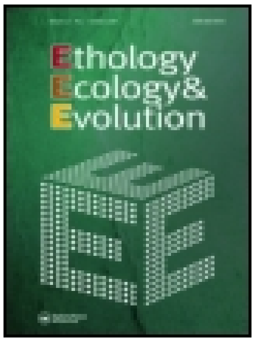

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Only fear the fatal foe: predation risk assessment by eastern newts (*Notophthalmus viridescens*) in response to common snapping turtles and other potential predators

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Many organisms utilize toxic or noxious compounds as a means of deterring predation. Eastern newts (*Notopthalmus viridescens*), along with other species in the family Salamandridae, possess a potent neurotoxin called tetrodotoxin (TTX). Although TTX can serve as an effective antipredator mechanism in species of newts with high concentrations (e.g., *Taricha*), eastern newts have relatively low levels of toxicity in comparison to those species, and it may not serve as an effective antipredator mechanism against all threats. In this case, they may benefit rather by utilizing behavioral changes to avoid initial contact with predators. We tested for predator-avoidance behavior in newts by exposing individuals to kairomones from various predators. We recorded activity patterns of newts when they were exposed to cues from potential predators including bullfrogs (*Lithobates catesbeiana*), water snakes (*Nerodia sipedon*) and snapping turtles (*Chelydra serpentina*), as well as a non-predator (bullfrog tadpoles), and a control (deionized water). Newts reduced activity when exposed to snapping turtle stimuli, but did not change activity when exposed to any other chemical cues. We verified that newts interact with this predator by trapping snapping turtles found in ponds from which newts were collected. Finally, we used turtles caught during this sampling to test whether they are an actual predator of newts and whether newts shift microhabitat use when exposed to this predator. In each replicate, turtles consumed newts, and newts spatially avoided the snapping turtle, relative to a control. The results of these experiments indicate newts rely on predator-avoidance behavior to reduce the probability of being consumed by snapping turtles, but do not reduce activity in response to other potential predators that may only consume them rarely.

KEY WORDS: *Notophthalmus viridescens*, tetrodotoxin, antipredator mechanism, predator avoidance, kairomone.

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INTRODUCTION

In aquatic systems, it is common for organisms to rely on the detection of chemical cues from conspecifics or heterospecifics in order to acquire information about their surroundings (Ferrari et al. 2010). This ability to assess nearby individuals is often used to learn about foraging opportunities and to locate conspecifics or mates, and it is very prominent in predator-prey interactions for both prey location and predator detection (Chivers & Smith 1998; Toyoda et al. 2004; Ferrari et al. 2010). Within the context of predator-prey interactions, if a prey is able to chemically detect a predator, it may be able to respond to danger by altering its behavior to increase its chance of survival (Lima & Dill 1990). During any such interaction, there are two distinct time periods: before the prey is within the perceptual field of the predator and after the prey has entered this perceptual field (Brodie et al. 1991). When a prey organism alters its behavior before it is initially perceived by the predator, it is utilizing predator-avoidance mechanisms; subsequent changes in behavior after the prey has entered the perceptual field of the predator are considered antipredator behaviors (Brodie et al. 1991). The detection of signals may occur during either of these periods and is crucial to the initiation of antipredator or predator-avoidance behaviors, especially in aquatic environments which offer limited visibility.

Not all organisms that a prey species encounters are predators. In addition to non-predators, many predatory species are likely to persist in the prey's habitat, including both potential and active predators. We describe potential predators as those that are capable of consuming the prey but do not prefer to do so if other prey items are abundant. Active predators are able and willing to consume prey regardless of the situation, and may prefer to eat that particular prey item. In aquatic environments chemical stimuli are often the most important means of information transfer, and organisms are constantly bombarded with chemical information from a suite of inorganic objects, as well as organisms including non-predators, potential predators and active predators (Solomon 1977). When the chemical cues are coming specifically from predators, they are called kairomones (Bronmark & Hansson 2000). Organisms that exhibit stimulus filtering (i.e., differentiation between these various chemical signals) and only respond to active predators are likely to have higher fitness relative to those who respond to all potential threats (Lima & Dill 1990). These individuals do not decrease their fitness by perceiving all predators as threats, and consequently responding by altering their behavior and decreasing time spent in other activities such as foraging or mating (Lima & Dill 1990).

