CADDISFLY LARVAE (LIMNEPHILIDAE) AS PREDATORS OF NEWT (*TARICHA GRANULOSA*) EGGS: ANOTHER PLAYER IN THE COEVOLUTIONARY ARMS RACE REVOLVING AROUND TETRODOTOXIN?

by

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ABSTRACT

Caddisfly Larvae (Limnephilidae) as Predators of Newt (*Taricha granulosa*) Eggs: Another Player in the Coevolutionary Arms Race Revolving Around Tetrodotoxin?

by

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Utah State University, 2012

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A coevolutionary arms-race between garter snakes (*Thamnophis sirtalis*) and newts (*Taricha granulosa*) is believed to be responsible for the presence of exaggerated phenotypes in these species. In this scenario, tetrodotoxin (TTX) resistance in garter snakes has led to the evolution of prey populations that are extremely toxic. Despite the wealth of information acquired on the interaction between these species, very little research has been conducted on possible interactions with other predators. I conducted a suite of experiments examining alternative predators on newts, specifically focusing on predators of the eggs and larvae. I tested for the presence of chemical communication in a potential egg predator (caddisfly larvae), investigated the basic ecological conditions of the interaction between caddisflies and newts, measured the toxicity of larval newts and tested for palatability to predatory dragonfly naiads, and more thoroughly explored the interaction between caddisfly larvae and newts. Caddisflies utilize chemical stimuli in their environment to detect and avoid potential predators, as well as locate and consume

newt eggs. Larval caddisflies are extremely abundant at one study site (775,000 caddisfly larvae / 0.2 ha pond), and appear to be resistant to the negative effects of ingesting tetrodotoxin. After hatching, larval newts retain substantial quantities of TTX and individuals with more TTX are more likely to be unpalatable to predatory dragonfly naiads. Ovipositing female newts respond to the presence of caddisflies by depositing their eggs at the top of the water column where they are out of the reach of most predatory caddisflies. When caddisflies do consume a newt egg, some of the toxin is sequestered and may be retained through metamorphosis. Finally, caddisflies preferentially consume newt eggs that contain less toxin. This has the potential to lead to selective pressure against newts with less toxin and ultimately drive an increase in toxicity in the adult population. Collectively, these findings indicate an additional player, caddisfly larvae, is likely involved in the arms-race revolving around tetrodotoxin.

(160 pages)

PUBLIC ABSTRACT

Caddisfly Larvae (Limnephilidae) as Predators of Newt (*Taricha granulosa*) Eggs: Another Player in the Coevolutionary Arms Race Revolving Around Tetrodotoxin?

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Some populations of newts (*Taricha granulosa*) possess large quantities of the neurotoxin tetrodotoxin (TTX) in their skin and eggs. Many populations of garter snake (*Thamnophis sirtalis*) are resistant to this toxin and can consume large numbers of newts with no negative effects. Despite the wealth of information acquired on the interaction between newts and their predator, garter snakes, very little research has been conducted on possible interactions between newts and other predators. I conducted a suite of experiments examining for the presence of other predators on newts, specifically focusing on predators of their eggs and larvae. I found a single predator, caddisfly larvae were capable of consuming the toxic eggs. Larval caddisflies are extremely abundant at one study site (775,000 caddisfly larvae per pond), and appear to be resistant to the negative effects of ingesting tetrodotoxin. After hatching, larval newts retain substantial quantities of TTX and most are unpalatable to predatory dragonfly naiads. Ovipositing female newts respond to the presence of caddisflies by depositing their eggs at the top of the water column where they are out of the reach of most predatory caddisflies. When caddisflies do consume a newt egg, some of the toxin is retained in their body tissues.

Finally, caddisflies consume more newt eggs when those eggs contain less toxin versus eggs that contain large amounts of TTX. This may cause newt eggs that contain low quantities of TTX to more likely to die of predation which could ultimately drive an increase in toxicity of the adult population over time. Collectively, these findings indicate an additional player, caddisfly larvae, is a major predator of newts and could be involved in the evolution of tetrodotoxin toxicity in newts.

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CHAPTER 1

INTRODUCTION

Coevolution requires reciprocal selection between closely interacting species, which may ultimately lead to complementary evolutionary changes (Thompson 1994). Reciprocal selection between predator and prey results in a coevolutionary "arms race" when antagonistic interactions lead to exaggerated counter-adaptations in both organisms. The trait or suite of traits that moderates each species' adaptations is known as the phenotypic interface of coevolution (Brodie and Brodie 1999), and is consequently the feature that becomes exaggerated in coevolutionary processes. In one of the most well documented coevolutionary arms races, this phenotypic interface centers on the potent neurotoxin, Tetrodotoxin (TTX).

In this system, the snake predator, *Thamnophis sirtalis*, have evolved increasing TTX resistance to counter elevated toxicity in their toxic newt prey (*Taricha granulosa*) (Brodie and Brodie 1990, 1991). Throughout the range of the prey, different populations of newts and snakes have varying levels of toxicity and resistance (Hanifin et al. 1999; Brodie et al. 2002) resulting in a geographic mosaic of coevolution (Thompson 2005). Although some population phenotypes are well matched, possibly resulting in strong reciprocal selection for these traits, others are mis-matched leading to escape from the arms race by the predator (Hanifin et al. 2008).

Surprisingly, snake-newt interactions are not limited to *Thamnophis sirtalis* and *Taricha granulosa*. Similar patterns of coevolution, all revolving around the phenotypic interface of TTX toxicity and resistance, have apparently evolved repeatedly and independently between several snake species and their newt prey in North America. In

these cases, both *Th. couchii* and *Th. atratus* have developed resistance to separate species of newts including *Ta. granulosa, Ta. sierrae, and Ta. torosa* (Brodie et al. 2005; Feldman et al. 2009). With each interaction, the evolution of resistance in the predators is the result of similar modifications to the amino acid sequence of the sodium channel protein. Newts of the genus *Taricha* are not the only amphibians that produce TTX, and recent work has found that several other TTX producing amphibian species (including two frogs and a salamander) are each preyed upon by different snake species that are resistant to TTX via similar molecular patterns (Feldman et al. 2012). These interactions involve multiple species, evolved independently, and occur on multiple continents (Feldman et al. 2012).

The most toxic populations of newts are exploited by snakes so resistant as to not suffer negative effects of TTX ingestion (Hanifin et al. 2008). Yet, these newts are tremendously toxic, containing up to 16 mg of TTX, enough to kill 56,000 mice or at least 8 humans. Although the evolution of extreme toxicity in modern newt populations may be due to coevolution with garter snakes, the origin of tetrodotoxin in this group suggests alternative selective agents must be considered for the existence and exaggeration of this phenotype. Modern newts (members of the family Salamandridae) all possess TTX, yet they originated in the middle cretaceous (100 mya), well before the origin of garter snakes (50 mya). This timeline suggests predation by snakes is unlikely to be responsible for the initial evolution of TTX in this group and that alternative predators should be examined for their potential to influence toxicity.

Tetrodotoxin is primarily located in the skin of adult newts (Mosher et al. 1964; Wakely et al. 1966; Brodie et al. 1974; Hanifin et al. 1999) and successfully repels every

known predator except garter snakes (Brodie 1968). However, TTX can also be found in the ovaries and ova of adult females, as well as recently deposited eggs (Twitty 1937; Wakely et al. 1966). TTX has been assumed to serve an antipredator function in the eggs as well (Twitty 1966; Daly et al. 1987), but experimental evidence of a defensive function is lacking. The amount of TTX in newt eggs was recently established by Hanifin et al. (2003), who measured the level of TTX in individual eggs from several females. This study found high levels of TTX in individual eggs as well as variation between clutches from different females. There was little variation in TTX per egg within a clutch and clutch toxicity was highly correlated with the skin toxicity of the corresponding female. The correlation between female and egg toxicity led the authors to conclude that female toxicity could be influenced via indirect selection on the eggs or vice versa. This correlation indicates that this pathway has the potential to be a major factor in the evolution of toxicity in newts.

The predation regime on newt eggs and larvae has received very little attention, but given the high levels of TTX in individual eggs it seems unlikely that many predators will be able to consume the eggs. Nevertheless, two egg predators have been identified. One of those predators, adult newts, will readily cannibalize eggs or larvae (Chandler 1918). Given that female newts are the origin of the egg toxin and appear to be resistant to it (Brodie 1968; Brodie and Brodie 1991), their ability to cannibalize eggs is not surprising. The only other documented predators of newt eggs are caddisfly larvae (order: Trichoptera) (Lehman and Campbell 2007; see Chapter 3 herin). Except for a select few species that are secondarily adapted for life on land, caddisfly larvae are entirely aquatic (Wiggins and Currie 2008). Although most larvae are primarily

considered benthic detritivores, they have been documented consuming the eggs of other amphibians and fishes (Murphy 1961; Dalrymple 1970; Fox 1978; Rowe et al. 1994; Richter 2000). In addition, Anderson (1976) demonstrated that animal matter is important for normal caddisfly growth and development, and that caddisfly larvae are likely facultative carnivores, consuming animal matter whenever available.

The goal of this research is to elucidate the predator-prey dynamics between newt eggs and larvae and their predators in an effort to determine the potential for these interactions to influence the evolution of toxicity in newts. Specifically, I will examine the interaction between caddisflies and newt eggs, as well as between larval newts and predatory dragonfly nymphs.

Chapter 2. Due to structural complexity and turbidity, communication in aquatic environments is often dominated by the use of chemical cues (Ferrari et al. 2010). These cues are used for all manner of communication including locating food or mates and avoiding predators (reviewed by Ferrari et al. 2010). Chemical stimuli may therefore be an important vector for the transmission of information between newt eggs and predators, including caddisfly larvae. It is unknown if chemical cues are used by caddisflies (order Trichoptera) to acquire information in their environment. This chapter will examine whether caddisflies detect and avoid predators solely through the use of chemical stimuli, as well as test for the presence of a chemical alarm cue, which would further enhance their avoidance behavior. Because selection by predators may be one of the most intense interactions an organism experiences, utilization of chemical cues by caddisflies in this context would necessitate the expansion of our analysis of their use during interactions with toxic newt eggs.

Chapter 3. Almost nothing is known regarding the suite of predators capable of consuming newt eggs. This chapter will identify the aquatic invertebrates that consume newt eggs in the laboratory. Tetrodotoxin inhibits the propagation of action potentials (Narahashi et al. 1967); therefore growth may be negatively affected in caddisflies that consume these eggs. I will also provision caddisflies with eggs and measure a metric of growth (change in case length) over time to determine whether egg consumption is likely to have a positive or negative effect on caddisfly fitness. In addition, I will estimate the abundance of caddisflies at a study site to determine the scale at which this interaction could take place in a natural pond, as well as the potential for caddisflies to be a selective force on the newt population through egg predation.

Chapter 4. The focus of this chapter will be measuring tetrodotoxin in newt larvae and evaluating how toxicity influences interactions with predatory dragonfly nymphs. Indirect experimental evidence from Twitty and Johnson (1934) and Twitty (1937) indicates tetrodotoxin levels decline rapidly in newt larvae, likely rendering them palatable to potential predators. Using a competitive inhibition enzymatic immunoassay (CIEIA), I will measure the amount of tetrodotoxin present in newt larvae at three different developmental stages, as well as recently metamorphosed juveniles. Tetrodotoxin is believed to serve a defensive function in most organisms, and successfully repels almost all predators of adult newts (Brodie 1968). I will also expose newt larvae to predatory dragonfly nymphs (*Anax junius*) to determine if sufficient quantities of TTX remain after hatching to function in defense.

Chapter 5. This chapter will more deeply explore the interaction between newts and caddisflies to determine their potential to influence toxicity in adult newts.

Specifically, I will address four questions. First, do caddisflies respond behaviorally to newts? I will expose caddisflies to a variety of chemical stimuli they would likely encounter during a predatory interaction with newt eggs to determine if these cues are used to locate eggs. Second, do newts possess strategies that limit predation on their eggs? Many species of amphibians and insects detect and avoid egg and larval predators when depositing eggs (e.g. Chesson 1984; Resetarits and Wilbur 1989). I will examine the behavior of ovipositing female newts in response to predatory caddisflies and determine whether newts avoid depositing eggs in microhabitats that are relatively risky for their offspring. The fitness advantage of this behavior will be verified in field trials. Third, is the TTX present in newt eggs sequestered by caddisflies? Caddisflies will be provisioned with newt eggs to determine if the toxin is sequestered. These results will be verified by collecting and freezing caddisflies in the field and measuring the amount of TTX present in these wild-caught specimens. Finally, what is the potential for caddisflies to indirectly select for elevated toxicity in newts? Caddisflies will be provisioned with eggs of varying toxicity to determine if a preference exists for lower toxicity eggs. The presence of such a preference could lead to selection on the newt population for higher toxicity (Hanifin et al. 2003), thereby demonstrating the potential for reciprocal selection between newts and caddisflies.

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CHAPTER 2

BEHAVIORAL AVOIDANCE OF INJURED CONSPECIFIC AND PREDATORY CHEMICAL STIMULI BY AQUATIC CADDISFLY LARVAE (*HESPEROPHYLAX OCCIDENTALIS*)[1]

Prey animals often encounter situations that hinder their ability to conduct normal fitness-enhancing behaviors. Mating and foraging are frequently interrupted by predator vigilance and avoidance, and antipredator behavior. Many caddisfly larvae build protective cases that are carried with them throughout the aquatic lifecycle. However, they are still vulnerable to predation, yet it is unknown the extent caddisflies use chemical cues for predator recognition and avoidance. We exposed larval caddisflies *Hesperophylax occidentalis* (Banks, 1908) to predatory, conspecific, and heterospecific chemical cues to determine if caddisfly larvae can use chemical stimuli alone for predator recognition and avoidance. Exposure to predator and injured conspecific chemicals elicited significant decreases in activity, while exposure to injured and uninjured heterospecific chemicals yielded no significant change in activity. The extended latency to move following exposure to predator kairomones indicates larval caddisflies utilize chemical cues for predator recognition and avoidance, and a similar decrease in movement associated with exposure to injured conspecifics suggests the presence of a chemical alarm cue.

[1] Coauthored by Brian G. Gall and Edmund D. Brodie, Jr. Reprinted with permission of NRC Research Press from the Canadian Journal of Zoology Vol. 87, pages 1009-1015, 2009.

INTRODUCTION

Prey animals must often balance activities such as foraging and reproduction with predator avoidance (Lima and Dill 1990; Lima 1998). Predator avoidance and corresponding antipredator defenses are inherently costly because prey must forgo activities that would otherwise enhance fitness (Lima and Dill 1990; Lima 1998). Therefore, prey should be able to accurately measure the current level of predation risk and react accordingly to minimize the costs of this trade-off (see reviews in Lima and Dill 1990; Lima 1998).

Prey use a variety of behaviors to measure and respond to the threat of predation. In aquatic ecosystems where predators are often cryptic and sedimentation or dense vegetation frequently reduces visual acuity, chemical cues can provide reliable information in the absence of all other stimuli (see review in Kats and Dill 1998). Specifically, aquatic prey often use chemical cues such as kairomones and alarm cues to assess predation risk and decrease their probability of predation. Kairomones are chemical stimuli that elicit beneficial behavioral changes in heterospecific receivers (Brown et al. 1970). When exposed to predator kairomones, aquatic organisms often reduce activity, increase drift, increase shelter use, or a host of other predator avoidance behaviors (see reviews in Wooster and Sih 1995; Kats and Dill 1998). Alternatively, alarm cues are chemical stimuli released by injured individuals which elicit similar responses as predator kairomones when received by nearby conspecifics (Smith 1992; Chivers and Smith 1998). Alarm cues function to warn conspecifics of immediate danger (Smith 1977; Smith 1992; Mathis and Smith 1993a; Chivers and Smith 1998) or attract

additional predators that disrupt the predation event (Mathis et al. 1995; Chivers et al. 1996a; Chivers and Smith 1998). More recently, Chivers et al. (2007) demonstrated that alarm cues may also provide protection against pathogens, parasites, and ultraviolet-B radiation (at least in fishes). Some species have evolved to respond to alarm cues from sympatric heterospecifics that occupy the same prey guild (Mathis and Smith 1993b; Brown et al. 2001; Mirza and Chivers 2001a). This cross-species response is most likely to occur in species occupying the same prey guild and microhabitat, and having the same predators. Under these conditions, responses to heterospecific alarm cues should provide similar survival benefits as a response to conspecific alarm cues. In fact, several studies have documented the increased survival benefits experienced by organisms responding to chemical alarm cues. For example, Wisenden et al. (1999) demonstrated that the amphipod *Gammarus minus* Say, 1818, survives encounters with green sunfish (*Lepomis cyanellus* Rafinesque, 1819) longer when they are simultaneously exposed to conspecific alarm cues. Similar fitness benefits experienced by prey responding to alarm cues have also been found in amphibians and fishes (Hews 1988; Mathis and Smith 1993a; Chivers et al. 2002).

Invertebrates use chemical cues extensively, yet little is known about the use of chemical cues in one of the largest orders of aquatic insects. Caddisflies (Trichoptera) are one of the most widely distributed aquatic insect orders with 1400 recognized species in the United States and Canada alone (Merritt et al. 2008). Except for a few species, caddisfly larvae (henceforth caddisflies) are entirely aquatic and occupy a great diversity of freshwater habitats (Merritt et al. 2008). Many species of caddisflies construct portable cases that function as defense against some predators (Otto and Svensson 1980;

Johansson 1991; Nislow and Molles 1993; Wissinger et al. 2006) and may increase the efficiency of respiration (Williams et al. 1987).

Even with defensive cases, caddisflies are still vulnerable to a variety of vertebrate and invertebrate predators, and previous work on caddisfly-predator interactions has primarily focused on the direct influence of predation (e.g. Otto and Svensson 1980; Kohler and McPeek 1989; Johansson 1991; Johansson and Nilsson 1992; Nislow and Molles 1993; Johansson and Englund 1995; Otto 2000; Wissinger et al. 2006). Nevertheless, some empirical evidence exists to suggest caddisflies use chemical cues to detect predators. Kuhara et al. (2001) found that caddisflies exposed to sculpin *Cottus nozawae* Snyder, 1911 stimuli reduced activity and food intake during the most risky time of day. However, the experimental design in that study permitted both chemical and hydrodynamic cues. Malmqvist (1992) examined caddisfly activity in response to chemical cues from predators, but got conflicting results between the two caddisfly species tested. In addition, the study was weakly replicated and statistical analyses were not performed on the caddisfly data. Pestana et al. (2009) exposed caddisflies to predatory chemical cues and a pesticide and found respiration rates decreased with pesticide exposure but increased when exposed to predators. Experiments by Boyero et al. (2006) eliminated all but chemical cues and found that larvae adjusted their selection of case type according to the specific predatory threat. Although these studies suggest caddisflies utilize chemical cues, the extent to which chemical cues are used for immediate predator recognition and predator avoidance behavior is unknown. In addition, it is unknown if caddisflies possess chemical alarm cues which would further enhance their ability to detect and avoid predators.

To determine if caddisflies utilize chemical cues in immediate predator avoidance behavior and to determine if caddisflies possess a chemical alarm cue, we examined the behavioral response of the caddisfly *Hesperophylax occidentalis* (Banks, 1908) to chemical stimuli from (1) a potential predator (rainbow trout, *Oncoryncuss mykiss* (Walbaum, 1792)), (2) injured conspecifics, (3) injured heterospecifics (the amphipod *Gammarus lacustris* G.O. Sars, 1863; henceforth amphipod) known to possess chemical alarm cues (Wudkevich et al. 1997), (4) uninjured heterospecifics (*G. lacustris*) and (5) a blank control.

MATERIALS AND METHODS

Animal Collection and Maintenance. Caddisflies used in this study were collected on 29 October and 19 November 2008 from a single pond (41.5709ºN, -111.8485ºW) in Paradise, Utah. Caddisflies were collected by dip net and placed in plastic buckets for transport to Utah State University. Larvae were then transferred to a 37-L glass aquarium containing 15-L of tapwater filtered by reverse osmosis (henceforth "tapwater"). Larvae were transferred to the holding aquarium by draining most of the water in the buckets and dumping all larvae into the holding aquarium. This method was used to transfer test larvae to prevent acclimation to the simulated predation event (see below). The aquarium was kept in an environmental chamber at 16.5 °C and a 12 h light : 12 h dark cyle. Larvae were fed *ad libitum* on timothy hay pellets (Bunny Basics/T, Oxbow Pet Products, Murdock, Nebraska, USA).

While collecting caddisflies, we sampled (hook and line) and visually identified the presence of rainbow trout in the collection pond. We therefore assume the caddisflies used in this study were not naïve, but were experienced with trout predators.

Amphipods were collected from the same pond as the caddisfly larvae on 19 November 2008. The amphipods and a small amount of detritus (food) were transferred to a 37 L aquarium with 15 L of tap water and maintained in the same environmental chamber as caddisfly larvae.

Stimulus Preparation. Hesperophylax occidentalis larvae were tested with five different stimuli: (1) blank tap water control (N = 20), (2) uninjured amphipods (N = 20), (3) injured amphipods (N = 20), (4) injured caddisfly larvae (N = 20), and (5) rainbow trout (N = 20; Table 2.1). All stimuli (except rainbow trout stimulus, which was frozen following preparation) were prepared with tap water maintained at 16.5 °C with an aerator and used immediately following preparation. The rainbow trout treatment was prepared by catching four rainbow trout (fork length, range: 24.0–25.5 cm) by hook and line and placing two each into 39-L containers with 20-L of tap water. The trout were removed from the container 1 hr after introduction, and immediately returned to the pond of capture after stimulus collection. The stimuli from the two containers was homogenized and frozen at -80 C in 2-L aliquots after collection. Although rainbow trout were sampled from a separate pond as the caddisflies, caddisflies were present in the pond with stimulus trout. We thus can not rule-out the presence of dietary cues in the trout stimulus.

Immediately prior to testing, the trout stimulus was thawed and maintained at 16.5 °C with an aerator. The injured caddisfly treatment was prepared by crushing 20

Table 2.1. Treatments tested in experiments 1 and 2, including sample size, mean mass (g) of larval caddisfly *Hesperophylax occidentalis* in each treatment, mean latency to emerge (s), and mean latency to move (s).

			Latency (s)	
Treatment	Sample Size	Mass (g)	Emerge	Move
Experiment 1				
Blank Control	20	0.157 ± 0.02	23.00 ± 5.34	55.10 ± 11.41
Rainbow Trout	20	0.173 ± 0.02	102.50 ± 14.16	117.40 ± 15.60
Experiment 2				
Blank Control	20	0.163 ± 0.06	42.70 ± 8.97	49.15 ± 8.36
Uninjured Amphipod	20	0.169 ± 0.02	32.20 ± 6.73	45.00 ± 7.70
Injured Amphipod	20	0.174 ± 0.02	56.10 ± 10.55	62.40 ± 11.35
Injured Caddisfly	20	0.177 ± 0.07	95.35 ± 17.00	101.10 ± 16.73

Note: Values are mean ± SE.

caddisflies in 800 ml of tap water with a mortar and pestle. The caddisfly larvae were starved for seven days prior to stimulus collection to remove any food cues. Stimulus from injured amphipods was prepared by grinding 30 individual amphipods, starved for 72 hrs, in 800 ml of tap water using a mortar and pestle. The uninjured amphipod stimulus was prepared by placing 30 individuals, without food, in a 1-L plastic container with 800 ml of tap water for 72 hrs prior to experimentation. All solutions were filtered through 100% polyester (Poly-Fil, Fairfield Processing Corp., Danbury, Connecticut, USA) to remove large solid particles. Stimuli were coded prior to experimentation so the observer was blind to treatment selection.

