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Predatory Caddisfly Larvae Sequester Tetrodotoxin from Their Prey, Eggs of the Rough-Skinned Newt (*Taricha granulosa*)

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Abstract Caddisfly larvae (*Limnophilus* spp.) are important predators of eggs of the rough-skinned newt (*Taricha granulosa*). Newts may possess extremely large quantities of the neurotoxin tetrodotoxin (TTX) in their skin, and females may provision this toxin in their eggs. Using a competitive inhibition enzymatic immunoassay, we examined TTX-resistant caddisflies, sympatric with the known most toxic population of newts, for the presence of TTX. We found that caddisflies sequester TTX after consuming eggs in the laboratory. Caddisfly larvae that were frozen immediately after collecting in the wild possessed TTX. Finally, wild-caught larvae reared on a TTX-free diet in the laboratory retained TTX for up to 134 days, through metamorphosis and into the adult stage.

Keywords Coevolution · Toxicity · TTX · Sequester · *Limnephilus flavastellus*

Introduction

The rough-skinned newt, *Taricha granulosa*, possesses a powerful neurotoxin in its skin (Mosher et al., 1964; Hanifin et al., 1999). This toxin, tetrodotoxin (TTX), functions by blocking voltage-gated sodium channels in nerve and skeletal muscle (Kao, 1966; Narahashi et al., 1967). The presumed function of TTX in many organisms is as an antipredator defense. TTX appears to deter most potential predators of newts (Brodie, 1968). A link between toxicity and deterence has been demonstrated in *T. granulosa* (Williams et al., 2010; Gall et al., 2011b). Newt toxicity is highly variable, both within and among populations (Hanifin et al., 1999, 2008), with newts from some populations containing extremely large quantities of TTX. One individual from Benton Co., Oregon possessed more than 28 mg of TTX (unpublished data), which is the lethal oral dose for 14–56 humans (Yasumoto and Yotsu-Yamashita, 1996).

The presence of extreme quantities of TTX in newts is thought to be the result of a coevolutionary "arms race" between newts and one of their only known predators, garter snakes of the genus *Thamnophis* (see review in Brodie, 2010). These snakes have evolved resistance to the negative effects of TTX via changes to the pore region of the sodium channel (Geffeney et al., 2002, 2005). The level of resistance is dependent on the number, and position, of amino-acid substitutions to the sodium channel protein (Geffeney et al., 2005; Feldman et al., 2009, 2010). The phenotypic interface of coevolution between newts and snakes is TTX, with selection acting on toxin resistance in snakes, and toxicity in newts (Brodie et al., 2005). Some populations of snakes are actively involved in the "arms-race" process, while other populations have escaped the "arms race" and are highly resistant (Hanifin et al., 2008).

Recent evidence suggests that a second predator may also be involved in the coevolutionary process, thus driving elevated toxicity in newts (Lehman, 2006; Gall et al., 2011a). Caddisfly (Trichoptera) larvae are abundant in many aquatic habitats and, although traditionally viewed as detritovores, are now recognized as important predators of amphibian eggs (Wells, 2007). Newt eggs, like adults, contain large quantities of TTX (Wakely et al., 1966; Hanifin et al.,

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2003). The female provisions the TTX, with individual eggs containing more than 4 μg of TTX; an entire clutch (500 eggs) may contain 2 mg of TTX (Hanifin et al., 2003; Gall et al., 2012a). Despite their toxicity, large numbers of eggs may be consumed by caddisfly larvae with no apparent ill effects (Gall et al., 2011a). These results indicate that at least some species of caddisfly (e.g., *Limnephilus flavastellus*) are resistant to the toxin, although the mechanism by which resistance is conferred is unknown (Gall et al., 2011a).

