# Noxious newts and their natural enemies: Experimental effects of tetrodotoxin exposure on trematode parasites and aquatic macroinvertebrates

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## ABSTRACT

The dermal glands of many amphibian species secrete toxins or other noxious substances as a defense strategy against natural enemies. Newts in particular possess the potent neurotoxin tetrodotoxin (TTX), for which the highest concentrations are found in species within the genus *Taricha*. Adult *Taricha* are hypothesized to use TTX as a chemical defense against vertebrate predators such as garter snakes (*Thamnophis* spp.). However, less is known about how TTX functions to defend aquatic-developing newt larvae against natural enemies, including trematode parasites and aquatic macroinvertebrates. Here we experimentally investigated the effects of exogenous TTX exposure on survivorship of the infectious stages (cercariae) of five species of trematode parasites that infect larval amphibians. Specifically, we used dose-response curves to test the sensitivity of trematode cercariae to progressively increasing concentrations of TTX (0.0 [control], 0.63, 3.13, 6.26, 31.32, and 62.64 nmol $L^{-1}$) and how this differed among parasite species. We further compared these results to the effects of TTX exposure (0 and 1000 nmol $L^{-1}$) over 24 h on seven macroinvertebrate taxa commonly found in aquatic habitats with newt larvae. TTX significantly reduced the survivorship of trematode cercariae for all species, but the magnitude of such effects varied among species. *Ribeiroia ondatrae* — which causes mortality and limb malformations in amphibians — was the least sensitive to TTX, whereas the kidney-encysting *Echinostoma trivolvis* was the most sensitive. Among the macroinvertebrate taxa, only mayflies (Ephemeroptera) showed a significant increase in mortality following exogenous TTX exposure, despite the use of a concentration 16x higher than the maximum used for trematodes. Our results suggest that maternal investment of TTX into larval newts may provide protection against certain trematode infections and highlight the importance of future work assessing the effects of newt toxicity on both parasite infection success and the palatability of larval newts to invertebrate predators.

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## 1. Introduction

Tetrodotoxin (TTX) is a naturally occurring neurotoxin found in at least 140 animal species spanning a broad range of taxa (Lorentz et al., 2016). Flatworms (genus *Planocera*), the eggs of horseshoe crabs (genus *Limulus*), pufferfish (genus *Fugu*), xanthid crabs (genus *Xantho*), blue-ringed octopi (genus *Hapalochlaena*), and newts (family Salamandridae) are all known to possess TTX (Brodie III and Brodie Jr, 1990; Geffeney et al., 2005; Hanifin, 2010; Lorentz et al., 2016; Miyazawa et al., 1986; Noguchi et al., 1984; Ritson-Williams et al., 2006), which is recognized as an exceptionally deadly poison to many vertebrates (Guzmán et al., 2007). Among the 12

species of newts in the family Salamandridae known to possess TTX (Hanifin, 2010), the highest average dermal concentrations ($25 \, \mu g \, cm^{2 \, -1}$) occur in members of the genus *Taricha* (Wakely et al., 1966). Concentrations in other genera such as *Notophthalmus* and *Cynops* often range from 1 to 4 $\mu g$ TTX $g^{-1}$, while only trace amounts have been detected in *Triturus* (Wakely et al., 1966; Yotsu et al., 1990). Adult *Taricha* are hypothesized to use TTX as a chemical defense against predators, including birds, fishes, and garter snakes (Brodie III et al., 2005; Brodie Jr, 1968; Farner and Kezer, 1953; Storm, 1948). Predation on newts has often been studied as part of a coevolutionary framework positing that adult *Taricha* have evolved greater toxicity in response to predation by garter snakes, which can develop genetic resistance to the toxin (Brodie III et al., 2005; Brodie III and Brodie Jr, 1999; 1990; Toledo et al., 2016; Williams et al., 2003).

Considerably less is known about the role of TTX in defending larval newts against natural enemies, including macroinvertebrate predators, competitors, and infectious parasites. Although all newts appear to possess TTX, it is not clear how they produce the neurotoxin. Specifically, it may be endogenously controlled through a biosynthetic pathway or the result of bacterial symbionts. As a result, it is unclear how newts evolved TTX, leaving open many questions about its evolutionary origins and potential ecological roles in ecosystems. While TTX concentrations tend to be highest in adult newts (Wakely et al., 1966), maternal investment of the toxin into eggs is hypothesized to help defend embryos and larvae against enemy attack during aquatic development (Gall et al., 2011; Hanifin, 2010). In experimental studies, *Taricha* embryos and larvae were relatively unpalatable to dragonfly nymphs (Gall et al., 2011) and experimental exposure to TTX from adult newts impaired their feeding behavior (Bucciarelli and Kats, 2015). However, other aquatic invertebrate predators such as caddisfly larvae show remarkably high tolerance to TTX (Gall et al., 2012).