Behavioral Bioassays. Two experiments were performed between 1300 and 2000 on 5 and 26 November 2008. In experiment 1, caddisflies were exposed to rainbow trout stimuli and a blank control to determine whether larvae utilize predatory chemical cues in predator recognition and avoidance. These trials were conducted in a plastic container (9cm × 9cm, 6cm height) with 140 ml of stimulus. In experiment 2, caddisflies were exposed to a blank control, as well as stimulus water from uninjured amphipods, injured

amphipods, and injured conspecifics. Experiment 2 was conducted to determine the presence of chemical alarm cues in caddisfly larvae, as well as whether cross species responses to heterospecific alarm cues occur in this system. To ensure a strong concentration of stimuli and to minimize the unnecessary sacrifice of caddisflies and amphipods, these trials were conducted in 35mm × 10mm round plastic dishes with 10 ml of stimulus. All trials were conducted inside an environmental chamber at 16.5 °C. Prior to each trial, the test container was rinsed with tap water and a randomly chosen treatment was poured into the test container. A caddisfly was selected from the holding aquarium, grasped between the thumb and forefinger, and held for 3 seconds. The larva was then dropped into the center of the test container from 1 cm above the stimulus water and the trial began. This method simulates a predatory attack (Johansson 1991; Johansson and Englund 1995) and is similar to that used in Lefcort et al. (2000); larvae retreated into their protective case when grasped. After dropping the larva into the test container, the latency to emerge from the case (time between start of trial and emergence of the head, but not legs) and latency to begin moving (time between start of trial and emergence/movement of the legs) were recorded. Upon completion of testing, larvae were weighed to the nearest 0.01g and maintained in a holding aquarium. There was no difference in the mass of larvae in the six treatments (Kruskal-Wallis: $H = 7.80$, $P = 0.10$). Individual larvae were tested only once.

Statistical Analysis. Data from experiment 1 were analyzed with a Mann-Whitney rank-sum test (SigmaPlot version 15; Systat Software, Inc., San Jose, California, USA). Data from experiment 2 were analyzed by Kruskal-Wallis tests (Minitab version 15; Minitab Inc., State College, Pennsylvania, USA) followed by nonparametric multiple

comparisons (WINKS SDA version 6.0; Texasoft, Cedar Hill, Texas, USA). We performed linear regressions (SigmaPlot version 11; Systat Software, Inc., San Jose, California, USA) to test for differences in response time between different-sized caddisflies.

RESULTS

Caddisflies significantly increased their time to emerge from cases (Mann-Whitney *U* test, $U = 53$, $P < 0.001$; Fig. 2–1A) and begin moving (Mann-Whitney *U* test, $U = 96.5$, $P = 0.005$; Fig. 2–1B) when exposed to chemical stimuli from rainbow trout compared with the blank control (experiment 1, Table 2.1). In experiment 2, there were significant differences among larval responses to the four stimuli for both response variables (latency to emerge: Kruskal-Wallis test, $H = 13.37$, $P = 0.004$; Fig. 2–2A; latency to move: Kruskal-Wallis test, $H = 10.04$, $P = 0.018$; Fig. 2–2B). When exposed to chemicals released by injured conspecifics, caddisflies also showed a significant increase in latency to emerge (Nonparametric multiple comparisons: $Q = 2.73$, $P < 0.02$), and significantly increased the latency to begin moving (nonparametric multiple comparisons: $Q = 2.55$, $P < 0.05$), compared with the blank control (Figs. 2–2A, 2–2B; experiment 2, Table 2.1). When larvae were exposed to chemical stimuli from injured amphipods, they exhibited responses intermediate of the blank and trout or injured caddisfly treatments; however, this response was not significantly different from the blank control for either response variable (all *P* values >0.50; Figs. 2–2A, 2–2B; experiment 2, Table 2.1). Finally, larvae exposed to water containing uninjured

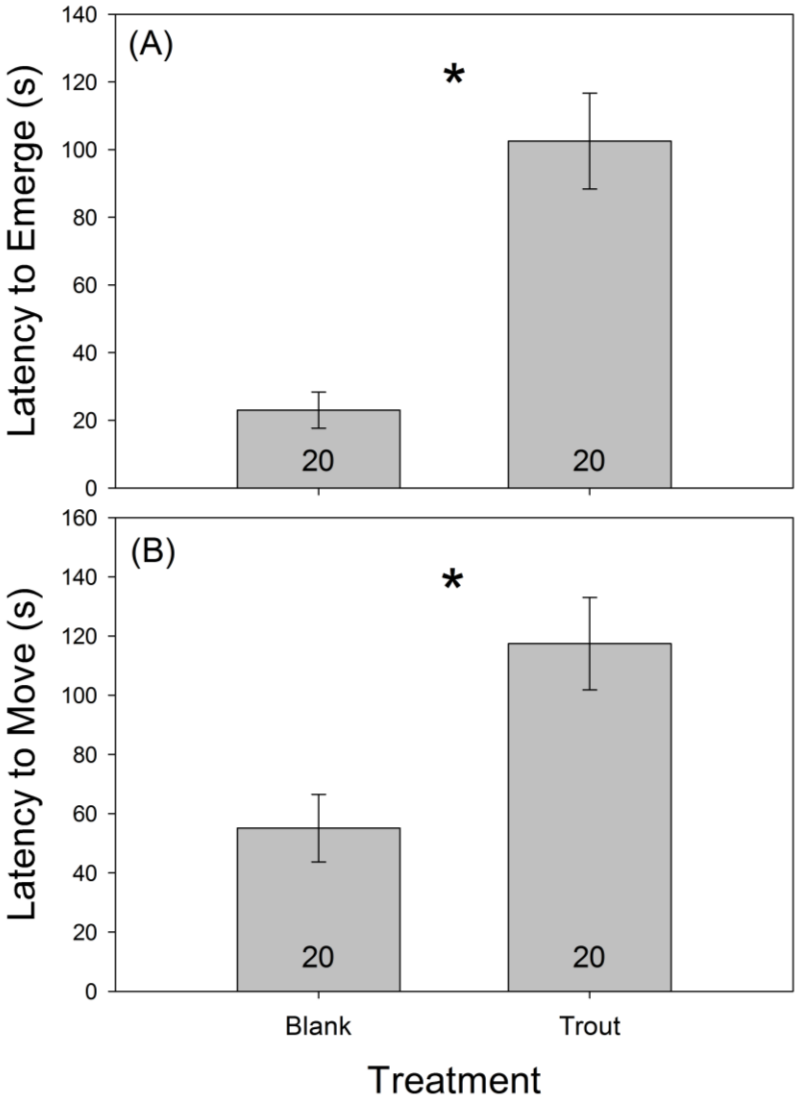

Fig. 2–1. Responses of larval caddisfly *Hesperophylax occidentalis* exposed to chemical stimuli from a predator (rainbow trout, *Oncoryncuss mykiss*) and a blank control. (A) The latency (mean ± 1 SE) for the heads of caddisflies to emerge from the cases. Mann–Whitney *U* test: *, $P < 0.001$, (B) The latency (mean ± 1 SE) for caddisflies to emerge from cases and begin moving. Mann–Whitney *U* test: *, $P < 0.005$. Sample sizes are given within the bars.

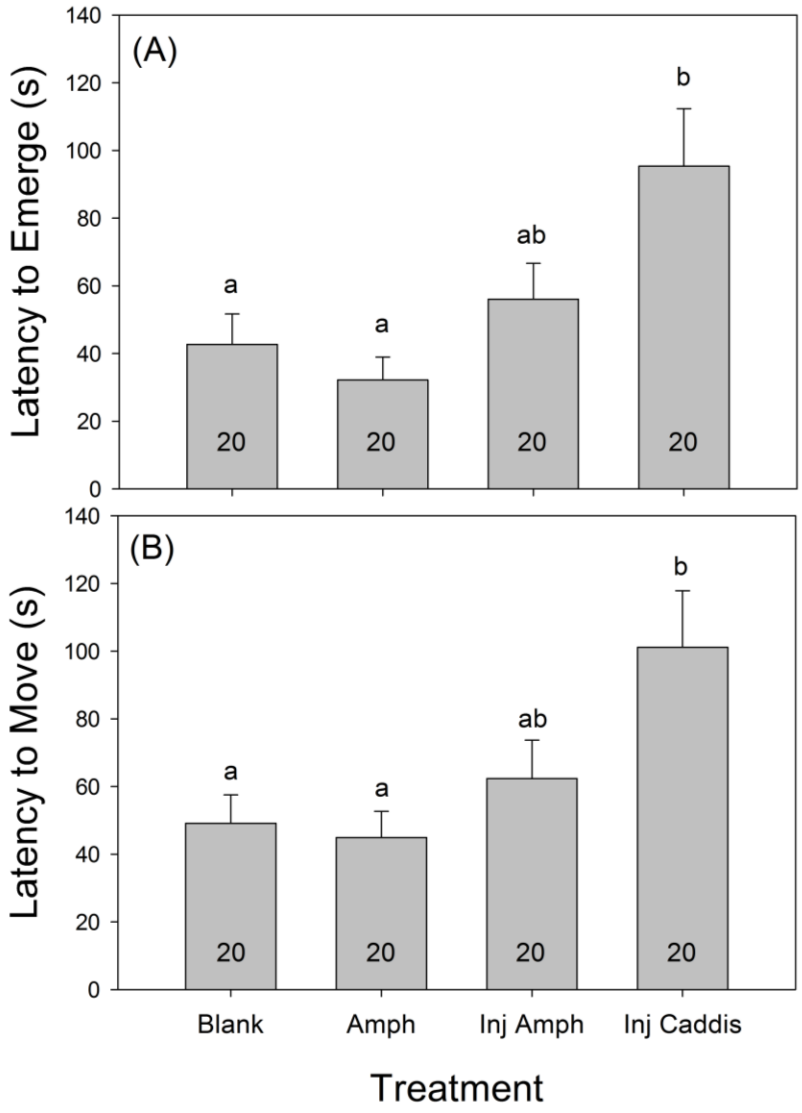

Fig. 2–2. Behavioral responses of larval caddisfly *Hesperophylax occidentalis* exposed to chemical stimuli from injured conspecifics (Inj Caddis), injured heterospecifics (amphipod *Gammarus lacustris*; Inj Amph), uninjured amphipods (Amph), or a blank control (Blank). (A) The latency (mean ± 1 SE) for the heads of caddisflies to emerge from cases. Kruskal–Wallis ANOVA: $P = 0.004$. (B) The latency (mean ± 1 SE) for caddisflies to emerge from cases and begin moving. Kruskal–Wallis ANOVA: $P = 0.018$. Different letters above bars indicate significant differences between treatments ($P < 0.05$). Sample sizes are given within the bars.

amphipods did not alter activity compared with the blank control (all P values >0.50; Figs. 2–2A, 2–2B; experiment 2, Table 2.1).

Larvae that emerge from the case but do not immediately begin moving may be attempting to acquire more information from the chemical stimuli or the surrounding environment. Therefore, the difference between latency to move and latency to emerge may indicate additional chemoreception or predator inspection behavior. The latency to emerge was subtracted from the latency to move for each test larva, and there was no significant difference between the treatments for this behavior (Kruskal-Wallis test: H = 6.12, P = 0.106).

Because the larvae varied in size within a treatment and because ontogenetic changes in antipredator response occur in other prey species (Puttlitz et al. 1999; Mathis and Vincent 2000; Brown et al. 2001; Marcus and Brown 2003), we used linear regression to look for differing responses between larvae of different sizes in each treatment; where necessary, data were transformed with a square-root function to meet assumptions of normality. A significant correlation between mass and either latency behavior was detected in only one treatment. Within the injured caddisfly treatment, larger larvae took longer to emerge from cases and begin moving (emerge: $F_{[1,18]}$ = 6.19, P = 0.02; move: $F_{[1,18]}$ = 4.28, P = 0.05; Figs. 2–3A, 2–3B).

DISCUSSION

Caddisflies showed a significant increase in time to egress from their protective cases when exposed to chemical cues from predatory rainbow trout. The threat of

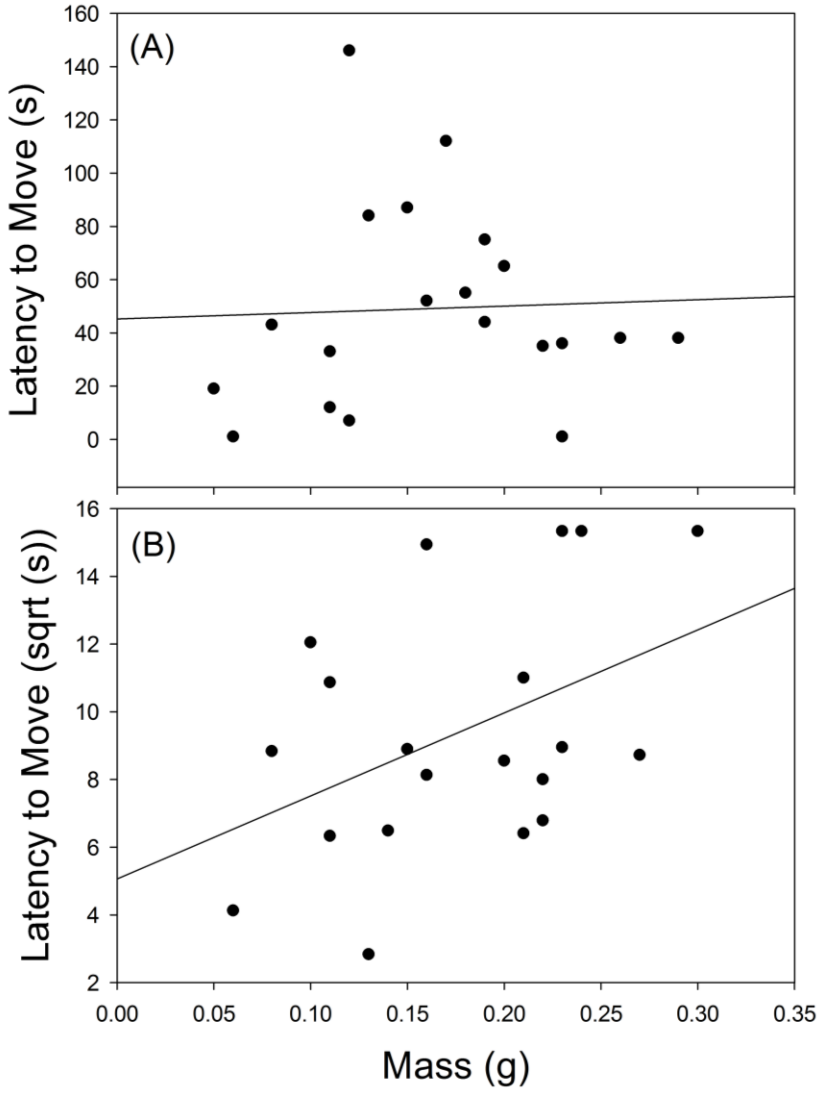

Fig. 2–3. Mass (g) of larval caddisfly *Hesperophylax occidentalis* in the (A) blank treatment in relation to the latency to move ($R^2 < 0.002$, $P = 0.86$) and the (B) injured caddisfly treatment compared with latency to move ($R^2 = 0.19$, $P = 0.05$). Data for injured caddisfly treatment were transformed with a square-root function.

predation is not constant, and the level of risk prey experience can fluctuate extensively (Lima and Dill 1990). Caddisflies that are able to adjust their behavior to the immediate level of predation risk should have a higher probability of survival. Larvae remaining inside their protective case after a predator has been chemically identified should reduce the likelihood of being detected and attacked. Rainbow trout are active predators that extensively utilize visual and hydrodynamic (i.e. lateral line) cues during foraging in clear and turbid waters (Montgomery et al. 2002, Rowe et al. 2003). The prey capture efficiency of rainbow trout decreases with reductions in prey movement (Ware 1973), and caddisflies that remain motionless and feign death after a predatory attack do indeed increase their probability of survival (Johansson 1991; Johansson and Englund 1995). The combination of chemical information clearly enhances this antipredator response and should result in an even greater reduction of predation risk.

The use of predator kairomones by caddisflies is not limited to immediate changes in activity. When exposed to chemical cues from three different predators (dragonfly niads, larval fire salamander (genus *Salamandra* Laurenti, 1768), and brown trout (*Salmo trutta* L., 1758)), caddisflies adjusted their choice of case type according to the level of predation risk (Boyero et al. 2006). When larvae were removed from their cases and given a choice between mineral and organic cases (mineral cases provide greater protection from many predators; Otto and Svensson 1980), those exposed to chemical cues from potential predators entered cases faster and consistently chose mineral cases over organic cases (Boyero et al. 2006). The combination of immediate responses to perceived predatory threat and adaptive long-term behavioral responses should minimize the risk of predation for caddisfly larvae.

The decrease in activity exhibited by caddisfly larvae in response to injured conspecifics was similar to the response to trout kairomones, indicating the presence of a chemical alarm cue. Alarm cues have previously been documented in a variety of aquatic organisms (reviewed by Chivers and Smith 1998), but not in Trichoptera. The presence of a chemical alarm cue increases the probability of prey detecting a predator that has recently attacked a conspecific. Alternatively, as demonstrated by Mathis et al. (1995), if the alarm cue also attracts additional predators, the predation event might be disrupted and the sender may survive the encounter. This model seems problematic in a prey organism that is swallowed whole; however, fishes often swallow caddisflies and subsequently spit them out in an effort to dislodge them from their protective case (Johansson 1991; Johansson and Englund 1995). This process can be repeated multiple times, and may provide ample opportunity for the release of the alarm cue and attraction of other predators. Nevertheless, how the alarm cue functions in wild caddisfly populations remains to be studied.

It is likely the caddisflies used in this study were experienced with trout, and larger caddisflies reduced activity to a greater extent than small larvae when exposed to the alarm cue (Fig. 2–3B). Large caddisflies are at a higher instar than smaller individuals and should therefore be older and presumably more experienced with local predation threat. The increased antipredator response with increasing mass (i.e. age) suggests that some component of learning may be involved in the larvae's response to the alarm cue. Other freshwater insects have been shown to use learning in predator recognition and avoidance. By pairing the alarm cue with novel predatory chemical stimuli, damselfly and mosquito larvae can learn to avoid novel predators (Wisenden et

al. 1997; Ferrari et al. 2007). With learning an important component of predator recognition and avoidance, further research should focus on its role, as well as predator diet, on caddisfly antipredator behavior.

Caddisflies did not significantly increase the latency to emerge from their case in response to amphipod alarm cue. However, a trend toward reduced activity is present (Figs. 2–2A, 2–2B). If caddisflies were to reduce activity toward amphipod alarm cues it would likely confer survival benefits because receivers would increase the chance of detecting nearby predators. On the other hand, if a predator is consuming heterospecifics, the presence of heterospecific alarm cues may indicate some degree of safety from predation and result in a weaker antipredator response. Predator recognition is often influenced by the diet of the predator (Gelowitz et al. 1993; Wilson and Lefcort 1993; Chivers et al. 1996b; Chivers and Mirza 2001; Mirza and Chivers 2001a), and some organisms do not respond to predator cues when the predator has only been eating heterospecifics (Gelowitz et al. 1993; Stabell and Lwin 1997; Belden et al. 2000). Nevertheless, the role heterospecific alarm cues have in caddisfly antipredator behavior needs further investigation.

Prey fitness is increased from responding to conspecific and heterospecific alarm cues by decreasing the probability of encountering the predator or increasing the chance of escape after detection (Mirza and Chivers 2001b). Chemical alarm cues are abundant in aquatic ecosystems and many species of gastropods, insects, crustaceans, amphibians, and fishes possess analogous systems where antipredator behavior is elicited from injured conspecific chemical stimuli (reviewed by Chivers and Smith 1998). Among freshwater invertebrates, the presence of such alarm systems has been well documented and includes

amphipods (genus *Gammarus* Fabricius, 1775; Williams and Moore 1985; Wisenden et al. 1999), daphnia (*Daphnia magna* Straus, 1820; Pijanowska 1997), crayfish (genus *Orconectes* Cope, 1872; Hazlett 1994), mosquito larvae (*Culex pipiens* L., 1758; Sih 1986), mayfly larvae (order Ephemeroptera; Scrimgeour et al. 1994; Huryn and Chivers 1999), and damselfly larvae (genus *Enallagma* Charpentier, 1840; Chivers et al. 1996b; Wisenden et al. 1997).

The survival of prey organisms is dependent upon accurate and reliable information about predation risk. In aquatic environments, chemical cues can provide the information necessary for prey to respond to the threat of predation and increase their probability of survival. Our study indicates that caddisflies utilize chemical cues for immediate predator recognition and antipredator behavior. Moreover, the caddisfly *H. occidentalis* appears to possess and utilize chemical alarm cues expanding our knowledge of chemical alarm cues in aquatic taxa.

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CHAPTER 3

SURVIVAL AND GROWTH OF AQUATIC CADDISFLIES AFTER PREDATION ON TOXIC NEWT EGGS (*TARICHA GRANULOSA*)[2]

The rough-skinned newt (*Taricha granulosa* (Skilton, 1849)) possesses a powerful neurotoxin tetrodotoxin in the skin that is secondarily deposited in the ova. Although assumed to serve an antipredator function in the eggs, empirical evidence of the toxin's role in preventing egg predation is lacking. In this study, we characterized the aquatic macroinvertebrate community at a location sympatric with extremely toxic newts and estimated the abundance of the caddisflies. We tested aquatic macroinvertebrates sympatric with toxic newts for their capacity to consume the toxic eggs, and examined the propensity of egg predation and its effect on growth of the only known predator of newt eggs, caddisfly larvae. Limnephilid caddisfly larvae were the only invertebrate observed to consume substantial quantities of toxic newt eggs. Survival and growth of the caddisfly, *Limnephilus flavastellus* (Banks, 1918), continued when larvae consumed toxic eggs and did not differ from *L. flavastellus* that also had access to an alternative food source (detritus). *Limnephilus flavastellus* that had access to eggs + detritus consumed a similar number of eggs compared with those provided with eggs only. These results, combined with the abundance of caddisflies, suggest caddisflies are important predators of eggs of *Taricha granulosa*.

[2] Coathored by Brian G. Gall, Edmund D. Brodie, III., and Edmund D. Brodie, Jr. Reprinted with permission of NRC Research Press from the Canadian Journal of Zoology Vol. 89, pages 483-489, 2011.

INTRODUCTION

Toxicity is a common antipredator strategy and empirical evidence indicates that toxic prey often escape predatory encounters uninjured, thus conferring a selective advantage to those individuals (Fisher 1930; Paradise and Stamp 1991; Williams et al. 2003; see Chapter 4). Some toxic or distasteful species secondarily provide the same protection to their offspring by depositing the chemical defenses in the ova. These compounds protect the developing embryo until hatching, whereupon behavioral defenses can reduce the risk of predation or the juvenile can sequester its own toxin.

Although embryonic toxins may prevent predation by some predators, it is unlikely that any defense will confer complete protection (Orians and Janzen 1974). For example, Licht (1968, 1969) established that eggs of toads (genus *Bufo* Laurenti, 1768) are unpalatable to a variety of potential predators including leeches (genus *Batrachobdella* Viguier, 1879), larval Northwestern Salamanders (*Ambystoma gracile* (Baird, 1859)), and fishes (black bullhead, *Ameiurus melas* (Rafinesque, 1820); longear sunfish, *Lepomis megalotis* (Rafinesque, 1820); green sunfish, *Lepomis cyanellus* Rafinesque, 1819; threespine stickleback, *Gasterosteus aculeatus* L., 1758; cutthroat trout, *Oncorhynchus clarkia* (Richardson, 1836)). However, the Common Garter Snake (*Thamnophis sirtalis* (L., 1758)) frequently eats adult bufonids and is not deterred from consuming toad eggs (Licht 1968). Eggs of the saddled toby (*Canthigaster valentine* (Bleeker, 1853)) are unpalatable to two species of sympatric reef fish, but are consumed by a closely related species (Gladstone 1987). Nonetheless, egg toxicity clearly limits the

scope of predators that can consume the eggs and likely confers a fitness advantage to those organisms that supply their embryos with this defense.