Caddisflies appear to be important predators on a toxic early life-stage of *Taricha* and may, thereby, be involved in the evolutionary escalation of toxicity. Many species sequester toxic compounds from their prey (e.g. Hutchinson et al., 2007), with sequestration of toxins among insects being fairly common (Duffey, 1980; Nishida, 2002). Thamnophine garter snakes, the only other predator of newts, also sequester TTX (Williams et al., 2004). The resistance to TTX exhibited by caddisflies, coupled with their ability to consume a large number of highly toxic eggs in a brief period of time, presents an excellent opportunity to examine for TTX sequestration by caddisflies. We conducted a series of experiments and observations to examine: (1) whether caddisflies sequester TTX when they consume newt eggs in the laboratory: (2) whether wild-caught caddisflies contain TTX or not: and (3) if so, how long TTX is retained by wild-caught caddisflies when reared on a TTX-free diet in the laboratory.

Methods and Materials

Animal Collection Newt (*T. granulosa*) eggs were obtained from females in reproductive condition, collected by hand in March 2011 from Soap Creek ponds in the central Willamette Valley (Benton County, Oregon, USA). Newts were transported immediately to Utah State University (USU) and housed individually in 5.7 l plastic containers with 3 l of filtered tap water. They were maintained on a 12:12 (L:D) photoperiod at 6 °C, to prevent spontaneous egg deposition, and fed blackworms, *Lumbriculus variegatus*, weekly.

Caddisfly larvae, *Limnephilus flavastellus*, were collected by dip-net, from the same ponds as *T. granulosa* (see above). Caddisflies were transported to USU and housed in groups of 100 individuals in 11 l plastic tubs with 4 l of water and an aerator. All tubs were held in an environmental chamber at 6 °C under a 12: 12 (L:D) photoperiod. Caddisflies were fed maple-leaf detritus, prepared by placing maple, *Acer grandidentatum*, leaves in aerated aquaria with a small amount of pond detritus to facilitate the buildup of beneficial bacteria and fungi. All materials used in detritus preparation were collected from an area where newts do not occur and no TTX-bearing organisms are known [Cache Valley, Utah; see Gall et al. (2011a) for a detailed description of detritus preparation].

Do Caddisflies Sequester TTX? To determine whether or not caddisflies sequester TTX, we quantified the amount of TTX retained in the tissues of caddisflies 24 h after they consumed 5 newt eggs in the laboratory. Wild-caught caddisflies were collected around the beginning of the newt breeding season in March 2010. Individual caddisflies were housed in mesh-bottom cups (236 ml) positioned inside larger cups (500 ml). The mesh-bottom cup was elevated off the floor of the larger container to ensure that fecal material did not accumulate inside the test container. The cups were filled with 400 ml of water and maintained in an environmental chamber at 16 °C under a 12: 12 (L:D) photoperiod.

Each caddisfly was randomly assigned to either the control (N=12) or egg-fed (N=12) treatment; there was no difference in caddisfly mass between control and egg-fed treatments (df=22, t=-0.785, P=0.44). Caddisflies in the egg-fed treatment were given 5 eggs from 1 of two randomly chosen female newts that had recently begun depositing eggs. We monitored the caddisflies daily and recorded the number of eggs consumed by each caddisfly. Most individuals consumed the 5 eggs in 9 d or less (maximum of 12 d). Twenty four hours after consuming the fifth egg, each caddisfly was removed from the mesh cup and extracted from its case with a probe. The length of the case was measured and the larva weighed to the nearest 0.01 g, before being frozen at −80 °C for later TTX analysis. At the same time, a control larva that had not been fed eggs was removed from its case, weighed, and frozen. We compared the amount of TTX present in control and egg-fed caddisflies with a t-test; data were square-root transformed to meet the assumption of normality. We also tested the relationship between the amount of TTX sequestered and caddisfly mass and case length with linear regression.

Do Wild-Caught Caddisflies Possess TTX? To determine whether caddisflies under natural conditions contained TTX, we collected wild caddisflies and immediately froze them. Caddisflies were collected from Soap Creek ponds in April 2011. *Taricha granulosa* begin breeding in February (Nussbaum et al., 1983; Petranka, 1998), and by early March many females are depositing eggs in the Soap Creek ponds (personal observation). Therefore, caddisflies were collected well after newts had begun breeding and, presumably, had access to ample egg resources. Immediately after collection, 32 caddisflies were removed from their cases, weighed to the nearest 0.01 g, and frozen on dry ice. We assessed the relationship between body mass and total TTX and TTX concentration (ng TTX/mg body mass) in the wild-caught caddisflies, using nonlinear regression with a polynomial inverse first-order equation in Sigmaplot v11.0 (Systat Software, Inc). We used an inverse first-order equation because both total TTX and TTX

concentration exhibited a descending curve that started at zero. This model fitted the equation to the data, estimated the values of each of the two parameters in the equation, and computed a *t* statistic and *P*-value for each parameter.