Whether TTX in the skin of larval newts affects their susceptibility to water-borne parasites, such as trematodes, remains an unexplored question. Adult newts are known to support a diversity of parasites, including nematodes (*Rhabdias tarichae*, *Cosmocercoides variabilis*, *Megalobatrachonemea moraveci*, *Hedruris siredonis*), protozoans (*Eimeria tarichae*, *Tritrichomonas* sp., *T. augusta*, *Hexamita ovatus*, *Karotomorpha swezi*, *Trypanosoma ambystomae*, and *T. granulosa*), trematodes (*Ribeiroia ondatrae*, *Clinostomum* sp., *Megalodiscus microphagus*, *M. americanus*, *Brachycoelium salamandrae,* and *Glypthelmins* sp.), and an acanthocephalan (*Neoechinorhynchus* sp.) (Bolek, 1997; Goldberg et al., 1998; Johnson et al., 2013, Johnson and Hoverman, 2012; Kuzmin et al., 2003; Lehmann, 1954; Macy, 1960; Parkinson, 2010; Richardson and Adamson, 1988; Vanderburgh and Anderson, 1987). In a recent study of macro- and microparasites in adult *T. granulosa* and *T. torosa*, Johnson et al. (personal communication) reported a negative correlation between host TTX concentration and both the presence of several microparasites and the total load of macroparasites. Encysted trematodes in the skin were notably rare, despite the fact that larval newts are exposed to many different trematodes (e.g., Caffara et al., 2014; Etges, 1961; Miller et al., 2004; Owen, 1946). Because water-borne trematode larvae (cercariae) often penetrate the skin of larval amphibians and encyst within host tissues, they may be exposed to higher concentrations of TTX relative to parasites within the gastrointestinal tract, where toxicity levels are relatively lower. However, little is known about the direct sensitivity of parasites exposed to exogenous TTX.

In this study, we experimentally tested how exposure to waterborne-TTX affected the survivorship of larval trematodes and aquatic macroinvertebrates. Specifically, we compared the sensitivity of cercariae representing five trematode species known to infect larval amphibians to progressively increasing concentrations of TTX. We then compared these results with the effects of TTX exposure on seven common macroinvertebrate taxa found in aquatic ecosystems. This work has implications for understanding the degree to which TTX provides protection from parasites generally and especially virulent trematodes, such as the trematode *R. ondatrae*, which causes limb malformations and elevated mortality in many amphibians (Johnson et al., 2012). Our results also underscore the importance of future work to assess the effects of TTX on parasite infection success *in vitro* and on the palatability of larval newts to invertebrate predators.

## 2. Materials and methods

### 2.1. Trematode bioassays

Between May and August of 2015, we collected freshwater snails (*Helisoma trivolvis* and *Physa* spp.) using dip-nets (45.7 cm D-frame with 1.2 mm mesh), seines (1.2 × 1.8 m), or by hand from pond ecosystems in California and Oregon (California: Alameda, Contra Costa, and Santa Clara counties; Oregon: Multnomah and Washington counties). These snails function as the first intermediate hosts for many trematode parasites known to infect pond-breeding amphibians. Snails were screened for infection using methods described in Calhoun et al. (2015) and Paull et al. (2012) and identified using morphological features (Bray et al., 2008; Gibson et al., 2005; Johnson et al., 2004; Jones et al., 2005; Schell, 1985, 1970). Once identified, we housed snails in 720 ml plastic containers with dechlorinated, UV-sterilized, and carbon-filtered tapwater (hereafter referred to as 'treated' water). We replaced water every other day and fed snails ad libitum a mixture of Tetramin™, agar, and calcium. Air temperature within the environmental chambers ranged between 21 and 23 °C.

