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A Study of the Sensitivity of *Plethodon cinereus* (Caudata: Plethodontidae) to Damage-Released Cues from Conspecifics

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ABSTRACT.—Some prey species vary the intensity of antipredator responses according to the perceived level of threat associated with different concentrations of chemical cues related to predation. Here, we examine whether Red-Backed Salamanders (*Plethodon cinereus*) respond to different concentrations of damage-released cues from the tails of conspecifics in a threat-sensitive manner. We exposed salamanders either to a control or to damage-released cues from conspecifics at one of three different concentrations. We found that salamanders exposed to damage-released cues significantly decreased their activity compared to salamanders exposed to a control. However, the intensity of the responses was not related to the concentration of the cue, suggesting that individuals of *P. cinereus* do not respond in a threat-sensitive fashion at the concentrations tested.

Many organisms evaluate the risk of predation through the detection and use of chemical cues related to predation (Chivers and Smith, 1998; Kats and Dill, 1998). The source(s) of these cues includes individuals that have been injured or distressed by predators and the predators themselves (Wisenden, 2000). The use of chemical cues to assess the threat of predation may be especially advantageous for species in habitats where visual cues are limited (Smith, 1992; Dodson et al., 1994) or whose predators are difficult to detect visually (e.g., Hickman et al., 2004). In addition, unlike most visual and acoustic stimuli, the use of chemical cues does not require that prey species be in close proximity (spatially or temporally) to their predators to glean useful information related to the threat of predation.

The responses of prey to chemical cues from predators may contribute to an increase in survival but also might have negative effects on fitness (Sih, 1987; Lima and Dill, 1990; Kats and Dill, 1998; Lima, 1998) because responding to chemical cues from predators may result in a decrease in foraging activity or efficiency (Abrahams and Dill, 1989; Moses and Sih, 1998; Whitham and Mathis, 2000; Maerz et al., 2001). In addition, responding to cues from a predator may result in lost mating opportunities and a subsequent reduction in the overall fitness of some prey (Berglund, 1993; Forsgren and Magnhagen, 1993; Matity et al., 1994; Polis et al., 1998). Such nonlethal effects of predation on prey species demonstrate that the complexity of interactions among predators and prey extends beyond simply removing prey individuals from a population.

Because of the trade-offs associated with many defensive behaviors, prey species may benefit by grading the intensity of their antipredator responses according to the level of threat perceived. In other words, the magnitude or intensity of the defensive response may be positively correlated with the level of threat perceived by the prey. This tenet is the primary component of the threat-sensitivity hypothesis (Helfman, 1989) and has been corroborated by data from a number of diverse, primarily aquatic, systems (Kats et al., 1994; Puttlitz et al., 1999; Amo et al., 2004; Kesavaraju et al., 2007). These studies suggest that prey respond more strongly to increased numbers of predators, more dangerous predators, or higher concentrations of chemical cues from predators or predation events. For instance, Kusch et al. (2004) show that fathead minnows that

have experienced cues from predatory pike use concentration of predator cue as a risk assessment tool. They display low-intensity responses to low concentrations of predator cues and high-intensity responses to higher concentrations. Additional studies in aquatic organisms demonstrate a similar behavioral trend in response to increased concentrations of cues related to predation (Dupuch et al., 2004; Ferrari et al., 2005).

This study was designed to examine the tendency of a terrestrial amphibian to respond to different concentrations of damage-released cues from conspecifics in a threat-sensitive manner. Damage-released cues are generally released during predator attack or capture and are thought to be indicative of an actively foraging predator and an immediate risk of predation (Hews, 1988). The use of these cues by aquatic amphibians to evaluate predation risk has been widely documented (see reviews by Wisenden, 2000; Ferrari et al., 2010); however, relatively few studies have examined the responses of terrestrial amphibians (e.g., Lutterschmidt et al., 1994; Marvin and Hutchinson, 1995; Chivers et al., 1996; Hucko and Cupp, 2001). Our study species, the Red-Backed Salamander (*Plethodon cinereus*), provides an excellent model for studies of chemically mediated predator-prey interactions in terrestrial systems because this species possesses well-developed chemosensory organs (Dawley and Bass, 1988, 1989) and has been shown to respond to chemical traces from both predators (Madison et al., 1999; Sullivan et al., 2004, 2005) and injured conspecifics (Sullivan et al., 2003) although Murray and Jenkins (1999) did not observe avoidance of cues from dead conspecifics. In addition, population densities of *P. cinereus* can be relatively high (Burton and Likens, 1975; Jaeger, 1980); thus, the detection of cues associated with predation may provide reliable and useful information about the proximity of predators or the level of threat presented. Furthermore, the detection and accurate assessment of the level of predation threat may be important in the reduction of costs associated with antipredator behavior because Red-Backed Salamanders may avoid potential predation by moving to areas inaccessible to predators or remaining beneath cover objects, with the potential for a corresponding decrease in foraging opportunities (Sullivan et al., 2002; Roberts and Liebgold, 2008).