There are many potential behavioral changes associated with predator avoidance including reduced activity, spatial avoidance and temporal avoidance (Kats & Dill 1998). The success of each of these mechanisms is likely dependent on the particular organism and environmental context. For example, a study by Relyea (2001) demonstrated that leopard frog tadpoles (*Rana pipiens*) respond to caged predators [Mudminnows (*Umbra limi*) and dragonfly naiads (*Anax* spp.)] with both reduced activity and spatial avoidance depending on the particular threat. Furthermore, among the six anuran species tested in his study, there was variation in which predators each species responded to, as well as variation in the specific behavioral mechanisms used by each species. Common predator-avoidance mechanisms for aquatic animals in murky water are reduced movement to avoid detection and spatial avoidance to evade contact (Wisenden 2000), but prey do not necessarily rely on only one behavior and, as a result, responses vary depending on the context of the situation (McCarthy & Fisher 2000).

Eastern newts (*Notophthalmus viridescens*) are known to produce small amounts of tetrodotoxin (TTX), a highly potent neurotoxin (Brodie 1968b). A close relative to eastern newts, rough-skinned newts (*Taricha granulosa*), contain much higher concentrations of TTX, and in this species the toxin acts as an effective antipredator mechanism (Brodie 1968a). The effectiveness of TTX as an antipredator mechanism in eastern newts is ambiguous, but research suggests that even the sub-lethal dose found in this species can cause minor ill effects if consumed (Brodie 1968b). For some predators, this may be enough to cause them to avoid consuming newts if other prey items are available (hence forcing them to be potential predators). However, studies have suggested that some predators can evolve resistance to newt chemical defenses (Brodie & Brodie 1991, 1999; Brodie et al. 2002, 2005; Jansa & Voss 2011; Rowe et al. 2013), or can be functionally resistant to small quantities of toxins (Wilson et al. 2014; Wilson & Williams 2014).

We tested for predator-avoidance behavior in eastern newts against three likely predators that were selected based on general aquatic diet and habitat overlap: bullfrogs (*Lithobates catesbeiana*), water snakes (*Nerodia sipedon*) and snapping turtles (*Chelydra serpentina*). The first experiment tested for predator-avoidance behavior (quantified as time spent active) by exposing newts to chemical stimuli from all three predators, as well as a non-predator and a blank control. Next, we collected survey data at newt sampling locations to verify that there was a potential for predator-prey interactions between the tested species. The final experiment tested for spatial predator avoidance (quantified as distance from predator) as well as predator willingness to consume newts.

METHODS

Animal collection and maintenance

Four bullfrogs (mean mass ± SD, 100.6 ± 45.6 g) and 15 bullfrog tadpoles (8.54 ± 1.19 g) were collected from a lake near Hanover, Indiana, using dip-nets and immediately transported to Hanover College in plastic containers. Bullfrogs were housed in 37-L tanks with 8 L of deionized water (henceforth water) and sphagnum moss. Bullfrog tadpoles were housed in 9.5-L tanks with 5 L of water and supplied with aerators. Four water snakes (51.05 ± 44.74 g) were collected along streams near Hanover, Indiana, and housed individually in 37-L tanks with newspaper substrate and a glass bowl (10 cm × 4 cm) filled with 0.3 L of water. Four snapping turtles (8.0 ± 2.1 kg) were collected from a lake near Hanover, Indiana, and were housed in plastic containers of various sizes such that each individual could turn freely inside the container. Based on previous observations, all stimulus animals (with the exception of bullfrog tadpoles) were likely large enough to consume adult newts (authors' pers. obs.). Each tub containing a turtle was filled with water until the water level reached the bottom of the plastron. Bullfrogs, tadpoles, snakes and turtles were collected from ponds that do not contain newts. The adult newts (2.75 ± 0.60 g) used in each experiment were collected from adjacent small fishless ponds near Madison, Indiana. Newts were housed in communal 37-L tanks filled with 8 L of water, and fed bloodworms (*Lumbriculus variegatus*) ad libitum. All animals were collected in June 2014.

