

Do Experience and Body Size Play a Role in Responses of Larval Ringed Salamanders, *Ambystoma annulatum*, to Predator Kairomones? Laboratory and Field Assays

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Abstract

Prey may experience ontogenetic changes in vulnerability to some predators, either because of changes in morphology or experience. If prey match their level of antipredator behavior to the level of predatory threat, prey responses to predators should reflect the appropriate level of threat for their stage of development. For larval salamanders, responses to predators may change with body size because larger larvae are less vulnerable to predation by gape-limited predators or because fleeing responses by large salamanders may be more effective than for smaller salamanders. In a field experiment, small larval ringed salamanders, *Ambystoma annulatum*, responded to chemical stimuli ('kairomones') from predatory newts, *Notophthalmus viridescens*, with an antipredator response (decreased activity). Laboratory-reared larvae decreased their activity following exposure to newt kairomones, indicating that larval ringed salamanders do not require experience with newts to recognize them as predators. In both experiments, larvae distinguished between chemical stimuli from newts and stimuli from tadpoles (non-predators) and a blank control. In a third experiment, field-caught (experienced) larvae showed a graded response to newt kairomones based on their body size: small larvae tended to decrease their activity while larger larvae showed no change or an increase in activity. This graded response was not observed for neutral stimuli, indicating that it is predator-specific. Therefore, ringed salamander larvae exhibit threat-sensitive ontogenetic changes in their response to chemical stimuli from predatory newts.

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Introduction

Relatively little attention has been given to condition-dependent plasticity of prey responses to predators. Plasticity might be beneficial if risk of predation from particular predator species is not always constant. For many animals, including small aquatic vertebrates, the range of potential predators is broad and the composition of the predator guild often varies seasonally. In addition, the vulnerability of the prey to some predators may not be the same at all life stages. For example, prey may outgrow the gape/handling limits of some predators (Brodie and Formanowicz 1983) or may develop defenses, such as toxins, that provide protection (Mathis and Vincent 2000).

Detection of chemicals produced by predators ('kairomones') can enhance survival of prey (Kats and Dill 1998). Kairomones can be particularly useful cues because they travel through many visual barriers, such as vegetation or sediments, and they can be available in the dark or when predators are visually cryptic. Moreover, chemical cues often are detected relatively early in the predation sequence when avoidance maneuvers are most effective (Lima and Dill 1990). For some aquatic vertebrates, chemical cues appear to be more important than visual cues in predator recognition (Stauffer and Semlitsch 1993; Kiesecker et al. 1996; Brown et al. 1997; Mathis and Vincent 2000). We used laboratory experiments to examine the role of experience and body size in recognition of predator kairomones by a larval salamander. We also confirmed that larval response to kairomones occurs under natural conditions.

Larval ringed salamanders, *Ambystoma annulatum*, are endemic to the Ozarks region of the US where they are typically found in fishless farm ponds. Their predators include a number of species of aquatic insects, aquatic salamanders such as newts or other *Ambystoma*, snakes, and wading birds. Responses of larval ringed salamanders to predator kairomones have not been studied, but they have been shown for some other species in the family Ambystomatidae (Kats et al. 1988; Sih and Kats 1994).

Ringed salamanders hatch in the early fall and overwinter in the ponds as larvae (Hutcherson et al. 1989). Central newts, *Notophthalmus viridescens louisianensis*, are predators of young larvae, but their foraging rates in laboratory studies declined with increasing prey size, presumably because larger larvae exceeded their gape limits (Wilson 1993). In Wilson's study, the larvae were completely safe from predation by newts by Mar., about 1 mo before metamorphosis. Another possible explanation for increased survival of larger larvae is that their predator-evasion skills are greater than those of small larvae, either because of increased experience or because of anatomical differences, such as larger muscle mass, promoting more efficient evasive maneuvers.

To understand potential ontogenetic changes in responses of ringed salamander larvae to newt kairomones, we tested the following hypotheses: (1) Under natural conditions, small ringed salamander larvae respond to newt kairomones with a fright response and distinguish between stimuli from newts and non-predatory stimuli. Field tests of responses to predator kairomones are

rare, particularly for ectothermic vertebrates (e.g. Kats et al. 1988; Sullivan et al. 2002); (2) Small larvae do not require experience with newts to recognize and respond to newt kairomones; (3) Larger larvae respond differently to predator kairomones than smaller larvae.