One of the most toxic vertebrates, newts of the genus *Taricha* Gray, 1850, deposit large quantities of the neurotoxin tetrodotoxin (TTX) in the ova (Twitty 1937; Mosher et al. 1964; Wakely et al. 1966; Hanifin et al. 2003). Although the toxin is also located in the skin and serves an antipredator function in adult newts (Brodie 1968; Brodie et al. 1974), it is unknown if the toxin confers similar protection to the eggs. Tetrodotoxin is an extremely powerful toxin, blocking the pore of voltage-gated sodium channels thereby stopping the propagation of action potentials and rapidly leading to asphyxiation and death (Kao 1966; Narahashi et al. 1967). Tetrodotoxin quantities range between 672 to 2767 ng in a single egg (Hanifin et al. 2003), enough to kill up to 14 mice (20 g each) in 10 minutes. Although it is unlikely many predators consume these eggs, Lehman (2006) observed larval caddisflies (order: Trichoptera) consuming newt eggs at some localities. Furthermore, caddisflies are known to consume the eggs of other amphibians (Murphy 1961; Dalrymple 1970; Richter 2000; Romano et al. 2008). If caddisflies exhibit the same susceptibility to TTX as mice, a single egg contains enough TTX to kill 500 to 3700 caddisflies.

We evaluated the possible interactions between newt eggs and potential predators by first characterizing the macroinvertebrate community at one of our study sites and providing these invertebrates with toxic eggs in the laboratory. Because caddisflies have been observed to consume newt eggs we specifically examined if consumption of newt eggs prevents larval caddisfly growth and development. Finally, we estimated the abundance of caddisflies to determine their potential impact on the newt population.

MATERIALS AND METHODS

Study Area and Macroinvertebrate Sampling. Invertebrate sampling took place at a series of eight man-made ponds (Soap Creek ponds) located in the Central Willamette Valley, near Corvallis, Oregon, USA. Located on the eastern edge of the Coast Range Mountains, the surrounding vegetation consists primarily of coniferous forest. The ponds are arranged in two rows of four, with the upper row 4 m higher than the lower ponds. Each pond is 22.86 m × 91.44 m square and gently slopes to a depth of 3-4 m at the easternmost edge. The ponds within a row are separated by 2 m wide berms.

For the present study, we haphazardly selected four ponds to serve as sampling sites. Invertebrate sampling occurred on 11 March 2010. We used two sampling techniques to document the diversity of the macroinvertebrate community and the abundance of caddisflies. Caddisfly abundance was estimated using a 19 L bucket (0.285 m diameter) with the bottom cut out. The bottomless bucket was rapidly pushed into the substrate and mesh strainers (aperture = 1 mm^2) were used to move all the contents into shallow trays. The contents of the trays were sorted and all macroinvertebrates were preserved in formalin-acetic acid-alcohol (FAA). Five within-pond samples were collected from each pond by haphazardly selecting a location at a depth up to 37 cm and proceeding at 2 m intervals across the pond from this location. An estimate of caddisfly abundance was calculated by dividing the area of the pond by the area of the bucket then multiplying by the mean number of caddisflies collected in the five samples collected per pond. Although this technique makes the assumption of even distribution across the pond, it gives a rough estimate of density and allows us to evaluate the potential influence of

caddisflies on the newt populations. The process of moving to the bucket sampling location may have slightly disturbed the water around the sampling site, resulting in mobile invertebrates being underrepresented in the samples. Therefore, we also sampled macroinvertebrates by dip-netting each pond for 1 h to further assess the diversity of the macroinvertebrate community. These samples were visually examined and subsamples were transferred to ice-chests and transported to Utah State University for identification and egg consumption experiments.

Gravid female Rough-skinned Newts (*Taricha granulosa* (Skilton, 1849); henceforth newts) were collected by hand from Soap Creek ponds for the collection of eggs (see below) in March 2009 and 2010. Newts were transported to Utah State University where they were housed in 5.7 L containers with 2 L of tap water filtered by reverse osmosis. To initiate egg deposition and ensure eggs were of similar developmental stage, females were injected with 2 µl/g of luteinizing hormone releasing hormone [(de-Gly^{10}, d-His(Bzl)^{6}]-LH-RH ethylamide; Sigma #L2761). Eggs were collected 24 h after the initiation of deposition and frozen (caddisfly growth experiment) or immediately given to potential predators (egg predation by aquatic invertebrates).

Consumption of Newt Eggs by Aquatic Invertebrates. We tested macroinvertebrates from 5 different orders and 11 families collected from Soap Creek ponds for their propensity to consume recently deposited newt eggs (Table 3.1). The sample sizes of potential egg predators tested was unequal because of differences in availability. Invertebrates were housed individually in clear plastic cylinders (9 cm

Table 3.1. Macroinvertebrates collected at Soap Creek Ponds and tested for their propensity to consume toxic newt eggs of Rough-Skinned Newts.

Class	Order	Family	Species	No. invertebrates offered eggs	Eggs consumed
Insecta	Odonata	Libellulidae		11	No
Insecta	Odonata	Aeshnidae		1	No
Insecta	Odonata	Coenagrionidae		6	No
Insecta	Hemiptera	Belostomatidae		1	No
Insecta	Hemiptera	Notonecta		2	No
Insecta	Hemiptera	Nepidae		NA	Not Tested
Insecta	Coleoptera	Noteridae		3	No
Insecta	Coleoptera	Dytiscidae		1	No
Insecta	Trichoptera	Limnephilidae	*Limnephilus flavastellus*	3	Yes
Insecta	Trichoptera	Limnephilidae	*Limnephilus concolor*	3	Yes
Insecta	Trichoptera	Limnephilidae	*Limnephilus occidentalis*	15	Yes
Insecta	Trichoptera	Limnephilidae	*Grammotaulius betteni*	2	Yes
Gastropoda	Lymnophila	Physidae		4	No
Gastropoda	Lymnophila	Lymnaeidae		2	No
Gastropoda	Lymnophila	Planorbidae		4	Yes

Note: NA, not available. "No" indicates that eggs were neither damaged nor consumed, whereas "yes" indicates that eggs were consumed.

diameter × 4 cm tall) with a plastic cap affixed to the bottom. Small holes were punched in the sides to allow aerated water to pass into the cylinder. Invertebrates were randomly assigned to 1 of 10 cylinders placed inside a shallow tray filled with 3 L of filtered tap water and two aerators. Each tray was maintained in an environmental chamber at 6 °C.

Recently deposited newt eggs were collected and combined from five female newts. Five of these eggs were placed in each cylinder and monitored daily for egg consumption for 16 days. The eggs were examined upon termination of the experiment for signs of predation. To ensure lack of egg consumption was not the result of aversion to feeding in the test containers, each invertebrate was provided with alternative prey, bloodworms or conditioned detritus (see below), at the conclusion of testing. All potential predators fed on at least one of the alternative diets within the confines of the test tanks.

Consumption of Newt Eggs by Caddisflies. Because caddisflies appeared to be the only major predator of *Taricha* eggs (see Results), we tested whether caddisflies would feed on eggs when a detritus food source was available. The case-making caddisflies (suborder Integripalpia) construct portable cases out of plant or mineral material that aid in respiration and defense (Wiggins and Currie 2008). As a larva grows, additional material is added to the anterior end of the case and the change in case length over time can be used to estimate growth.

Fifty-four *Limnephilus flavastellus* Banks, 1918 were measured to the nearest 0.01 mm (case length) using digital calipers, individually placed in mesh bottom cups, and randomly assigned to one of three 37 L aerated aquaria with 15 L of filtered tap water at 12 ºC. Each *L. flavastellus* was then randomly assigned to one of three treatments: detritus only (n = 18), newt eggs only (n = 18), or eggs + detritus (n = 18) with an equal number of each treatment occurring within each aquarium. The median length of *L. flavastellus* in each treatment was not significantly different at the start of the experiment (Kruskal–Wallis one-way ANOVA; $H_{[2]} = 0.224$, $P = 0.894$). Several thin strips of artificial silk leaves were added to each cup to provide *L. flavastellus* with media for case construction.

Each *L. flavastellus* in the egg-only and egg + detritus treatment was given five newt eggs. Eggs were previously collected from 15 female newts, combined, and killed by freezing (-80 ºC) to ensure toxicity was similar throughout the experiment and between treatments; TTX concentration may decrease as development proceeds (Twitty 1937). Freezing is a common technique to preserve tissue samples for TTX analysis (Hanifin et al. 1999; Hanifin et al. 2003). Although not tested during this experiment,

caddisflies also consume live eggs (B.G. Gall, unpublished data). Animals in the detritus and egg + detritus treatments were given three conditioned maple leaves. Conditioned maple leaves were prepared by collecting maple leaves (*Acer grandidentatum* Nutt.) shortly after abscission in October 2009 and maintained dry until further use. In January 2010, leaves were placed in aerated aquaria with filtered tap water and a small amount of pond detritus to facilitate the build-up of beneficial bacteria and fungi (henceforth conditioned detritus). The number of eggs consumed by each *L. flavastellus* was recorded every 24-48 h, upon which the consumed eggs were replaced. All eggs and detritus were replaced every seven days. Detritus was replaced more frequently when large quantities were consumed. Because of the difficulties in quantifying small pieces of detritus and because our goal was to provide an excess of an alternative food source, we did not attempt to quantify the amount of detritus consumed. All caddisflies with access to detritus (including *L. flavastellus* in the egg + detritus treatment) consumed this resource; partially consumed leaves were frequently replaced. Eggs and detritus were never reused. Case length was measured at the completion of trials, providing an estimate of developmental rate (Anderson 1974). Trials were terminated after two weeks and caddisflies were not retested.

Two *L. flavastellus* in the egg-only treatment pupated before the completion of trials and were excluded from the analyses. To determine whether an alternative food source affected egg consumption, we compared the number of eggs eaten between the egg-only and egg + detritus treatments with a Student's *t* test. We compared the change in growth (increase in case length) between *L. flavastellus* in the three treatments by subtracting the initial length from the final length and comparing the change in length

between the treatments with a randomized complete block design with subsamples. Tank was used as the blocking factor and cups within tank as subsamples. The Tukey-Kramer multiple comparisons method was used to identify differences between treatments. Because of the reduced power from blocking by tank and to account for the conservative nature and limited ability to detect small differences between means of Tukey-Kramer post hoc comparisons, we used an adjusted α value (determined a priori) for post hoc comparisons of 0.10 (Day and Quinn 1989). Analysis was performed with GLIMMIX in SAS version 9.2 (SAS Institute Inc., Cary, North Carolina, USA) with the nobound option. Finally, we examined the role that the size of *L. flavastellus* had on the number of eggs a larvae consumed by combining the egg-only and egg + detritus treatments and comparing the number of eggs consumed to initial length using linear regression. All data met assumptions for parametric statistics.

RESULTS

Macroinvertebrate Sampling. Invertebrates from 18 genera and 13 families were collected in samples from the three ponds (Table 3.1). No invertebrates were present in the bucket samples that were not found in the dip-net samples. A total of 494 invertebrates were collected during bucket sampling from all four ponds. The most abundant invertebrate in the bucket samples were caddisflies, comprising 95.7% of the invertebrates collected. Four species of caddisfly were present, with *Limnephilus flavastellus* comprising the majority (Table 3.2). In total, as many as 774,000 caddisflies (Table 3.2) may populate each 2,090 m^2 pond.

Table 3.2. Species and estimated population sizes of caddisflies (Trichoptera) sympatric with a population of toxic Rough-skinned Newts (*Taricha granulosa*).

Species	Total collected	Mean number per sample	Estimated population size per pond
Limnephilus flavastellus	430	21.5	704484
Limnephilus concolor	29	1.5	47512
Limnephilus occidentalis	10	0.5	16383
Grammotaulius betteni	4	0.2	6553

Note: All species consumed living and freeze-killed newt eggs in the laboratory.

Egg Predation by Macroinvertebrates. Caddisflies were the only invertebrate to consume newt eggs, except a single Planorbid snail, which consumed 1 egg (Table 3.1). Each caddisfly species tested consumed newt eggs (Table 3.1).

Egg Predation by L. flavastellus. All *L. flavastellus* offered newt eggs consumed at least one egg over the 14 day trial. *Limnephilus flavastellus* in all three treatments increased in size during the trials; however change in growth was significantly different between the treatments ($F_{[2,4]} = 6.19$, $P = 0.059$; Fig. 3–1). Consumption of eggs did not hinder growth but actually facilitated it; *L. flavastellus* in the egg-only and egg + detritus treatments grew more than *L. flavastellus* in the detritus-only treatment (egg only: $t_{[4]} = -2.87$, $P = 0.095$; egg + detritus: $t_{[4]} = -3.16$, $P = 0.072$; Fig. 3–1). There was no significant difference in the growth between *L. flavastellus* provisioned with eggs only or eggs + detritus ($t_{[4]} = -0.14$, $P = 0.99$). In addition, *L. flavastellus* with access to an alternative food source (detritus) consumed a similar number of eggs (eggs consumed = 14.72 ± 2.02 (mean ± SE)) compared with *L. flavastellus* that had access to eggs only (eggs consumed = 12.94 ± 2.12) ($t_{[32]} = -0.61$, $P = 0.55$; Fig. 3–2). Larger *L. flavastellus* consumed more eggs than smaller L. flavastellus ($F_{[1,32]} = 17.94$, $R^2 = 0.359$, $P < 0.001$; Fig. 3–3).

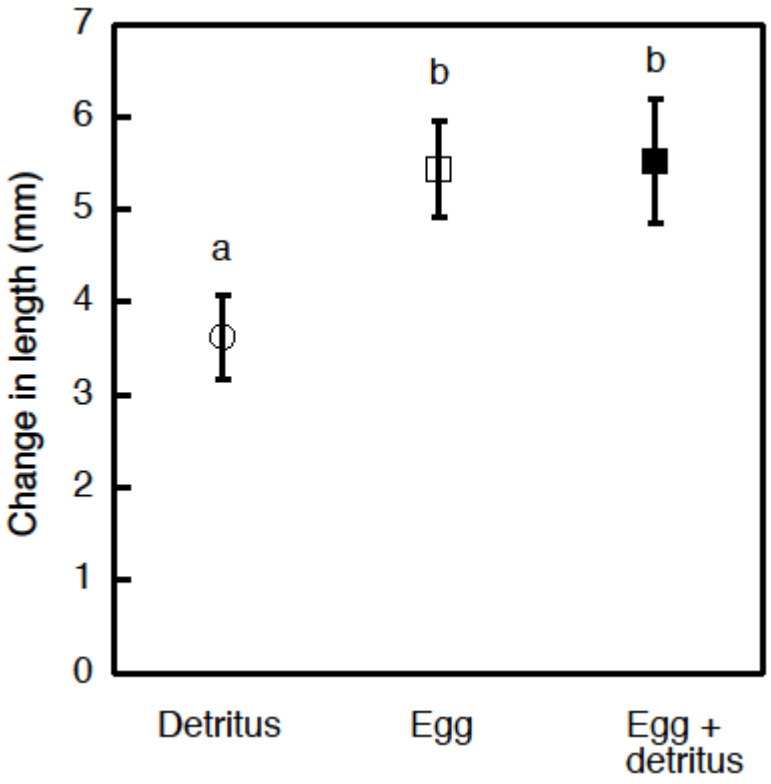

Fig. 3–1. Growth (mm) of the caddisfly *Limnephilus flavastellus* observed in larvae fed detritus only, eggs of Rough-skinned Newts (*Taricha granulosa*) only, or eggs + detritus. Growth was measured as the change in case length. Different letters indicate significant differences ($P \leq 0.1$) between treatments. Values are means ± SE.

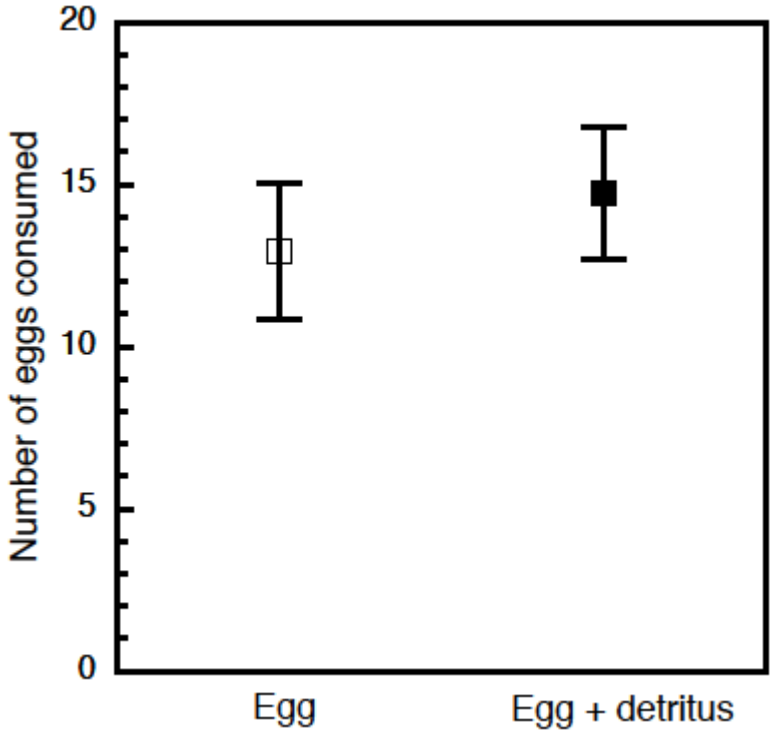

Fig. 3–2 The caddisfly *Limnephilus flavastellus* with access to an alternative food source (detritus) consumed a similar number of eggs of Rough-skinned Newts (*Taricha granulosa*) (eggs consumed = 14.72 ± 2.02) as *L. flavastellus* that had access to eggs only (eggs consumed = 12.94 ± 2.12) (Student's *t* test, $t = -0.61$, $P = 0.55$). Values are means ± SE.

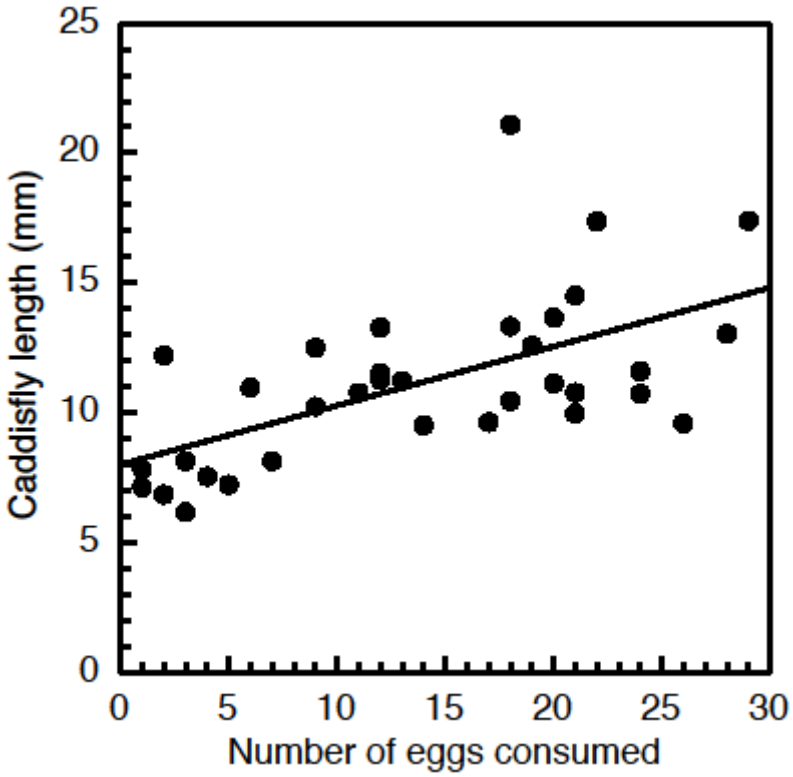

Fig. 3–3. Regression of the number of eggs of Rough-skinned Newts (*Taricha granulosa*) consumed by individual caddisfly *Limnephilus flavastellus* versus the initial length of *L. flavastellus* in the egg-only and egg + detritus treatments (treatments were combined because no significant difference in number of eggs consumed was detected). Larger *L. flavastellus* consumed more eggs over the course of the experiment than smaller *L. flavastellus* ($F_{[1,32]} = 17.94$, $R^2 = 0.359$, $P < 0.001$). Values are means ± SE.

DISCUSSION

The presence of TTX in the eggs of newts has been postulated to serve as a defense against egg predators (Twitty 1966; Daly et al. 1987). The eggs from the population we sampled contain the highest levels of TTX in an egg known in the animal kingdom (1.53 µg TTX/egg; Hanifin et al. 2003), yet a single invertebrate, sympatric caddisflies (a single planorbid snail also consumed one egg), were able to consume these toxic eggs. Moreover, the presence of an alternative food resource (detritus) did not affect the number of eggs eaten by *L. flavastellus*, indicating egg predation may be common in an ecological context. *Limnephilus flavastellus* given a diet of eggs only or eggs + detritus grew equally well, and outgrew *L. flavastellus* that had access to detritus only. These data indicate that ingestion of TTX does not kill *L. flavastellus* predators, but these individuals continue to grow and develop (this study) and may eventually pupate and eclose as functional adults (B.G. Gall, personal observation).

Ingestion of toxic foods is often assumed to have physiological costs that negatively affect growth of an organism (Freeland and Janzen 1974; Behmer et al. 2002). Detoxifying dangerous compounds requires energy and nutrients that could otherwise be used for growth or reproduction (Sorensen et al. 2005). Consuming toxic eggs may entail a cost relative to some other animal based resource, yet consuming this immobile and plentiful resource is beneficial relative to a detritus-only diet. *Limnephilus flavastellus* continued to grow when provided with toxic eggs, suggesting individuals from this locality are resistant to TTX. At present it is unknown what metabolic pathway is utilized to excrete, detoxify, or sequester TTX.

Although Limnephilid caddisflies are primarily detritivores, their food is limited in resources important for growth (e.g. lipid, protein, carbohydrate; Suberkropp et al. 1976; Cummins and Klug 1979), and field and laboratory observations indicate that they are opportunistic scavengers and predators of animal matter (Brusven and Scoggan 1969; Gallepp 1974; Wissinger et al. 1996). Adult caddisflies reared exclusively on a diet of detritus are often smaller and have more wing abnormalities than field caught specimens (Anderson 1976), and experimental evidence indicates consumption of animal matter is important for normal growth and development. Anderson (1976) conducted laboratory experiments on the growth of caddisfly larvae fed diets of detritus and detritus supplemented with animal matter. He found that supplementing animal matter reduced developmental time and yielded 50% larger larvae compared to caddisflies fed detritus only. Majecki and Majecka (1996) demonstrated that growth of the larval caddisfly *Oligotricha striata* (L., 1758), was accelerated when they consume eggs of European Frogs (*Rana temporaria* L., 1758) compared with *O. striata* fed detritus only from their natural habitat. This increase in growth and decrease in developmental time was attributed to additional protein or lipid resources in the supplemented food, and alternative foods containing these compounds are likely important to caddisfly growth in natural environments (Anderson 1976; Cummins and Klug 1979).

Both lipids and protein are major components of amphibian eggs (Duellman and Trueb 1994), and caddisfly larvae have been observed eating the eggs of several amphibian species (Murphy 1961; Dalrymple 1970; Rowe et al. 1994; Majecki and Majecka 1998; Richter 2000). At our study site, newt populations can reach local densities of 6,000 individuals per pond (Isaac and Bond 1963; Smith 1967), and a single

female may deposit 400 to 650 eggs (Hanifin et al. 2003). Although sex-ratios of *T. granulosa* from our study site are unknown, Janzen and Brodie (1989) documented a male:female sex ratio of 3.8:1 at a pond in Lane County, Oregon, with many more females present on land that had not yet entered the pond. At our study site, if 2,000 females lay, on average, 500 eggs each, then one million eggs would be deposited in a single year, in a single pond. This massive reproductive output by newts is temporally limited, typically occurring from March through July (Nussbaum et al. 1983; B.G. Gall, personal observation). The final stage of rapid caddisfly growth (when larvae are large and consume the greatest number of eggs) coincides with newt oviposition, and ingestion of protein-rich foods during this phase is particularly important for proper development (Anderson 1976; Anderson and Cummins 1979; Cummins and Klug 1979). Moreover, caddisflies are very dense at this locality, possibly as many as three quarters of a million animals populating each pond. If each animal consumes, on average, 10 eggs, then as many as 30 times the reproductive output of the newt population could be consumed in a single season. Newt eggs are clearly not available to caddisflies in the same manner used in our experiments; newts attach their eggs to vegetation and may fold leaves around them (Miaud 1994). Nevertheless, caddisflies are likely major predators of newt eggs.