Stimulus collection

Adult bullfrogs and bullfrog tadpoles were weighed and placed in individual plastic containers with 10 mL of water per gram body mass and an aerator, and left for 24 hr. Dividers were placed around the containers to reduce any external visual stimuli. After 24 hr, the bullfrogs and tadpoles were removed and stimuli from individual donors within a treatment were combined to reduce any variation in individual cues. The stimuli were then frozen in 50-mL centrifuge tubes in 25-mL aliquots at − 20 °C. Because the snakes used in this study are not fully aquatic, stimulus was collected according to Gall et al. (2011) and Johnson et al. (2013). Snakes were weighed and placed individually in 3.7-L plastic containers with 35 mL of water. Dividers were placed around the containers to reduce any external visual stimuli. After 24 hr, snakes were removed and water was added to each container so that the concentration reached 10 mL of water per gram body mass. Turtles were weighed with a Tanita digital scale. Each turtle was placed in 7 L of water for 24 hr, after which the turtle was removed. Water was then added to each container so that the concentration reached 10 mL water per gram body mass. Stimuli from individual donor snakes or turtles were then combined to reduce any variation in individual cues, and the resulting solutions were stored at − 20 °C in 25-mL aliquots.

Experiment 1: response to predator kairomones

In Experiment 1, we tested for predator-avoidance behavior in eastern newts by exposing newts to chemical cues from predators, non-predators and a control. The experimental chamber consisted of a 9.5-L aquarium and was modified slightly from that used by Mathis (2003). Airline tubing (0.5 cm diameter) was glued to the side and bottom of the tank such that the tube extended 10 cm along the bottom of the tank. Approximately 20 cm of excess tube remained at the top of the tank to serve as a site for stimulus injection (see below). The tank was then rinsed thoroughly with hot tap water and water.

The experimental procedure was modified slightly from Mathis (2003). A newt was haphazardly selected from a holding tank and introduced into the experimental tank which had previously been filled with 1 L of water. An acclimation period of 20 min was initiated, after which 50 mL of water was drawn from the tank using a 50-mL syringe. This syringe was set aside and 50 mL of conditioned water was injected into the tank using a new syringe. The contents of the first syringe were then injected into the tank to disperse the conditioned water. After the injection process, a pre-stimulus period of 10 min was initiated, where the total time the newt spent moving was recorded. Movement was defined as any movement other than breathing movements. After the pre-stimulus period was complete, 50 mL of a randomly determined treatment solution was injected using the same injection process as described above, only instead of using 50 mL of conditioned water, 50 mL of a random treatment (blank control, tadpoles, bullfrogs, snakes or turtles) was used. All treatments were coded by the injector so that the observer recording newt movements was blind to the treatments. A post-stimulus period of 10 min was initiated, where the total movement by the newt was recorded. After the trial was over, the mass and sex of each newt were recorded. At the conclusion of the trial, the tank and tubing were cleaned with hot tap water for 30 sec, followed by rinsing with water for 30 sec.

We calculated the change in activity between the pre-stimulus and post-stimulus periods for each newt by subtracting the time spent moving in the pre-stimulus period from the time spent moving in the post-stimulus period. If newts reduced activity after stimulus exposure this resulted in a negative value, and if newts increased activity after stimulus exposure a positive value was recorded. The change in total movement among treatments was analyzed with a Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks followed by post-hoc multiple comparisons. Sex was initially included in the model as a fixed factor; however, it had no effect on the results and was removed from the analysis.