How Long Do Caddisflies Retain TTX? To determine the length of time that caddisflies retained TTX, we reared wild-caught caddisflies in the laboratory. Caddisfly larvae were collected in April 2011 and transported to USU. Larvae were maintained in mesh-bottom cups under the same conditions as described above. Caddisflies were given a diet of maple-leaf detritus and wheat grains (*Triticum* sp.) to facilitate growth and metamorphosis. Subsets of larvae were frozen at −80 °C, for TTX quantification, 13–44 d after collection. Seven larvae pupated, and the resulting adults were frozen immediately after emergence, 81–142 d following collection. We used linear regression to compare the length of time in captivity to the amount of TTX retained by caddisflies. In addition, 4 caddisfly larvae (*Hesperophylax occidentalis*) allopatric with newts (collected from Paradise, UT), as well as samples of all food items (wheat grains and detritus), were tested for the presence of TTX.

TTX Quantification Frozen caddisfly samples were extracted for analysis using previously described techniques (Hanifin et al., 2002). Briefly, each sample was macerated in 600 μl of 0.1 M acetic acid, placed in a boiling water bath for 5 min, and centrifuged at 13000 RPM for 20 min. The supernatant was transferred to a filter tube (500 μm) and centrifuged for an additional 20 min. The extracted sample was transferred to a screw-top microcentrifuge tube and frozen at −80 °C until analysis. TTX 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 also to bind to the plate, resulting in a high absorbance reading. The absorbance value is used to calculate TTX concentration off 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 undiluted and run against standards in 0.1 M acetic acid. Plates were read at 405 nm.

Results

Caddisflies that consumed five newt eggs in the laboratory had higher (df=22, t=5.06, P<0.001, Fig. 1) levels of TTX in their tissues (mean total TTX±SE=18.75±2.78 ng) than caddisflies that did not consume newt eggs in the laboratory (mean total TTX=5.27±0.91 ng). There was no relationship (case length: R^2<0.001, P=0.78; mass: R^2<0.001, P=0.692) between caddisfly mass and the amount of TTX sequestered. Many of the caddisflies frozen immediately after collection from the Soap Creek ponds possessed TTX. However, total TTX (R^2=0.65, P<0.001, Fig. 2a) and TTX concentration (R^2=0.79, P<0.001, Fig. 2b) were both negatively correlated with body mass. All parameter estimates in the polynomial inverse equations used to model these relationships were highly significant (P<0.001), except for parameter one in the equation for total TTX, which was non-significant (P=0.089).

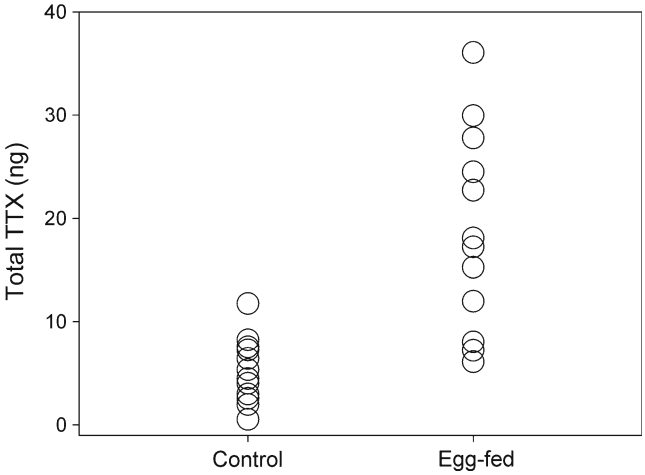

Fig. 1 Total tetrodotoxin (TTX) present in larval caddisflies (*Limnephilus flavastellus*) that consumed five rough-skinned newt eggs (Egg-fed) or were maintained on a TTX-free diet (Control). Twenty-four hours after consuming eggs, caddisflies had greater quantities of TTX in their tissues than control animals (df=22, t=5.06, P<0.001)