To test the sensitivity of different trematode species to TTX, we collected cercariae by placing infected snails in 50 ml conical tubes filled with 40 ml of treated water (*A. marcianae*, *E. trivolvis*, *M. syntomentera*, *R. ondatrae*, and *Cephalogonimus* sp.). Cercariae for each species were collected within 4 h of release from snails to ensure viability. We purchased commercially pure TTX (Fisher Scientific, Acros Organics) and prepared following manufacturer's instructions to rehydrate to 1 mg ml$^{-1}$. To begin, we made a stock solution of TTX at 10 $\mu g$ ml$^{-1}$, which was used to complete five serial dilutions (0.0, 0.63, 3.13, 6.26, 31.2, and 62.64 TTX nmol L$^{-1}$). Finally, we prepared individual wells on sterile, 96-well plates for trials by adding 0.1 $\mu g$ ml$^{-1}$ of TTX prepared at various concentrations to 0.4 ml of treated water achieving our final doses. One $\mu l$ of treated water was used as the control solution. We pipetted individual cercariae into each well with a minimal transfer of water (<0.1 $\mu l$) using an automatic 0.1 $\mu l$ micropipette (Fisher Scientific, Eppendorf™) and no pipette contact to the well solution. Cercariae were collected from a single snail when possible, otherwise pooled from no more than three snails (*A, marcianae* [n = 140 cercariae], *E. trivolvis* [n = 168 cercariae], *M. syntomentera* [n = 144 cercariae], *R. ondatrae* [n = 474 cercariae], and *Cephalogonimus* sp. [n = 168 cercariae]). Each plate included replicates of all treatments to control for plate effects and contained only a single parasite species. Finally, we used alternating rows to limit the risk of cross-contamination of parasites or doses. Each treatment was replicated at least 24 times per parasite taxon. Once all cercariae were added, plates were covered to prevent evaporation and maintained at 22 °C for 24 h or until all cercariae had died. We examined each well every 2 h using an Olympus SZX10 and recorded the status of the cercaria (alive and swimming, alive but not swimming, or dead). The typical lifespan of trematode cercariae is < 24 h (Singh, 2015), such that our experiment sought to evaluate how different concentrations of TTX altered the survivorship of cercariae relative

to the control treatment. Cercariae were considered dead when they failed to respond to a probe or when the tail detached from the body, as described previously (Calhoun et al., 2015; Paull et al., 2012; Reddy et al., 2004; Rohr et al., 2008).

## 2.2. Invertebrate bioassays

We collected macroinvertebrates from three adjacent streams in the Santa Monica Mountains (California: Los Angeles County) using either D-frame nets (30 cm wide) or by hand from the underside of rocks (Peck et al., 2006). We separated macroinvertebrates by order into Nalgene™ containers filled with 750 ml of stream water, transported them in a cooler to the laboratory, and housed them in acrylic containers (30 × 15 × 12 cm, l x w x d) until bioassays commenced (~24 h). Specific invertebrate taxa included caddisflies (order Trichoptera, *Lepidostoma* sp.), dragonflies (order Odonata, *Anax* sp.), damselflies (order Odonata, *Enallagma* sp.), hellgrammites (order Megaloptera, *Corydalus* sp.), mayflies (order Ephemeroptera, *Paraleptophlebia* sp.), stoneflies (order Plecoptera, *Malenka* sp.), and toe biters (order Hemiptera, *Abetus* sp.). Although we have high confidence that all macroinvertebrates within a genus were the same species, we are limited by the taxonomic resolution in some species that require adult sex organs to identify the species. Because some orders of macroinvertebrates in our experimental bioassays undergo hemimetabolism, we measured the sclerotized head capsule of each specimen in the orders of Ephemeroptera, Hemiptera, Odonata, and Plecoptera. We evaluated if measurements were all within one standard deviation of the mean for each order (genera for Odonata) to determine if development differed between specimens. Any individuals that exceeded one standard deviation were excluded. In our statistical analysis, we subdivided order Odonata into the suborders Zygoptera and Anisoptera. We fabricated individual chambers from translucent acrylic tubing and sheet material to house individual insect larva for the 24 h bioassay period. Chamber diameters ranged from 2 to 6 cm and were scaled to accommodate various-sized larvae. We rinsed all chambers with HCl and then molecular grade water prior to use. The TTX solution of 1000 nmol TTX was mixed using 1 mg TTX standards (sodium citrate, Fisher Scientific) and 5-μm filtered, dechlorinated, tapwater. Filtered water was used as the control solution. We used an upper concentration of 1000 nmol $L^{-1}$, rather than the dose-response curve used for the cercariae assays, both because this is lethal to many invertebrates and vertebrates (Bucciarelli et al., 2016) and because we hypothesized that the invertebrates might have a higher tolerance of TTX given that they live a large part of their life cycle exposed to TTX in the water.

We performed bioassays in a climate-controlled, walk-in room where chambers were placed on a numbered matrix affixed to a workbench. Trichoptera (n = 65), Zygoptera (n = 38), Anisoptera (n = 38), Megaloptera (n = 15), Ephemeroptera (n = 59), Plecoptera (n = 22), and Hemiptera (n = 38) were collected from their containers, assigned a random number, and placed in the corresponding pre-filled chamber. All chambers were filled with TTX solution or treated tapwater (control). We monitored invertebrates hourly for 24 h for movement of limbs or gills or signs of mortality following protocols outlined in Brown (1980), whereby we used a paintbrush tip to touch the water surface or the animal itself. If the macroinvertebrate was not moving or unresponsive to the stimulation, we tested whether it had died or was sublethally poisoned using a Doppler flow probe (Conforma DP-NSP1, Cook Medical, Vandergrift, PA) that monitored whether hemolymph was moving within the circulatory system. If alive, we returned the macroinvertebrate to the experiment and continued monitoring.