Experiment 1: Field experiment

Methods

This experiment used a field assay to test whether small larval *A. annulatum* responded differently to chemical stimuli from (1) predatory central newts, (2) non-predatory tadpoles, *Rana sphenocephala*, and (3) a blank control of de-chlorinated tap water (hereafter, 'water'). We conducted the experiment in two small fishless ponds in Stone County, Missouri, between late Nov. and mid-Dec. of 2001. Water temperatures ranged from 6–16°C. We conducted observations after sunset (between 19:00 and 24:00 hrs) because that is when *Ambystoma* larvae typically are most active (Branch and Altig 1981; C. Hickman, pers. obs.).

We collected newts and tadpoles in Ozark, Stone and Webster Counties in southwestern Missouri and held them in the lab in 60-l aquaria on a 14h:10 h light:dark cycle at 19–22°C. Tadpoles were fed commercial spirulina discs and newts were fed blackworms (*Lumbriculus variegates*) ad libitum.

Test larvae were exposed to water from tanks containing either 18 newts, 16 tadpoles, or no animals (blank). We kept stimulus animals in conspecific groups in 60-l aerated aquaria with the water volume adjusted so that there was approximately 2.7 ml of water per gram of stimulus animal (± 1 SD: newts, 1.7 ± 0.31 g; tadpoles, 2.5 ± 0.63 g). We fed the stimulus animals just prior to introduction to the aquaria, but did not feed them during the stimulus-collection period. An additional aquarium contained only aerated water for use as a blank control stimulus.

After approximately 120 h, we placed water from the stimulus aquaria into 1-l glass jars and placed the jars on ice for transport to the field site. The jars were coded so that the observations were blind. During each trial, individual larvae were exposed to 50 ml of a randomly chosen stimulus (newt, tadpole, or blank). We introduced the stimuli into the pond via polyethylene tubing (105 cm) attached to a 60-ml syringe. The tubing was fastened to a bamboo splint, which allowed us to precisely position the tubing in the water. The rate of diffusion in a pond varies because of unpredictable variables such as currents or local temperature. Therefore, we added one drop of green food coloring to the stimulus water in each trial so that we could determine the point at which the stimuli came in contact with the test animal. We used flashlights for illumination, and covered the lights with red cellophane to reduce the intensity of the light and minimize disturbance.

We located individual test animals at stations around the shoreline of the pond. The stations were approximately 1 m apart. We estimated that there were hundreds of larvae in the pond during the trial (see also quantitative data for these

ponds in Peterson et al. 1991). Most larvae were foraging and appeared to restrict their activity to small areas as they searched for prey. Therefore, we felt that it was extremely unlikely that a test larva from one station would be re-tested at another station.

At each station, we chose a focal larva that appeared relatively small (<25 mm total length, based on estimate by eye) to ensure that they would be vulnerable to predation by newts. We observed the focal larva for 2–3 min and proceeded with testing only if the larva moved at least once during that period. This criterion minimized the possibility that the presence of the observer alone had caused an extreme fright response (freezing). Movements by larvae typically were fairly discrete events (stop and start). During a period when the focal larva was not active, we slowly lowered the end of the stimulus injection tubing (on the bamboo splint) into the water approximately 20 cm in front of the focal animal. The stimulus water was injected slowly (<1 ml/s) until all of the stimulus water was released. We recorded latency to move after the green-tinted stimulus contacted the snout of the focal animal. A maximum latency score of 300 s was recorded for focal animals that did not move within that time period. In rare instances, the focal larva moved before the stimulus contacted its snout; these trials were aborted. We compared latencies among the three treatments using a Kruskal–Wallis ANOVA followed by non-parametric multiple comparison tests (Zar 1984), with $n = 18$ for each treatment.

Results

There was a significant difference in latency to move among treatments ($H = 13.45$, $p = 0.001$) (Fig. 1). Larvae had longer response latencies to the newt stimulus than to either the tadpole ($Q = 4.06$, $p < 0.025$) or the blank ($Q = 4.83$, $p < 0.005$) treatments. There was no significant difference between the tadpole and blank treatments ($Q = 0.77$, $p > 0.50$).