Although functioning in the last stage of a predation event, toxicity has the potential to reduce the number of predators capable of consuming a particular prey species. However, antipredator defenses are unlikely to deter all potential predators and even extremely toxic *Taricha* eggs are vulnerable to predation. The abundance of caddisflies, apparent importance of protein based-food resources on growth, and the

continued growth and development after consuming tetrodotoxin indicate that caddisflies are major predators on the toxic egg stage of *T. granulosa*.

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CHAPTER 4

TETRODOTOXIN LEVELS IN LARVAL AND METAMORPHOSED NEWTS (*TARICHA GRANULOSA*) AND PALATABILITY TO PREDATORY DRAGONFLIES[3]

Abstract

Some populations of the newt *Taricha granulosa* possess extremely high concentrations of the neurotoxin tetrodotoxin (TTX). Tetrodotoxin is present in adult newts and their eggs, but has been assumed to be absent from the larval stage. We tested larval and metamorphosed juveniles for the presence of TTX and evaluated the palatability of these developmental stages to predatory dragonfly nymphs. All developmental stages retained substantial quantities of TTX and almost all individuals were unpalatable to dragonfly nymphs. Tetrodotoxin quantity varied greatly among individuals. When adjusted for mass, TTX concentrations declined steadily through metamorphosis. Several juveniles were palatable to dragonflies and these individuals had significantly lower TTX levels than unpalatable juveniles. These results suggest that despite previous assumptions, substantial quantities of TTX, originally deposited in the embryo, are retained by the developing larvae and metamorphosed juveniles and this quantity is enough to make them unpalatable to some potential predators.

[3] Reprinted from Toxicon, 57, Brian G. Gall, Amber N. Stokes, Susannah S. French, Elizabeth A. Schlepphorst, Edmund D. Brodie, III., and Edmund D. Brodie, Jr., Tetrodotoxin levels in larval and metamorphosed newts (*Taricha granulosa*) and palatability to predatory dragonflies, 978-983, Copyright (2011), with permission from Elsevier.

1. Introduction

The neurotoxin tetrodotoxin (TTX) is found in an enormous range of taxa including marine bacteria, ribbon worms, flatworms, mollusks, and puffer fishes as well as several species of terrestrial amphibians (reviewed by Hanifin, 2010; Miyazawa and Noguchi, 2001; Williams, 2010). Tetrodotoxin is an extremely powerful toxin, binding to sodium channels in nerve and skeletal muscle thereby blocking the propagation of action potentials resulting in asphyxiation and death (Kao, 1966; Narahashi et al., 1967). The ecological function of TTX is likely diverse, and TTX may function in both intraspecific (Matsumura, 1995; Zimmer et al., 2006) and interspecific (Hwang et al., 2004; Ito et al., 2006) communication, prey immobilization and capture (Ritson-Williams et al., 2006), or as an antipredator mechanism (Brodie, 1968; Williams et al., 2010).

The role of TTX in deterring predation has been well documented in adult rough-skinned newts (*Taricha granulosa*). Newts may contain extremely high concentrations of TTX in the skin (Hanifin et al., 1999), and cutaneous extracts are lethal to almost all potential predators (Brodie, 1968). Moreover, newts with higher toxicity are more likely to survive encounters with potential predators, thus conferring a fitness advantage to toxicity (Williams et al., 2010).

The presence of TTX and its role in other life-history stages including eggs, larvae, and metamorphosed juveniles is less clear. Substantial quantities of TTX are present in the ovaries and ova of adult females, as well as recently deposited eggs (Hanifin et al., 2003; Wakely et al., 1966). Maternal toxicity is highly correlated with egg toxicity and there is little variation in toxicity within a clutch (Hanifin et al., 2003). Due to their sedentary nature, embryos are vulnerable to predation (Orians and Janzen, 1974),

and the TTX present in newt eggs may limit the scope of predators capable of consuming them (Daly et al., 1987; Twitty, 1966; see Chapter 3). Twitty and Johnson (1934) and Twitty (1937) conducted the only experiments investigating the toxicity of late stage embryos and larvae. When *Taricha torosa* embryos were grafted to *Ambystoma tigrinum* the host became paralyzed. The paralysis persisted until the yolk was absorbed (shortly after hatching), whereupon mobility was regained. Extracts from macerated embryos (developmental stages: neurula to active swimming) were also injected into larval *Ambystoma* yielding similar results. Compared to embryonic extract, the length of paralysis was reduced when *Ambystoma* was injected with extract from larvae that had recently started feeding, and injection of extract from developmentally advanced larvae yielded no locomotor impairment.

These experiments led to the conclusion that the toxin is primarily located in the yolk, and prevailing views assume that newt larvae retain no toxin. Nevertheless, specific toxicity levels of larvae and juveniles have not been measured. Moreover, these developmental stages have not been presented to potential predators to determine whether toxicity (if present) functions to deter predation. We reared *Taricha* from egg deposition to metamorphosis in the laboratory and tested the palatability of four developmental stages to predatory dragonflies (*Anax junius*). We also screened each developmental stage for the presence of TTX using a Competitive Inhibition Enzymatic Immunoassay (CIEIA).

2. Material and Methods

2.1 Animal Collection

Twelve gravid female newts were collected by hand from a series of manmade ponds near Corvallis OR in March 2010. Newts were transported to Utah State University where they were housed in 5.7-L containers with 2 L tap water filtered by reverse osmosis (henceforth: filtered water) in an environmental chamber at 6 °C. Females were transferred to a separate environmental chamber at 17 °C and injected with 2 µl/g LHRH (de-Gly10, [d-His(Bzl)6]-Luteinizing Hormone Releasing Hormone Ethylamide; Sigma #12761) to stimulate egg deposition. Some species of *Taricha* (e.g. *Taricha torosa*) attach clusters of eggs to stones or vegetation, however, *T. granulosa* deposit eggs singly on aquatic vegetation over several weeks in nature (Petranka, 1998). Eggs were collected 72 h after the initiation of deposition and maintained in 9 cm diameter plastic dishes with 50 ml filtered water until hatching. Upon hatching, larvae were transferred to 5.7 L plastic containers with 2 L filtered water. Larvae were maintained in these containers without food until the yolk was completely absorbed (3-5 days). Larvae were then fed *Daphnia pulex* ad libitum for one month. After this period, clutches were transferred to 38-L aquaria filled with 35 L of pond water, detritus, and aquatic vegetation (*Myriophyllum spicatum*); this mixture contained abundant quantities of *Daphnia* and other microorganisms. The tank mixture was supplemented with blackworms (Order: Lumbriculida; *Lumbriculus variegatus*) weekly. Blackworms were obtained from a commercial supplier (Aquatic Foods, Fresno CA). Immediately prior to testing 15 week old larvae (see below), the tanks were drained and the larvae were transferred to 5.7 L containers with 2 L filtered water to monitor development. Larvae were fed blackworms

ad libitum until the initiation of metamorphosis (see below), whereupon they were transferred to a 1.2 L container filled with damp sphagnum moss and fed fruit flies (*Drosophila melanogaster*) until testing.

Dragonfly nymphs (*Anax junius*) are major predators of anuran and caudate larvae (Buskirk, 1988; Storfer and White, 2004; Yurewicz, 2004). We used dragonflies from a population (near Preston ID) that does not co-occur with newts to ensure predators had no prior experience with newt larvae; sympatric dragonfly nymphs may learn to avoid newt larvae if they are unpalatable. Nymphs were maintained individually in 275 ml glass bowls with 200 ml filtered water and a small stone (perch site) in an environmental chamber at 17 °C. Dragonflies were fed blackworms ad libitum.

2.2 Testing

We tested the palatability of larvae (three age classes) and recently metamorphosed juvenile *T. granulosa* to predatory dragonfly nymphs (*A. junius*). (1) First stage larvae (hatchlings: 0 weeks old) hatched two days prior to testing at developmental stage 39-41 (Harrison, 1969) and were tested on 3 June 2010. (2) Second stage larvae (4 weeks old) hatched on 27 to 30 April 2010 and were tested at developmental stage 46 (Harrison, 1969) on 27 May 2010. (3) Third stage larvae had fully formed front and back limbs, fully developed digits, and were tested 15 weeks after hatching (hatched: 27 to 30 April 2010; tested: 14 August 2010). (4) Metamorphosed juveniles were identified based on a reduction of the gills, change from smooth larval skin to granulated adult skin, and development of orange pigmentation on the venter. Because larvae initiated metamorphosis at different times, small groups of metamorphs

were tested in November and December 2010 (roughly 28 weeks old, hatched on 27 to 30 April 2010). Juveniles were tested exactly 25 days after the initiation of metamorphosis. Half the individuals in each group were used to test palatability while the other half served as controls.

Each trial consisted of two 20 minute periods during which a nymph was provided either a single blackworm (control) or a newt. Each period was separated by 40 minutes and the order of testing (control or newt) was randomly determined before the start of each trial. Twenty dragonfly nymphs were randomly chosen from a pool of individuals and fed three blackworms three days prior to testing to standardize hunger level between individual nymphs. The dragonfly holding containers were cleaned prior to each test period. Individual newts within a treatment (i.e. developmental stage) were collected from a minimum of five families. A prey (control or newt) was introduced to a bowl with a pipette and we recorded whether the prey was seized, rejected or consumed, and injured, uninjured, or dead. Trials were terminated after 20 minutes if the nymph did not consume the prey, or after a prey was seized and either rejected or consumed. Seizure usually occurred within the first minute. Dragonfly nymphs and newt larvae and metamorphosed juveniles were never retested.

To ensure injuries incurred during the predation event were not due to handling, we tested the survival of each newt compared to control larvae that were treated the same except no predator was present in the bowls. The tested and control larvae were transferred to glass bowls and the number of uninjured, injured, and dead larvae was recorded after 48 h. Each larva was removed from the bowl and nudged with the pipette. If the larva responded immediately by swimming in a general linear path it was classified

as uninjured. Injured larvae often had difficulty swimming or swam in convoluted patterns. We verified the health of metamorphs by placing them on their back and recording the time to right themselves (<5.0 s, classified as uninjured). Of 78 control larvae 76 were uninjured and two died; this variable did not affect the results and these data were removed from the analysis. At the termination of the experiment all unpalatable larvae were frozen at -80 ºC for TTX analysis. Larvae that were seized and partially consumed were removed from the predator after 1 hour and frozen at -80 °C to quantify TTX so the toxicity of palatable larvae could be compared to larvae that were rejected.

2.3 TTX Analysis

Tetrodotoxin was quantified using a Competitive Inhibition Enzymatic Immunoassay (CIEIA) as in Stokes et al. (submitted for publication). This assay is highly specific and works by binding anti-TTX monoclonal antibodies to TTX. In the absence of TTX or in low concentrations of TTX, the antibodies bind to the conjugate on the plate allowing secondary antibodies to also bind to the plate, resulting in a high absorbance reading. This value is then used to calculate the TTX concentration using the 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., submitted for publication). Anti-TTX antibody concentrations for all plates were 0.391 μg/mL. All samples were diluted 1:2, 1:4, 1:8, or 1:16 depending on the total TTX concentration, to assure they were within range of the standard curve. Blanks (no antibody), negative controls (blackworms), and a standard curve (100, 75, 50, 25, 10 ng) were included on each plate.

All plates were read at 405 nm. The average coefficient of variation for each plate was between 4.55 and 6.55%.

2.4 Statistical Analysis

We compared the total and mass corrected (ng TTX/mg body mass, log transformed to meet assumptions of normality) amounts of TTX (ng) in individuals from each developmental stage with ANOVAs followed by Holm-Sidak multiple comparisons. Finally, we compared the concentration of TTX in unpalatable and palatable metamorphosed juveniles with a *t*-test.

We determined whether the frequency of palatable and unpalatable individuals was the same for the four developmental stages using a contingency table and a chi-square test. The survival data were analyzed by combining the number of injured and dead larvae into a single category (wounded) and comparing the frequency of uninjured and wounded larvae in the four developmental stages with a contingency table and a chi-square test.

3. Results

Tetrodotoxin levels in unpalatable larvae and juveniles ranged between 229 and 2354 ng TTX per individual or 0.463–3344 ng TTX/mg body mass, with hatchlings exhibiting the highest concentration and toxicity generally decreasing with increasing developmental stage (Table 4.1). Palatable juveniles contained 296 ± 103 ng TTX or 0.33 ± 0.08 ng TTX/mg body mass (Table 4.1). There were significant differences in the total amount (ng) of TTX from the different developmental stages (df = 3, $F = 5.02$, $P < 0.008$, Fig. 4–1), with toxicity greatest in hatchlings, declining at 4 weeks old, and remaining

Table 4.1. The quantity (total and mass corrected) of TTX present in unpalatable larvae and juveniles from four developmental stages and palatable (palat) 28 week old juveniles. Conc.: concentration. Values are ± SE.

Treatment	N	mass ± SE (mg)	Total TTX (ng)	Range	TTX conc. (ng/mg)	Range
0 Week	6	5.9 ± 1.3	1076.3 ± 272.5	488-2354	700.02 ± 530.9	55.18-3344.36
4 Week	6	77.6 ± 4.3	420.3 ± 57.5	259-580	5.67 ± 1.1	3.24-9.88
15 Week	6	203.0 ± 41.1	427.5 ± 55.9	229-518	2.56 ± 0.6	1.14-5.17
28 Week	9	681.8 ± 71.5	509.7 ± 68.5	316-929	0.78 ± 0.1	0.46-1.24
28 Week (Palat)	4	836.5 ± 130.2	296.0 ± 103.4	90-583	0.33 ± 0.08	0.12-0.48

relatively constant for the remainder of development. There was also a significant difference between developmental stages in the concentration of TTX (ng TTX/mg body mass) (df = 3, $F = 65.1$, $P < 0.001$, Fig. 4–1), whereby TTX concentration decreased after hatching and was the lowest after metamorphosis (Fig. 4–1). Palatable metamorphs had significantly lower TTX concentrations relative to unpalatable metamorphs (df = 11, $t = 2.83$, $P = 0.016$, Fig. 4–2, Table 4.1). The negative control (blackworms) contained no TTX and was not different from the BSA control.

The frequency of palatable and unpalatable individuals was not the same for the four developmental stages (df = 3, $\chi^2 = 10.22$, $P < 0.025$) whereby the palatability of newts generally increased with developmental stage (Table 4.2). Hatchling and four week old larvae were completely unpalatable, whereas one 15 week old larva was palatable and four juveniles were consumed (Table 4.2).

The frequency of uninjured and wounded individuals was not the same for the four developmental stages (df = 3, $\chi^2 = 16.89$, $P < 0.001$). Younger larvae were more likely to be wounded after being seized by predatory dragonflies than older larvae (Table 4.2).

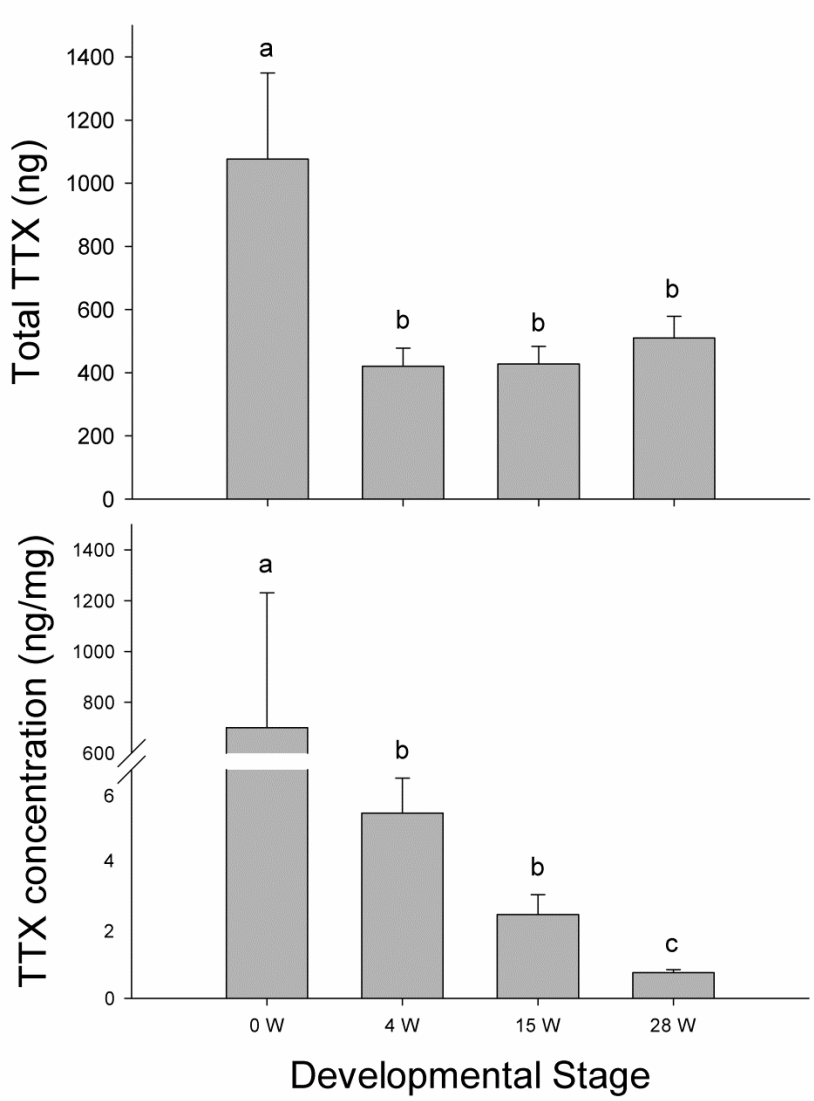

Fig. 4–1. (top) Mean (± SE) total amount of TTX (ng) in newt larvae from four developmental stages. (bottom) Mass corrected (mean (± SE) ng TTX/mg body mass) TTX in newt larvae from four developmental stages. 0 W = Larvae that had recently hatched, 4 W = Four week old larvae, 15 W = fifteen week old larvae, 28 W = Larvae that had recently completed metamorphosis. Different letters indicate significant differences between treatments ($P < 0.015$).

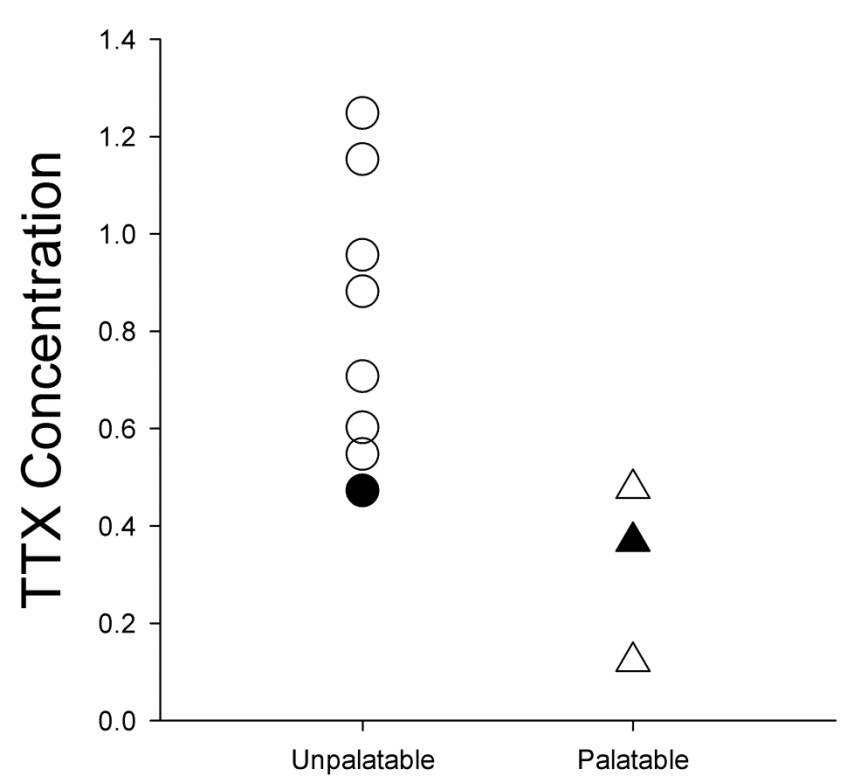

Fig. 4–2. TTX concentration (ng TTX/mg body mass) present in unpalatable and palatable individual juvenile newts (approx. 28 Weeks post hatching; exactly 25 days post metamorphosis). Palatable juveniles had significantly lower TTX concentrations relative to unpalatable juveniles (df = 11, t = 2.83, P = 0.016). Circle: unpalatable juvenile newts. Triangle: palatable juvenile newts. Filled symbols indicate two overlapping values.

Table 4.2. Number and survival of newt larvae from four developmental stages presented to predatory dragonfly nymphs. H = Hatchling, M = Metamorph.

Developmental Stage	N	Seized	Rejected	Uninjured	Injured	Dead	Consumed
0 Week	20	19	19	9	6	5	0
4 Week	20	19	19	16	1	3	0
15 Week	20	15	14	17	1	1	1
28 Week	18	17	13	13	0	0	4

4. Discussion

T. granulosa larvae and metamorphosed juveniles possess substantial quantities of TTX and most are unpalatable to predatory dragonfly nymphs. Female newts deposit large quantities of TTX in their eggs prior to deposition (Hanifin et al., 2003), and it was previously unknown how toxicity changed ontogenetically. Recently deposited eggs may contain as much as 2767 ng of TTX per egg (Hanifin et al., 2003), and early experiments suggested toxicity decreased with increasing development, ultimately resulting in nontoxic larvae (Twitty, 1937; Twitty and Johnson, 1934). Our study indicates toxicity does decline slightly during development (mean egg toxicity = 1528 ng (Hanifin et al., 2003); mean hatchling toxicity = 1072 ng), however, larvae remain extremely toxic at hatching and all developmental stages, including metamorphosed juveniles, possess substantial quantities of TTX. The total toxicity of a single larva at hatching is 1076 ± 272 ng of TTX. Total individual toxicity declines between hatching and four weeks old (420 ± 57 ng), and then remains stable through metamorphosis. These results contradict previous assumptions about the ontogenetic change of toxicity in *Taricha* and indicate some TTX is retained at least through metamorphosis.

When adjusted for body mass, TTX continues to decline through metamorphosis, despite no change in total toxicity. Due to their small size, hatchlings remain extremely toxic relative to eggs. However, by four weeks old, total toxicity decreased and body mass increased resulting in a 100 fold decrease in the amount of TTX (per mg body mass) relative to hatchlings.

Egg toxicity is correlated with maternal toxicity (Hanifin et al., 2003), and the TTX present in larval and metamorphosed juveniles is likely residual from the quantity initially deposited in the eggs. At our study site, TTX quantity is highly variable among individual adults (Hanifin et al., 1999, 2003). Because larvae for the current study were collected from at least five different clutches, it is likely initial clutch variability is driving some of the variation in larval and juvenile toxicity that we observed. This large amount of variation suggests the quantity of TTX in larval and juvenile newts may be correlated to the initial investment of TTX in the embryo. If female toxicity is correlated with all ontogenetic changes in toxicity, a selective advantage would likely be conferred to all age groups if derived from a highly toxic lineage.

Taricha larvae are unpalatable to one of the most pervasive predators of amphibian larvae. For TTX to function as an antipredator mechanism, individuals with greater toxicity must be more likely to survive a predatory encounter. Williams et al. (2010) directly tested the role of TTX toxicity on the survival probability of encounters between adult newts and toxin resistant garter snakes. The authors found that newts that survived the encounters with predatory snakes had higher TTX concentrations than newts that were eaten, thereby directly testing the efficacy of TTX as an antipredator mechanism. In this study, unpalatable juveniles exhibited higher toxicity than palatable

individuals suggesting TTX toxicity also is directly related to probability of survival for larval newts.