## 2.3. Statistical analyses

For the parasite data, we used Cox proportional hazards regression models to assess the effects of TTX concentration (a continuous variable), trematode species identity (a factor with five levels), and their interaction on survivorship (hours to death, measured in 2 h intervals) (Fox and Weisberg, 2011). Parasites that survived the entire observational period (typically 24 h) were classified as 'censored' (although the majority of parasites died within this time frame, as expected for short-lived infectious stages (Paull et al., 2012). Because we detected a significant interaction between parasite species and TTX concentration, we subsequently subset the dataset by trematode species and evaluated the effects of TTX on each individually. The estimated coefficients and their standard errors from these analyses were used to compare sensitivity to TTX among parasites. Because cercariae typically die within 24 h, using coefficients was preferable to metrics such as LC50s because we sought to quantify the effect of TTX on survival relative to the control treatment. For the macroinvertebrate data, we used a similar approach to analyze survivorship with the only difference being that individuals experienced only two dosages (0 and 1000 nmol TTX $L^{-1}$) and their status was assessed hourly. All analyses were conducted using the R statistical program (2012) with the function *coxph* in the package *survival* (Therneau and Lomley, 2017). For analyses of invertebrate survivorship, which sometimes exhibited the problem of monotone likelihood because no individuals died within the 24 h period, we used *coxphf* to implement Cox regression with Firth's penalized likelihood (Heinze and Ploner, 2016).

## 3. Results

In total, 974 cercariae representing five species of trematode were tested for their sensitivity to TTX exposure. Consistent with the short lifespan of these free-living stages, 98.9% of all cercariae died over the course of the observation period, with censored cases (living more than 24 h) manifesting only for *M. syntomentera* (which exhibited high survivorship in many treatments, including 11 of 145 cercariae). Based on the overall analysis, we detected significant effects of TTX concentration, parasite identity, and their interaction on cercariae time-to-death (Log rank test = 562.2, df = 9, n = 974, P < 0.00001). TTX strongly reduced the survivorship of cercariae overall (TTX coefficient: 0.022156 ± 0.004152 SE, z = 5.336, P < 0.000001; Fig. 1). The model including the interaction effect had a much lower Akaike Information Criterion (AIC) value, which is a measure of the relative quality of a model for which lower values are better (▲AIC: 66.54).