Experiment 2: Role of experience

Methods

The purpose of this experiment was to determine whether experience with predators is required for predator recognition by small larvae. We collected several dozen *A. annulatum* egg masses in Stone County, Missouri, in Oct. of 1997 approx. 1–2 d after they were laid. Some eggs were used in an unrelated experiment; the remainder were placed in water and maintained from Oct. to Jan. at 5°C to slow development and simulate natural conditions. In Jan., we placed larvae that had hatched from the mixed egg masses into individual 400 ml plastic containers (10.5 × 10.5 × 8.5 cm), and raised the temperature to 15°C. Larvae were fed live *Daphnia* sp. ad libitum and were kept in an environmental chamber on a 12h:12h light:dark cycle. At the time of testing, the larvae were 15–21 mm in total length (± 1 SD = 17.7 ± 1.27 mm).

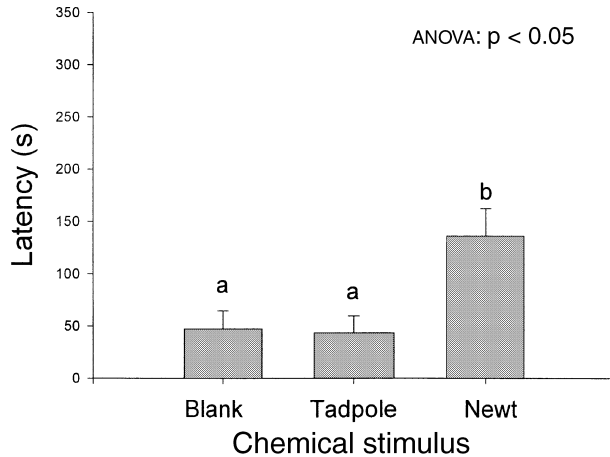


Fig. 1: Latency to move (± 1 SE) for larval ringed salamanders exposed to de-chlorinated tap water (blank), and to chemical stimuli from *Rana* tadpoles (neutral stimulus) and newts (predator stimulus) in a field experiment. Probability value is for a non-parametric Kruskal–Wallis ANOVA. Different letters indicate significant differences according to non-parametric multiple comparisons tests

In this experiment, we exposed lab-reared larvae to the same treatments as in the field experiment: chemical stimuli from adult central newts (predators), southern leopard frog tadpoles (non-predators), or to a chemical ‘blank’ of only water. We collected adult newts from Greene County, Missouri, kept them in separate 400-ml plastic containers ($10.5 \times 10.5 \times 8.5$ cm), and fed them frozen brine shrimp (*Artemia* sp.) weekly. We collected tadpoles from Stoddard County, Missouri, housed them together in 1-l plastic containers ($21 \times 15 \times 8.5$ cm), and fed them commercial spirulina discs ad libitum. Stimulus animals were kept at room temperature ($14\text{--}19^\circ\text{C}$) on a 14h:10h light:dark cycle.

The stimulus treatments consisted of water from aerated collecting chambers holding one adult newt (predator treatment), one tadpole (non-predator treatment), or only water (blank treatment). We determined volume of newts and tadpoles by displacement in water. Mean volumes (± 1 SD) were 1.3 ± 0.27 ml for newts ($n = 10$) and 1.4 ± 0.71 ml for tadpoles ($n = 7$); some stimulus animals were used for more than one trial. To maintain similar chemical concentrations between treatments and among trials, a concentration of approximately 240 ml of water per 1 ml of stimulus animal was maintained in the collecting chamber. We placed the stimulus animals in the collecting chamber approximately 24 h prior to the start of each trial.

Trials were conducted under fluorescent lighting between 08:30 and 16:40 hrs. Testing chambers were plastic containers ($21 \times 15 \times 8.5$ cm) filled with 1 l of water. We attached a plastic tube to the center of one side of each chamber for use as a stimulus introduction tube, with the end of the tube approximately 2 cm below the surface of the water. Wooden panels surrounded the testing chambers on three sides to minimize background disturbance. We placed a single randomly-chosen larva in each testing chamber 20–30 min before testing.

We randomly assigned the three treatments for each trial. Immediately before each trial, we withdrew 60 ml of water from the appropriate stimulus chamber with a plastic syringe. Trials consisted of a 10-min pre-stimulus observation period and a 10-min post-stimulus observation period.

