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Source: Northwestern Naturalist, 96(1):13-21.
Published By: Society for Northwestern Vertebrate Biology
URL: http://www.bioone.org/doi/full/10.1898/NWN13-19.1

OTTER PREDATION ON *TARICHA GRANULOSA* AND VARIATION IN TETRODOTOXIN LEVELS WITH ELEVATION

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ABSTRACT—Tetrodotoxin (TTX) is a low molecular weight neurotoxin that is found in a wide variety of taxa. Tetrodotoxin blocks voltage-gated sodium channels, preventing the propagation of action potentials and inducing paralysis in susceptible animals. *Taricha granulosa* have been documented to possess TTX in high quantities and are preyed upon by snakes of the genus *Thamnophis*. However, recent observations of predation events on *T. granulosa* by otters were documented in a high-elevation population just outside of Crater Lake National Park, Oregon. We quantified TTX levels in this population as well as 3 populations in Crater Lake National Park using a Competitive Inhibition Enzymatic Immunoassay. We further compared these high elevation populations to a known high-toxicity population from Benton County, Oregon. We found that the populations in Crater Lake have lower levels of TTX relative to populations outside of the lake, and that all high-elevation locations have relatively low levels of TTX. We then analyzed previously published whole-newt TTX levels and elevation, and found that there is a significant negative relationship. However, there is a non-significant relationship between whole-newt TTX levels and elevation when examining elevations below 500 m. This further exemplifies the potential for novel predation and previously unidentified selective pressures in high-elevation newt populations.

Key words: Crater Lake National Park, *Lontra canadensis*, North American River Otter, predation, Rough-skinned Newt, *Taricha granulosa*, tetrodotoxin

Tetrodotoxin (TTX) is one of the most potent neurotoxins known (Mosher and others 1964; Wakely and others 1966; Brodie 1968; Brodie and others 1974; Hanifin and others 1999). Tetrodotoxin acts by blocking voltage-gated sodium channels in nerve and muscle tissues, thereby preventing propagation of action potentials (Narahashi and others 1967). A fatal dose of TTX is usually between 0.5 to 2 mg for an adult human (Yasumoto and Yotsu-Yamashita 1996), and death usually occurs due to paralysis of the diaphragm and asphyxiation (Brodie 1968). Tetrodotoxin is unusual in that it is found in a wide array of taxa, from marine bacterial species to terrestrial vertebrates (as reviewed by Miyazawa and Noguchi 2001; Chau and others 2011). One of the best studied species that possesses TTX is the Rough-skinned Newt, *Taricha granulosa* (Mosher and others 1964; Wakely and others 1966; Brodie 1968; Daly and others 1987; Hanifin and others 1999).

The only well-documented predators of adult *T. granulosa* are the gartersnakes *Thamnophis sirtalis* (Brodie 1968; Brodie and others 2002), *T. couchii* (Brodie and others 2005), and *T. atratus* (Green and Feldman 2009). Newts and gartersnakes are involved in a coevolutionary arms race with reciprocal selection acting upon toxicity in newts and resistance in snakes (Brodie and Brodie 1990, 1991; Hanifin and others 2008). Populations of thamnophine snakes that are sympatric with newts have evolved varying levels of resistance to TTX via as little as 1 amino acid change in the pore region of the voltage-gated sodium channel (Geffeney and others 2002, 2005; Feldman and others 2009, 2012). These adaptations allow snake predation on newts when individual snakes are resistant enough to tolerate the toxin load present in the local newt population, which leads to variation in newt toxicity across their range (Hanifin and others 2008).

Early experiments using *T. granulosa* have shown that TTX is lethal to nearly every potential predator (Brodie 1968), and mortality has been documented following predation attempts on *Taricha* in several species of birds (Storm 1948; Pimentel 1952; Mobley and Stidham 2000). However, there are some more recent examples of successful non-snake predation on newts. Great Blue Herons (*Ardea herodias*) in northern California have been observed eating newts with no apparent ill effects (Fellers and others 2008). Additionally, a population of newts in Santa Rosa, California, has been heavily preyed upon over many years (Stokes and others 2011), although the predator, which has never been positively identified, appears to circumvent the toxin in the skin by eviscerating the newts and only eating the internal organs.