After subsetting the dataset, each trematode species exhibited significantly elevated mortality in response to progressively higher TTX concentrations (range of $R^2$: 0.086 [*R. ondatrae*] to 0.558 [*E. trivolvis*]; Table 1). At TTX concentrations of 31.32 nmol $L^{-1}$, for instance, only 47% of cercariae survived longer than 2 h, relative to 82% in the control treatment. However, sensitivity varied considerably among trematode species (Fig. 1). *Echinostoma trivolvis* exhibited the greatest increase in mortality associated with TTX exposure (coefficient: 0.066164 ± 0.006058 SE, z = 10.92, P < 0.0001), such that each 1.0 nmol $L^{-1}$ increase of the toxin enhanced the risk of death by 6.6%. This was followed by *Cephalogonimus* sp. and *A. marcianae*, which had coefficients of 0.034850 ± 0.004483 SE (z = 7.773, P < 0.00001) and 0.022845 ± 0.004525 SE (z = 5.049, P < 0.00001), respectively. *Manodistomum syntomentera* and *R. ondatrae* exhibited the weakest responses to TTX (*M. syntomentera* TTX coefficient: 0.017673 ± 0.003664 SE, z = 4.823, P < 0.00001; *R. ondatrae* TTX coefficient: 0.015223 ± 0.002314 SE, z = 6.579, P < 0.00001) (Fig. 2).
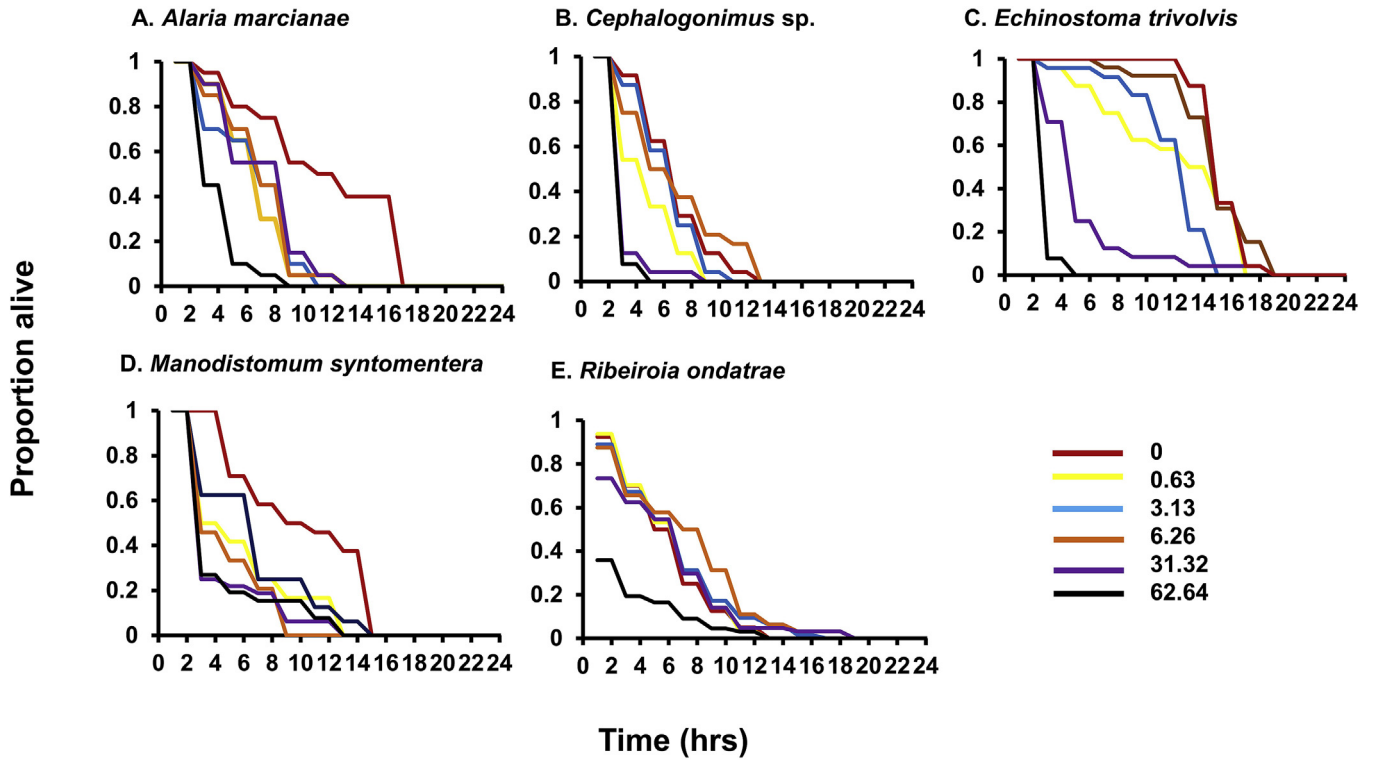
**Fig. 1.** Survivorship curves for cercariae of five trematode species exposed to increasing concentrations of tetrodotoxin (0.0, 0.63, 3.13, 6.26, 31.2, and 62.64 TTX nmol L$^{-1}$) over 24 h. Presented is the proportion of surviving cercariae every 2 h throughout the exposure period. For sample sizes and statistical results, see Table 1.

**Table 1**
Survivorship results for freshwater trematode cercariae exposed exogenously to different concentrations of tetrodotoxin (TTX). For each trematode species, we present the number of cercariae included in trials, the encystment location of the next life stage within the amphibian hosts, and model statistics for the best-fitting Cox proportional hazards model, including R$^2$ value and coefficient of TTX (value ± 1 SE).

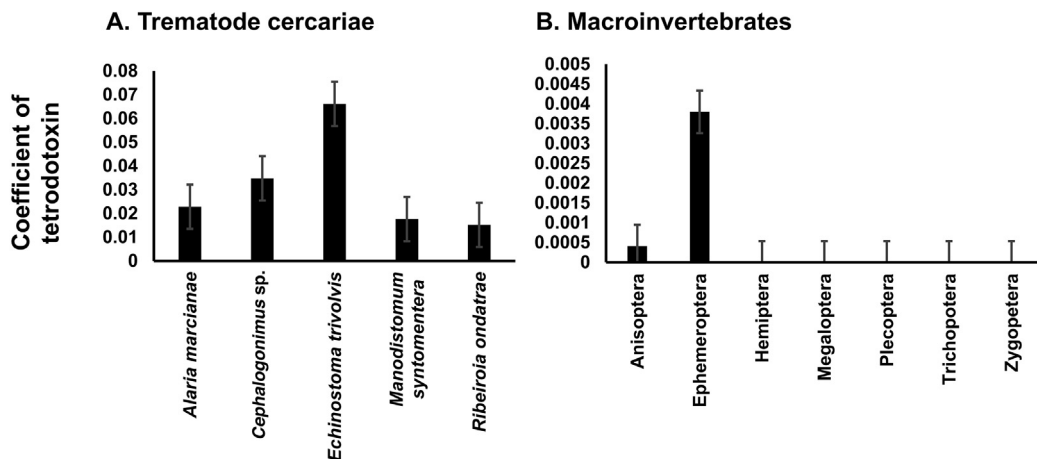| Trematode taxon | N (cercariae tested) | Encystment location | R$^2$ value | Coefficient of TTX ± 1 SE |
|---|---|---|---|---|
| *Alaria marcianae* | 120 | subcutaneous, mandible,tongue, and muscle | 0.17 | 0.022845 ± 0.004525 |
| *Cephalogonimus* sp. | 144 | subcutaneous and muscle | 0.312 | 0.034850 ± 0.004483 |
| *Echinostoma trivolvis* | 144 | kidneys | 0.558 | 0.066164 ± 0.006058 |
| *Manodistomum syntomentera* | 144 | subcutaneous | 0.134 | 0.017673 ± 0.003664 |
| *Ribeiroia ondatrae* | 422 | subcutaneous | 0.086 | 0.015223 ± 0.002314 |