During the pre-stimulus period, we recorded total time swimming along the substrate (indicated by tail movement) and total number of discrete movements (either 'nips' at the substrate, rapid head turns, or movements to the surface of the water). These latter movements appeared to be feeding responses or, in perhaps in some cases, supplementary air breathing. 'Discrete moves' made negligible contributions to swimming time (often < 1 s each), and were counted separately because these rapid movements seemed likely to be especially conspicuous to predators. After 10 min, we injected the 60 ml of stimulus water through the stimulus injection tube at a rate of approx. 2 ml/s. We then resumed behavioral observations for an additional 10 min. We interpreted decreases in activity following exposure to the stimulus to be an indication of a fright response.

Our hypothesis was that the largest decrease in activity would be to the newt stimulus. Rather than performing separate analyses of swimming and discrete moves, we combined these two response variables into a single 'activity index' to minimize the experiment-wise error rate. Swimming and discrete moves were correlated ($r_s = 0.52$, $p < 0.001$). We ranked the data for each variable (swimming and discrete moves) to standardize them, and then we averaged the two ranks. The activity index was calculated by subtracting the pre- and post-stimulus data for each trial; positive indices indicate increases in activity and negative indices indicate decreases in activity in response to the stimulus. Decreased activity is a common response to predatory stimuli by amphibians and can lead to increased survival (e.g. Azevedo-Ramos et al. 1992; Skelly 1994; Lefcort 1996). We compared the indices using a Kruskal–Wallis ANOVA followed by non-parametric multiple comparison tests (Zar 1984). For the tadpole and newt treatments, $n = 16$; for the blank treatment, $n = 15$.

Results

There was a significant difference among activity indices for the three treatments ($H = 6.26$, $p = 0.04$) (Fig. 2). The largest decrease in activity was in response to the newt stimulus. The response to the newt stimulus was significantly different from the response to both the blank stimulus ($Q = 2.94$, $df = 2$, $p < 0.01$) and the tadpole stimulus ($Q = 3.38$, $p < 0.001$). The blank treatment did not differ significantly from the tadpole treatment ($Q = 0.49$, $p > 0.50$).

Both variables (swimming time and number of discrete movements) made similar contributions to the activity indices. For descriptive purposes we present the absolute activity scores here as average pre/post behavior followed by average percentage decrease. For both variables, the largest decrease in activity was in response to the newt stimulus (swimming = 21.3/12.4 s, 41.8%; discrete moves = 6.9/3.9, 43.5%). Smaller decreases in activity were observed in

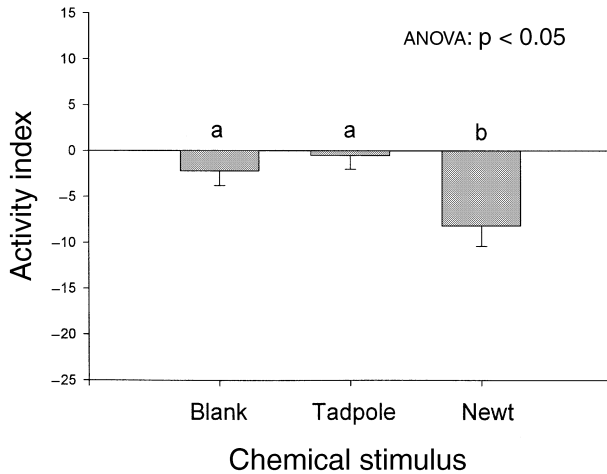


Fig. 2: Activity indices (± 1 SE) of larval ringed salamanders exposed to de-chlorinated tap water (blank), and to chemical stimuli from *Rana* tadpoles (neutral stimulus) and newts (predator stimulus). The horizontal line indicates no change between pre- and post-stimulus activity. Indices are based on a combination of swimming behavior and number of 'discrete moves' (see text for calculation of index). Probability value is for a non-parametric Kruskal–Wallis ANOVA. Different letters indicate significant differences according to non-parametric multiple comparisons tests

response to the blank (swimming = 18.5/14.1 s, 23.8%; discrete moves = 3.8/2.9, 23.7%) and tadpole (swimming = 22.1/20.8 s, 5.9%; discrete moves = 8.3/6.7, 19.3%) treatments.