A diet study conducted on North American River Otters (*Lontra canadensis*) in Oregon reported that an otter ingested 1 *T. granulosa* with apparently no ill effects (Toweill 1974). However, there were no analyses of TTX levels of newts in this region and there have not been further reports since. Here we present further documentation of predation on newts by *Lontra canadensis* at Lake in the Woods, a small, high-altitude lake in Douglas County, Oregon. On 6 August 2007, we (EP and DP, pers. obs.) observed an otter (*L. canadensis*) eating more than 1 *T. granulosa*; and on 27–28 October 2007, 1 adult and 2 juvenile otters were observed eating approximately 20 salamanders in 1 d. The otters were eating both *T. granulosa* and Northwestern Salamanders, *Ambystoma gracile* (Fig. 1). The typical sequence of symptoms due to TTX exposure includes muscular weakness that is especially apparent in the hind limbs, loss of righting reflex, convulsions, gasping, gaping, regurgitation, total paralysis, and a drop in blood pressure (Brodie 1968). After several hours of observation of the otters feeding, at no time did any of the otters display any of these signs or symptoms of TTX intoxication. River Otters are opportunistic predators (Melquist and others 2003; Kruuk 2006), and their diets change depending on the season and availability of food sources (Melquist and others 2003). In addition to a diet consisting mostly of fish, they have been documented feeding on several amphibian species including Coastal Giant Salamanders (*Dicamptodon tenebrosus*), Two-toed Amphiumas (*Amphiuma means*), and several ranid frog species. The observation of otters eating toxic prey like *T. granulosa* can lead to 1 of 2 immediate hypotheses: either the newts in that locality have little to no TTX, or the otters are in some way resistant to TTX. We investigated the apparent success of this predation event by testing TTX levels in *T. granulosa* from

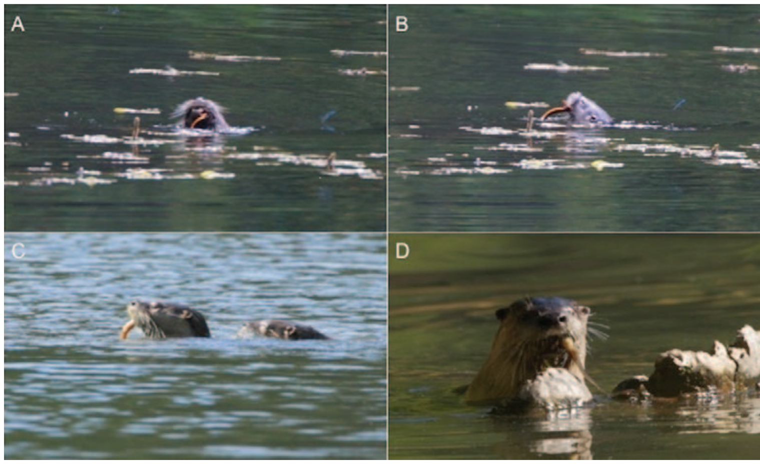

FIGURE 1. *Lontra canadensis* feeding on *Taricha granulosa* (A–B) and *Ambystoma gracile* (C–D) at Lake in the Woods, Oregon. Photo credit: Dale and Elva Paulson © 2014.

the Lake in the Woods population. Furthermore, we tested TTX levels in nearby high-elevation lakes just outside of and within Crater Lake National Park and compared them to a well-known high-toxicity population from Benton County, Oregon. Given these results, we explored the relationship between elevation of each population and whole-newt TTX levels using our data set and previously published whole-newt TTX level data.