**Fig. 2.** Results of Cox proportional hazard regression models to analyze the experimental effects of tetrodoxtoxin exposure on (A) five trematode species (n = 974) known to infect larval newts at increasing concentrations (0.0, 0.63, 3.13, 6.26, 31.2, and 62.64 TTX nmol L$^{-1}$) over 24 h, and (B) seven macroinvertebrates taxa (n = 296) known inhabit similar habitats as larval aquatic newts at two concentrations (0 vs. 1000 TTX nmol L$^{-1}$). Presented is the coefficient estimate ±1 SE that characterizes the proportional increase in mortality associated with each 1.0-unit increase in TTX nmol L$^{-1}$.

**Table 2**
Survivorship results for freshwater macroinvertebrates exposed exogenously to tetrodotoxin (TTX). For each taxonomic order or suborder, we present the number of individuals included in trials, and the model statistics for the best-fitting model, including the coefficient of TTX (value ± 1 SE). Because several invertebrate groups experienced no mortality, we used Cox regression with Firth's penalized likelihood.

| Macroinvertebrate taxon | Common name | N (individuals tested) | Coefficient of TTX ± 1 SE |
|---|---|---|---|
| Trichoptera | caddisflies | 64 | 0 ± 0 |
| Zygoptera | damselflies | 38 | 0 ± 0 |
| Megaloptera | hellgrammites | 15 | 0 ± 0 |
| Ephemeroptera | mayflies | 59 | 0.0038 ± 0.0015 |
| Plecoptera | stoneflies | 22 | 0 ± 0 |
| Hemiptera | toe biters | 38 | 0 ± 0 |
| Anisoptera | dragonflies | 38 | −0.00041 ± 0.0014 |

It is important to note, however, that while cercariae mortality generally increased with TTX concentration, this effect was not always linear (Fig. 1). For example, *E. trivolvis* average mortality at dose 3.13 nmol $L^{-1}$ was 11 h whereas at 6.26 nmol $L^{-1}$ it was 13.37 h. Such non-monotonic dose-response patterns are not uncommon in toxicological studies (Birnbaum, 2012; Conolly and Lutz, 2004; Vandenberg, 2014), particularly because of reactions in a complex biological system (Conolly and Lutz, 2004).

Among the six orders of macroinvertebrates tested (n = 296 total individuals), we observed very little mortality over the 24 h trials, regardless of TTX exposure. Only 21 Ephemeroptera (36%) and 2 Anisoptera (5%) died over 24 h. Specifically, 1 mayfly died in the first hour, 11 died over the next 15 h, and 7 died in final 8 h (for a total of 21). All invertebrates that died during testing were in the TTX exposure treatment, except one control Anisoptera. Ninety-two percent of tested invertebrates survived for 24 h. Based on the analysis, we detected a significant, negative effect of TTX (coefficient: 0.0026 ± 0.00087 SE, P = 0.000065) on survivorship with a main effect of invertebrate taxon (Table 2). More specifically, the effects of TTX were significant only in trials involving Ephemeroptera (coefficient: 0.0038 ± 0.0015 SE, P = 0.000014, n = 59) (Fig. 2).

