Female newts (*Taricha granulosa*) produce tetrodotoxin laden eggs after long term captivity

Brian G. Gall [a],*, Amber N. Stokes [b], Susannah S. French [b], Edmund D. Brodie III [c], Edmund D. Brodie Jr. [b]

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

ARTICLE INFO

ABSTRACT

We investigated the presence of tetrodotoxin (TTX) in the eggs of wild-caught newts (*Taricha granulosa*) at capture and again after one, two, and three years in captivity. Females initially produced eggs that contained quantities of TTX similar to previous descriptions of eggs from wild-caught adults. After the first year in captivity, the egg toxicity from each female declined, ultimately remaining constant during each of the successive years in captivity. Despite declining, all females continued to produce eggs containing substantial quantities of TTX during captivity. The decline in toxicity can not be attributed to declining egg mass but may be the result of the abbreviated reproductive cycle to which the captive newts were subjected in the lab. Finally, an estimate of the amount of TTX provisioned in the entire clutch from each female is similar to the quantity of TTX regenerated in the skin after electrical stimulation. These results, coupled with other long-term studies on the maintenance and regeneration of TTX in the skin, suggests an endogenous origin of TTX in newts.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

One of the most deadly naturally occurring compounds is tetrodotoxin (TTX). This neurotoxin is a non-proteinaceous water-soluble guanidinium ion, consisting of a complex carbon ring-structure with associated amine and aminal groups (Mosher et al., 1964; Narahashi et al., 1967). Organisms in 17 different orders distributed across eight phyla possess TTX (Chau et al., in press; Miyazawa and Noguchi, 2001), yet the only clear phylogenetic pattern in its occurrence falls within a small number of families (e.g. Salamandridae, Tetraodontidae). This seemingly "random" distribution has made characterizing the mechanism by which organisms acquire the toxin, as well as the biosynthetic pathway by which TTX is produced exceedingly difficult, and little solid evidence has been gathered on either of these fronts.

Three major hypotheses exist to explain the acquisition of TTX in vertebrates and invertebrates; (1) symbiotic bacteria produce TTX which is then sequestered by the host organism, (2) tetrodotoxin toxicity occurs through bio-accumulation via the food chain, (3) TTX is produced endogenously. The model widely accepted for marine organisms involves the production of TTX by symbiotic bacteria (Simidu et al., 1987; Wang et al., 2010). However, many of the marine organisms found to possess TTX are eaten by larger vertebrates, such as pufferfish, that are resistant to the toxin (Venkatesh et al., 2005). In these cases, it is believed that TTX toxicity occurs through bio-accumulation through the food chain (Noguchi et al., 2006). Finally, some organisms, amphibians in particular, do not appear to fit either of these models and may be able to directly synthesize TTX (reviewed by Hanifin, 2010).

* Corresponding author. Tel.: +1 217 653 3037.
  E-mail address: gall@hanover.edu (B.G. Gall).

Until the pathway for TTX production is discovered, researchers must utilize indirect evidence on the distribution of TTX to infer where and how this toxin is formed. Regardless of the mechanism by which TTX is acquired, understanding temporal and ontogenetic changes in TTX toxicity would allow a more in-depth assessment of the hypotheses for TTX production. Despite decades of research, ontogenetic or temporal changes in toxicity are poorly understood in most organisms. For example, the puffer fish (Order: Tetraodontiformes) have been intensively studied for over 100 years (Chau et al., in press; Fuhrman, 1986) and although TTX is present in high concentrations in the liver, ovaries, and skin of wild-collected adult puffer fish (e.g. Jang and Yotsu-Yamashita, 2006), it is virtually unknown if toxicity changes over time in adults or larvae/juveniles (but see Nunez-Vazquez et al., 2012).

Individual and ontogenetic changes in toxicity are probably best understood in the newts (Salamandridae). In newts large quantities of TTX are located in the skin, while minute amounts can be found in other tissues such as muscle and blood (Wakely et al., 1966). There is tremendous within and between population variation in tetrodotoxin toxicity across large spatial scales in *Taricha granulosa*, which is believed to be the result of coevolution with a snake predator (Brodie and Brodie, 1990; Brodie et al., 2002; Feldman et al., 2012; Geffeney et al., 2002; Hanifin et al., 2008, 1999; Williams et al., 2010a). Long-term lab studies indicate that the toxicity of the skin not only increases over time but can be regenerated after excretion, despite being maintained on a diet that does not contain TTX (Cardall et al., 2004; Hanifin et al., 2002).