METHODS

Animal Collection and Care

Taricha granulosa (hereafter referred to as newts) were collected by hand or using minnow traps from 4 populations including Skell Channel (n = 19, Klamath County), Phantom Ship (n = 5, Klamath County), Spruce Lake (n = 19, Klamath County), and Lake in the Woods (n = 6, Douglas County), Oregon (Fig. 2). Two of these locations (Spruce Lake, 1451 m; Lake in the Woods, 920 m) are relatively high-elevation lakes located outside of Crater Lake, while 2 locations (Skell Channel, 1887 m; Phantom Ship, 1895 m) are prominent features located in Crater Lake. Thirty-four newts were also collected by hand from the Soap Creek ponds (344 m) in Benton County, Oregon. All newts were transported back to Utah State University, and housed in 5.7-L containers with 2 L carbon-filtered water in an environmental chamber at 6°C. Newts from the high- elevation sites (>500 m) were later euthanized with MS-222, and skin punches were taken following the methods of Hanifin and others (2002) for TTX quantification. Newts from Benton County were anesthetized using 1% MS-222 to take skin punches, but were not euthanized and were used in subsequent experiments.

Elevation Data

Mean whole-newt TTX levels for several populations throughout Oregon were obtained from Hanifin and others (2008), Ridenhour (2004), and the high- and low- elevation data collected in the present study (Table 1). The average whole newt TTX levels summarized for the Soap Creek ponds population and used in this analysis were from recent collections that were part of this study rather than from previous studies (for example, Hanifin and others 1999). We determined the elevation of each collection site for each population by entering latitude and longitude coordinates into Google Earth and National Geographic Topo! software (Version 4.2.8, 2006). Locations were classified as low-elevation (<300 m) and high-elevation (>700 m).

TTX Quantification

TTX was extracted from skin tissues using the methods of Hanifin and others (2002). Quantification of TTX was done using a Competitive Inhibition Enzymatic Immunoassay as in Stokes

FIGURE 2. The 4 high-elevation locations in Oregon where *Taricha granulosa* were collected. Note that *Lontra canadensis* predation was observed at Lake in the Woods.

and others (2012). We used the linear range of the standard curve, which was between 10 and 500 ng/mL. The minimum level of detection in this assay is 10 ng/mL. Values <10 ng/mL are referred to as below detectable limits (BDL) and considered as zero in our analyses. Due to TTX levels that fell within the linear range of our standard curve, newt samples from high-elevation sites were not diluted and were run against standards diluted in 0.1 M acetic acid. Newt samples from Soap Creek were diluted 1:120, 1:300, 1:500, 1:800, and 1:1000 in a 1% Bovine Serum. This assay was run in 96-well Nunc Maxisorp plates (Fisher Scientific), and samples were run against standards diluted in BSA. The average coefficient of variation on each plate was between 4.72 and 7.03%. Whole newt TTX was calculated using the methods of Hanifin and others (2004).

Statistical Analyses

Comparisons of newt TTX levels among populations in the current study were done using non-parametric Wilcoxon/Kruskal-Wallis tests. This was followed with Wilcoxon comparisons for each pair. The relationship between log-transformed elevation and mean population TTX concentrations for populations <300 m elevation was examined using linear regression. We used studentized residuals to help identify outlier populations following the principle that residuals exceeding ± 2 may be considered ''discrepant observations'' and should be evaluated as possible outliers (Fox 1991). Finally, we binned newt populations into 2 elevational cohorts (<300 m and >700 m) and compared log-transformed mean population TTX levels from the current and previous studies using one-way analysis of variance and a Tukey HSD test to control for all pairwise comparisons. Analyses were performed using JMP 9 (SAS Institute, Inc.) or the regression wizard feature in SigmaPlot v 10.0 (Systat Software, Inc.).