## 4. Discussion

Chemical forms of defense conventionally studied as antipredator strategies have increasingly been recognized for their potential to limit or prevent infections by parasites ranging from tachinid flies to protozoans (Bos et al., 2015; de Roode et al., 2011; Huffman, 2003; Singer et al., 2009). For example, de Roode et al. (2008) found that consumption of milkweed-containing cardenolides by monarch caterpillars reduced the probability of subsequent infection by the apicomplexan parasite, *Ophryocystis elektroscirrha*. Infected monarchs have a shorter life span than uninfected individuals, suggesting that foraging on milkweed provides a positive fitness influence. Among amphibians, newts in the genus *Taricha* contain high concentrations of tetrodotoxin, which helps deter predation by vertebrates such as snakes, birds, and mammals (Brodie III and Brodie Jr, 1990; Brodie Jr, 1968). However, the effects of this toxin on infection by parasites remains relatively unexplored. Results of the current study indicate that exogenous exposure to TTX caused significant, dose-dependent mortality in trematode free-living stages (cercariae). This effect was true for all five species tested, each of which is known to infect or encounter larval newts in aquatic environments (Johnson et al., 2013). By comparison, TTX had no detectable effects on 6 of the 7 tested macroinvertebrate taxa, even at a substantially greater concentration. The only group that showed a significant response was mayflies, which experienced 27% mortality within 24 h, relative to zero mortality in the control treatment. This result is consistent with the overall sensitivity of mayfly larvae to chemicals and low oxygen availability previously established in the literature (Courtney and Clements, 2000; Hickey and Vickers, 1992; Johnson et al., 2010).

Among the trematode species included in this study, the magnitude of TTX-mediated reductions in survivorship varied by roughly 5-fold. All species exhibited a significant mortality response as TTX dosage increased. This effect was greatest in the kidney-infecting species *E. trivolvis*, for which a 1.0 increase in TTX nmol $L^{-1}$ was associated with a 6.8% increase in mortality risk, and weakest for *R. ondatrae*, for which the equivalent increase in TTX led to a 1.5% increase. *Cephalogonimus* sp. exhibited the second strongest response, while *A. marcianae* and *M. syntomentera* had intermediate sensitivity values and were not appreciably different from each other (Fig. 2). This variation in response could relate to differences among the parasites with respect to the type of infectious stages utilized or the encystment location within the host. For instance, four of the five parasites (*R. ondatrae*, *M. syntomentera*, *E. trivolvis*, *Cephalogonimus* sp.) form metacercariae within amphibian hosts, whereas *A. marcianae* form mesocercariae (Schell, 1985). Metacercariae are protected by a cyst wall that varies in thickness and is the product of both parasite and host tissue (Fried, 1993; Sukhdeo and Sukhdeo, 1994), which may help reduce exposure to toxins or host immune defenses (Bryan-Walker et al., 2007; Morley et al., 2003; Siddall, 1992). In contrast, mesocercariae lack a protective coating and are more mobile within intermediate hosts, making it somewhat surprising that *A. marcianae* was not more sensitive. A second salient factor is the location of infection, which could affect the amount of TTX experienced by a parasite and whether they have developed any tolerance to the toxin. *Echinostoma trivolvis* cercariae travel up the cloaca of larval amphibians to encyst within the developing kidneys, which have low concentrations of TTX relative to the skin (Wakely et al., 1966). Skin-encysting trematodes such as *R. ondatrae* likely experience higher TTX exposure (Cardall et al., 2004; Mosher et al., 1964; Tsuruda et al., 2001) and may therefore have greater tolerance to the toxin. An important next step will be to couple these *in vitro* experimental results with *in vivo* infection trials using newt hosts that vary in TTX concentration, which will also offer insights into natural patterns of infection by different trematodes observed in larval newt populations.

Based on the current results, larval trematodes were more sensitive to TTX relative to common aquatic invertebrates that co-occur with newt larvae in freshwater ecosystems. Among the 7 invertebrate taxa, we detected reductions in survivorship only within mayfly larvae, for which exposure to 1000 nmol $L^{-1}$ was associated with a 36% decrease in survivorship. Because the lifespan of trematode cercariae is short, the most relevant comparison is of the coefficients from the Cox proportional hazards models on survivorship. While the 1.0 unit (nmol $L^{-1}$) effect of TTX on mayfly mortality was 0.0038 ± 0.0015, the overall effect on trematode mortality was 0.0236 ± 0.00153 – a 6-fold increase (Fig. 2). This value accounts for the inherently shorter lifespan of cercariae. The

lesser sensitivity of macroinvertebrates is consistent with the ability of some insect taxa to buffer the effects of poisons by investing energetic resources into cuticle deposition, thereby decreasing the need to upregulate transcription of more enzymes to catalyze metabolism (Wood et al., 2010). Correspondingly, several macroinvertebrate taxa have been shown to consume larval or embryonic *Taricha* without notably ill effects, including larvae of Trichoptera, Zygoptera, and Anisoptera (Gall et al., 2012, 2011; Toledo et al., 2016). For instance, caddisfly larvae consume the eggs of *T. granulosa* (maximum of 1.53 µg TTX/egg) (Gall et al., 2012; Mebs et al., 2016) while dragonfly nymphs will eat larvae of both *T. granulosa* and *T. torosa* (0.296 ± 0.103 µg TTX/larva) (Bucciarelli and Kats, 2015; Mebs et al., 2016). Further consistent with our results, mayfly larvae — which are not predators of newt larvae — are often cited as bioindicator taxa sensitive to adverse environmental conditions, including hypoxia and contaminants (Gerhardt, 1996; Hodkinson and Jackson, 2005; Menetrey et al., 2008; O'Halloran et al., 2008).