Experiment 3: Role of body size

Methods

In this experiment we tested whether larvae of different sizes, but with similar levels of experience with predators, responded differently to predator kairomones. We collected larvae of *A. annulatum* from ponds in Stone County, Missouri, in late Oct. of 1996. At the time of testing in early Nov., the larvae were 15–30 mm in total length (± 1 SD = 22.9 ± 3.23 mm). *A. annulatum* is an explosive breeder whose eggs are laid in bouts of 1–2 d. Embryos hatch at a size of 7–8 mm (Wilson 1993) and it takes several weeks for larvae to reach the minimum size of larvae in our experiment (Hutcherson et al. 1989). Therefore, all of our focal larvae had been free-swimming for similar lengths of time and thus were likely to have similar experience with predators. We immediately placed individual larvae into 400-ml holding containers (10.5 \times 10.5 \times 8.5 cm) and fed them frozen brine shrimp (*Artemia* sp.) every few days. We kept the larvae at room temperature (15–20°C) on a 12h:12h light:dark cycle.

We maintained stimulus animals (newts and tadpoles) as in the previous experiment at room temperature (15–20°C). Newts (predators, $n = 3$) ranged

from 3.8–4.0 cm snout-vent length (SVL) and tadpoles (non-predators, $n = 6$) ranged from 2.5–5.0 cm total length.

Tests were conducted between 16:00 and 19:15 hrs and methods were the same as in the previous experiment with the following exceptions. First, each larva was exposed to both the blank stimulus and to one of the other treatments (newt or tadpole) in random order with a range of 8–12 d between tests of the same larva. There was no pre-stimulus observation period because the response to the blank stimulus was considered as baseline. Secondly, the only response variable that was recorded was total time swimming.

We calculated an activity response index for each larva as time swimming for the treatment (newt or tadpole) minus the blank (baseline). Positive numbers indicate increased activity relative to baseline and negative numbers indicate decreased activity relative to baseline.

We tested the hypothesis that large body size was associated with decreased response to the predatory stimuli by calculating a Spearman rank correlation coefficient for larval total length and the activity response index for the newt treatment. To determine whether the effect of body size is a specific response to predatory stimuli or a generalized response to any chemical stimulus, we also calculated the Spearman rank correlation coefficient for larval total length and the activity response index for the tadpole treatment.

Results

There was a significant positive correlation between larval body size and response to the newt stimulus ($r_s = 0.53$, $n = 16$, $p < 0.05$, two-tailed; Fig. 3). In contrast, the correlation between larval body size and the activity response index was not significant for the tadpole stimulus ($r_s = 0.19$, $n = 17$, $p > 0.20$, two-tailed; Fig. 3).

Discussion

Under field conditions, larval ringed salamanders responded to chemical stimuli from predators (kairomones) with decreased activity. This response is consistent with an adaptive antipredator response because it lowers the risk of detection for visually-oriented predators and can lead to increased survival (e.g. Azevedo-Ramos et al. 1992; Skelly 1994; Lefcort 1996). Studies of responses to predator kairomones in natural habitats are relatively rare, particularly for ectothermic vertebrates. Field experiments have shown that larval *A. texanum* (Kats et al. 1988), tadpoles of *R. sylvatica* (Petranka & Hayes 1998), and adults of the terrestrial salamander *Plethodon cinereus* (Sullivan et al. 2002) distinguish between predator kairomones and blank controls. Our field experiment includes the additional step of demonstrating that larval ringed salamanders distinguish between predatory and non-predatory heterospecifics.

Larval ringed salamanders do not require experience to use chemical cues to detect predatory newts. Larvae distinguished between stimuli from predators