RESULTS

Each of the high-elevation populations had newts with some TTX, although with very low concentrations (Fig. 3), and individuals from Soap Creek had >1000 times more TTX on average than any of the high-elevation populations ($P < 0.001$). We found that there were significant differences in TTX concentrations between several of the populations (Table 2). Pairwise comparisons demonstrated that populations outside of Crater Lake (Soap Creek, Lake in the Woods, and Spruce Lake) had significantly greater levels of TTX relative to populations within Crater Lake. No differences in TTX levels, however, were detected between the Spruce Lake and Lake in the Woods populations outside of Crater Lake, and between the Skell Channel and Phantom Ship populations in Crater Lake.

The empirical relationship between mean whole body population TTX (mg) and elevation is presented in Fig. 4a. The majority (65%) of sampled populations occupied sites at elevations approximately ≤265 m (Fig. 4a hatched box). Additionally, there is a discontinuous distribution of sampled populations that resulted from a lack of published toxicity levels at all available elevations. For that reason, we binned populations into the 2 elevation cohorts, and a t-test comparing the populations at <300 and >700 m was highly significant ($P < 0.001$;

TABLE 1. Complete data set for the elevation analyses. Elevation category is separated into either low (<300 m) or high (>700 m) elevation sites. Also listed are the elevation (m), the location in Oregon, and the average TTX levels (mg). The sources of the data used for our analyses are listed in the Publication column: Hanifin = Hanifin and others (2008); Ridenhour = Ridenhour (2004); Stokes = the current publication.

Elevation category	Elevation (m)	Location	TTX (mg)	Publication
Low	3.05	Hunter Creek, Curry Co.	1.414	Ridenhour
	6.10	Tahkenitch Lake, Douglas Co.	0.197	Ridenhour
	7.62	Warrenton, Clatsop Co.	0.787	Hanifin
	9.14	Euchre Creek, Curry Co.	2.811	Ridenhour
	19.81	Ten Mile Creek, Coos Co.	2.366	Ridenhour
	24.38	Chetco River, Curry Co.	1.207	Ridenhour
	30.48	Edson Creek, Curry Co.	3.108	Ridenhour
	60.96	Morgan Creek, Douglas Co.	0.708	Ridenhour
	91.44	Alsea, Benton Co.	4.021	Ridenhour
	104.85	Benton, Benton Co. (Soap Creek)	8.551	Stokes
	140.21	Elk River, Curry Co.	2.035	Ridenhour
	152.40	Stayton, Marion Co.	0.312	Ridenhour
	213.36	McGribble, Curry Co.	3.803	Hanifin
	235.92	Ten Mile, Lane Co.	1.628	Hanifin
	265.18	Mill City, Linn Co.	2.632	Ridenhour
High	701.04	Highway 22 Ponds, Linn Co.	0.776	Ridenhour
	920.50	Lake in the Woods, Douglas Co.	0.003	Stokes
	960.12	Lost Lake, Hood River Co.	0.025	Ridenhour
	1318.87	Parsnip Lakes, Jackson Co.	0.002	Hanifin
	1451.46	Spruce Lake, Jackson Co.	0.008	Stokes
	1463.04	Scott Lake, Lane Co.	0.000	Hanifin
	1886.71	Skell Channel, Klamath Co.	0.000	Stokes
	1895.25	Phantom Ship, Klamath Co.	0.001	Stokes