Experiment 2: trapping

To verify that snapping turtles were a relevant threat to the newt populations (see results), we trapped for snapping turtles in four ponds using hoop nets. A hoop net was baited with 8–12 eviscerated bluegill (*Lepomis macrochirus*) which were placed inside a small container with holes to ensure that scent was dispersed. Traps were checked daily for turtles. We trapped for a minimum of 5 days in each pond, and if bait was not eaten in this time frame, the traps were moved to another pond and the bait was replaced.

Experiment 3: predation by turtles and spatial avoidance of predators

In Experiment 3, we tested for newt avoidance of turtles, as well as whether turtles are active predators of newts (preliminary trials with bullfrogs and water snakes indicated they were unwilling to consume newts in the lab). Three turtles (2.8, 4.9 and 2.3 kg) were collected from newt ponds by trapping with hoop nets (see Experiment 2). Once a turtle was collected, it was transported to the lab in a large tub with approximately 5 cm of pond water. Newts were collected from these same ponds with dip nets and transported in small coolers filled with pond water. Before beginning a trial, each turtle was housed for 24 hr in a large plastic container that allowed it to turn freely. The container was filled with water such that it reached the bottom of the plastron. During this time, newts were housed in 37-L tanks with 8 L water.

The experimental chambers consisted of two hard plastic swimming pools (100 cm diameter, 18 cm deep). To provide support for the lid, a 2 L beaker was glued to the bottom of each pool in the center and filled with aquarium gravel. Chambers were filled with water to a depth of 12.5 cm. Two large panes of plexiglass were used to cover each chamber to ensure that neither the newts nor the turtles could escape.

After the chambers were prepared, 20 newts were randomly selected from the holding tanks and placed into the experimental chambers (10 newts per chamber, five males and five females). Newts were allowed a 1-hr acclimation period. Following this acclimation, a snapping turtle was weighed and placed into a chamber. A rock (obsidian) of similar mass was also selected and was randomly placed in the other chamber to serve as a control. Each day at approximately 15:00, we measured the distance between the closest point of either the rock or the turtle to each newt. We also recorded when newts were consumed by the turtle or were dead in the chamber. Data were collected during the 8 days following the initiation of trials, or until all the newts had been consumed. A total of six trials were conducted sequentially (three turtle, three control).

We used a repeated-measures ANOVA to determine whether newts exhibit avoidance behavior toward predatory snapping turtles across time. Each trial was treated as a random factor while treatment (control or turtle) and trial day were treated as fixed effects. We fit the model to multiple covariance structures and chose the most appropriate model based on the lowest AIC value [ARMA (1,1)]. Assumptions of normality and homoscedasticity were assessed with graphical analysis of residuals; all assumptions appeared to be adequately met for all response variables. Analyses were obtained using the PROC MIXED procedure in SAS v. 9.1 (SAS Institute Inc., Cary, NC, USA). In addition, we used a Chi-squared test (1:1) to determine whether newt survivorship was lower in the presence of snapping turtles when compared to the control.

RESULTS

Experiment 1: newt response to kairomones

There was a significant difference between treatments (H = 12.201, df = 4, $P = 0.016$; Fig. 1), and newts significantly decreased activity in response to snapping turtle stimulus ($n = 18$, Q = 3.41, $P < 0.05$) compared to the control, but did not significantly alter their activity when they were exposed to tadpole ($n = 17$, Q = 1.25, $P > 0.05$), bullfrog ($n = 17$, Q = 1.58, $P > 0.05$) or water snake stimulus ($n = 15$, Q = 1.84, $P > 0.05$).