Tetrodotoxin is primarily located in the skin of adult newts (Mosher et al., 1964; Wakely et al., 1966; Brodie et al., 1974), and in the yolk of embryos (Twitty, 1937). The location of the toxin in larval newts is unknown; however, rejected larvae and juveniles were struck on all regions of the body (tail, limbs, head, torso). Dragonflies slowly consume pieces of their prey (rather than whole). The greater palatability of older larvae is likely due to a reduction in TTX per unit mass in larger individuals as well as greater sensitivity to TTX concentration rather than total quantity in predatory dragonflies.

Although toxicity makes newt larvae unpalatable to potential predators, this antipredator mechanism functions after the prey has been seized and when the risk of injury is high (Endler, 1986; Brodie et al., 1991). Tetrodotoxin toxicity cannot confer a selective advantage if the prey still dies from injuries received during the attack. Dragonfly nymphs possess palpal lobes on the labium that terminate in movable grasping hooks. These hooks function to pierce and subjugate prey (Corbet, 1999). Larvae in early developmental stages (especially hatchlings) are vulnerable to injury or death from these predatory attacks. However, numerous individuals did survive these encounters, thus conferring a selective advantage to unpalatability. The predators used in this study had no prior experience with newts, requiring each individual to sample and reject the prey. Odonates are capable of learning (e.g. Chivers et al., 1996), and may be able to learn to avoid dangerous prey (O'Donnell, 1996). If dragonfly nymphs learn to avoid chemical or visual cues associated with newt larvae, the effective advantage of unpalatability increases, especially for those individuals that survive the initial encounter.

Despite previous assumptions, *Taricha* larvae and most metamorphosed juveniles were unpalatable to predatory dragonfly nymphs. All age classes retained TTX, yet palatable juveniles possessed reduced levels of TTX relative to their unpalatable cohorts. A wide range of organisms possess tetrodotoxin, and understanding the ontogenetic changes in toxicity and the role TTX plays in the various life-history stages is paramount to determining the function of TTX for these organisms.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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CHAPTER 5

COMPLEX INTERACTIONS BETWEEN NEWTS AND CADDISFLIES AND IMPLICATIONS FOR THE EVOLUTIONARY ARMS-RACE BETWEEN SNAKES AND NEWTS

Ecological interactions rarely involve only two species, yet this restriction is often inferred in coevolution. One of the most well documented coevolutionary interactions, the arms-race between toxic salamanders and toxin-resistant predatory snakes, has received little attention regarding additional predators. I document the interactions between a second class of predator, caddisfly larvae, and their prey, the toxic eggs of the rough-skinned newt. Caddisflies are major predators on newt eggs and possess behavioral adaptations (attraction to cues from gravid female newts and eggs) that likely facilitate the localization of prey. Females however are not defenseless, and avoid predatory caddisflies by ovipositing high in the water column. Caddisflies generally, and especially large individuals, do not utilize this microhabitat, and field experiments confirmed that oviposition avoidance renders newt eggs relatively free from predation. In addition, caddisfly larvae sequester some of the tetrodotoxin after consuming eggs and retain similar quantities for more than 135 days, including through metamorphosis to the winged-adult stage. Finally, caddisflies offered eggs with varying quantities of TTX consumed fewer eggs with higher levels of toxin, indicating caddisflies have the potential to indirectly drive the evolution of skin toxicity in adult newts. These results indicate that reciprocal selection between caddisflies and newts is possible, and the coevolutionary

arms-race between snakes and newts may involve an additional player, predatory caddisfly larvae.

INTRODUCTION

The degree of reciprocal selection between two coevolving species can be dependent on the ecological characteristics and community in which the interaction takes place (Thompson 1994, Thompson 2005). For example, both spatial and temporal variation in the pattern of coevolution can alter the dynamics between the coevolving species and lead to broad scale differences in the outcome of the interaction (Gomulkiewicz et al. 2000; Nuismer et al. 2003; Thompson and Fernandez 2006). The inclusion of additional species can also modify the interaction between a coevolving pair and have dramatic influences on the evolution of the phenotypes revolving around the interface. For example, interactions between a pollinating floral parasite, the moth *Greya politella*, and their host plant *Lithophragma parviflorum* are mutualistic in populations where the moth is the primary pollinator (Thompson and Cunningham 2002). However, in other populations the presence of additional pollinators modifies the interaction resulting in commensalistic and, at the extreme, antagonistic interactions between the coevolving species (Thompson and Pellmyr 1992; Thompson and Fernandez 2006). Similarly, the presence of multiple species of pollinating yucca moth may destabilize the coadaptation between the moths and yucca plants leading to the evolution of "cheater" moths (Pellmyr et al. 1996), and the presence of cryptic species of wasp in fig-wasp interactions has the potential to alter the perceived one-to-one coevolutionary relationship between these species, potentially leading to a parasitic interaction (Molbo et al. 2003). These examples suggest that the nature of reciprocal selection between coevolving species is likely to be heavily influenced by other players in the system.

The arms-race analogy is often invoked to explain the presence of extreme phenotypes in predators and prey (Brodie and Brodie 1999). In this case, a predator causes selection on properties that make their prey less likely to be caught and consumed which leads to adaptations that increase foraging efficiency in the predator. For example, gastropods in Lake Tanganyika in Africa appear to have evolved thick shells to counter the well-developed crushing chelae of predatory crabs (West et al. 1991), and similar patterns have been inferred from the fossil record of marine species (Vermeij 1977). Recently, Jansa and Voss (2011) have demonstrated that the adaptive evolution of venom-resistance in opossums (Didelphidae) that prey on pitvipers (Viperidae) may be the result of an evolutionary arms-race. Like interactions between plants and pollinators however, interactions between predators and prey are complex, and it is unlikely that only one predator and one prey influence the degree of reciprocal selection, and therefore the evolution of a trait, between two species. For example, Benkman et al. (2003) demonstrated that selection by crossbills has likely led to larger lodgepole pine cones, which has in turn led to changes in bill morphology. The interaction becomes more complex where cone borer moths (*Eucosma recissoriana*) occur. Predation by moths leads to smaller cones with relatively few seeds, thereby changing the coevoluationary dynamic between crossbills and trees (Siepielski and Benkman 2004). Another seed predator, the red squirrel (*Tamiasciurus hudsonicus*), changes the selective environment further and competitively excludes crossbills, thereby terminating the coevolutionary process between these species (Benkman 1999). Despite this multispecies interaction, understanding the role of multiple predators on coevolution is difficult, and the role of

alternative predators in shaping coevolutionary arms-races has not been well documented.

One of the most well documented evolutionary arms-races involves gartersnake predators (*Thamnophis*) and their newt prey (*Taricha*). Newts of the genus *Taricha* possess the powerful neurotoxin tetrodotoxin (TTX) that successfully repels almost all potential predators (Brodie 1968). Tetrodotoxin functions by blocking voltage-gated sodium channels in nerve and skeletal muscle thereby inhibiting the propagation of action potentials resulting in muscle failure, asphyxiation, and death (Kao 1966; Narahashi et al. 1967). Although *Taricha* toxicity is geographically variable (Hanifin et al. 1999; Hanifin et al. 2008), newts in some populations may contain up to 16 mg of TTX, or enough to kill at least eight humans. The evolution of extreme toxicity in *Taricha* is believed to be the result of coevolution with several species of Thamnophiine garter snakes (Brodie and Brodie 1990; Brodie et al. 2002; Brodie et al. 2005; Williams et al. 2010). These snakes exhibit resistance to TTX due to changes in the amino acid sequence of the sodium channel protein NaV1.4 (Geffeney et al. 2002; Geffeney et al. 2005). Snakes vary in resistance, and some populations are able to consume newts with no negative physiological effects (Brodie et al. 2002).

Although TTX is primarily located in the skin of adult newts, large quantities are also present in the ovaries, ova, and recently deposited eggs (Wakely et al. 1966; Hanifin et al. 2003). The eggs of *T. granulosa* are oviposited singly on aquatic vegetation over several weeks or months in early spring (Petranka 1998). In the eggs, TTX is believed to serve an antipredator function (Twitty 1966; Daly et al. 1987). Several types of aquatic invertebrates, including species of Odonata, Hemiptera, Coleoptera, Ephemeroptera, and

Gastropoda avoid consuming the toxic eggs (see Chapter 3). Nevertheless, toxicity is unlikely to successfully repel all potential predators. Caddisfly larvae (Trichoptera) have been found to consume large quantities of eggs and are major predators on this life-history stage (see Chapter 3). Moreover, these caddisflies appear to be at least partially resistant to the negative effects of ingesting tetrodotoxin (see Chapter 3). Understanding the evolution of toxicity in newts requires a broader analysis of the selective forces shaping this phenotype, and one of the most important questions of this interaction is what role other predators may be having on the arms-race. I explored the behavioral, ecological, and selective forces occurring between newts and caddisflies to determine the potential for reciprocal selection between these species and how this might impact the arms-race between snakes and newts.

MATERIALS AND METHODS

Several experiments were conducted to elucidate the interactions occurring between newts and predatory caddisflies. I addressed the following questions: (1) Do caddisflies respond behaviorally to newts? (2) Do newts possess strategies that limit predation on their eggs? (3) Is the TTX present in newt eggs sequestered by caddisflies? (4) What is the potential for indirect selection by caddisflies to lead to elevated toxicity in newts?

ANIMAL COLLECTION

Female newts (*Taricha granulosa*) in reproductive condition were collected in March 2009, 2010, and 2011 from Soap Creek ponds in the central Willamette Valley

(Benton County, Oregon). Newts were transported to Utah State University (USU) and housed individually in 5.7-l plastic containers with 3 l of filtered tap water. They were maintained at 6 ºC to prevent spontaneous egg deposition and fed blackworms (*Lumbriculus variegatus*) weekly.

Caddisfly larvae (henceforth: caddisflies) were collected from the same ponds as *Taricha*. Caddisflies were housed in 37-l aerated aquaria with 20 l filtered tap water at 6 ºC. Caddisflies were fed maple-leaf detritus (see Chapter 3 for description of detritus preparation). Mayfly larvae (Baetidae; henceforth: mayflies) were used as a nonpredatory control. Mayflies co-occur with *Taricha* at Soap Creek ponds, but at low densities. Mayflies were collected near Paradise, UT, and housed in a 37-l aerated aquarium with a small amount of detritus. No caddisfly, mayfly, newt, or newt egg was reused for any experiment, except when serving as the source of chemical stimuli for behavioral trials (see below).

1. DO CADDISFLIES RESPOND BEHAVIORALLY TO NEWTS?

The behavioral responses of predators to their prey can influence the level of selection between two coevolving species, especially when the prey are dangerous (Brodie and Brodie 1999; Williams et al. 2003). I examined the behavior of two species of caddisflies to stimuli that they may be exposed to before or during a predatory encounter with newt eggs. Using two types of choice experiments, caddisflies were exposed to stimuli from (1) a blank control, detritus (food), male newts, and gravid and "spent" female newts, as well as (2) newt eggs and agar containing TTX.

The first set of trials exposed caddisflies to flowing chemical cues from a treatment or a blank control, depending on the position of the caddisfly within the test chamber. *Limnephilus flavastellus* was exposed to chemical stimuli from a control (double blank), detritus (food), recently deposited newt eggs, male newts in reproductive condition, gravid female newts, and "spent" female newts that had completed egg deposition (N = 10 per treatment). I also tested the response of *L. concolor* to a control (double blank), newt eggs, male newts, and gravid female newts in these same test tanks (N = 10 per treatment). The test chamber was a gravitational flow-through system consisting of a series of vertically positioned containers modified from Petranka et al. (1987). Briefly, water from two 5.7-l containers housing the treatment stimulus drained via plastic tubing into two sides of a test container separated by a plastic partition that prevented the stimuli from mixing until they passed through mesh and into the experimental chamber (4 × 16.6 × 5 cm). A chemical gradient was produced preventing the stimuli from mixing while permitting caddisflies to move freely between the two chemical zones.

I recorded the position (control or chemical stimulus) of the caddisfly in the experimental chamber every 30 sec and the number of times the caddisfly crossed the center line. Observations were made for 20 minutes. The number of observations spent on each side of the test tank (control or treatment) was tabulated for each caddisfly and divided by the total number of possible observations. The proportion of observations on the stimulus side of the test container was compared to a random distribution of 0.50 using a one-sample t-test. The number of times that the center line was crossed in each treatment was compared using an ANOVA followed by Holm-Sidak comparisons.

A second set of trials were conducted in an arena containing static water with the stimulus source placed directly in the tank. These trials were used to verify caddisflies' response to newt eggs and determine whether TTX was used as a cue to locate the eggs. Caddisflies were exposed to 30 newt eggs and a blank control (N = 26), or agar containing 46 µg TTX (equivalent to the average amount of TTX present in 30 newt eggs) and control agar (N = 20). The test chamber consisted of a 9 × 3 × 2.3 cm opaque plastic container with a line drawn across the middle to separate it into two halves. The caps from two 1.5 ml screw-cap microcentrifuge tubes were inverted and glued 1.5 cm from each end of the container. Sixty small holes were punched in each tube to permit the passage of chemical stimuli but prevent caddisflies from accessing the eggs or agar inside.

Because of the presence of TTX in our experimental eggs I added TTX to agar to determine if caddisflies are specifically attracted to this toxin. The average amount of TTX/egg, excluding the jelly coat, from the Soap Creek newt population is 1.528 µg. The volume of an egg (excluding the jelly coat) from this population ranges from 4.92 µl to 8.67 µl (C. Hanifin, personal communication); I used a volume of 7 µl per egg to calculate the average volume and amount of TTX in 30 eggs.

I made both control and TTX containing agar using Ionagar No. 2 (Consolidated Laboratories, Inc., Chicago Heights, Illinois, U.S.A.). Control agar was made by mixing 1.5 g agar with 100 ml boiling distilled water and mixing the solution thoroughly. After the solution had partially cooled, it was poured into a Petri dish. The agar was then allowed to cool and solidify, at which time it was placed in a refrigerator. Because an extremely large quantity of TTX is present in the eggs of newts from this population, I

made substantially less TTX containing agar compared to the blank control. I mixed 2 mg TTX with 1 ml distilled water, then boiled 9 ml distilled water and added 0.15 g agar. After the boiled water-agar solution had cooled, but not solidified, I added the TTX solution, mixed the solution thoroughly, and poured it into a Petri dish. It was then allowed to solidify at which time it was refrigerated.

A punch was used to remove a section of agar that was equal to the volume of 30 newt eggs. The agar (control and TTX containing) was then inserted into separate centrifuge tubes and randomly assigned to one of the caps in the test chamber; separate punches were used for the two agars. The agar was refrigerated between punch removal.

The test chamber was filled with 50 ml filtered tap water. One of the centrifuge tubes was filled with either 30 newt eggs or agar containing TTX and screwed to a randomly chosen cap. The second centrifuge tube was screwed to the other cap but left empty (if paired with newt eggs) or filled with control agar (if paired with agar containing TTX) to serve as a control. After ten minutes, a caddisfly was placed inside an acclimation cylinder (2.7 cm diameter) in the center of the test chamber and a three minute acclimation period was initiated. The cylinder was then removed and a 20-minute trial started. I recorded the position of the caddisfly every 30 seconds and the number of times the caddisfly crossed the center line.

2. DO NEWTS POSSESS STRATEGIES THAT LIMIT PREDATION ON THEIR EGGS?

To determine if newts possess behavioral strategies that limit predation on their eggs, I recorded the oviposition behavior of female newts in response to caddisflies,

examined the microhabitat use of larval caddisflies, and conducted a field study to determine whether the behavioral strategy employed by female newts increased egg survival.

Oviposition choice

A choice test was used to determine the propensity of female newts to avoid ovipositing near predatory caddisflies (N = 8) and nonpredatory mayflies (N = 6). Female newts were tested in one half of a 74-l aquarium divided lengthwise by a piece of opaque plexi-glass that prevented water exchange between the two halves of the test tank (Fig. 5–1A). A piece of screen (10 cm × 15 cm, 1.5 mm mesh) was glued 7 cm from each end. A 5 × 10 cm piece of polyester fiber was anchored to a suction cup at each end of the middle compartment to serve as egg deposition sites. Females were able to move freely between fiber blocks and choose between oviposition sites.

Each test tank was filled with 6 l of filtered tap water. Ten caddisflies or mayflies were randomly assigned to one of the small compartments with the second compartment remaining empty creating a treatment side and a control side. The second test tank (within the same aquarium) was assigned the opposite treatment structure as the first tank. Invertebrates had been fed only detritus.

A female newt was injected with 2 µl/g LHRH and immediately placed in the test tank. Females were monitored every 2 hours from 0800 hrs to 2000 hrs for the beginning of egg deposition. Newts were removed from the test tank 24 hours after the beginning of

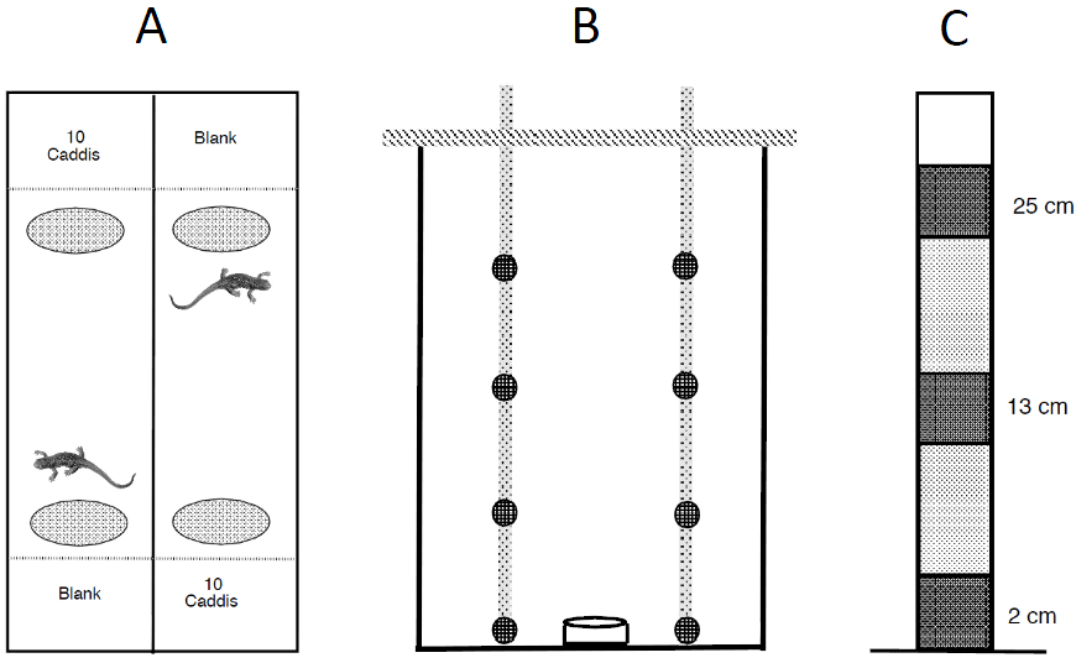

Figure 5–1. (A) Experimental chamber used to test ovipositing female newts responses to the presence of caddisflies and mayflies. Screen (dashed line) prevented the invertebrates from interacting with the female or consuming eggs. Clumps of polyester fiber (ovals) were provided for oviposition sites. (B) Vertical chamber used to test the responses of ovipositing female newts to the presence of caddisflies, mayflies or a blank control. Oviposition sites consisted of polyester clumps (dark circles) that were attached to willow (*Salix amygdaloides*) branches at 0, 9.5, 19.0, and 28.5 cm above the floor of the chamber. Invertebrates were maintained in two clear cylinders with screen tops on the bottom of the bucket. (C) Experimental stake used in field experiment testing newt egg survival at three different heights above the pond substrate. Eggs were attached to one of three turf squares (dark stippling) and separated by rectangular pieces of turf (light stippling). The stake was pushed into the pond such that the bottom of the lowest square rested on top of the substrate.

egg deposition, and the number of eggs on each piece of filter fiber was counted. To ensure that the number of eggs on each fiber block was based on 24 hours of oviposition, I subtracted the number of eggs on each piece of fiber at the start of the trial from the total number of eggs deposited after 24 hours. I made an a priori decision to remove females from the analysis if fewer than 50 eggs were deposited in the 24 hour test period because these females may not have completely entered oviposition. The number of eggs deposited on the control and treatment sides of the test tank were counted for each female and divided by the total number of eggs deposited. I compared the number of eggs deposited on the control and treatment sides of the test container for each treatment with a paired t-test. Assumptions for parametric statistics were met by these data.

Oviposition behavior in vertical chamber

I tested the responses of ovipositing females in a vertical test chamber to chemical stimuli from predatory caddisflies (N = 10), nonpredatory mayflies (N = 10), and a blank control (N = 10). Each test chamber consisted of a 19 l opaque bucket. Oviposition sites were provided at 0, 9.5, 19.0, and 28.5 cm along a vertical axis starting at the bottom of the bucket (Fig. 5–1B). Each oviposition site consisted of a 1g piece of polyester fiber glued to a willow (*Salix amygdaloides*) branch at the appropriate height. Two branches with attached oviposition sites were present in each bucket (Fig. 5–1B). A bucket was filled to a height of 32 cm with filtered tap water.

Caddisflies or mayflies were placed in two holding containers at the bottom of the test chamber (Fig. 5–1B). Holding containers consisted of 3 cm long pieces of clear plastic tubing (8.9 cm diameter) with a plastic cap on the bottom. Each container was

filled with eight conditioned maple leaves (*Acer grandidentatum*) and five of the appropriate invertebrates or no invertebrate (control). A piece of fiberglass window screen was fixed to the top of the container with an elastic band. Two of these containers of the appropriate treatment were placed in the bottom of each bucket. A gravid female newt was randomly assigned to one of the three treatments, injected with 2µl/g LHRH, and placed in the bottom of the bucket. Each experimental chamber was assembled immediately prior to the start of each trial to prevent individual females from experiencing different gradients of chemical cues at the beginning of each trial. Trials were conducted inside an environmental chamber at 11ºC with a 12:12 light:dark regime. Females were monitored daily for the beginning of egg deposition. After approximately 50 eggs had been deposited, the female was removed and the total number of eggs on each fiber block was counted. When trials were terminated (24–48 hours after the start of oviposition), all females had deposited more than 50 eggs.

To compare egg deposition between treatments, I analyzed the proportion of eggs deposited at each height relative to the total number of eggs deposited. Data were analyzed with a generalized linear mixed model using number of eggs as the response and (log-transformed) total number of eggs deposited as an offset, with a negative binomial distribution and a log link. The design structure partitioned variance between and within females in a split-plot design, with female as the whole plot unit, a repeated measure (within a female) as the subplot unit, and treatment and height as the two fixed-effects whole plot and subplot factors, respectively. Analyses were obtained using the GLIMMIX procedure in SAS 9.2 (SAS Institute Inc., Cary, NC, USA). I also compared the mean total number of eggs deposited in each treatment with an ANOVA.

Caddisfly distribution in relation to oviposition behavior

I examined the vertical space use of *L. flavastellus* in aquatic vegetation. Three different species of plants were used; however, plant type had no effect on the results and will not be discussed further. The test chamber consisted of a 3.8 l glass jar with 4.5 cm of course sand and 3.5 l of filtered tap water. Four lines were drawn around the jar every 4.5 cm from the top of the sand, resulting in four zones of increasing height, as well as the ground zone (located on the substrate). Three plants of a single species were placed in the sand in a triangular array. Four conditioned maple leaves and five caddisflies were placed on the substrate. After a 20 min acclimation period, I recorded the position of each caddisfly within each zone [ground (0cm), 0–4.5 cm, 4.5–9 cm, 9–13.5 cm, 13.5–18 cm] every 20 minutes for 5 hours. Two replicates were conducted per plant species resulting in six experimental chambers. The total number of caddisfly observations at each height was summed for each experimental container. These data were analyzed by a two-way ANOVA with plant type and height as the two fixed factors. Data were square-root transformed to meet assumptions of normality.