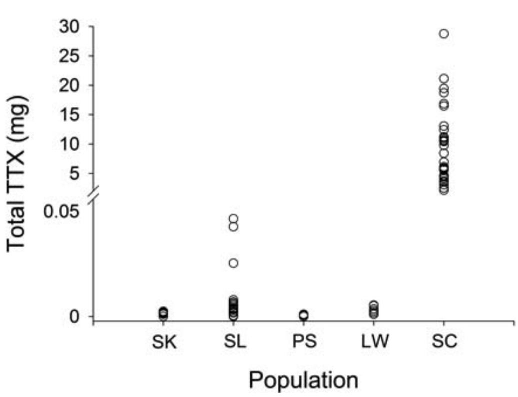

FIGURE 3. Total whole-body TTX (mg) for each individual newt (*Taricha granulosa*) from each of 5 populations in western Oregon. Population labels are abbreviated as follows: SK = Skell Channel (n = 19; $\bar{x}$ = 0.00049 mg; SEM = 0.00018); SL = Spruce Lake (n = 19; $\bar{x}$ = 0.00819 mg; SEM = 0.00279); PS = Phantom Ship (n = 5; $\bar{x}$ = 0.00053; SEM = 0.00016); LW = Lake in the Woods (n = 6; $\bar{x}$ = 0.00290; SEM = 0.00064); SC = Soap Creek (n = 34; $\bar{x}$ = 8.55110; SEM = 1.15029). Note that the scale of the y-axis changes beyond the break.

Fig. 4b). The lack of sites across all available elevations also limited our ability to mathematically describe the relationship between elevation and TTX using a single continuous function. For this reason, we restricted our analysis to the 15 populations sampled from sites located at elevations <300 m that had measurable concentrations of whole body TTX (Fig. 4c). From this restricted analysis, we found that the relationship between elevation and mean population TTX is weakly positive but not significant ($P = 0.209$). Even if we remove populations from sites that appear to be outliers (sites with studentized residuals that exceed $|2|$ and circled on Fig. 4c), the resulting regression equation is still not significant ($P = 0.093$). The results emphasize that below 300 m there appears to be no statistical trend in TTX levels that can be explained by elevation alone.

DISCUSSION

Newts from the high-elevation regions of Oregon outside of Crater Lake ranged from BDL to 0.042 mg TTX, while newts in Crater Lake ranged from BDL to 0.00214 mg TTX. The toxicity levels of newts in the high-elevation

TABLE 2. Results from Wilcoxon pairwise comparisons for high-elevation populations. Stars designate significant differences. Populations are abbreviated as follows: SL = Spruce Lake; SK = Skell Channel; PS = Phantom Ship; LW = Lake in the Woods. Populations with + superscript indicates that they are within Crater Lake.

Comparison	Score mean difference	Standard error difference	Z	*P*-value
SL to SK^+	15.2632	3.493339	4.36922	<0.0001*
SL to PS^+	8.5895	3.551001	2.41889	0.0156*
SL to LW	3.1798	3.445880	0.92279	0.3561
SK^+ to PS^+	−2.6526	3.183194	−0.83332	0.4047
PS^+ to LW	−5.3167	2.008316	−2.64733	0.0081*
SK^+ to LW	−10.4167	3.196192	−3.25909	0.0011*

lakes are low enough that otters, and possibly other predators such as fish (Swanson and others 2000), likely can eat at least some individuals with very few, if any, side effects. Many predators of toxic prey are able to circumvent the harmful toxin(s) either through changes in behavior (Williams and others 2003), including development of novel predation strategies (Stokes and others 2011), or adaptation to the toxin itself (Geffeney and others 2002, 2005; Phillips and Shine 2006; Feldman and others 2012). Gartersnakes have been shown to ''taste'' and subsequently reject newts that are excessively toxic (Williams and others 2003). It is possible that although we did not observe otters rejecting prey, otters, similarly to gartersnakes, may also be able to determine whether or not a newt is safe to eat by ''tasting'' the newt and rejecting ones that are too toxic.