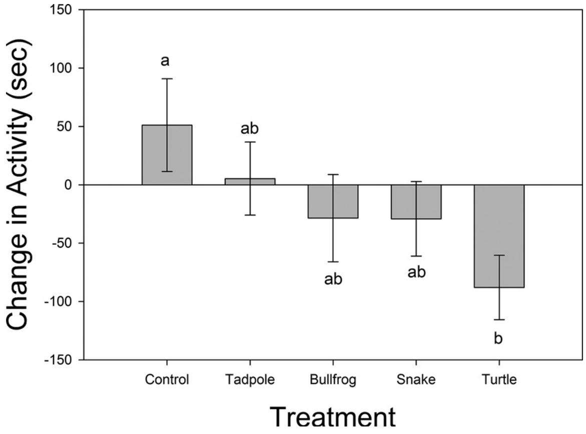

Fig. 1. — Mean (± SE) time spent moving (sec) by newts when they were exposed to a control (deionized water), bullfrog tadpole (tadpole), adult bullfrog (bullfrog), water snake (snake) and snapping turtle (turtle) chemical cues. There was a significant difference between treatments (H = 12.201, df = 4, $P = 0.016$), with newt activity during exposure to turtle kairomones being significantly different from the control. Activity during exposure to all other stimuli was not significantly different from the control group. Different letters indicate significant differences between treatments ($P < 0.05$).

Experiment 2: turtle trapping

Turtle trapping was conducted for a total of 27 trap days in four separate ponds. The number of days for trapping in each pond was as follows: 6, 5, 11 and 5. Turtles were only caught in pond number 3. Newts are present in all ponds, but were most dense in pond 3.

Experiment 3: newt avoidance in turtle presence

A total of 15 newts were consumed in three separate trials with snapping turtles. One turtle consumed nine newts in 24 hr. The tenth newt survived by climbing the wall of the experimental chamber; however, this individual was consumed the following day. The remaining two turtles consumed two and three newts, respectively, across the 8-day trial. Evidence of successful predation by turtles was abundant and included small pieces of tail, limbs and skin, whole newts with large gashes or chunks removed, and missing newts. Survivorship in the presence of snapping turtles was significantly lower than in the control ($\chi^2 = 7.5$, df = 1, $P = 0.006$). Newts maintained a significantly greater distance from the turtles than from the control rock ($F_{[1,4]} = 79.05$, $P = 0.0009$; Fig. 2).

DISCUSSION

We found that when newts were exposed to snapping turtle kairomones, they reduced activity, thereby displaying predator-avoidance behavior in response to these cues. Newts did not respond to chemical cues from non-predatory tadpoles, adult bullfrogs or water snakes. The finding that newts alter their behavior in response to

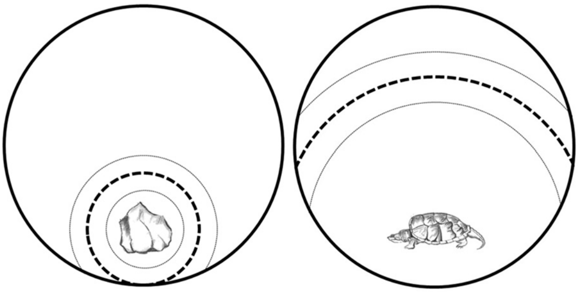

Fig. 2. — Mean (± SD) distance (cm) between newts and the outer edge of either the control rock (left circle) or snapping turtle (right circle) during 8 consecutive days of three replicates. Thick dashed line represents mean, light dashed line represents ± one standard deviation. Newt distance from turtles was significantly different ($F_{[1,4]} = 79.05$, $P = 0.0009$) from the distance maintained from the control rock. Illustration of the rock and snapping turtle provided by Tara M. Nastoff.