Sullivan et al. (2003) demonstrated a defensive response by *P. cinereus* to damage-released stimuli with a concentration of 1.0 g macerated tissue per 50.0 mL water, and our unpublished pilot data suggest that *P. cinereus* will respond to a more dilute stimulus (1.0 g macerated tissue per 150.0 mL water), but no

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direct comparison was made regarding the intensity of the defensive responses in these two experiments. In this study, we examine the sensitivity of *P. cinereus* to damage-released cues from conspecifics and determine whether members of this species respond to these cues in a threat-sensitive manner.

MATERIALS AND METHODS

We collected 120 adult Red-Backed Salamanders from the woodlands of the Houghton College campus (Allegany County, NY) from 4–18 October 2006 for use in behavioral trials. Individual salamanders with a snout–vent length >40 mm were considered adult (Sayler, 1966). Each salamander was housed individually in a 15 cm–diameter × 1.5 cm–height Petri dish lined with unbleached paper towels saturated with reverse osmosis (RO) water. Salamanders were maintained at a 13 : 11 L : D photoperiod, with a daytime temperature of 7.2°C and nighttime temperature of 1.6°C to closely approximate field conditions at the time of collection. Test salamanders were maintained in the laboratory for no more than 10 days prior to the beginning of the experiment and were not fed prior to trials. Each salamander was tested once and released at the site of capture. We collected eight additional *P. cinereus* (four males and four females) from the same site to serve as stimulus donor animals. Stimulus donor animals also were housed individually in 15 cm–diameter Petri dishes lined with moistened paper towels.

The damage-released cues were prepared by forcing the stimulus donor salamanders to autotomize their tails by grasping them with forceps approximately 1 cm posterior to the cloacal vent. Autotomized tails were combined with enough water to produce a stimulus concentration of 1 g of tail tissue per 150 mL of RO water. The tails were macerated in a blender for five minutes and then filtered through glass wool to remove large particles. A portion of the 1 g/150 mL mixture was diluted to produce 1 g/1,500 mL and 1 g/15,000 mL concentrations to be used in the behavioral trials. After the dilutions had been prepared, the stimulus solution was divided into 50-mL aliquots in conical-bottom tubes and frozen until needed. We used autotomized tails as the source of the cue in our study (rather than whole-body macerates) because this method of cue preparation allowed us to collect the stimulus for our bioassay without killing the donor animals and has been demonstrated to be effective in eliciting antipredator responses in other plethodontid salamanders (Hucko and Cupp, 2001). We did not include a “biological” control (e.g., stimulus from non-damaged conspecifics) in this study in an effort to reduce the confounding factors associated with the sex of the donors and test subjects as well as seasonal influences on the production of pheromones. In addition, previous work shows that test subjects were neither attracted to nor avoided the chemical traces from uninjured conspecifics in a similar bioassay (Sullivan et al., 2003).

The behavioral bioassay to test for treatment responses was similar to those described by Sullivan et al. (2003) in which two filter paper semicircles were placed on opposite sides of each 15-cm Petri dish such that a 3-mm gap was maintained between each semicircle. Within each dish, we added 1.5 mL of the appropriate dilution to saturate one semicircle and RO water to saturate the other semicircle using a 10-mL tuberculin syringe. In the case of the control treatment, each filter paper semicircle within a dish received 1.5 mL of the RO water. We tested each of the 120 salamanders once, in one of six trials, which were spread

evenly over three consecutive nights (i.e., two trials per night). We tested 20 salamanders per trial and, therefore, tested 40 salamanders each night. Test dishes were placed randomly on a 4 × 5 grid placed on the floor of our experimental room, and the treatments were randomly stratified over the three nights such that 10 replicates of each treatment were repeated each night for a total of 30 replicates in each of the four treatments (1 g/150 mL, 1 g/1,500 mL, 1 g/15,000 mL, and the control). We transferred each salamander from its home dish to the assigned experimental dish with a cotton swab, and placed a 15-mm collar of brown paper around the dish to isolate visually each salamander within test dishes during the set-up period. After all salamanders were distributed (~8 min), the lights were turned off, and each trial was recorded for 15 min with a Sony DVD Camcorder equipped with an infrared light source. We began recording trials between 2200 h and 2300 h each night.