Most caddisfly larvae collected at the Soap Creek ponds and reared in the laboratory retained TTX, despite being maintained on a TTX-free diet (Fig. 3). Five out of seven larvae that pupated and eclosed retained similar TTX levels through to adulthood, with some individuals retaining TTX for at least 134 days after collection (Fig. 3). One adult caddisfly that emerged 108 days after collection as a larva had a large quantity of TTX (163.3 ng) relative to other individuals. There was a positive relationship ($F_{[1,71]}$=7.1, R^2=0.078, P=0.01) between the amount of TTX and the time (days) after collection. However, this trend was driven by the adult caddisfly with 163 ng of TTX; after removing this datum point, no relationship ($F_{[1,71]}$=0.61, R^2<0.001, P=0.44) between toxicity and time after collection was detected. Four caddisfly larvae (*H. occidentalis*) from Utah, and the samples of food items (detritus and wheat), contained no TTX (data not shown).

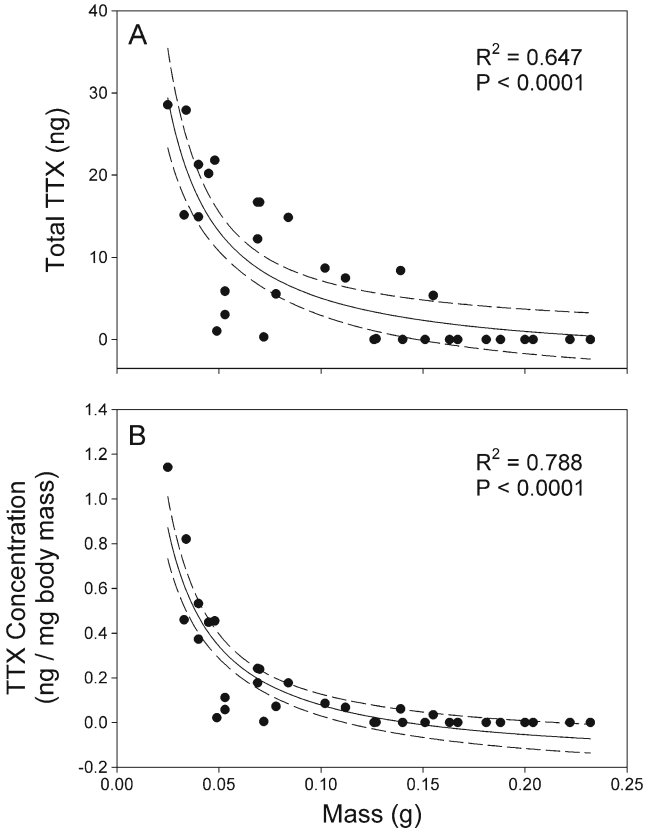

Fig. 2 Non-linear regression (solid line) of larval caddisfly (*Limnephilus flavastellus*) mass (without case) and (**a**) total amount (ng) of tetrodotoxin (TTX), and (**b**) TTX concentration (ng/mg body mass). Larval caddisflies were frozen immediately after collection in the field. Dashed lines are 95 % confidence intervals. Curves are polynomial inverse first order

Discussion

Caddisflies are major predators of eggs of *T. granulosa*, with individual larvae consuming as many as 29 eggs in 14 days