In newts, high levels of TTX are also found in the ovaries, ova, and recently deposited eggs (Hanifin et al., 2003; Mosher et al., 1964; Wakely et al., 1966). Although several recent studies have expanded our understanding of changes in TTX during early-life history stages (Gall et al., 2011b; Tsuruda et al., 2002), only one study has quantified the toxicity of individual eggs from females (Hanifin et al., 2003). Further, it is unknown if toxicity changes between successive clutches from a female or whether TTX is deposited in the eggs when females are reared in captivity. To better understand the lability of TTX production in female newts, we quantified the amount of TTX in newt eggs immediately after gravid females were collected from the wild and after one, two, and three years in captivity. Additionally, we estimated the total amount of toxin provisioned in a clutch by each female and compare this to published reports of TTX regeneration in newts from the same population.

2. Materials and methods

2.1. Animal collection and maintenance

We quantified the amount of TTX in a subset of eggs from each of 3 or 4 clutches of eggs deposited in successive years by female *T. granulosa*. Gravid female newts (*T. granulosa*) were collected in March 2009 ($N = 3$) and 2010 ($N = 5$) from Soap Creek ponds in the central Willamette Valley, OR. This population is well studied (Gall et al., 2011a,

2011b; Hanifin et al., 2002, 1999, 2003), and includes the most toxic known newts; a single individual may contain up to 28 mg of tetrodotoxin (Stokes et al., submitted) which is the oral lethal dose for as many as 56 humans (Yasumoto and Yotsu-Yamashita, 1996). After collection, 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 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. In nature *T. granulosa* deposit eggs singly on aquatic vegetation over several weeks (Petranka, 1998). In the lab, 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. A small subset of eggs from each female was collected less than 48 h after deposition and frozen at −80 °C for TTX quantification. The mass of each egg was recorded prior to freezing (2011 & 2012) or after freezing (2009 & 2010). To account for a change in mass due to freezing, 20 eggs that were weighed prior to freezing were thawed and re-weighed. The change in mass was calculated for each of these re-weighed eggs and the mean change (−7.18%) was added to each egg collected in 2009 and 2010. These adjusted values were used in all analyses.

After a female had deposited all of the eggs from this initial clutch (approximately 3 weeks) the polyester fiber was removed and a small piece of foam was placed in the container to provide a terrestrial refuge. Females were provided blackworms (*Lumbriculus variegatus*) weekly, which were rarely supplemented with earthworms (suborder: Lumbricina). Neither blackworms (Gall et al., 2011b), nor earthworms (ANS, unpublished data) possess TTX. Although we did not standardize the amount of food given to each female, only rarely were all the blackworms consumed within one week and we therefore assume newts had continuous access to food.

To examine TTX levels in newt eggs after an extended period in captivity, eggs were sampled from these captive females after one, two, and three years in the lab. Females were maintained at 17 °C until mid-December at which point the temperature was slowly dropped to 8 °C. Newts were maintained at this temperature until early-February, whereupon the temperature was slowly increased to 17 °C. Each female was then placed in a 75 L tank with a recently collected male newt that was in reproductive condition. The pair was observed for 72 h for signs of amplexus and mating, at which point the female was removed and injected with 2 μL/g LHRH. A small amount of polyester fiber was then added to the container to serve as an oviposition site. A small subset of eggs was frozen for TTX analysis and the husbandry process was repeated (as described above). Females were maintained in this manner for 2 or 3 years after initially being collected.

2.2. 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 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 (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%. This immunoassay has been proven useful in quantifying TTX in newts of the genus *Taricha* (Stokes et al., submitted), and has yielded concentrations within the expected range of newts quantified using HPLC previously (this study; unpublished data).