I also measured the vertical space use of individual caddisflies to determine what role size (mass, case length, and case width) had on the height obtained in aquatic vegetation (*Egeria densa*). The experimental chamber and test procedure were the same as previously described, except that a single caddisfly was placed in each jar. At the conclusion of testing, I recorded the length and diameter of the case as well as the mass of the caddisfly (without case). I calculated the mean height obtained by the caddisfly during each trial by assigning each zone a value based on the distance from the middle of that zone to the substrate and averaging all the observations from the five-hour trial. I

compared case length and diameter and caddisfly mass with the mean height obtained during the trials with linear regression.

Field experiment on egg survival

I tested the survival of newt eggs positioned at one of three heights (2, 13, or 25 cm) above the substrate in a natural pond. Gravid female newts (N = 10) were collected from Soap Creek ponds and transported to Corvallis, OR. Each female was injected with 20 µL LHRH. A female was placed in a 15-l tub with approximately 5 l of pond water. Small squares (3.8 x 3.8 cm) of artificial turf were glued to ceramic tiles and placed in the bottom of the tub for females to oviposit on. After females had deposited 5 eggs on a small square, the square was removed and placed in a separate tub until transportation to the field site. At 0900 hrs, all squares (collected either at 2000 hrs the previous day or 0700 hrs that morning) were transported to the pond. The tub containing the experimental squares was placed in the pond to acclimate the eggs to the lower temperature.

One small square of turf containing five eggs was randomly assigned to the bottom, middle, or top of a 59 cm long wooden stake (Fig. 5–1C). Two small squares (without eggs) were stapled to the remaining empty positions, and two rectangular pieces of turf (3.8 x 17.5 cm) were stapled in the gaps, thus creating a continuous piece of turf with 5 eggs at the appropriate height (Fig. 5–1C). An imaginary grid (7 rows and 3 columns; each square was approximately 3 × 3 m) was created across the pond and a stake was randomly assigned to one of the 21 squares. Stakes were pushed into the substrate (approximately 20 degrees from vertical) until the bottom of the lowest square rested on the substrate. Trials were initiated in three separate ponds (N = 5 or 6

stakes/treatment/pond). After 25 hours, the stakes were removed and the number of surviving eggs was recorded.

I compared the number of surviving eggs on each stake among the three heights using a general linear model followed by REGWQ multiple comparisons in SAS v 9.1. Stake was treated as the experimental unit, with the number of surviving eggs summed for each stake. Height was treated as a fixed effect factor while pond was incorporated into the model as a random factor. These data were squared to meet assumptions of normality.

3. IS THE TTX PRESENT IN NEWT EGGS SEQUESTERED BY CADDISFLIES?

Caddisflies likely ingest a large amount of tetrodotoxin when consuming newt eggs. To determine if caddisflies sequester this toxin, I tested whether (1) caddisflies provisioned with eggs in the lab possess more TTX than control animals, (2) if wild-caught caddisflies harbor the toxin and (3) how long TTX is retained when wild-caught caddisflies are reared in the lab.

To determine whether caddisflies sequester tetrodotoxin, I tested the amount of toxin retained in their tissues 24 hours after consuming five eggs in the laboratory. A caddisfly was housed in a mesh-bottom cup (236 ml) placed inside a larger cup with 500 ml of water. The mesh cup was elevated off the floor of the second cup ensuring that fecal material did not accumulate inside the test container. The cups were maintained in an environmental chamber at 16 C on a 12 hour light: 12 hour dark cycle. Each caddisfly was randomly assigned to either the control (N = 12) or egg-fed (N = 12) treatment. Caddisflies in the egg-fed treatment were given five eggs from one of two female newts

that had recently begun depositing eggs. I recorded the number of eggs consumed by each caddisfly daily. Twenty-four hours after consuming the fifth egg, a caddisfly was removed from the mesh cup and extracted from its case. The larva was weighed (0.01g) and frozen at -80 C for TTX analysis. At the same time, a control larva that had not been fed eggs was removed, weighed, and frozen. Tetrodotoxin was quantified using a competitive inhibition enzymatic immunoassay (CIEIA), as described in Chapter 4. The amount of TTX present in control and egg-fed caddisflies was compared with a t-test. Data were square root transformed to meet assumptions of normality.

To determine the length of time caddisflies retain TTX, I collected caddisflies from Soap Creek ponds after newts had begun breeding. Immediately after collection, 32 individuals were removed from their case, weighed to the nearest 0.01g, and frozen on dry ice. A group of caddisflies was also transported to Utah State University and maintained in mesh cups under the same conditions as described above. Caddisflies were maintained on a diet of detritus and wheat grains (*Triticum* sp.) to facilitate growth and obtain winged-adults. Subsets of larvae were frozen 13-44 days after collection. Several larvae successfully pupated, and adult caddisflies were frozen between 81 and 142 days following collection. Four caddisfly larvae (*Hesperophylax occidentalis*) allopatric with newts (collected from Paradise, UT) were also tested for the presence of TTX.

The relationship between body mass and TTX quantity was assessed using linear regression on the subset of caddisflies frozen immediately after collection. To determine whether caddisflies retain TTX over time, I compared the amount of TTX retained by caddisflies with the days post collection.

4. WHAT IS THE POTENTIAL FOR INDIRECT SELECTION BY CADDISFLIES TO LEAD TO ELEVATED TOXICITY IN NEWTS?

Caddisflies were provided with newt eggs containing low (569 ng; N = 43), medium (676 ng; N = 13), or high (1108 ng; N = 29) levels of TTX to determine whether caddisflies can influence the selective regime on the newt population by consuming more eggs with less TTX. Three female newts were injected with 2µl/g LHRH and allowed to oviposit for 24 hrs. Five eggs from each female were frozen. The protocol for housing caddisflies (mesh-bottom cups) is the same as that described above. Each caddisfly was provided with five eggs from one female; variation in TTX between eggs within a clutch (i.e. one female) is extremely low (Hanifin et al. 2003). The experimenter was blind to the toxicity of the females and eggs. After 24 hours the number of eggs that had been consumed was recorded for each caddisfly, and the cups were re-stocked with eggs from the appropriate female. Caddisflies were allowed to consume eggs over an additional 24 hour period, whereupon the number of eggs consumed was recorded. At the conclusion of testing eggs from a single female were combined, macerated, and extracted for TTX analysis using CIEIA (see Chapter 4).

The sample size between treatments was highly variable because of the dependence on the number of eggs deposited by each female over 24 hrs. The females in the high and low TTX treatments deposited substantially more eggs than the female in the medium TTX treatment. The total number of eggs consumed over the 48 hr period was calculated for each caddisfly. The mean number of eggs consumed in the three treatments (low, medium, and high TTX) were compared with a one-way ANOVA followed by Holm-Sidak multiple comparisons. Statistical analysis was performed blind with respect

to egg and female toxicity. Data were square root transformed to meet assumptions of normality and homoscedasticity.

RESULTS

1. DO CADDISFLIES RESPOND BEHAVIORALLY TO NEWTS?

Limnephilus flavastellus exposed to cues from gravid female newts and a blank control spent significantly more time on the side of the test container associated with the gravid female newts ($t = 4.385$, $df = 9$, $P = 0.002$; Fig. 5–2). However, several weeks after these females had deposited all of their eggs, caddisflies spent a similar portion of time on the control and stimulus sides of the test tank ($t = 0.187$, $df = 8$, $P = 0.857$; Fig. 5–2). When exposed to control, detritus, recently deposited eggs, and male newts, caddisflies exhibited a random distribution within the test tank, spending similar amounts of time on both sides (all $P > 0.09$; Fig. 5-2). There was no significant difference between treatments in activity levels as measured by the number of lines crossed ($H = 2.3$, $df = 5$, $P = 0.06$).

When exposed to cues from a control, recently deposited newt eggs, male newts, and gravid female newts, *L. concolor* spent a similar amount of time on both sides of the test container (all $P > 0.21$). However, there was a significant difference between treatments in activity levels as measured by the number of lines crossed ($df = 3$, $F = 5.245$, $P = 0.004$). *Limnephilus concolor* was least active in response to cues from a control [mean lines crossed (LC) ± SE = 3.8 ± 1.2], male newts (mean LC = 1.9 ± 0.48), and newt eggs (mean LC = 4.6 ± 1.14), but crossed significantly more lines in response to

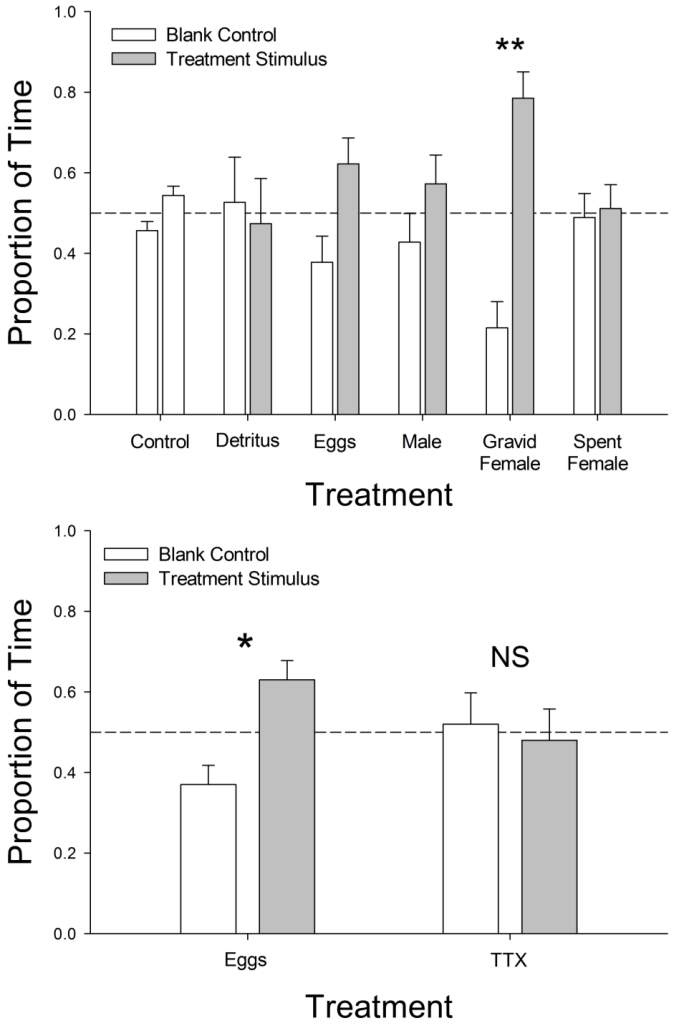

Figure 5–2. Do caddisflies respond behaviorally to newts? (Top) Proportion (Mean ± SE) of time caddisflies (Limnephilus flavastellus) spent on the control and treatment side of a test chamber during choice-trials. Dashed line indicates a random distribution. **P = 0.002, all other P > 0.09. (Bottom) Mean (± SE) proportion of time caddisflies (Limnephilus concolor) spent on the control and treatment side of a test chamber during choice-trials. Dashed line indicates a random distribution. *P < 0.05, NS = nonsignificant.

cues from gravid female newts (mean LC = 8.4 ± 1.6) compared to control or male newt cues.

Limnephilus flavastellus exposed to newt eggs and a blank control in a stagnant-water container spent significantly more time in the portion of the test chamber containing newt eggs ($t = 2.73$, $df = 25$, $P = 0.011$; Fig. 5–2). However, caddisflies were not specifically attracted to agar containing 46 µg of TTX ($t = -0.261$, $df = 19$, $P = 0.80$; Fig. 5–2). There was no significant difference in the number of lines crossed between caddisflies exposed to eggs or agar with TTX ($t = 0.53$, $df = 44$, $P = 0.60$).

2. DO NEWTS POSSESS STRATEGIES THAT LIMIT PREDATION ON THEIR EGGS?

Oviposition choice

Ovipositing female newts responded strongly to caddisflies, depositing just 25 % of their eggs near the caddisflies. The proportion of eggs deposited on the control side of the test chamber (0.75) was significantly higher than the proportion of eggs deposited near caddisflies (0.25; $t = 3.233$, $df = 7$, $P = 0.014$; Fig. 5–3). However, egg deposition between the two sides of the test tank (Mayfly: 0.51; Control: 0.49) in response to nonpredatory mayflies did not differ from random ($t = 0.096$, $df = 5$, $P = 0.927$; Fig. 5–3).

Oviposition behavior in vertical chamber

Significant main effects were detected for both treatment ($F_{[2,28]} = 7.51$, $P = 0.0024$) and height ($F_{[3,80]} = 65.71$, $P < 0.0001$). A significant interaction effect between treatment and height, however, was also identified ($F_{[6,80]} = 3.79$, $P = 0.0023$, Fig. 5–3).

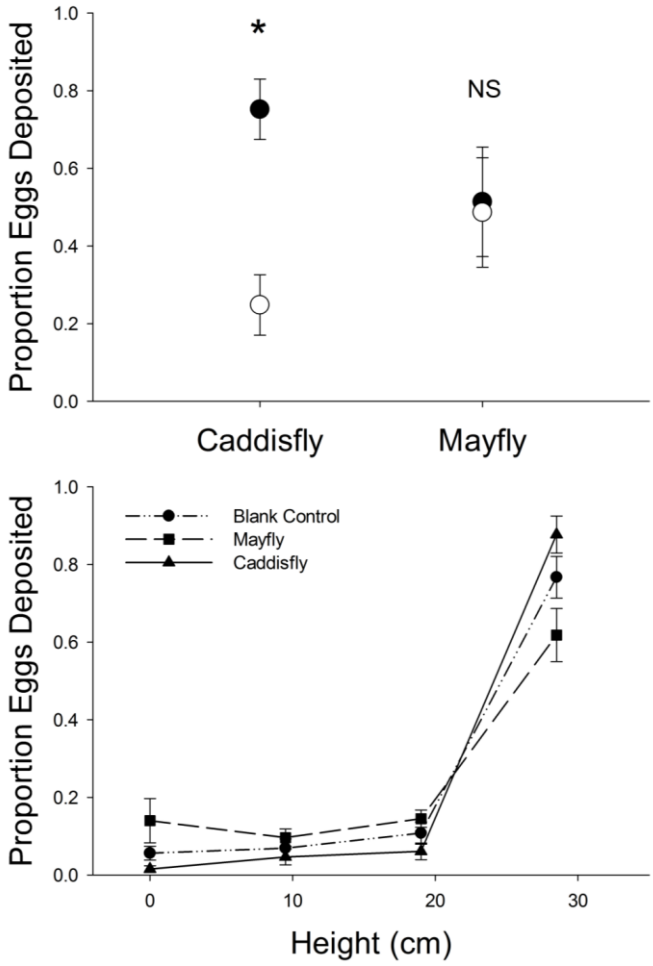

Figure 5–3. Do newts possess strategies that limit predation on their eggs? (Top) Mean (± SE) percentage of eggs deposited by female newts on oviposition sites either next to (open circles) or away from (closed circles) predatory caddisflies or nonpredatory mayflies. *P = 0.014; NS, P = 0.93. (Bottom) Mean (±SE) proportion of eggs deposited at four different heights (cm) by female newts exposed to predatory caddisflies (triangle), nonpredatory mayflies (square), or a blank control (circle). Newts decrease the number of eggs deposited near the bottom and deposit more eggs near the top of the water column when an egg predator (caddisfly larvae) is placed near the bottom of the test chamber.

When exposed to caddisflies, ovipositing females shifted deposition upward relative to females exposed to mayfly and control treatments (Fig. 5–3). Females exposed to caddisflies oviposited just 1.5% of all eggs on the bottom fiber block compared with 5.6% and 14.0% in the control and mayfly treatments, respectively. This shift away from the bottom resulted in 87.7% of all eggs being deposited at the top when exposed to caddisflies compared with 61% in the nonpredator mayfly treatment and 76% in the control treatment. There was no significant difference between treatments in the mean total number of eggs deposited (Caddisfly: 81.2 ± 7.5; Mayfly = 77.5 ± 3.1; Control: 76.8 ± 3.7; $F = 0.21$, $P = 0.81$).

Caddisfly distribution in relation to oviposition behavior

There was a significant main effect of height on the distribution of caddisflies throughout the plants ($F_{[4,29]} = 54.93$, $P < 0.001$, Fig. 5–4). Caddisflies primarily utilized the substrate and lowest sections of vegetation and few were observed in the upper sections of vegetation (Fig. 5–4). The type of plant that caddisflies were offered had no effect on the distribution of caddisflies during the trial ($F_{[2,29]} = 0.94$, $P = 0.412$). There was no significant interaction between plant type and height ($F_{[8,29]} = 2.34$, $P = 0.074$). When compared to egg deposition by newts, caddisflies generally did not utilize areas where newt eggs were likely to be deposited (Fig. 5–4).

Smaller caddisflies climbed higher in aquatic vegetation than large caddisflies, which remained on or close to the substrate (Fig. 5-5). There was a significant negative correlation between caddisfly mass ($F_{[1,24]} = 7.7$, $R^2 = 0.24$, $P = 0.01$, Fig. 5–5), case

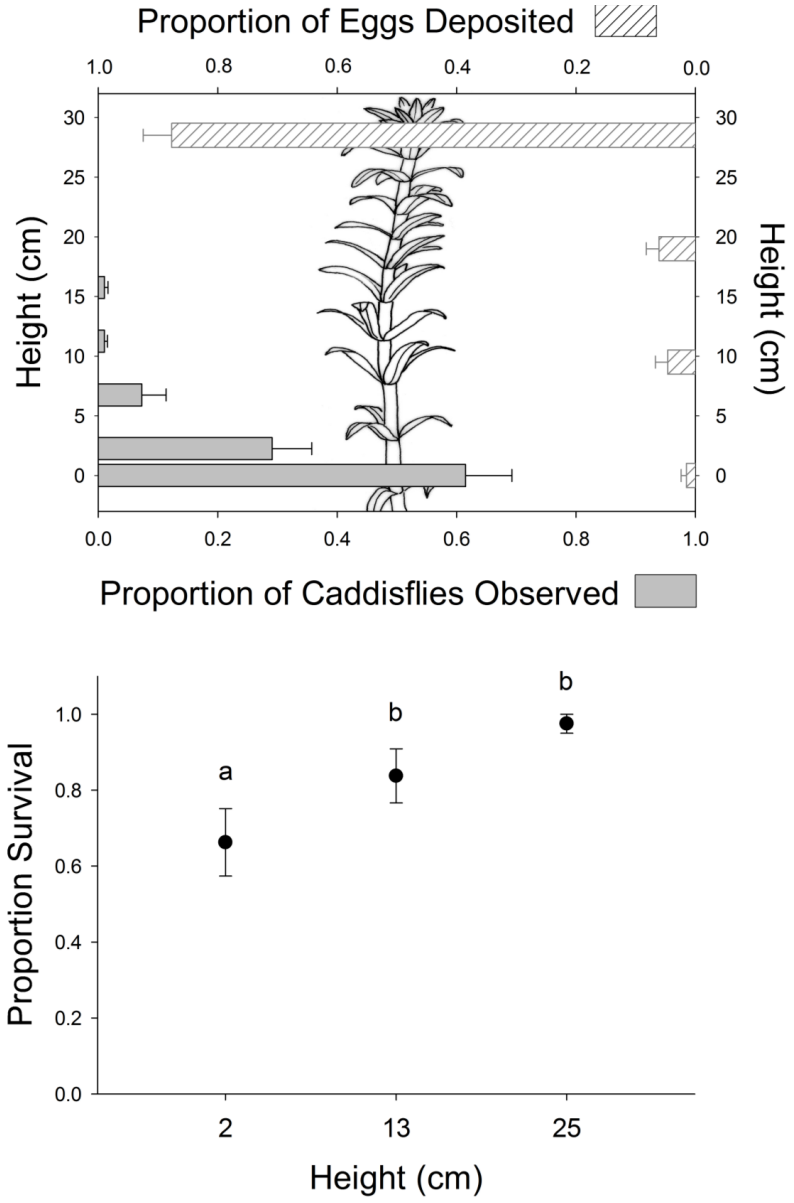

Figure 5–4. Do newts possess strategies that limit predation on their eggs? (Top) The number of caddisflies observed at five heights in aquatic vegetation over a five-hour period compared with the proportion of eggs deposited at four different heights by female newts exposed to caddisflies during vertical oviposition trials. All trials were conducted in the lab. *Elodea* line drawing provided by the University of Florida, Center for Aquatic and Invasive Plants. (Bottom) Mean (± SE) proportion of eggs that survived over a 25 hour period when placed at one of three different heights (cm) above the substrate in a natural pond. Different letters indicate significant differences between treatments ($P < 0.05$).

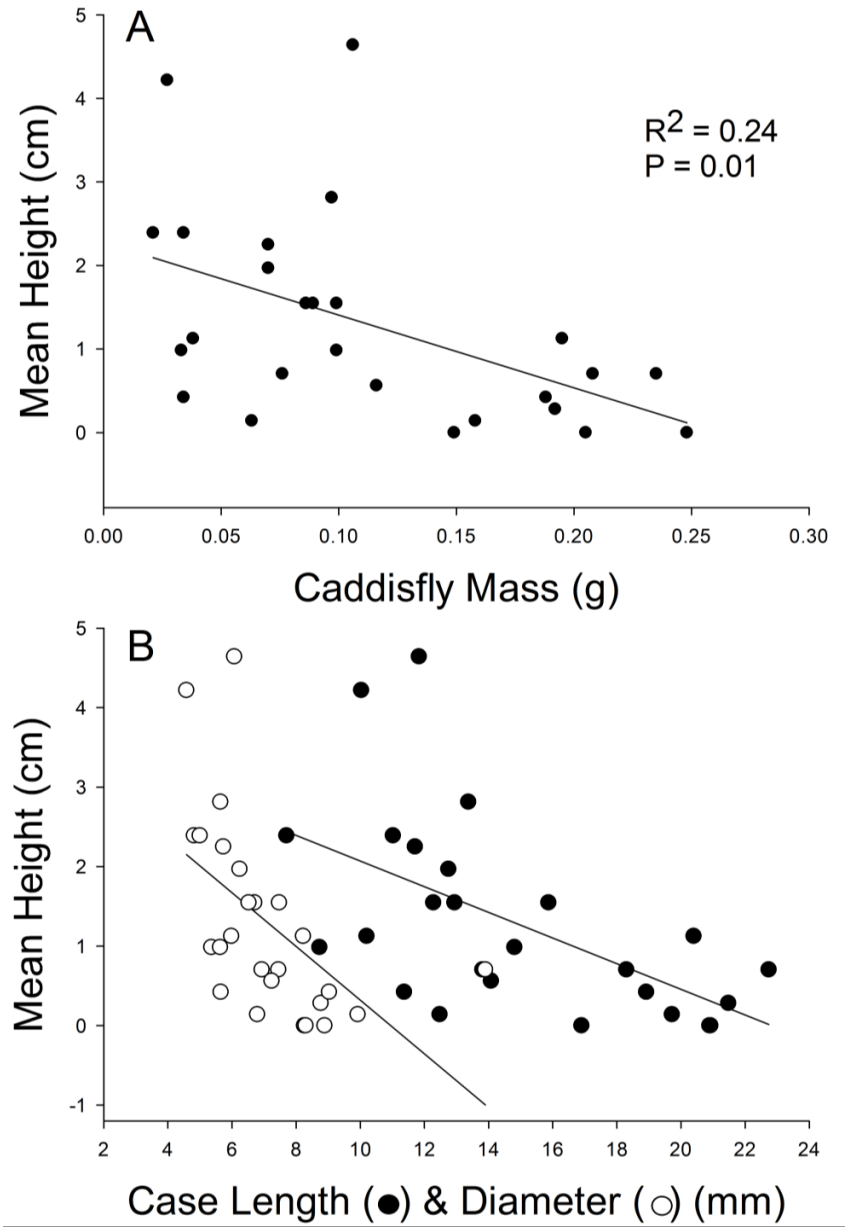

Figure 5–5. The mean height obtained by larval caddisfly *Limnephilus flavastellus* in relation to (A) Larval mass ($F_{[1,24]}$ = 7.7, R2 = 0.24, P = 0.01), (B) case length (solid circles; $F_{[1,24]}$ = 11.4, R2 = 0.32, P = 0.003), and case diameter (hollow circles; $F_{[1,24]}$ = 10.1, R2 = 0.30, P = 0.004).

length ($F_{[1,24]}$ = 11.4, R^2 = 0.32, P = 0.003, Fig. 5–5), and case diameter ($F_{[1,24]}$ = 10.1, R^2 = 0.30, P = 0.004, Fig. 5–5) and the height obtained in vegetation.

