Ecol.: Prog. Ser. 406, 71–78.

Lorentz, M.N., Stokes, A.N., Rößler, D.C., Lötters, S., 2016. Tetrodotoxin. Curr. Biol. 26, R870–R872.

Mailho-Fontana, P.L., Jared, C., Antoniazzi, M.M., Sciani, J.M., Pimenta, D.C., Stokes, A. N., Grant, T., Brodie, E.D., III., Brodie, E.D., Jr Variations in tetrodotoxin levels in populations of *Taricha granulosa* are expressed in the morphology of their cutaneous glands. Sci. Rep. 9, 1-8.

Mebs, D., Yotsu-Yamashita, M., 2021. Acquiring toxicity of a newt, *Cynops orientalis*. Toxicon 198, 32–35.

Miyazawa, K., Noguchi, T., 2001. Distribution and origin of tetrodotoxin. J. Toxicol. Toxin Rev. 20, 11–33.

Mosher, H.S., Fuhrman, F.A., Buchwald, H.D., Fischer, H.G., 1964. Tarichatoxin-tetrodotoxin: a potent neurotoxin. Science 144, 1100–1110.

Narahashi, T., Moore, J.W., Poston, R.N., 1967. Tetrodotoxin derivatives: chemical structure and blockage of nerve membrane conductance. Science 156, 976–979.

Stokes, A.N., Ducey, P.K., Neuman-Lee, L.A., Hanifin, C.T., French, S.S., Pfrender, M.E., Brodie III, E.D., Brodie, J., E. D., 2014. Confirmation and distribution of tetrodotoxin for the first time in terrestrial invertebrates: two terrestrial flatworm species (*Bipalium adventitium* and *Bipalium kewense*). PLoS One 9, e100718.

Stokes, A.N., Williams, B.L., French, S.S., 2012. An improved competitive inhibition enzymatic immunoassay method for tetrodotoxin quantification. Biol. Proced. Online 14, 1–5.

Strand, M., Hedström, M., Seth, H., McEvoy, E.G., Jacobsson, E., Göransson, U., Andersson, H.S., Sundberg, P., 2016. The bacterial (Vibrio alginolyticus) production of tetrodotoxin in the ribbon worm Lineus longissimus—just a False Positive? Mar. Drugs 14, 63.

Twitty, V.C., 1961. Second-generation hybrids of the species of Taricha. Proc Natl Acad Sci USA 47, 1461–1486.

Uveges, B., Fera, G., Moricz, A.M., Kruzselyi, D., Bokony, V., Hettyey, A., 2017. Age-and environment-dependent changes in chemical defences of larval and post-metamorphic toads. BMC Evol. Biol. 17, 1–10.

Vaelli, P.M., Theis, K.R., Williams, J.E., O'Connell, L.A., Foster, J.A., Eisthen, H.L., 2020. The skin microbiome facilitates adaptive tetrodotoxin production in poisonous newts. Elife 9, e53898.
Williams, B.L., 2010. Behavioral and chemical ecology of marine organisms with respect to Tetrodotoxin. Mar. Drugs 8, 381–398.
Williams, B.L., Hanifin, C.T., Brodie Jr., E.D., Caldwell, R.L., 2010. Ontogeny of tetrodotoxin levels in Blue-ringed Octopuses: maternal investment and apparent independent production in offspring of *Hapalochlaena lunulata*. J. Chem. Ecol.
Woodward, R.B., 1964. The structure of tetrodotoxin. Pure Appl. Chem. 9, 49–74.
Yang, G., Xu, J., Liang, S., Ren, D., Yan, X., Bao, B., 2010. A novel TTX-producing Aeromonas isolated from the ovary of *Takifugu obscurus*. Toxicon 56, 324–329.
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-Yamashita, M., Toennes, S.W., Mebs, D., 2017. Tetrodotoxin in asian newts (Salamandridae). Toxicon 134, 14–17.