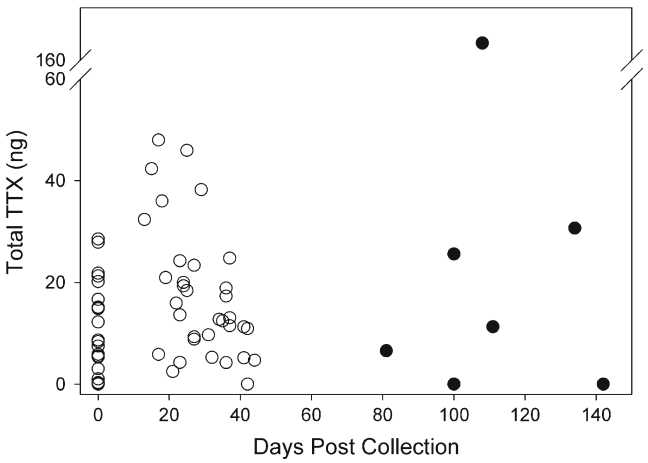

Fig. 3 Total amount (ng) of tetrodotoxin (TTX) present in larval (open circles) and adult (closed circles) caddisflies (*Limnephilus flavastellus*) collected as larvae from the wild and reared in the laboratory on a diet of detritus and wheat (*Triticum* sp.)

(Gall et al., 2011a). Newts deposit large quantities of TTX in their eggs (Wakely et al., 1966; Hanifin et al., 2003), thereby exposing caddisfly larvae to large amounts of TTX during predation. In this study, caddisfly larvae sequestered TTX in their tissues after consuming five toxic eggs. Moreover, TTX levels did not decline in caddisflies reared in the laboratory, with some individuals retaining TTX through to adulthood, up to 134 days after collection.

The discovery that caddisflies sequester TTX is not entirely surprising, given that other predators of TTX-bearing organisms also sequester the toxin. For example, adult *T. granulosa* harbor large quantities of TTX in their skin (Mosher et al., 1964; Wakely et al., 1966; Hanifin et al., 1999), yet are readily eaten by TTX-resistant garter snakes (Brodie, 2010). After consuming a toxic newt, the garter snake *T. sirtalis* sequesters TTX in its liver (Williams et al., 2004, 2011b). Measurable quantities of TTX are in the liver of adult snakes 30 days after newt ingestion, with at least 73 days being estimated for the liver to be devoid of the toxin (Williams et al., 2004). When neonate snakes were provided a mass-adjusted oral dose of pure TTX, the toxin half-life was estimated at 8.1 days, yielding a comparable estimate of 61 days to clearance (Williams et al., 2011b). Sequestration of TTX also has been demonstrated in marine systems. For example, the echinoderm *Astropecten scoparius* sequesters TTX from its prey, which includes bivalves and gastropods (Lin and Hwang, 2001), and non-toxic puffer fish sequester TTX in the liver when fed a diet containing TTX (Matsumoto et al., 2007; Kono et al., 2008).

The toxicity of adult newts and their eggs is correlated and highly variable (Hanifin et al., 2003). Although the toxicity of individual eggs used in our experiment was unknown, the eggs came from the most toxic population of newts known (TTX amounts range from 672 to almost 3000 ng per egg; Hanifin et al., 2003; Gall et al., 2012a). Based on the average egg amounts from Soap Creek (1528 ng TTX/egg, Hanifin et al., 2003), the caddisflies sequestered approximately 0.25 % (range: 0.08–0.47 %) of the available TTX in the eggs they consumed. By comparison, *T. sirtalis* sequestered between 0.68–3.4 % (mean= 1.6 %) of available TTX seven days after consuming a newt (Williams et al., 2004). The similarity in percentage of toxin sequestered by caddisflies and snakes is intriguing. TTX-binding proteins have been identified in the liver and plasma of puffer fish (Kodama et al., 1983; Matsui et al., 2000; Yotsu-Yamashita et al., 2001), as well as the hemolymph of the shore crab, *Hemigrapsus sanguineus* (Nagashima et al., 2002). Williams et al. (2004) hypothesized that a binding protein may be responsible for TTX sequestration in snakes. We suspect that there may be a functional limit in the amount of TTX caddisflies can sequester due to a similar process.