Hanifin and others (2008) showed that TTX levels vary greatly both within and between populations of Rough-skinned Newts along the Pacific coast of North America. Specifically, in Oregon they found that newts from Benton County have very high levels of TTX with a mean of 4.695 mg TTX. Our results for Benton County newts ($\bar{x}$ TTX = 8.551 mg) nearly doubled this previously reported mean. The newts from Benton County have been shown to be highly variable, ranging from 0 mg TTX (Williams and others 2004) to the upper limit of 28 mg TTX, collected during this study. This large degree of variation may allow for changes in the mean as we collect more data. Furthermore, Hanifin and others (2008) measured TTX levels in newts from Parsnip Lakes (Jackson County) and Scott Lake (Lane County), Oregon, both above 1200 m elevation. Both locations were found to have very low average levels of TTX, with means of 0.002 and 0.000 mg TTX, respectively. All other populations from Oregon that were measured in that study were <300 m elevation, with the lowest mean TTX level being 0.787 mg. Similarly, Ridenhour (2004) demonstrated that *T. granulosa* at lower elevations in Washington and central and coastal Oregon tend to have higher levels of TTX than those at higher elevations. In his study, newts at elevations <300 m ranged in mean TTX levels from 0.197 to 4.695 mg; at elevations >300 m TTX levels ranged from 0.025 to 0.776 mg TTX. The one previously documented instance of otter predation on newts did not report where the predation occurred (Toweill 1974). Many locations from that study were from high-elevation sites in western Oregon; however, we cannot determine whether that observation supports ours.

It seems possible that the apparent differences in TTX levels in newts from high- elevation sites versus those in lower-elevation sites may be due to relaxed selection from a low abundance of snake predators. It has been shown that reptile diversity on elevational gradients is most greatly correlated with temperature (McCain 2010). Therefore, snake diversity generally decreases with increasing elevation, and this effect is stronger on wetter than on drier mountains. However, *Thamnophis sirtalis* (Common Gartersnakes) are not uncommon in Crater Lake National Park and are often found on the lakeshore (elevation approximately ≥1900 m) as well as in other areas of the park (Farner and Kezer 1953). Yet, without a detailed measure of relative abundance of snakes in this area, it is unclear how selective pressures may be altered by elevation in these systems. Furthermore, from the present study, it is clear that other predators like otters have the potential to affect selection on newt toxin levels. However, it is

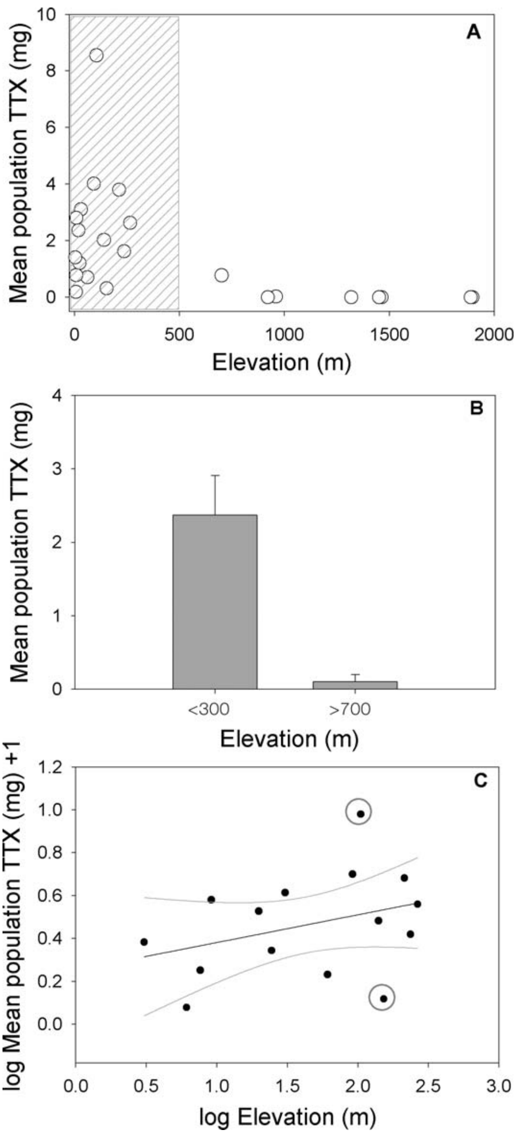

FIGURE 4. Relationship between elevation and TTX levels across populations from Hanifin and others (2008), Ridenhour (2004), and the present study. (A) The empirical relationship between mean whole body population TTX (mg) and elevation, with the majority of sample sites highlighted with the hatched box. (B) Comparison of binned data for elevations <300 m and >700 m. (C) Regression analysis of populations <265 m elevation (n = 15); circles represent outliers that were removed to confirm the analysis.

important to note that we do not know how long otters have been feeding on newts at this location, nor do we know how often otters feed on newts. This lack of data makes it difficult to make conclusions about the selective forces, either in terms of strength or identity, on this population, and will require further investigation.