To evaluate *P. cinereus* responses to the different chemical treatments, we used the number of times that salamanders crossed the 3-mm gap as an estimate of activity. Salamanders were said to cross from one side to the other when the midpoint of the animal had passed the gap between filter paper semicircles. Within a given treatment, the activity of salamanders did not vary among the six trials (Kruskal-Wallis: $H = 16.82$; $P = 0.266$); hence, the data collected over the three consecutive days were pooled for analysis. We used the nonparametric Kruskal-Wallis ANOVA to compare the number of side changes among our treatment groups because the data were not distributed normally. Pairwise comparisons were performed using the Bonferroni-Dunn test to determine where significant differences between treatments occurred (Sheskin, 2007).

RESULTS

Salamander activity, as measured by the number of times that individuals crossed the center of the test dishes, differed significantly among treatments (Kruskal-Wallis: $H = 26.60$; $P < 0.001$). Pairwise comparisons revealed that test subjects exposed to damage-released cues were, on average, less active than were individuals exposed to the control treatment (control vs. g/150 mL cue, $P < 0.001$; control vs. 1 g/1,500 mL, $P < 0.001$; control vs. 1 g/15,000 mL, $P < 0.001$). The activity levels were not graded according to the concentration of the chemical cues (Fig. 1).

DISCUSSION

Our results show that individual Red-Backed Salamanders exposed to damage-released stimuli from injured conspecifics reduced their activity significantly when compared to individuals exposed to a control (RO water). In addition, salamanders in this study detected and responded to cues less than 1/300 of the relative concentration of previous work that evaluated plethodontid responses to injured conspecifics (Hucko and Cupp, 2001; Sullivan et al., 2003). However, our study offers no indication of threat-sensitive, or graded, antipredator, behavior in Red-Backed Salamanders despite exposing salamanders to a serial dilution of the damage-released stimulus (ranging from 1 g tissue/150 mL water to 1 g tissue/15,000 mL water). Although the exact concentration of the cue(s) responsible for inhibiting salamander activity is unknown, the relative difference between our most concentrated stimulus and most dilute should represent a 100-fold difference in cue concentration because the treatments were derived from the same “stock”

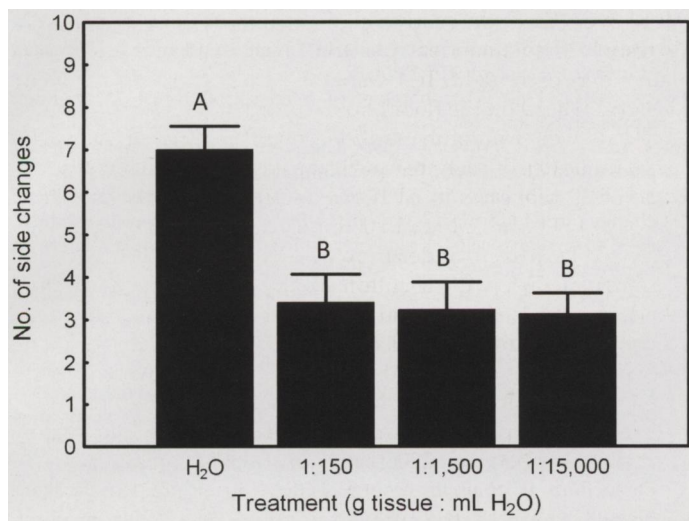


FIG. 1. Average (\pm SE) activity scores for Red-Backed Salamanders exposed to a control treatment (H₂O) and three different concentrations (g tissue/mL water) of a damage-released cue derived from the macerated tails of conspecifics. Activity scores are the number of times that individual salamanders crossed the midline of test dishes. Letters indicate significant differences among responses to chemical treatments. $N = 30$ for each treatment.

solution prepared from the simultaneous maceration of tails collected from four male and four female salamanders. These data are congruent with the study by Sullivan et al. (2003) who showed that Red-Backed Salamanders avoid chemical stimuli associated with whole-body macerates but do not avoid stimuli from noninjured conspecifics. Additionally, the reduction in activity in response to stimuli associated with predation is consistent with the antipredator responses of a number of other salamander species in laboratory and field studies (e.g., Brodie et al., 1974; Ducey and Brodie, 1983; Maerz et al., 2001). Collectively, these data suggest that the behavioral changes observed in salamanders exposed to injured conspecific stimuli is not an avoidance response to territorial pheromones or competitor odors but a response to a chemical cue(s) released upon damage to salamander tissues that indicates an increased threat of predation.