2.3. Statistical analysis

We used repeated-measures ANOVA to examine for changes in the total egg toxicity, TTX concentration (ng TTX/mg mass), and egg mass among the four years. Female was treated as a random factor while year was treated as a fixed effect. We fit the model to multiple covariance structures and choose the most appropriate model based on the lowest AIC value (total TTX: autoregressive moving average; TTX concentration: autoregressive; egg mass: autoregressive). Assumptions of normality and homoscedasticity were assessed with graphical analysis of residuals; all assumptions appeared to be adequately met for all response variables. A Kendall's tau rank correlation was used to determine if total TTX, TTX concentration, and egg mass were consistent between females across years. Year three of captivity was excluded from this analysis because only 2 females deposited eggs, which did not permit a complete assignment of ranks. Analyses were obtained using the PROC MIXED procedure in SAS 9.1 (SAS Institute Inc., Cary, NC, USA).

3. Results

The amount of Tetrodotoxin in newt eggs varied significantly across years, with total TTX (per egg) and TTX concentration (ng TTX/mg egg mass) declining between the first clutch collected from wild-caught females (W) and subsequent clutches in the lab (total TTX: $F_{[3,15]} = 32.7$, $P < 0.0001$, Fig. 1a; TTX conc: $F_{[3,15]} = 34.8$, $P < 0.0001$, Fig. 1b). There was no significant difference in total TTX or TTX concentration among the first, second, and third years in the lab. Despite declining after the initial clutch, newts continued to produce eggs containing large quantities of TTX in all years in captivity (Fig. 1). There was no significant correlation between total TTX and year (Kendall's tau = 0.356, $P > 0.25$) or TTX concentration and year (Kendall's tau = 0.321, $P > 0.25$) indicating that the females did not produce eggs that were consistently the same toxicity across years (i.e. the most toxic females did not necessarily produce the most toxic eggs in all three years).
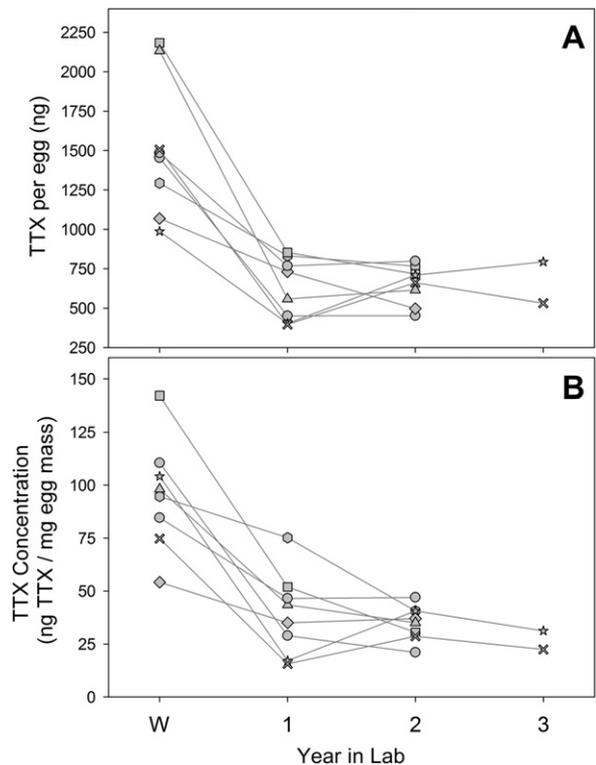

Fig. 1. Mean total TTX (A) and TTX concentration (ng TTX/mg egg mass) (B) present in eggs from eight different female *Taricha granulosa*. A sample of eggs was taken immediately after collection from these wild-caught females (W), and after one, two, and three years in captivity. There was a significant decline in total TTX ($F_{[3,15]} = 32.7$, $P < 0.0001$) and TTX concentration ($F_{[3,15]} = 34.8$, $P < 0.0001$) after the first year in captivity, yet substantial quantities of toxin were present and remained relatively constant in the following three years.