Field experiment on egg survival

Caddisflies were observed on the experimental stakes and evidence of predation by caddisflies (torn egg jelly and consumed yolk) was identified on most stakes. The height of newt eggs in the pond had a significant effect on their survival (df = 2, F = 7.51, P = 0.002, Fig. 5-4), with eggs placed near the substrate suffering the greatest predation and survival increasing with increasing height (Fig. 5-4). Block (pond) had no effect on egg survival (df = 2, F = 1.44, P = 0.25).

3. IS THE TTX PRESENT IN NEWT EGGS SEQUESTERED BY CADDISFLIES?

Caddisflies that consumed five newt eggs in the laboratory had significantly higher levels of tetrodotoxin in their tissues than caddisflies that did not consume newt eggs (df = 22, F = 5.06, P < 0.001, Fig. 5–6). Many of the caddisflies frozen immediately after collection from Soap Creek ponds had elevated levels of TTX. However, total TTX (ng) was negatively correlated with body mass (N = 32, F = 55.86, R^2 = 0.65, P < 0.001, Fig. 5–6). Caddisfly larvae collected at Soap Creek ponds but reared in the lab retained TTX despite being maintained on an egg-free diet (Fig. 5-6). Larvae that successfully pupated and eclosed in the laboratory also retained similar TTX levels, with some individuals retaining TTX for at least 135 days after collection (Fig. 5–6). Nine larvae and nine adult caddisflies from Utah (*H. occidentalis*) did not possess tetrodotoxin.

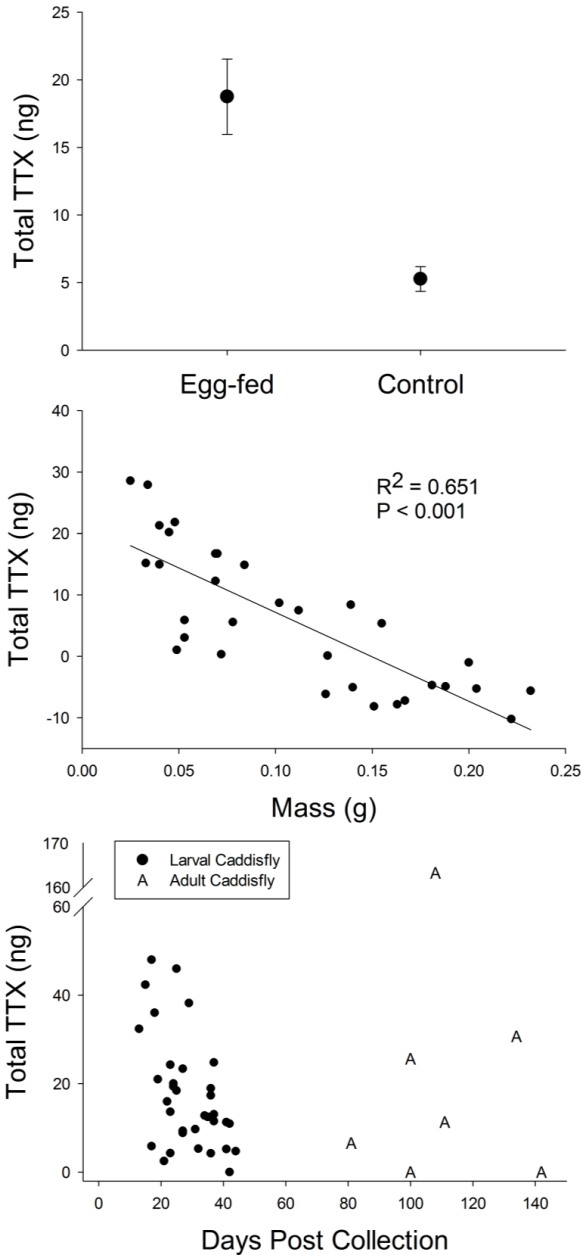

Figure 5–6. Is the TTX present in newt eggs sequestered by caddisflies? (Top) Mean (± SE) total amount of TTX (ng) present in the tissues of caddisflies that had consumed five eggs in the laboratory. Caddisflies that consumed eggs had significantly higher levels of tetrodotoxin than caddisflies that did not consume eggs (df = 22, F = 5.059, P < 0.001). (Middle) The total amount of tetrodotoxin (ng) in the tissues of larval caddisfly *Limnephilus flavastellus* in relation to body mass (N = 32, F = 55.86, R^2 = 0.651, P < 0.001). Caddisflies were frozen immediately after collection from a pond containing actively breeding newts. (Bottom) Total amount of TTX (ng) present in caddisflies collected from a pond with actively breeding newts and reared in the lab.

4. WHAT IS THE POTENTIAL FOR INDIRECT SELECTION BY CADDISFLIES TO LEAD TO ELEVATED TOXICITY IN NEWTS?

Caddisflies consumed progressively fewer eggs as the amount of TTX in the eggs increased (Fig. 5–7). There was a significant difference in the number of eggs consumed by caddisflies when those eggs contained low (569 ng), medium (676 ng), or high (1108 ng) quantities of TTX ($F_{[2,82]} = 6.75$, $P = 0.002$, Fig. 5–7). In the low and medium TTX treatments, 28% and 31% of caddisflies failed to consume any eggs, respectively. In contrast, 55% of caddisflies in the high TTX treatment failed to consume a single egg. Further, in the low TTX treatment 49% of caddisflies consumed two or more eggs, whereas 31% and 14% of caddisflies in the medium and high TTX treatments consumed two or more eggs. The maximum number of eggs consumed (maximum possible was 10) in the low, medium, and high TTX treatments was 10, 3, and 2, respectively.

DISCUSSION

EVOLUTION OF BEHAVIOR IN PREDATOR AND PREY

Caddisflies are major predators of newt eggs and appear to be resistant to the negative effects of tetrodotoxin poisoning (see Chapter 3). In addition, caddisflies possess a series of behavioral responses that likely increase access to newt eggs. One species of caddisfly, *L. concolor*, responded to the presence of gravid female newts by increasing activity. The most abundant species (*L. flavastellus*) was specifically attracted to chemical cues emanating from gravid female newts, as well as the presence of recently deposited eggs. Surprisingly, this response disappeared after the female newts had

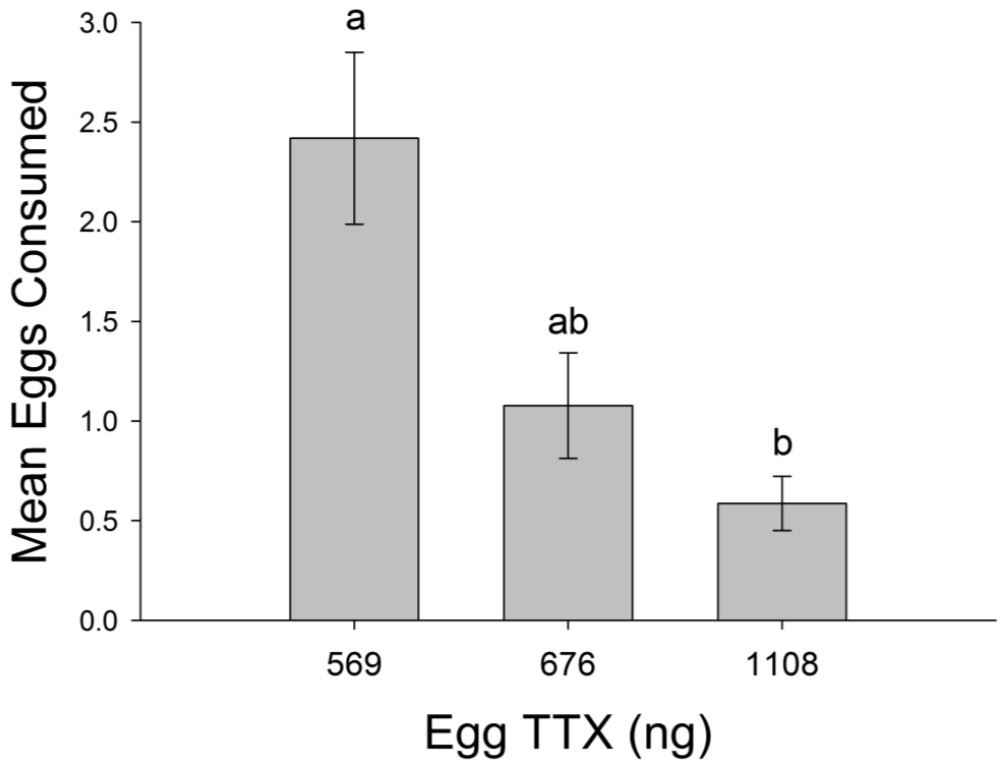

Figure 5–7. What is the potential for indirect selection by caddisflies to lead to elevated toxicity in newts? Mean (± SE) number of eggs consumed by caddisflies containing low (569 ng), medium (676 ng), or high (1108 ng) concentrations of TTX. Different letters indicate significant differences between treatments ($P < 0.05$).

completed egg deposition. These results, combined with the lack of response to male newts and TTX in an agar solution, indicates that a response is contingent upon a specific cue present during gravidity that is currently unknown.

Previous studies have determined that caddisfly larvae can detect and respond to water-born chemical stimuli (Boyero et al. 2006; see Chapter 2), yet in these cases, changes in behavior were the result of exposure to predators. Spanhoff et al. (2005) examined the response of caddisflies with intact and excised antennae to patches of algal biofilm. The authors found that random movement, rather than attraction to chemical cues from those patches, was the method used to locate food. Unlike periphyton or detritus, eggs provide a resource rich in lipids and protein, and caddisflies are often found in aggregations on dead fish and egg clusters from fish and amphibians (Murphy 1961; Brusven and Scoggan 1969; Fox 1978). Caddisflies attain greater sizes when they consume eggs in the laboratory (see Chapter 3), and larger sizes have been correlated with greater egg production in female caddisflies (Spanhoff 2005; Jannot 2009). Caddisflies that respond to cues indicating the presence of eggs would likely consume more eggs in the wild, thereby gaining a fitness advantage. Further, both strategies for finding eggs would likely increase the rate at which caddisflies find and consume newt eggs in nature, further strengthening their role as a selective agent on the newt population.

Given the potential for caddisflies to influence selection on newts via egg predation, one would predict the evolution of behavioral strategies by newts that limit predation on their progeny. In this laboratory study, female newts avoided egg predators by ovipositing in microhabitats relatively inaccessible to predatory caddisflies. Caddisfly

locomotion is generally limited by the presence of a portable case (Dodds and Hisaw 1925), and most species are restricted to benthic habitats (Betten 1934; Mackay and Wiggins 1979). In the laboratory, caddisfly abundance decreased with increasing plant height, indicating that *L. flavastellus* does not commonly use the upper portions of aquatic vegetation. Larger caddisfly larvae, which consume greater numbers of eggs in the laboratory (see Chapter 3), are even less prone to climb vegetation than are smaller larvae, and larger wild-caught caddisflies possessed lower quantities of TTX, further indicating large larvae are not consuming eggs in the wild and may be inefficient predators on newt eggs. This spatial isolation has yielded a microhabitat that serves as an optimal oviposition site for female newts.

Discrimination between oviposition sites has been shown to affect offspring survival and parental fitness. For example, phytophagous insects often exhibit strong preference for specific host plants, and offspring survival on these plants is often higher than on non-preferred species (Rausher 1980; Thompson and Pellmyr 1991). For prey with access to discrete oviposition sites that vary in predation risk, avoidance of habitats containing egg or larval predators may greatly increase offspring survival. For example, mosquitos avoid depositing eggs in habitats containing predatory notonectids, amphibians, and fish (Chesson 1984; Petranka and Fakhoury 1991). Several treefrog species (*Hyla*) deposit more eggs in artificial ponds that lack predatory conspecifics, salamanders, and fish than in ponds containing these predators (Resetarits and Wilbur 1989; Crump 1991; Resetarits and Wilbur 1991; Resetarits 1996). In these cases, predator dispersal is limited (e.g. fishes), and successful oviposition is dependent on the presence of habitats that completely lack predators (e.g. Hopey and Petranka 1994). Caddisflies are

highly mobile as adults (winged) and inhabit almost all freshwater ecosystems (Wiggins and Currie 2008). It is therefore unlikely that female newts would find a completely new pond that lacked these predators. However, because predation pressure from caddisflies varies spatially within a pond, newts are able to reduce the risk of predation on their eggs by selecting oviposition sites within a breeding habitat that are less accessible or unsuitable to caddisflies.

Spatial variation in predation pressure likely has driven the evolution of behavioral responses to avoid egg predators and increase female fitness. For this behavioral strategy to be effective, eggs deposited high in the water column must be more likely to survive. Results from our field experiment indicate that eggs deposited higher in the water column are indeed subjected to reduced predation and may therefore be more likely to survive to hatching. Therefore, female newts increase total lifetime fitness by shifting oviposition upward in the water column.

POTENTIAL FOR SELECTION

Maternal toxicity is correlated with egg toxicity in newts (Hanifin et al. 2003). This relationship implicates predation on newt eggs as a potential pathway for indirect selection on toxicity, one that could ultimately yield elevated TTX levels in adult newts. It was previously unclear whether egg consumption by a predator was capable of influencing selection on toxicity. For selection to operate on the toxicity of adult newts caddisflies must preferentially consume more eggs that contain lower quantities of TTX. In no-choice trials caddisflies consumed almost five times more eggs that contained lower (500 ng), as opposed to higher (1100 ng), quantities of TTX. This difference in

preference suggests that they have the potential to indirectly drive the selective environment of the newt population.

It is unclear how caddisflies distinguish between palatable eggs (low TTX) and less palatable eggs (high TTX) without sampling the contents of each egg. There is very little variation in toxicity level within a clutch of eggs from a female newt (Hanifin et al. 2003), and sampling one egg would provide a reliable measure of the toxicity of the other eggs. Similarly, the Bella moth (*Utetheisa ornatrix*) provisions its eggs with pyrrolizidine alkaloids, which are then deposited in clusters on the larval food plant (Dussourd et al. 1988). A predator of those eggs, larvae of the green lacewing (*Ceraeochrysa cubana*), sample one or a few eggs from a clutch and reject clusters that contain high concentrations of the toxin (Eisner et al. 2000). However, in our study more than half of the caddisflies given the most toxic eggs avoided the eggs entirely, indicating that some other method of discrimination is likely utilized by caddisfly larvae to assess the toxicity of newt eggs.

Caddisflies attain larger sizes when they consume toxic eggs in the laboratory compared to control individuals that do not consume eggs (see Chapter 3), and it is possible that retaining the TTX present in these eggs may provide additional fitness benefits. Caddisflies that consume eggs in the laboratory sequester small concentrations of TTX in their tissues. Sequestration of toxins from prey occurs in a variety of organisms including insects (Eisner et al. 1997; Nishida 2002), New Guinea passerine birds (Dumbacher et al. 1992; Dumbacher et al. 2004), dendrobatid frogs (Daly et al. 1994), and bufophagous snakes (Hutchinson et al. 2007). Similarly, garter snakes that consume toxic newts sequester TTX in the liver (Williams et al. 2004). Tetrodotoxin was

found in the tissues of wild-caught caddisflies and was retained by some individuals through metamorphosis. Organisms that sequester toxins are assumed to utilize it for defense (Brower and Glazier 1975; Dussourd et al. 1988), and the TTX present in caddisflies may protect the larvae or winged-adults or could be secondarily transferred to the caddisflies' eggs or offspring (e.g. Eisner et al. 2000; Hutchinson et al. 2008). If TTX does serve a defensive function in caddisflies, individuals that are able to consume and sequester greater quantities of toxin may receive a fitness advantage, leading to greater tolerance and preference for TTX laden eggs.

IMPLICATIONS FOR COEVOLUTION

The phenotypic interface of the coevolutionary arms-race between newts (*Taricha*) and garter snakes (*Thamnophis*) is the potent neurotoxin tetrodotoxin. In this well characterized system newts have evolved extreme toxicity, which has been countered in snakes via modifications to the amino acid sequence of the sodium channel protein, thereby conferring resistance to TTX poisoning (see review in Brodie 2010). The level of selection on each population is geographically variable, with one of the most intense interactions occurring in the Willamette Valley of Oregon (Hanifin et al. 2008). Newts originated in the early Cretaceous (approximately 100 million years ago; Zhang and Wake 2009), whereas the ancestor of modern colubrid snakes (Colubridae), including garter snakes (Thamnophis), did not originate until at least 50 million years later (Pyron and Burbrink 2011). All modern newts possess tetrodotoxin (Hanifin 2010); therefore its evolution in salamanders preceded the origin of garter snakes. There may be multiple selective influences on the evolution of toxicity, and understanding the origin of extreme

toxicity in the arms-race requires analyzing the role of alternative predators in toxin evolution.

The interaction between caddisflies and newts is complex, and the question remains: Are caddisflies and newts involved in a coevolutionary interaction? For this process to occur, natural selection imposed by caddisflies must lead to defensive adaptations in newts, which further increases selective pressure on caddisflies to exploit their prey (i.e. reciprocal selection). Over three-quarters of a million caddisflies may occupy a single pond from central Oregon and that under optimal conditions they have the potential to consume the entire reproductive output of a newt population in as little as 36 hrs (see Chapter 3). Further, caddisflies possess behavioral adaptations that likely increase their ability to find and consume newt eggs. This may be partially moderated however by behavioral adaptations of female newts to reduce predation on their eggs. Although the details are unknown, caddisflies appear to be at least partially resistant to the negative effects of TTX (see Chapter 3), indicating that selection (newt → caddisfly) may occur. The preference that caddisflies exhibit for lower toxicity eggs has the potential to lead to the elevation of toxicity (caddisfly → newt). Although the results of this study do not demonstrate reciprocal selection between newts and caddisflies, the interaction between these species is multifaceted and could potentially involve this process.

Ultimately, the predator-prey arms-race between snakes and newts may be influenced by caddisflies if the correlation between egg toxicity and maternal toxicity, combined with selective egg predation, leads to indirect selection on the toxicity of newts. At the extreme, snake resistance, believed to be due to selection from newts, could

be due to correlational selection with caddisflies if the primary selective pressure driving adult toxicity is egg predation. In this case, snake resistance may be "hitchhiking" on an arms-race between caddisflies and newts. Nevertheless, the reciprocal pathway is also possible, and caddisflies may be consuming toxic eggs only as a byproduct of coevolution between snakes and their toxic prey. Certainly, each player may be influencing the traits of the other to some degree, and only additional research will begin to elucidate the extent of interaction between these three players in the evolution of the arms-race between toxicity and resistance.

Most organisms have multiple predators, and understanding the evolution of an arms-race requires an analysis of the selective role of all potential predators. The ancestors of caddisflies (Trichoptera) arose approximately 225 million years ago (Wiggins 2004), well before the origin of newts; recent work has demonstrated that caddisflies may be major players in the arms-race revolving around tetrodotoxin (see Chapters 3 and 5). Specifically, newts and caddisflies exhibit a series of behavioral adaptations that limit egg predation and facilitate egg consumption, respectively, that is redolent of a behavioral arms-race. Moreover, the TTX sequestered by caddisflies and the preference they exhibit for eggs with less toxin indicate reciprocal selection between newts and caddisflies is possible, and that this interaction may influence the coevolutionary process between newts and snakes.

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CHAPTER 6

SUMMARY

The goal of this research was to provide a greater analysis of the predator-prey interactions between newt eggs and larvae and their predators (Table 6.1). Understanding these relationships begins to fill a major gap in our knowledge of the evolutionary arms-race revolving around tetrodotoxin (TTX) by defining the role egg and larval predation may have on the toxicity of adult newts (Table 6.1; Brodie 2010).

I began (Chapter 2) by determining that larval caddisflies respond to chemical stimuli from potential predators (Rainbow trout; *Oncorhynchus mykiss*) with predator avoidance behavior (see Chapter 2). Chemical cues are widely used by aquatic vertebrates and invertebrates for all aspects of their ecology including foraging, reproduction, and predator avoidance (Wisenden and Chivers 2006; Ferrari et al. 2010). It was previously unclear if caddisflies responded to chemical stimuli (Malmquist 1992), yet data presented here indicate caddisflies use chemical cues to acquire information from their environment (see Chapter 2), which is similar to other aquatic insects (e.g. Sih 1986; Huryn and Chivers 1999; Blaustein et al. 2005). In addition, caddisflies modified their behavior in response to cues from injured conspecifics indicating the presence of a chemical alarm cue in caddisflies, the first demonstration of an alarm cue in Trichoptera (see Chapter 2). The behavioral responses exhibited by caddisflies toward predators provided a validation of techniques and suggested testing their responses to other chemical cues, such as those emanating from gravid female newts and their eggs (see Chapter 5) would be a fruitful area of research.

Table 6.1. Summary of the known attributes of the interaction between caddisflies, newts, and snakes.

Caddisflies	
Extremely abundant	(This Study, Chapter 3)
Feed on newt eggs	(This Study, Chapter 3)
Resistant to negative effects of TTX	(This Study, Chapter 3)
Larger larvae consume more eggs in the lab	(This Study, Chapter 3)
Attracted to gravid female newts	(This Study, Chapter 5)
Attracted to recently deposited newt eggs	(This Study, Chapter 5)
Primarily benthic and do not climb vegetation	(This Study, Chapter 5)
Large larvae do not climb as high as small larvae	(This Study, Chapter 5)
Sequester tetrodotoxin after eating newt eggs	(This Study, Chapter 5)
Sequestered toxin retained through metamorphosis	(This Study, Chapter 5)
Prefer consuming eggs with less TTX	(This Study, Chapter 5)
Newts	
TTX stored in the skin, ovaries, and eggs	(Twitty 1937; Wakely et al. 1966)
Egg toxicity correlated with female toxicity	(Hanifin et al. 2003)
Avoid caddisflies by ovipositing at top of water column	(This Study, Chapter 5)
Eggs laid higher are more likely to survive	(This Study, Chapter 5)
TTX deposited in egg retained by larvae and juveniles	(This Study, Chapter 4)
Juveniles with more TTX more likely to survive predation	(This Study, Chapter 4)
Multiple newt species have entered an arms race	(Brodie et al. 2005)
Geographic variation in toxicity	(Brodie and Brodie 1991; Hanifin et al. 1999; Brodie et al. 2002)
Adults avoid chemical cues from newt-fed snakes	(Gall et al. 2011)
Newts with more TTX more likely to survive predation	(Williams et al. 2010)
TTX increases in long-term captive newts	(Hanifin et al. 2002)
Snakes	
Feed on adult newts	(Brodie 1968)
Resistant to TTX	(Brodie 1968; Brodie and Brodie 1990; Geffeney et al. 2002)
Amino acid substitutions to sodium channel protein	(Geffeney et al. 2005)
Some populations have escaped the arms-race	(Hanifin et al. 2008)
Multiple snake species involved in arms race	(Brodie et al. 2002; Feldman et al. 2009)
Ability to self-assess resistance	(Williams et al. 2003)
Geographic variation in resistance	(Brodie et al. 2002)
Trade-off between speed and resistance	(Brodie and Brodie 1999)
Sequester TTX in liver after consuming newts	(Williams et al. 2004)
Preadapted to TTX ingestion	(Motychak et al. 1999)
Allele variation in $Na_v1.4$ correlates with resistance	(Feldman et al. 2010)

In Chapter 3, I started to evaluate the interaction between newt eggs and their predators. First, I characterized the macroinvertebrate community at my study site and tested a suite of these invertebrates for their propensity to consume toxic newt eggs in the laboratory. I estimated that caddisflies were extremely abundant (approximately 775,000 per 0.21 hectare pond). Moreover, caddisflies were the only invertebrate to consume any number of eggs in the laboratory (see Chapter 3), confirming preliminary observations from previous researchers (Lehman 2006; Lehman and Campbell 2007). All four species of caddisflies that co-occur with *Taricha* at this site consumed newt eggs (see Chapter 3). Moreover, individual *Limnephilus flavastellus* grew substantially larger when they consumed eggs compared to when they had access to detritus only (see Chapter 3). This research confirms that caddisflies are resistant to the negative effects of ingesting TTX. The extreme abundance of caddisflies, their resistance to TTX, and greater growth when provisioned with eggs suggests caddisflies are an important predator of newt eggs and have the potential to be a major selective force on the newt population.