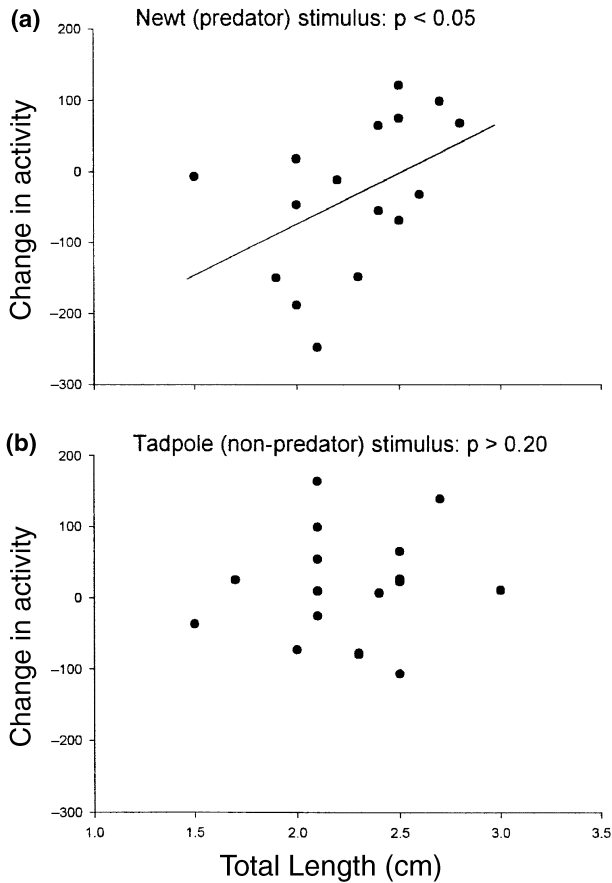


Fig. 3: Correlation between body size (total length) and change in activity in response to chemical stimuli from predatory newts (a) or non-predatory *Rana* tadpoles (b). Zero indicates no difference between responses to de-chlorinated tap water (baseline responses) and the stimulus treatment. Probability values are for the Spearman rank correlation coefficient

(newts) and non-predators (tadpoles) and showed greater decreases in activity in response to stimuli from predators. The response was present even though the larvae had been kept in the laboratory for several months prior to testing with no experience with newts. Because salamanders were collected as eggs that were laid in ponds under natural conditions, our data do not preclude the possibility that early embryonic experience could have influenced the subsequent behavior of the larvae (e.g. Hepper & Waldman 1992).

The ability to recognize major predators without experience may be common in larval amphibians (e.g. Kats et al. 1988; Sih & Kats 1994; Watt et al. 1997; Gallie et al. 2001). However, the results of some studies need to be re-examined in light of recent findings that predator diet can influence predator recognition by inexperienced prey (e.g. Mathis & Smith 1993; Laurila et al.

1997; Lefcort 1998). In many studies, it is difficult to determine whether larval responses are because of recognition of the chemical signature of the predator *per se* or to a chemical derived from the predator's recent diet. In many studies where inexperienced larvae respond to predatory stimuli, either amphibian larvae were included in the recent diet of the predator (e.g. Stauffer & Semlitsch 1993; Petranka & Hayes 1998; Laurila 2000; Nyström & Åbjörnsson 2000) or the predator's diet was not reported (e.g. Semlitsch and Gavasso 1992; Niecieza 1999). In our study, the observed decreased activity was not in response to chemicals derived from amphibians in the predator's diet because the stimulus animals had been maintained in the laboratory on a non-amphibian diet (worms or brineshrimp) for several weeks prior to testing. These prey were chosen because worms and crustaceans are part of the natural diet of larval *A. annulatum* (Nyman et al. 1993). Therefore, if the larvae in our study had responded to any diet-related chemicals, the response should have been manifested as increased activity (feeding response: Mathis and Vincent 2000; A. Mathis, unpubl. data). Therefore, the decreased activity observed in our study is consistent with a response to predator-specific chemicals (kairomones) rather than diet-related cues.

Some recent studies have indicated that salamander responses to predator chemical cues can vary depending on the time of day (Madison et al. 1999a,b; Maerz et al. 2001). Our laboratory tests were conducted during the day while the field assays were conducted after dark. The responses of the larvae were similar in all three of our experiments. Regardless of whether experiments were diurnal or nocturnal, larvae distinguished between predatory and non-predatory chemical stimuli in a manner that is consistent with antipredator behavior.

In our study, small larvae tended to decrease their activity in response to chemical stimuli from newts while larger larvae showed no change or increased activity in response to newt cues. This differential response by large and small larvae is specific to predator kairomones because individuals of all sizes responded similarly to neutral (tadpole) stimuli. The higher activity levels of larger larvae could be because they are less vulnerable to predation by newts (Wilson 1993), or because their larger muscle mass makes avoidance maneuvers, such as fleeing, more efficient. It is unlikely that the differential response is because of increased experience for larger larvae, because all larvae were approximately the same age. Regardless of the specific explanation for the differential responses, larval ringed salamanders appear to exhibit threat-sensitive changes in responses to predator kairomones over the course of their development. Body size also has been shown to influence antipredator response of anuran larvae to predators (Anholt et al. 1996; Bridges & Gutzke 1997; Puttlitz et al. 1999; Eklöv 2000) and juvenile bass to heterospecific alarm pheromones (Brown et al. 2001). Further study is required to determine whether threat-sensitive ontogenetic shifts in behavior are genetically programmed or are the result of experience with predators.

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