Despite relatively low levels, the amount of TTX sequestered by caddisflies may still fall within ecologically relevant levels. TTX functions as a potent antipredator defense (e.g. Brodie, 1968; Williams et al., 2010) and may function in this context for caddisflies. Dragonfly nymphs are important predators in freshwater pond communities (Corbet, 1999) and are present in the Soap Creek ponds. Nevertheless, these invertebrates reject larval newts possessing as little as 229 ng of TTX (whole body) or concentrations greater than 0.46 ng/mg body mass (Gall et al., 2011b). Some caddisflies in this study had TTX concentrations falling within this unpalatable range (0.0–1.14 ng/mg body mass), which could render them less vulnerable to predation. Susceptibility to TTX is highly variable among taxa (Brodie, 1968), and it is unknown if other predators of caddisflies, including freshwater invertebrates (e.g., Hemiptera) or fishes, are deterred from consuming prey containing TTX; adult newts do not consume caddisfly larvae if alternative prey is available (unpublished data). A recent study on the palatability of blue-ringed octopus, *Hapalochlaena lunulata*, paralarvae found that 200 ng of TTX were insufficient to deter predation by many marine stomatopods and fish (Williams et al., 2011a). The authors speculated that, despite negative results with some predators, this quantity of TTX may be sufficient to deter parasites or other untested predators.

Adult caddisflies are nocturnal and generally cryptically colored (Wiggins, 2004). Adult *L. flavastellus* are tan and do not possess conspicuous coloration or markings that could be considered aposematic (personal observation). Nevertheless, adults may occur in high densities (personal observation), and the presence of TTX post-metamorphosis could protect adults from potential predators. Alternatively, the toxin could be allocated into caddisflies eggs to increase offspring survival; similar processes have been demonstrated in other insects that sequester toxic compounds from their prey (e.g., Eisner et al., 2000).

The presence of TTX in field-caught caddisflies does not demonstrate definitively that the caddisflies acquired TTX from newt eggs; they could synthesize TTX directly or accumulate it through other dietary sources, such as detritus. Although plausible, we find these alternative explanations unlikely. First, the amount of TTX sequestered by caddisflies in the laboratory was not correlated with mass, despite a negative relationship between TTX quantity and mass in field-caught animals. This relationship suggests the presence or absence of TTX may be behaviorally mediated. Recent work has demonstrated that caddisfly larvae are attracted to chemical stimuli emanating from gravid female newts and newt eggs (Gall et al., 2012b). Moreover, the level of TTX in caddisflies in this study was highly variable, between 0–163 ng, with many field-caught individuals lacking TTX entirely. The primary food source of caddisfly larvae is detritus, and all individuals consumed detritus when it is was provided in the laboratory. If caddisflies synthesize TTX, or accumulate it through bacteria or other dietary sources such as detritus, we expect less variability in TTX amount or fewer individuals lacking TTX altogether. This variability in sequestered TTX might, however, correspond with the variability of caddisflies consuming newt eggs; in the laboratory, some individuals do not consume eggs, while others consume several dozen in a few days (Gall et al., 2011b). Additional research is necessary to demonstrate conclusively a relationship between caddisfly TTX and egg predation in the field, as well as to understand the variation in TTX in caddisfly populations.

The negative relationship between TTX and caddisfly body mass in wild-caught individuals may be explained by the primarily benthic habits of caddisflies and their inability/unwillingness to climb aquatic vegetation. Female newts attach their eggs to aquatic vegetation, with the presence of caddisfly larvae corresponding with an increase in the height at which eggs are deposited (Gall et al., 2012b). We have tested the behavior of caddisflies at different depths and found that caddisfly larvae generally are restricted to the benthic and low-lying areas within aquatic vegetation (Gall et al., 2012b). Further, larger caddisflies, which contained the least amount of TTX in our study, were even less prone to climb (Gall et al., 2012b). These results suggest that large caddisflies may not be consuming as many newt eggs as small caddisflies, which may climb vegetation to consume this resource.

In summary, we demonstrated that caddisflies sequester TTX from eggs of the rough-skinned newt, that caddisflies frozen immediately after field collection contained TTX, suggesting that they consumed newt eggs, and that caddisflies collected mid-way through the newt breeding season and reared in the laboratory maintained TTX levels over long periods of time (at least 134 days) and retained it through to adulthood.

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