That Red-Backed Salamanders responded to damage-released cues from conspecifics was somewhat expected given previous studies of the chemosensory ability of this species (Simons et al., 1994; Madison et al., 2002; Sullivan et al., 2003), but the current study demonstrated an antipredator response to crushed autotomized tails (rather than whole-body macerates) at a relative cue concentration that was substantially lower than tested previously. However, the consistency of the activity levels to the damage-released cues of decreasing concentration was unexpected given that other amphibian species respond to cues from predation in a threat-sensitive manner (e.g., Mathis and Vincent, 2000; Mirza et al., 2006; Ferrari et al., 2008). One possible explanation for the “all-or-nothing” response exhibited in this experiment may be that Red-Backed Salamanders do not respond to chemical cues from predation events in a threat-sensitive manner but rather respond with antipredator behavior when some hypothetical threshold level of chemical stimulus is detected (e.g., Mirza et al., 2006). Under natural conditions, this threshold may prevent the dependence by salamanders on cues that are too diluted to be reliable measures of predator proximity or threat. This may be especially relevant considering the potential costs associated with some predator avoidance

behavior by *P. cinereus*. For example, Roberts and Liebgold (2008) showed that individual Red-Backed Salamanders will occupy suboptimal foraging habitats in response to simulated predator attacks. In this scenario, responding to a “false alarm cue” can have a substantial impact on fitness as it relates to successful foraging. Alternatively, it is possible that salamanders have the potential to respond to chemical cues from predation in a threat-sensitive manner, but our least concentrated stimulus (1 g tissue/15,000 mL water) was perceptually equivalent to our most concentrated stimulus (1 g tissue/150 mL water). In such a scenario, even lower concentrations of the damage-released cue would be needed to elicit a graded antipredator response. Finally, the lack of an apparent threat-sensitive response in our study may be a function of our bioassay. It is conceivable that the quantification and comparison of more subtle behavioral responses (e.g., nose-tapping) by individuals of *P. cinereus* exposed to the damage-released cues could reveal a graded response to the different concentrations used here.

Currently, the answers to ecological questions relating to the sensitivity to different concentrations of cues, threshold levels required to elicit antipredator responses, and even interspecific differences with regards to cue sensitivity are unknown in part because the active component(s) of alarm cues have not been characterized in many vertebrate species. A notable exception is the identification of hypoxanthine-3-N-oxide, which appears to be a component of the chemical alarm signal, released from the damaged skin of some Ostariophysan fishes (Brown et al., 2000). In studies with fathead minnows (*Pimephales promelas*), Brown et al. (2001) found that only 15 nanomoles of synthetic hypoxanthine-3-N-oxide were required to elicit an overt response by minnows in a 35-L tank. A comparable model among amphibians is lacking, but recently a study of chemically mediated antipredator behavior in ranid tadpoles has shown that an alarm pheromone is produced by skin cells and actively released upon predator attack (Fraker et al., 2009). Although the chemical nature has not been fully described, Fraker et al. (2009) have demonstrated that the alarm pheromone derived from the skin consists of at least two components that must be combined to elicit behavioral inhibition among tadpoles. There has been no comparable study with terrestrial amphibians, although some suggest that the activity of alarm cues comes from the toxins produced by cutaneous glands and may serve the dual purpose of deterrent to predators and alarm cue for conspecifics (Lutterschmidt et al., 1994; Chivers et al., 1996).

An attempt to evaluate the behavioral responses by *P. cinereus* and other terrestrial amphibians in the context of the threat-sensitivity hypothesis will likely elucidate the mechanisms by which prey species balance the benefits of chemosensory detection of predators with the costs of avoidance or reduced activity. In addition, an understanding of the ecological and evolutionary mechanisms responsible for the observed results will allow for subsequent studies of salamander behavior that could lead to the characterization of the active component(s) of damage-released cues from prey.

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