chemical cues from a predator is not surprising given the great diversity of aquatic organisms that utilize these cues to acquire information from their surroundings (Ferrari et al. 2010). In addition, both larval and adult newts are fully aquatic, and previous studies support the assumption that chemical stimuli are a critical component of their predator-avoidance mechanisms. For example, adult newts have been shown to respond to chemical cues from larvae (a food source) by increasing foraging behavior (Mathis 2003), but avoid chemical stimuli from damaged conspecifics (Marvin & Hutchison 1995; Woody & Mathis 1997, 1998; Rohr et al. 2003). Similarly, larval newts reduce activity when exposed to cues from cannibalistic adults (Mathis 2003), and Mathis and Vincent (2000) have demonstrated that they also show antipredator responses toward kairomones from predatory ambystomatid salamanders. Results from these studies, combined with results from this study on differential responses to predatory kairomones by adult newts, indicate that newts may have relatively fine-scale discretion when dealing with chemical cues indicating predation risk.

Different prey organisms utilize different predator-avoidance behaviors based on their ecology. For example, when predation risk is high, larval tiger salamanders (*A. tigrinum*) shift their temporal patterns of microhabitat use by occupying certain depths of the water column at different times of day (Holomuzki 1986). Other organisms utilize only spatial avoidance, where foraging behavior is determined by the location of predators in the environment. This behavior is observed in snails (*Physa acuta*), which seek refuge in the presence of pumpkinseed sunfish and continue foraging if a safe distance is maintained (Turner & Montgomery 2003). Furthermore, other species benefit by reducing activity in order to avoid being detected by dangerous predators (Ferrari et al. 2010). The results from Experiment 1 show that newts significantly reduce activity when they are exposed to snapping turtle kairomones, whereas results from Experiment 3 suggest that newts also utilize spatial avoidance to prevent predation. This difference could be due to an intensification of the predator stimuli because both chemical and visual cues were present in Experiment 3. In addition to the added presence of visual stimuli, a study by Rohr and Madison (2001) on eastern newts found that newts exhibit reduced activity when there is a uniform distribution of predator stimulus, but demonstrate spatial avoidance when there is not a uniform distribution (i.e., a chemical gradient). Newts typically occupy murky habitats where visibility is limited and a uniform concentration of chemical stimuli is unlikely to be present. Therefore, although newts display reduced activity when they are exposed to a uniform concentration of chemical cues in a confined space, their primary behavior in a natural context is most likely to maintain spatial refuge upon detecting predator kairomones (present in a gradient), and a secondary behavior is to reduce activity if they perceive they are in close proximity to a predator.

Newts in this study exhibited differential responses to chemical stimuli from adult bullfrogs, water snakes and snapping turtles. All three of these species are generalist predators whose diets frequently include amphibians (Frost 1935; Pell 1940; Gove & Burghardt 1975; Mushinsky & Lotz 1980; King 1993), and all three species can be found at our study ponds from which newts were collected. However, the interactions between eastern newts and their predators are likely complicated by the presence of the powerful neurotoxin TTX that is found in the newts' skin (Brodie 1968b; Yotsu-Yamashita & Mebs 2001), and despite being potential predators, not all of these species may be equally likely to consume newts (i.e., active predators). The only systematic studies of the ability/willingness of each predator to consume these toxic amphibians are Brodie (1968b) and Hurlbert (1970). In these studies, each of these predators was fed eft (the terrestrial juvenile stage) or adult newts. The authors found ambiguous results with all three species. One adult bullfrog retained an eft and adult newt with no apparent ill effect, yet regurgitated two adult newts fed simultaneously the following day (Brodie 1968b). Hurlbert (1970) maintained a bullfrog on a diet of newts alone for several months; however, the frog regurgitated when fed multiple newts in rapid succession. A study by Marion and Hay (2011) found that when bullfrogs were offered one crayfish and one newt simultaneously in the lab, six out of seven bullfrogs consumed both species (one consumed only the crayfish); however, these frogs were never offered more than one newt simultaneously. In tests with adult water snakes, Brodie (1968b) found water snakes showed symptoms of TTX intoxication after consuming the eft stage, but did not exhibit ill effects after being force-fed a single adult newt. Nevertheless, TTX intoxication occurred after feeding them three adult newts, indicating sub-lethal toxicity from consuming adults, which may lead to an inherent avoidance of newts by these snakes. Support for the absence of a predator-prey interaction between these species also comes from the presence of ancestral levels of TTX resistance in these snakes (Motychak et al. 1999) and the lack of TTX-resistant mutations in these snakes' sodium channels (Feldman et al. 2009). Furthermore, a long-term dietary study in Virginia has failed to ever find a bolus containing a newt in the stomach of a water snake, despite an abundance of both species and a predator that forages exclusively on aquatic amphibians (E.D. Brodie III pers. comm.). Given these results it seems likely that adult bullfrogs and water snakes, although potentially capable of consuming newts, may avoid doing so under most conditions.