The decline in TTX within newt eggs after being collected from the wild can not be accounted for by a reduction in egg mass between this first year and subsequent years in captivity. The average mass of newt eggs remained constant among years ($F_{[3,15]} = 2.15$, $P = 0.136$, Fig. 2). There was no correlation between egg mass and year (Kendall's tau = 0.183, $P > 0.75$) indicating that the mass of a females eggs was variable across years (Fig. 2).

4. Discussion

Female newts continued to produce toxic eggs for up to three years in the laboratory, despite being exclusively fed a TTX-free diet. Although the amount of toxin in the eggs declined after the initial clutch was collected, each individual female continued to produce eggs with quantities of TTX that fall within (or slightly below) the naturally occurring range for this population (Hanifin et al., 2003). Given that newts reared in captivity maintain TTX over long periods of time (Hanifin et al., 2002) and are capable of replenishing depleted TTX stores (Cardall et al., 2004), these results are not entirely surprising. It is unknown whether our females synthesized or sequestered their own toxin or mobilized it from another area, such as the skin.
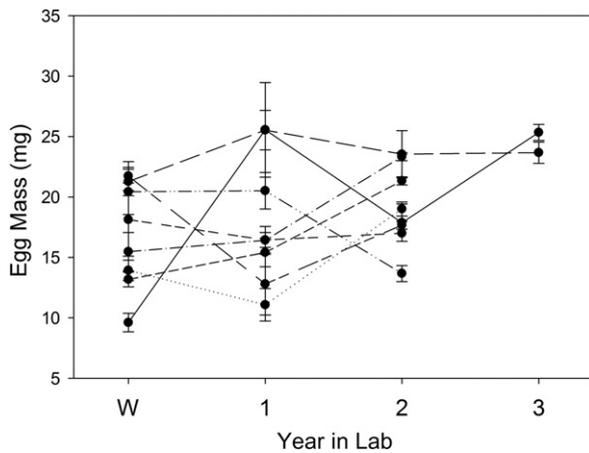
Fig. 2. Mean (±SE) mass (mg) of eggs from eight female newts (*Taricha granulosa*) from initial collection in the wild (W) through three additional years in captivity. There was no significant change in egg mass between years ($F_{[3,15]}$ = 2.15, P = 0.136).

Cardall et al. (2004) demonstrated that newts fed a TTX-free diet regenerated an average of 0.76 mg of skin toxin in nine months in the laboratory (Fig. 3). This replenishment of TTX in the skin was independent of sex, indicating that the newts were not mobilizing TTX from other tissues and transferring it to the skin (Cardall et al., 2004); other than the skin, the only tissues that contain substantial quantities of TTX are the ovaries in females, which are not available as a source of TTX to males (Hanifin et al., 2004;
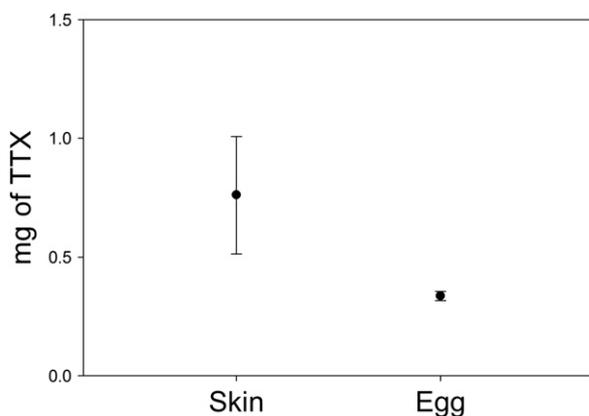
Fig. 3. Comparison of the mean (±SE) amount of TTX regenerated in the skin of adult newts (*Taricha granulosa*) over 9 months (Skin) versus the estimated mean (±SE) amount of TTX provisioned by eight female newts in an entire clutch of 525 eggs (Eggs). Regenerated skin TTX was calculated by estimating whole-body TTX from raw data in Cardall et al. (2004) and subtracting post-stimulus TTX from final TTX. An estimate of the total TTX provisioned in a clutch of eggs for each female reared in captivity was calculated by multiplying the average egg toxicity from years one, two, and three for each female by 525 [the average number of eggs produced by females from our study site (Hanifin et al., 2003)]. These values were then averaged to obtain an estimate of the amount of TTX (±SE) provisioned in an entire clutch of eggs in the laboratory. In both studies (Cardall et al., 2004; this study) the newts were fed a TTX-free diet. There is no significant difference between the amount of TTX regenerated in the skin and the amount of TTX provisioned in a clutch of eggs by newts in the laboratory (Mann–Whitney: U = 223, P = 0.25).