Consumption of TTX laden prey may introduce similar selective pressures on ecologically different predators. For example, garter snakes from the continental United States that prey on toxic newts (*Taricha*) have amino acid substitutions in the sodium channel protein that render them resistant to TTX intoxication (Geffeney et al. 2002; Geffeney et al. 2005; Feldman et al. 2009). Moreover, several species of snake from Southeast Asia, Japan, and South America prey upon TTX laden prey, and all of these species exhibit similar genetic changes rendering them resistant to the negative effects of TTX (Feldman et al. 2012).

Unlike the eggs, larval *Taricha granulosa* were assumed to be extremely vulnerable to predation. Experiments by Twitty and Johnson (1934) and Twitty (1937) found that *Taricha* eggs paralyzed their host when grafted to another species. The paralysis persisted until the yolk was absorbed. Therefore, *Taricha* larvae were believed to lack tetrodotoxin. The results of Chapter 4 however indicate that substantial quantities of TTX are retained by developing larvae (see Chapter 4). Although the amount of TTX in each individual does decline after hatching, larvae and recently metamorphosed juveniles retain approximately 400 nanograms of TTX (see Chapter 4). This amount of TTX was sufficient to repel one of the most voracious predators on amphibian larvae, predatory dragonfly nymphs (*Anax junius*). Analyzing the toxicity of metamorphosed juveniles that were palatable and unpalatable to dragonflies indicated that elevated toxicity levels conferred a survival advantage, which is consistent with previous studies on the survival advantage of TTX in newts (Williams et al. 2010). The amount of TTX present in larval and metamorphosed juvenile newts was highly variable and likely residual from the quantity initially deposited in the eggs. Because female toxicity is correlated with egg toxicity this relationship may continue between larvae and adult females (see Chapter 4). In addition to indirect selection on toxicity via egg predation (Hanifin et al. 2003), these results indicate that selection may also operate on adult toxicity indirectly through predation on the larvae.

The goal of Chapter 5 was to further assess the interaction between caddisflies and newts. Building on information acquired in Chapter 2, it was found that two species of caddisfly (*Limnephilus flavastellus* and *L. concolor*) were attracted to chemical stimuli emanating from gravid female newts and recently deposited newt eggs. These data,

combined with their propensity to consume eggs, greater growth, and great abundance (See Chapter 3) further implicate caddisflies as a major selective force on the newt population.

Because eggs are extremely vulnerable to predation (Orians and Janzen 1974) many female invertebrates and amphibians have evolved behavioral defenses enabling them to detect the presence of egg predators and shift oviposition to habitats that are relatively safe for their offspring (e.g. Chesson 1984; Resetarits and Wilbur 1989). Like many of these species, female newts are not defenseless toward predatory caddisflies. Gravid female newts responded to the presence of caddisflies by ovipositing away from caddisflies and shifting egg deposition to the upper portion of the water column. If this shift in microhabitat use is to be a successful strategy to prevent predation, caddisflies must be less likely to utilize this habitat and newt eggs must be more likely to survive in this area. Caddisflies are assumed to be benthic organisms, primarily utilizing the bottom of the pond and consuming fallen organic debri (Betten 1934; Mackay and Wiggins 1979). In the laboratory, caddisflies primarily utilized the substrate and lowest portion of vegetation indicating the microhabitat used as an oviposition site by female newts is unsuitable for predatory caddisflies in some way. Further, results from a field experiment indicate the second of these tenants, that newt eggs have a greater probability of surviving predation in this microhabitat, is also met. Combined, these results indicate complex behavioral strategies have evolved in caddisflies to procure newt eggs and in newts reduce the probability of predation on their offspring.

Organisms that eat toxic prey often sequester some of the toxin and utilize it for their own defense. For example, poison-dart frogs (*Dendrobates* sp.) in Central and

South America sequester large quantities of alkaloids from arthropods and other invertebrates they consume from the leaf litter (Daly et al. 1994; Saporito et al. 2007). These alkaloids function to repel predators (Daly and Myers 1967; Fritz et al. 1981). Relative to their size, caddisflies consume a large amount of TTX when they eat newt eggs. Laboratory experiments identified that caddisflies sequestered small quantities of TTX after consuming newt eggs. Samples from caddisfly larvae collected in the field also found similar levels of TTX indicating caddisflies consume newt eggs in nature. Further, several caddisflies retained the TTX through metamorphosis (up to 140 days). The role of sequestered TTX in caddisflies is unknown, although it may function in defense of the larvae or winged-adult, or be secondarily deposited in the caddisflies eggs. The amount of TTX retained by caddisflies is insufficient to kill avian and mammalian predators (Williams et al. 2004), but may deter other unknown predators [e.g. invertebrates; dragonfly larvae are far more sensitive to TTX than birds and mammals (see Chapter 4)]. Nevertheless, TTX is also a powerful emetic (Hayama and Ogura 1962; Kao 1966) and could function in this role for caddisflies.

Finally, to more directly assess the possible influence of caddisflies on newt toxicity via indirect selection, I provided caddisflies with eggs of varying toxicity and examined their preference for these eggs. Caddisflies consumed more eggs when those eggs contained the least amount of toxin. Moreover, there was tremendous variation between caddisflies in the number of eggs consumed indicating natural variation in either the ability or the willingness to consume newt eggs. This preference indicates caddisflies have the potential to drive the evolution of toxicity in adult newts by preying upon their eggs.

Additional research is necessary to fully understand whether reciprocal selection is occurring between the caddisfly and newt populations. Specifically, the following questions need to be addressed: (1) Is there variation in resistance among caddisflies within a population? (2) What patterns of behavior, resistance, and toxicity exist between caddisflies and newts across a large geographic scale? (3) Is the gene sequence of the sodium channel protein in caddisflies different from populations that do not consume newt eggs? (4) When a caddisfly sequesters TTX, where is it stored?

Is there variation in resistance between caddisflies within a population? Assessing individual caddisfly resistance to TTX is difficult; caddisflies cannot be injected with TTX and raced down a racetrack, and measuring LD_{50} is not cost effective. Therefore, the most effective method may involve a repeated measures design whereby an individual is provided with eggs of varying toxicities to determine the maximum amount of toxin the individual consumes. Although this technique does not enable the differentiation between ability and willingness, it may be the most effective way to understand the level of variation in resistance within a population. This question is critical to determining whether there is sufficient standing genetic variation for selection to operate on caddisfly resistance.

What patterns of behavior, resistance, and toxicity exist between caddisflies and newts across a large geographic scale? It is unknown whether the patterns observed between caddisflies and newts from my study population can be extrapolated across a wide geographic range. Therefore, it is necessary to examine this interaction in other populations, especially populations that exhibit lower toxicity, to fully investigate the possibility that predation on newt eggs is playing a role in the evolution of newt toxicity.

The first step will entail collecting gravid female newts and caddisflies from multiple populations across the western United States. These caddisflies will be offered newt eggs from their locality to determine if egg predation by caddisflies is widespread or restricted to central Oregon. I will use these data as the basis for examining the extent of interaction between caddisflies and newts across their range.

Is the gene sequence of the sodium channel protein in caddisflies different from populations that do not consume newt eggs? Caddisflies appear to be at least partially resistant to the negative effects of ingesting TTX, yet one of the most important questions yet to be addressed is what mechanism underlies this resistance? Garter snakes that consume toxic newts possess amino acid substitutions in the sodium channel protein that confer varying levels of resistance to TTX (Geffeney et al. 2005; Feldman et al. 2009, Feldman et al. 2010). Pufferfishes possess large quantities of TTX in the liver and ovary, and genetic deviations analogous to those found in TTX resistant snakes are responsible for resistance in these species (Venkatesh et al. 2005). Further, molluscs exposed to high concentrations of saxitoxin (STX) from algal blooms exhibit changes in the P-loop region of domain II of the sodium channel, thus conferring resistance to STX (Soong and Venkatesh 2006); STX is functionally similar to TTX. Thus, it seems probable that TTX resistance in caddisflies from Soap Creek ponds may be the result of similar action. The first critical phase to answering this question will be to compare the sodium channel gene sequences from caddisflies in sympatry and allopatry with toxic newts. I will also compare these sequences to sodium channels from organisms with known resistance to investigate whether caddisflies sodium channels possess similar genetic modifications that may confer resistance to TTX.

When a caddisfly sequesters TTX, where is it stored? Caddisflies sequester tetrodotoxin after consuming newt eggs, and substantial quantities are retained through metamorphosis. Yet, the location where the toxin is stored remains unknown. I will dissect and section adult caddisflies with specific focus on the wings and integument, ovaries, testes, and viscera. This information may provide valuable information about the ecological function of TTX in caddisflies.

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Williams, B. L., C. T. Hanifin, E. D. Brodie, Jr., and E. D. Brodie, III. 2010. Tetrodotoxin affects survival probability of rough-skinned newts (*Taricha granulosa*) faced with TTX-resistant garter snake predators (*Thamnophis sirtalis*). Chemoecology 20:285-290.

Wisenden, B. D., and D. P. Chivers. 2006. The role of public chemical information in antipredator behaviour. Pp. 259–278 *in* F. Ladich, S. P. Collins, P. Moller, and B. G. Kapoor, eds. Communication in fishes. Science Publishers, Enfield.

APPENDIX

PERMISSION LETTERS

UNIVERSITY of VIRGINIA
MOUNTAIN LAKE BIOLOGICAL STATION

February 20, 2012

Mr. Brian Gall
Department of Biology
Utah State University
Logan, Utah 84322-5305

Dear Brian,

You have my permission to use the following manuscripts in your dissertation:

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

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

In my opinion your contribution to these projects constituted clear independent and creative effort in leading the work from conception to completion. I am grateful to have been included as a collaborator on your work.

Sincerely,

Edmund D. Brodie III
Director, Mountain Lake Biological Station
Professor, Department of Biology

P.O. Box 400327 • 223 Gilmer Hall • Charlottesville, VA 22904-4327
Office: 434/982-5486 Fax: 434/982-5626
E-mail: mlbs@virginia.edu
www.mlbs.org

17 February 2012

Mr. Brian G. Gall
Department of Biology
Utah State University
Logan, UT 84322

Dear Brian,

You have my permission to use the following manuscript in your dissertation:

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

In my opinion you were the primary contributor to this work which constituted independent and creative effort in design, implementation, and publication. I am grateful to be included as a collaborator.

Sincerely,

Elizabeth A. Gall

Elizabeth A. Gall
ESL Teacher
Canyon Elementary School

17 February 2012

Mr. Brian G. Gall
Department of Biology
Utah State University
Logan, UT 84322

Dear Brian,

You have my permission to use the following manuscript in your dissertation:

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

In my opinion you were the primary contributor to this work, which constituted independent and creative effort in design, implementation, and manuscript preparation. I am grateful to be included as a collaborator.

Sincerely,

Amber N. Stokes

Amber N. Stokes
PhD Candidate
Utah State University

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CURRICULUM VITAE

Brian G. Gall

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278 W 725 N
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Work Address:
5305 Old Main HL
Logan, UT 84322
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Education

Doctor of Philosophy (Biology), expected completion: May 2012. Utah State University, Logan, Utah; GPA 4.00 / 4.00
Dissertation title: "Caddisfly Larvae (Limnephilidae) as Predators of Newt (*Taricha granulosa*) Eggs: A New Player in the Coevolutionary Arms Race Revolving around Tetrodotoxin?"
Advisor: Professor Edmund D. Brodie Jr.

Master of Science (Biology), August 2008. Missouri State University, Springfield, Missouri; GPA 4.00 / 4.00
Thesis title: "Predator-Prey Interactions among Hellbenders (*Cryptobranchus alleganiensis alleganiensis* and *C. a. bishopi*) and Native and Nonnative Predatory Fishes."
Advisor: Professor Alicia Mathis

Bachelor of Science (Environmental Studies/Biological Sciences), May 2006. Quincy University, Quincy, Illinois; GPA 3.49 / 4.00

Mentoring and Teaching Experience

Instructor, Herpetology (BIOL 5570), Spring semester 2012. Utah State University, Logan, Utah. Developed the general course curriculum, which examines the systematics, ecology, and behavior of the major groups of reptiles and amphibians. I use live-animal presentations to demonstrate key traits (e.g., stimulate a newt to observe antipredator behavior, feed snakes to observe skull kinesis and jaw "walking"). Students write research papers on a herpetology topic of their choice and present their findings to the class.

Instructor, Exploring Animal Behavior (BIOL 4060), Fall semester 2011. Utah State University, Logan, Utah. Developed a course that utilizes the principles of animal behavior to develop students' critical thinking and scientific inquiry skills.

Teaching Assistant, Herpetology (BIOL 5570; 2 semesters), Utah State University, Logan, Utah. Developed the curriculum for weekly laboratory sessions on the systematics and ecology of reptiles and amphibians.

Designed and implemented laboratory practicals. Graded all homework and tests. Presented lecture material when instructor was unavailable.

Laboratory Instructor, Principles of Biological Science (BIO 102; 2 semesters), Missouri State University, Springfield, Missouri. Taught weekly laboratory sessions to new biology majors. Designed weekly quizzes and evaluated student performance. Helped students develop research reports and provided feedback on oral presentations.

Tutor, Learning Resource Center (3 years), Quincy University, Quincy, Illinois. Tutored students in biology, basic physics, math, writing, and computer applications.

Adams County Scout Reach Coordinator, Boy Scouts of America (2 years), Quincy, Illinois. Planned and led meetings at local Cub Scout packs comprised of underprivileged children. Helped guide parents and volunteers into leadership positions within the pack. Helped youth develop into well-rounded, moral adults.

Honors and Awards

G. Andy Runge Award (2012) – awarded to the Ozark Hellbender Working Group by the Missouri Chapter of The Wildlife Society for significantly impacting wildlife conservation in Missouri. As a member of the working group I conducted research on the influence of native and nonnative fishes on Ozark Hellbender declines, and published several papers (see below) from this work.

School of Graduate Studies Dissertation Fellowship, Utah State University (2011-2012): $5,000 and waiver of resident tuition

Graduate Student Researcher of the Year, Department of Biology, Utah State University (2010-2011)

James A. and Patty MacMahon Scholarship, Utah State University (2009): $500

Research Vice President's Fellowship, Utah State University (2008): $15,000 stipend for the academic year and waiver of the nonresident tuition

Distinguished Thesis Award, Department of Biology, Missouri State University (2008)

Basil and JoAnn Boritzki Endowment Award, Missouri State University $2,500 (university-wide competition, 1 male, 1 female recipient, 2007)

Cum Laude honors, Quincy University (2006)

Eagle Scout, Boy Scouts of America (1999)

Publications: Peer-Reviewed Papers

1. Emily E. Ferry, Gareth R. Hopkins, Amber N. Stokes, Shabnam Mohammadi, Edmund D. Brodie Jr., and **Brian G. Gall**. Do all portable cases constructed by caddisfly larvae function in defense? In Review.

****Emily was a Senior at USU, I mentored Emily through this research as well as writing the manuscript.**

2. Gareth R. Hopkins, **Brian G. Gall**, and Edmund D. Brodie Jr. 2011. Ontogenetic shift in efficacy of antipredator mechanisms in a top aquatic predator, *Anax junius* (Odonata: Aeshnidae). Ethology 117: 1093-1100, DOI: 10.1111/j.1439-0310.2011.01963.x.

3. **Brian G. Gall**, Edmund. D. Brodie III, and Edmund D. Brodie Jr. 2011. Survival and growth of the caddisfly *Limnephilus flavastellus* after predation on toxic eggs of the Rough-skinned Newt (*Taricha granulosa*). Canadian Journal of Zoology: 89 (6): 483–489, DOI: 10.1139/z11-015.

4. **Brian G. Gall**, Amber N. Stokes, Susannah S. French, Elizabeth A. Schlepphorst, Edmund D. Brodie III., and Edmund D. Brodie Jr. 2011. Tetrodotoxin levels in larval and metamorphosed newts (*Taricha granulosa*) and palatability to predatory dragonflies. Toxicon 57: 978-983, DOI: 10.1016/j.toxicon.2011.03.020.

5. Joseph S. Wilson and **Brian G. Gall**. *Thamnophis elegans* (Western Terrestrial Garter Snake) feeding behavior, prey subjugation by drowning. 2011. Herpetological Review 42 (1): 103.

6. **Brian G. Gall**, Gareth R. Hopkins, and Edmund D. Brodie Jr. 2011. Mechanics and ecological role of swimming behavior in the caddisfly larvae *Triaenodes tardus*. Journal of Insect Behavior 24: 317–328, DOI: 10.1007/s10905-011-9260-1.

7. **Brian G. Gall** and Alicia Mathis. 2011. Ontogenetic shift in response to amphibian alarm cues by Banded Sculpins (*Cottus carolinae*). Copeia 2011 (1): 5-8, DOI: 10.1643/CE-09-229.

8. **Brian G. Gall**, Abigail A. Farr, Sophia G. A. Engel, and Edmund D. Brodie Jr. 2011. Toxic prey and predator avoidance: responses of toxic newts to chemical stimuli from a predator and injured conspecifics. Northwestern Naturalist 92 (1): 1-6, DOI: 10.1898/10-22.1.

   ****Abigail and Sophia were freshmen high-school students from Georgia who came to work in our lab during the summer of 2010.**

9. **Brian G. Gall** and Alicia Mathis. 2010. Response of native and introduced fishes to presumed antipredator secretions of Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*). Behaviour 147: 1769–1789, DOI: 10.1163/000579510X535749.

10. **Brian G. Gall**, Adam L. Crane, and Alicia Mathis. 2010. *Cryptobranchus alleganiensis alleganiensis* (Eastern Hellbender) Secretion Production. Herpetological Review 41 (1): 59.

11. **Brian G. Gall** and Alicia Mathis. 2010. Innate predator recognition and the problem of introduced trout. Ethology 116: 47–58, DOI: 10.1111/j.1439-0310.2009.01718.x.

12. **Brian G. Gall** and Edmund D. Brodie Jr. 2009. Behavioral avoidance of injured conspecific and predatory chemical stimuli by larvae of the aquatic caddisfly *Hesperophylax occidentalis*. Canadian Journal of Zoology 87 (11): 1009–1015, DOI: 10.1139/Z09-091.

Professional Presentations

1. Hopkins, Gareth R., **Brian G. Gall**, and Edmund D. Brodie, Jr. November 2011. Ontogenetic shifts in efficacy of antipredator mechanisms in a top aquatic predator, *Anax junius.* Entomological Society of America. Reno, Nevada

2. **Brian G. Gall**, Edmund D. Brodie III., and Edmund D. Brodie Jr. June 2011. Newts, Garter Snakes, and Caddisflies? Predation on early life-history stages and the implications for coevolution. Evolution 2011. Norman, Oklahoma.
   ****Featured in *The Loom*, blog by Carl Zimmer for Discover Magazine:** http://blogs.discovermagazine.com/loom/2011/06/21/a-beautiful-web-of-poison-extends-a-new-strand/

3. Stokes, Amber N., **Brian G. Gall**, Susannah S. French, Elizabeth A. Schlepphorst, Edmund D. Brodie III., Edmund D. Brodie Jr. June 2011. Tetrodotoxin levels in larval and metamorphosed juvenile newts (*Taricha granulosa*) and palatability to predatory dragonflies. Evolution 2011. Norman, Oklahoma.

4. Hopkins, Gareth R., **Brian G. Gall**, and Edmund D. Brodie, Jr. April 2011. Antipredator behavior of a top aquatic predator: the importance of size and ontogeny in a predatory dragonfly nymph. Intermountain Graduate Research Symposium. Logan, Utah.

5. **Gall, Brian G.**, Abigail A. Farr, Sophia G. A. Engel, and Edmund D. Brodie, Jr. August 2010. Toxic prey and predator avoidance: responses of toxic newts to chemical stimuli from a predator and injured conspecifics. Department of Biology Research Symposium. Paradise, Utah.
   ****Poster designed by Abigail and Sophia with my guidance**

6. **Gall, Brian G.**, Edmund D. Brodie Jr., and Edmund D. Brodie III. March 2010. Predator avoidance during oviposition: Females newts avoid depositing eggs near

invertebrate predators. Utah State University Graduate Student Research Symposium. Logan, Utah.

7. **Gall, Brian G.** and Alicia Mathis. July 2009. Innate predator recognition in larval hellbenders (*Cryptobranchus alleganiensis*) and the problem of introduced trout. Joint Meeting of Ichthyologists and Herpetologists. Portland, Oregon.

8. Mathis, A. and **Brian G. Gall.** June 2009. Predator recognition in larval hellbenders from Missouri and the problem of introduced trout. 4th Hellbender Symposium. Corbin, Kentucky.

9. **Gall, Brian G.** and Alicia Mathis. August 2008. Behavioral responses of larval hellbenders to native and introduced fish kairomones. Animal Behavior Society. Snowbird, Utah.

10. **Gall, Brian G.** June 2008. Predator-prey interactions between hellbenders (*Cryptobranchus alleganiensis alleganiensis* and *C. a. bishopi*) and native and nonnative fishes. Thesis defense presentation, Missouri State University, Springfield, Missouri.

11. **Gall, Brian G.** and Alicia Mathis. April 2008. Influence of stress secretions from adult Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*) on feeding behavior of native and nonnative predatory fishes. Southwest Association of Naturalists, Memphis, Tennessee.

12. **Gall, Brian G.** March 2008. Stress and Predation; the potential impact of introduced predatory trout on larval hellbenders. District Missouri Junior Academy of Science, invited speaker, Springfield, Missouri

13. Mathis, A., **Brian G. Gall**, and David Woods. February 2008. Behavioral bioassays; Faculty and student seminar. Utah State University, Springfield, Missouri.

14. **Gall, Brian G.** and Alicia Mathis. September 2007. Foraging behavior of native and nonnative fishes during exposure to chemical cues from stressed Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*). Missouri Herpetological Association, Branson, Missouri.

15. Mathis, A. and **Brian G. Gall.** August 2007. Understanding the impact of predatory fishes on Missouri hellbender population declines, current and future research. Missouri Department of Conservation, West Plains, Missouri.

Professional Organizations

Society for the Study of Evolution (2010 – present)

Animal Behavior Society (2008 – present)
Missouri Herpetological Association (2006 – present)
Society for the Study of Amphibians and Reptiles (2005 – present)

Student Involvement

Graduate Program Committee, Utah State University (2011-2012)
Biology Graduate Student Association, Utah State University, 2008 to present
2011 President, 2010 Treasurer
Ozark Biological Graduate Society, Missouri State University, 2006-2008
2007-2008 President

Manuscript Review

Animal Behaviour, Caldasia, Hydrobiologia, Marine Drugs

Science Outreach

Science Unwrapped, Utah State University, herpetology speaker (November 2011)
Herpetologist Guest Speaker, Edith Bowen Laboratory School (February 2011)
Science Fair, Biology Judge (February 2010)
Junior Academy of Science, Biology Judge (March 2008)
Science Olympiad, herpetology event coordinator and judge (February 2008)