Of relevance to conservation, trematode parasites can be harmful to amphibian development, fitness, and survival (Blaustein and Johnson, 2003; Koprivnikar et al., 2012, 2008). Previous research has found that exposure to *R. ondatrae* increases amphibian mortality as well as the risk of developing severe hindlimb malformations, which help to increase parasite transmission to bird definitive hosts via predation (Johnson and Lunde, 2005). In lab-based studies, parasite-induced malformations are associated with reduced jumping ability, lower prey intake, and reduced recapture rates (Goodman and Johnson, 2011a, 2011b). Similarly, infection by echinostome trematodes can inhibit osmoregulatory function in amphibians and reduce growth or survival (Holland et al., 2007; Johnson and McKenzie, 2009; Schotthoefer et al., 2003). With respect to newt hosts specifically, Johnson et al. (2012) found that *R. ondatrae* exposure reduced length and mass-at-metamorphosis of *T. torosa* larvae in a dose-dependent manner, although this species showed only a low frequency of parasite-induced malformations. The current results indicated that *R. ondatrae* was the least sensitive trematode to exogenous TTX exposure, consistent with the frequent detection of this parasite in larval newts in natural populations (e.g., (Johnson et al., 2013, 2002). Understanding whether TTX provides defense against such pathogenic trematodes and how it limits pathology is an important future research arena, including an examination of how infection susceptibility and TTX concentrations covary in populations (Johnson personal communication).

The effects of toxic pollutants on the functional biology of aquatic parasites has been a subject of increasing interest in recent years (Koprivnikar and Walker, 2011; Marcogliese, 2005). Notably, studies of contaminants have found a reduction in the production of trematode cercariae from molluscan hosts as well as their capacity to infect downstream hosts (Cross et al., 2001; Morley et al., 2003). For example, Rohr et al. (2008) reported an increase in cercariae mortality when *E. trivolvis* cercariae were exposed to atrazine at ecologically relevant concentrations. However, the effect of herbicide on cercariae mortality was smaller than the pesticide-induced increase in tadpole susceptibility to infections, leading to a net increase in total amphibian infection (Rohr et al., 2008). Alongside pesticides, heavy metals such as cadmium and zinc can decrease locomotion and survival of cercariae for multiple trematode species (Cross et al., 2001; Morley et al., 2003, 2001). Several researchers have suggested that, owing to their heightened sensitivity, parasites may function as effective bioindicators in the study of pollution in aquatic ecosystems (e.g., Bongers and Ferris, 1999; Huspeni et al., 2005; Marcogliese, 2005).

Overall, our study indicates that TTX exposure has a substantial effect on the mortality of trematode cercariae, which suggests that host toxicity has the potential to decrease infection in naturally occurring animals. An important subsequent step will involve *in vivo* parasite exposures of amphibian hosts that vary naturally in TTX concentration to investigate whether host toxicity can limit either initial infection, the temporal trajectory of parasite dynamics within the host, or overall pathology. Interestingly, our results indicate that macroinvertebrates exogenously exposed to TTX showed little to no changes in survivorship, at least over a period of 24 h. Beyond the implications of these findings for the infection or consumption of larval newts in aquatic ecosystems, they also raise interesting questions about the amount of waterborne-TTX to which aquatic organisms are exposed; particularly in systems or seasons with large breeding aggregations of adult *Taricha*, it is possible that concentrations of TTX within aquatic habitat could become non-trivial and thus have consequences for co-occurring vertebrate or invertebrate organisms.

### Author agreement/declaration

It is a statement to certify that all authors have seen and approved the final version of the manuscript being submitted. They warrant that the article is the authors' original work, hasn't received prior publication and isn't under consideration for publication elsewhere.

### Author contributions

DMC and PTJJ conceived, designed, and performed the parasite experiment; PTJJ analyzed the data; GMB, LBK, and RKZ conceived, designed, and performed the invertebrate experiment; DMC, GMB, and PTJJ wrote the paper and all authors provided editorial input and agreed approved final version of manuscript.

### Conflicts of interest

The authors declare no conflict of interest. The funding sponsors had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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