Wakely et al., 1966). Based on a clutch size of 525 eggs (Hanifin et al. (2003) reported clutch sizes of 400–655 eggs from newts from our study site), the females in this study provisioned their entire clutch with an average total of 0.34 mg of TTX (Fig. 3). This quantity of toxin, provisioned during years one, two, and three of this study, is slightly less than, but not significantly different from the amount of toxin regenerated in the skin of newts as demonstrated by Cardall et al. (2004) (Mann–Whitney: U = 223, P = 0.25, Fig. 3). These results suggest that females may develop/sequester new toxin rather than transfer it out of the skin.

One major question that remains is why egg toxicity declines after the first year? We hypothesized that the decrease in TTX observed in this study was due to a decrease in egg mass. However, we documented no change in egg mass between clutches (mean egg mass actually increased very slightly) indicating the decline in TTX is not a result of producing smaller eggs. Moreover, the allocation of TTX in newt eggs is under active maternal control and the amount of toxin in each egg is independent of egg volume (Hanifin et al., 2003). More likely, the decrease in egg toxicity is due to the abbreviated reproductive cycle to which these captive newts were subjected. In the wild, female newts do not breed for the first time until six to eight years of age (Petranka, 1998; Twitty, 1961, 1966). Eggs are deposited singly over a period of several weeks or months (Nussbaum et al., 1983; Petranka, 1998), and females eventually leave the pond to overwinter. Twitty (1966) conducted an extensive mark-recapture study on 5587 female *Taricha*. He found that although some individuals breed in successive years (approximately 1.4%), the vast majority of females do not return to breed again for at least two or three years (Twitty, 1966). This extended non-reproductive period may be necessary to acquire sufficient resources to produce eggs or to obtain large quantities of TTX with which to endow their eggs. We hypothesize that the synthesis or sequestration of TTX in newts may be time-limited.

Few studies have attempted to monitor temporal patterns of TTX production in other taxa. Another amphibian, a frog of the genus *Atelopus*, maintained a high level of TTX for more than three years in captivity (Yotsu-Yamashita et al., 1992). Puffer fish may contain large quantities of TTX in the skin, liver, and ovaries, and although cultured individuals have little or no TTX (Ji et al., 2011; Matsumura, 1996; Noguchi et al., 2006; Sasaki et al., 2008), no studies have monitored the toxicity of individuals or their egg clutches over time. Unlike temporal patterns, our understanding of the ontogenetic changes in toxicity during early developmental stages is slowly advancing. Tetrodotoxin toxicity appears to increase during embryonic development in one species of pufferfish (*Fugu niphobles*), as well as the blue-ringed octopus (Williams et al., 2010b). Nunez-Vazquez et al. (2012) examined the toxicity of multiple life-history stages in a cultured Mexican pufferfish and found very small quantities of TTX in juveniles, pre-adults, and adults. No TTX was identified in eggs, larvae, or post-larvae (Nunez-Vazquez et al., 2012).

The complexity of TTX production in these diverse taxa may be related to the pattern of TTX biogenesis observed in each group. Several groups of marine bacteria have been

identified that produce TTX in vitro (Simidu et al., 1987), and some of these bacteria have been isolated from the tissues of TTX-bearing marine organisms (Wu et al., 2005; Yu et al., 2004). The prevailing hypotheses for TTX toxicity in marine organisms is dietary sequestration through the food chain or symbiotic bacteria (see review in Williams, 2010). However, tetrodotoxin production in amphibians is more controversial. Wild-caught adult *Atelopus* sp. possess TTX (Kim et al., 1975, 2003; Yotsu-Yamashita and Tateki, 2010), yet two *Atelopus varius* reared from eggs in captivity did not possess TTX after 2 and 3 years, respectively (Daly et al., 1997). Nevertheless, Daly et al. (1997) questioned the dietary and bacteria hypotheses in *Atelopus* sp. that overlap in distribution and habitat characteristics yet possess different TTX congeners (Daly, 2004).