It is also possible that the differences in TTX levels between high- and low- elevation populations are due to dietary differences of the newts themselves. We do not yet know whether newts acquire TTX endogenously (produce the toxin themselves) or exogenously (sequester the toxin through diet) (Chau and others 2011). Other TTX- possessing species such as puffer fish (Tetraodontidae) have been shown to have virtually no TTX when raised in captivity, whereas they have very high levels of TTX in the wild (as reviewed by Williams 2010). This suggests some dietary source, possibly TTX-producing bacteria, for the acquisition of TTX in these species. If newts at low elevations have access to a dietary source for TTX that is not found at the high elevations, we may see great differences between low- and high-elevation populations. However, there is very little evidence to support exogenous acquisition of TTX in *Taricha* spp. at this time. Newts have been shown to increase their levels of TTX over time in the lab while being fed a TTX-free diet (Hanifin and others 2002). Furthermore, there has yet to be evidence of TTX-producing bacteria in *Taricha* spp. (Cardall and others 2004; Lehman and others 2004). These lines of evidence so far suggest endogenous production of TTX in these species.

The newts from Crater Lake are in contrast to those from Soap Creek, which are some of the most toxic individuals sampled. The most toxic individual in Soap Creek contained 28 mg TTX, enough to kill up to 56 humans (Yasumoto and Yotsu-Yamashita 1996). In contrast, the least toxic populations, such as the high-elevation populations we tested, have large numbers of individuals that lack detectable levels of TTX. Soap Creek newts are also one of the best examples of variation within a population of newts known to date, ranging from no detectable TTX (Williams and others 2004) to 28 mg TTX (present study). Interestingly, the newts in Crater Lake have significantly lower levels of TTX than the populations outside of Crater Lake. At this time it is not possible to definitively discern whether the variation between the populations inside Crater Lake and those outside of the lake is due to genetic isolation or dietary differences. This latter point is particularly important to consider, because low productivity in an ultraoligotrophic lake like Crater Lake may limit the energy available for endogenous production of TTX.

Given the lack of evidence for exogenous production of TTX in *T. granulosa*, however, it can be hypothesized that the variation seen within and between populations may be important to the evolution of this toxin, because variation is required for selection to act upon a trait (Fisher 1930). *Taricha granulosa* and gartersnakes have been one of the most well studied examples of a co-evolutionary arms race between predators and prey (Brodie and Brodie 1990, 1991; Geffeney and others 2002, 2005; Williams and others 2003; Hanifin and others 2008; Feldman and others 2009, 2012). Recent work, however, has shown that the evolution of TTX in these newts may be more complicated than a simple 2-species system. There is research showing that caddisfly larvae are capable of eating newt eggs, potentially influencing TTX concentrations at a very early life-history stage (Gall and others 2011). Additionally, the evidence in this study and elsewhere (Fellers and others 2008; Stokes and others 2011) demonstrates that gartersnakes are not the only predators of *Taricha granulosa*. Especially in newt populations with low toxicity, novel predators such as River Otters have the potential to put selective pressure on these populations of newts, favoring increased levels of TTX.

ACKNOWLEDGMENTS

The US Geological Survey and Crater Lake National Park provided support for this work. We would especially like to thank D Hering, S Girdner, M Parker, A Denlinger, and H Griffin for help in the field. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the US Government.

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Submitted 20 August 2013, accepted 26 May 2014. Corresponding Editor: Robert Hoffman.