Only two small snapping turtles have been tested for their ability to consume newts. One 580-g turtle was killed by a single adult newt in approximately 1 hr (Brodie 1968b), while a 50-mm turtle refused to eat an adult newt but did consume recently metamorphosed efts (Hurlbert 1970). In contrast, our study used turtles that were between 4 and 350 times larger than those tested previously, and we found strong support that snapping turtles will consume newts when given the opportunity. In Experiment 3, each turtle consumed a minimum of two newts (out of 10), and one consumed all 10 newts within 48 hr, indicating that at least some snapping turtles are likely active predators on newts.

There are several possible explanations for the high level of predation exhibited by snapping turtles in our study. First, newts from this particular population could fall below the average toxicity of other populations from the eastern United States. Although a systematic study of the toxicity of this population has not been completed, preliminary analysis suggests the adults do contain TTX and that these concentrations are not abnormal (B.G. Gall unpub.). Second, like garter snakes from the western United States (Brodie et al. 2002, 2005), snapping turtles may be resistant to the negative effects of TTX intoxication. Although this has not been tested, the regurgitation observed after turtles were fed multiple individuals by Brodie (1968b) and Hurlbert (1970) also suggests this is unlikely. A third hypothesis is that the large body size of the turtles renders them functionally resistant, despite not containing physiological adaptations that confer resistance to large quantities of TTX. This type of physiological/functional disparity in toxin resistance was nicely demonstrated in a recent study by Wilson et al. (2014). In this study the authors showed that crayfish are able to orally consume substantial quantities of TTX-laden newt eggs, despite being highly susceptible to intramuscular injections of TTX. The authors speculate that this discrepancy may be due detoxifying abilities in the crustaceans' gut or through reduced bioavailability when the eggs are processed for consumption (Wilson et al. 2014; Wilson & Williams 2014). In addition, in species for which TTX resistance has been thoroughly tested, resistance is a strict linear relationship related to mass (Brodie et al. 2002). Therefore, larger predators, simply due to their greater mass, will be able to consume greater quantities of TTX without showing symptoms of intoxication, relative to their smaller counterparts. Our study provides support for this hypothesis, given that all turtles were able to consume newts, but the largest turtle (4.9 kg) was able to consume 10 newts whereas the smaller turtles (2.8, 2.3 kg) consumed only two and three newts, respectively.

The ability to assess predation risk by detecting chemical cues is an important predator-avoidance mechanism that numerous aquatic organisms exploit in order to increase survivorship. Organisms that can detect discrete differences between potential and active predators are likely able to maintain greater fitness because foraging and mating success are not constantly hindered by unnecessarily responding to organisms that do not commonly consume them (Lima & Dill 1990). Our results demonstrate that eastern newts are able to detect chemical cues from an active predator (snapping turtles), but they either do not detect or do not respond to other predators that are less likely to consume them (water snakes and bullfrogs). These results combined with the finding that turtles willingly consume newts in a lab setting and newts spatially avoid turtles suggest that newts only responded to snapping turtles because they present a consistent predator threat.

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We dedicate this paper to the memory of Jennifer M. Lundergan, whose passion for science was immense and promise as a young researcher was unbounded.

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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

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