In newts, evidence is mounting that bacteria and dietary sequestration may not be involved in TTX production. The eggs of *Cynops pyrrhogaster* are provisioned with small quantities of TTX, which disappears during development (Tsuruda et al., 2002). The larvae are non-toxic, but toxicity begins to increase during the juvenile stage when the granular glands are forming (Tsuruda et al., 2002); the authors did not indicate if these newts were reared in the lab or wild-caught. The bacteria hypothesis in newts was questioned by Lehman et al. (2004) who found bacterial DNA in the intestines of newts (which contain very little TTX) but failed to find evidence for bacteria in extracts from the most toxic tissues. Additionally, when newts are maintained in captivity and fed a diet that does not include TTX, individuals maintain skin toxicity over long periods (Hanifin et al., 2002), continue producing toxic eggs (this study), and rapidly regenerate TTX after electrical stimulation (Cardall et al., 2004).

Because the metabolic pathway by which bacteria, marine organisms, and terrestrial vertebrates produce TTX is unknown, the mechanism by which toxicity is acquired in TTX-bearing organisms remains obscure. Understanding this mechanism requires analyses of ontogenetic and within-individual changes in TTX in wild and captive populations. The study presented here provides further evidence that TTX toxicity in newts is likely of endogenous origin, and adds to the growing body of literature that suggests newts may be able to directly synthesize TTX due to the large quantities produced in relatively short time-frames and under simple, TTX-free, diets.

Acknowledgments

Conflict of interest

The authors declare that there are no conflicts of interest.

References

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

Brodie Jr., E.D., Ridenhour, B.J., Brodie III, E.D., 2002. The evolutionary response of predators to dangerous prey: hotspots and coldspots in the geographic mosaic of coevolution between garter snakes and newts. Evolution 56, 2067–2082.

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

Chau, R., Kalaitzis, J.A., Neilan, B.A. On the origins and biosynthesis of tetrodotoxin. Aquat. Toxicol., in press.

Daly, J.W., 2004. Marine toxins and nonmarine toxins: convergence or symbiotic organisms?†. J. Nat. Prod. 67, 1211–1215.

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

Feldman, C.R., Brodie Jr., E.D., Brodie III., E.D., Pfrender, M.E., 2012. Constraint shapes convergence in tetrodotoxin-resistant sodium channels of snakes. Proc. Nat. Acad. Sci. 109, 4556–4561.

Fuhrman, F.A., 1986. Tetrodotoxin, tarichatoxin, and chiriquitoxin: historical perspectivesa. Ann. Ny. Acad. Sci. 479, 1–14.

Gall, B.G., Brodie III., E.D., Brodie Jr., E.D., 2011a. Survival and growth of the caddisfly *Limnephilus flavastellus* after predation on toxic eggs of the Rough-skinned Newt (*Taricha granulosa*). Can. J. Zool. 89, 483–489.

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

Geffeney, S., Brodie Jr., E.D., Ruben, P.C., Brodie III, E.D., 2002. Mechanisms of adaptation in a predator-prey arms race: TTX-resistant sodium channels. Science 297, 1336–1339.

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-skin newt, *Taricha granulosa*, are correlated with female toxicity. J. Chem. Ecol. 29, 1729–1739.

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., Brodie Jr., E.D., Brodie III., E.D., 2008. Phenotypic mismatches reveal escape from arms-race coevolution. Plos Biol. 6, 471–482.

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.

Jang, J., Yotsu-Yamashita, M., 2006. Distribution of tetrodotoxin, saxitoxin, and their analogs among tissues of the puffer fish *Fugu pardalis*. Toxicon 48, 980–987.

Ji, Y., Liu, Y., Gong, Q.L., Zhou, L., Wang, Z.P., 2011. Toxicity of cultured puffer fish and seasonal variations in China. Aquacult. Res. 42, 1186–1195.

Kim, Y.H., Brown, G.B., Mosher, F.A., 1975. Tetrodotoxin: occurrence in atelopid frogs of Costa Rica. Science 189, 151.

