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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 nmolL^{-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.

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

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species of newts in the family Salamandridae known to possess TTX (Hanifin, 2010), the highest average dermal concentrations (25 μg $cm^{2\ -1}$) 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 μg 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* embroys 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 μg 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 μg ml^{-1} of TTX prepared at various concentrations to 0.4 ml of treated water achieving our final doses. One μl of treated water was used as the control solution. We pipetted individual cercariae into each well with a minimal transfer of water (<0.1 μl) using an automatic 0.1 μl 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