Kim, Y.H., Kim, Y.B., Yotsu-Yamashita, M., 2003. Potent neurotoxins: tetrodotoxin, chiriquitoxin, and zetekitoxin from *Atelopus* frogs in Central America. J. Toxicol-Toxin Rev. 22, 521–532.

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.

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

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.

Noguchi, T., Arakawa, O., Takatani, T., 2006. Toxicity of pufferfish *Takifugu rubripes* cultured in netcages at sea or aquaria on land. Comp. Biochem. Physiol. D: Genomics and Proteomics 1, 153–157.

Nunez-Vazquez, E.J., Garcia-Ortega, A., Campa-Cordova, A.I., Parra, I.A., Ibarra-Martinez, L., Heredia-Tapia, A., Ochoa, J.L., 2012. Toxicity of

cultured bullseye puffer fish *Sphoeroides annulatus*. Mar. Drugs 10, 329–339.

Nussbaum, R.A., Brodie Jr., E.D., Storm, R.M., 1983. Amphibians and Reptiles of the Pacific Northwest. University of Idaho Press, Moscow, ID.

Petranka, J., 1998. Salamanders of the United States and Canada. Smithsonian Institution Press, Washington.

Sasaki, K., Takayama, Y., Tahara, T., Anraku, K., Ito, Y., Akaike, N., 2008. Quantitative analysis of toxin extracts from various tissues of wild and cultured puffer fish by an electrophysiological method. Toxicon 51, 606–614.

Simidu, U., Noguchi, T., Hwang, D.F., Shida, Y., Hashimoto, K., 1987. Marine bacteria which produce tetrodotoxin. Appl. Environ. Microbiol. 53, 1714–1715.

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.

Tsuruda, K., Arakawa, O., Kawatsu, K., Hamano, Y., Takatani, T., Noguchi, T., 2002. Secretory glands of tetrodotoxin in the skin of the Japanese newt *Cynops pyrrhogaster*. Toxicon 40, 131–136.

Twitty, V.C., 1961. Second-generation hybrids of the species *Taricha*. Proc. Nat. Acad. Sci. 54, 51–58.

Twitty, V.C., 1966. Of Scientists and Salamanders. Freeman, San Francisco, CA.

Venkatesh, B., Lu, S.Q., Dandona, N., See, S.L., Brenner, S., Soong, T.W., 2005. Genetic basis of tetrodotoxin resistance in pufferfishes. Curr. Biol. 15, 2069–2072.

Wakely, J.F., Fuhrman, G.J., 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.

Wang, J., Fan, Y., Yao, Z., 2010. Isolation of a *Lysinibacillus fusiformis* strain with tetrodotoxin-producing ability from puffer fish Fugu obscurus and the characterization of this strain. Toxicon 56, 640–643.

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., Brodie III., E.D., 2010a. Tetrodotoxin affects survival probability of rough-skinned newts (*Taricha granulosa*) faced with TTX-resistant garter snake predators (*Thamnophis sirtalis*). Chemoecology 20, 285–290.

Williams, B.L., Hanifin, C.T., Brodie Jr., E.D., Caldwell, R.L., 2010b. Ontogeny of tetrodotoxin levels in blue-ringed octopuses: maternal investment and apparent independent production in offspring of *Hapalochlaena lunulata*. J. Chem. Ecol.

Wu, Z., Yang, Y., Xie, L., Xia, G., Hu, J., Wang, S., Zhang, R., 2005. Toxicity and distribution of tetrodotoxin-producing bacteria in puffer fish *Fugu rubripes* collected from the Bohai Sea of China. Toxicon 46, 471–476.

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

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.

Yotsu-Yamashita, M., Tateki, E., 2010. First report on toxins in the Panamanian toads *Atelopus limosus, A. glyphus* and *A. certus*. Toxicon 55, 153–156.

Yu, C.F., Yu, P.H.F., Chan, P.L., Yan, Q., Wong, P.K., 2004. Two novel species of tetrodotoxin-producing bacteria isolated from toxic marine puffer fishes. Toxicon 44, 641–647.