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NOCTURNAL SHIFT IN THE ANTIPREDATOR RESPONSE TO PREDATOR-DIET CUES IN LABORATORY AND FIELD TRIALS

Aaron M. Sullivan, Dale M. Madison, and John C. Maerz*

1. INTRODUCTION

Prey species may react to predator chemical traces in the environment with a variety of antipredator behaviors (Weldon, 1990; Chivers and Smith, 1998; Kats and Dill, 1998). Such responses to predator chemical cues might reduce predation risk, but also may result in lost foraging or mating opportunities (Lima, 1998a,b), so it is not surprising that some species adjust their responses based on the degree of perceived predation threat. These modifications may be based on chemical information gathered directly from the predator or its recent prey (Madison et al., 1999a; Chivers and Mirza, 2001). The mosaic of chemical products released at a predation site, and dispersed by the predator, could allow nearby prey to assess predation threat and fine-tune their responses.

Among the chemical cues released from predators during and after a predation event, those associated with predator diet have emerged as an important factor in predator assessment by both aquatic (Chivers and Mirza, 2001) and terrestrial prey species (Madison et al., 1999a,b). However, in most circumstances it is unclear whether the active components of the cue are from the prey, the predator, or some combination of prey and predator cues (e.g., Madison et al., 2002). As one example of this complexity, the interaction between the terrestrial red-backed salamander, *Plethodon cinereus*, and its garter snake predator, *Thamnophis sirtalis*, shows that during the day salamander avoidance of snake cues occurs independent of snake diet, but late at night avoidance is apparently restricted to cues from snakes feeding on red-backed salamanders (Madison et al. 1999a,b). One limitation of these studies is that few diet treatments were tested, leaving the possibility that alternative diets might also elicit late night avoidance.

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We hypothesize that the late night discrimination by red-backed salamanders of predator diet cues is most likely the result of either the “phylogenetic relatedness” or “ecological relatedness” between the test subjects and the prey of the snake. Phylogenetic relatedness is the degree of genetic similarity between species, who share a similar chemical composition (Smith, 1992; Brown et al., 2000). According to this concept, one would predict that a target prey species would avoid chemical cues from predators feeding on a congeneric prey because of shared prey chemistry and defense mechanisms, despite possible allopatry between the test species and the prey species. On the other hand, the “ecological relatedness” hypothesis for diet discrimination may be defined as the degree of habitat and trophic overlap among sympatric prey species that likely share the same predators (Mathis and Smith, 1993; Chivers et al., 1995). For example, two species sharing similar refuges that are members of the same prey guild would have a high degree of ecological relatedness, despite not being closely related phylogenetically.

Our goals in this study were: 1) to test the efficacy of the phylogenetic relatedness hypothesis by determining whether the chemical component from salamanders that “labels” garter snakes as an increased risk is conserved within the salamander family Plethodontidae, 2) to examine the ability of red-backed salamanders to detect and discriminate among predator chemical cues under laboratory and field conditions, and 3) to detect and accurately describe the temporal shift in responsiveness of red-backed salamanders to predator diet cues during early and late night trials. To achieve these goals, we tested red-backed salamanders in the laboratory and field to chemical traces from garter snakes fed red-backed salamanders and two-lined salamanders, *Eurycea bislineata*.

2. METHODS

2.1. Collection and Maintenance of Test Animals

For laboratory experiments, we collected red-backed salamanders and two lined salamanders from the Binghamton University Nature Preserve, and the Binghamton University Natural Area (Broome Co., New York), respectively. All salamanders were maintained in individual petri dishes (15 cm diameter × 1.5 cm high) with moistened paper towels at 17°C and a 14L:18D photoperiod. We collected five adult garter snakes as predator stimulus donors and housed them in 38-L aquaria with a water dish, heating element, and crumpled paper towels for shelter. We kept snakes in a separate room at 22°C and a 14L:18D photoperiod. Snakes were fed salamanders of the appropriate species each week for three weeks prior to collecting treatment rinses.

2.2. Collection of Predator Chemical Cues

A distilled water rinse of each garter snake was used as the predator stimulus. Immediately prior to collecting a rinse, we fed source garter snakes three adult female and three adult male salamanders of the appropriate species, and then transferred the snake to a 4 L beaker that had been cleaned with Alcojet detergent and rinsed in 2% nitric acid and distilled water. After transfer, we covered the beaker with cheesecloth and placed the beaker on a heating block for 72 h. We removed the snake and rinsed the

beaker with 200 ml of distilled water. We filtered the rinse through a 0.45- μm filter to remove solid materials, froze samples in liquid nitrogen, and stored them in a freezer. The entire collection process was repeated to provide enough snake stimuli for laboratory and field experiments.

2.3. Experiment 1: Laboratory Study of Predator Cue Avoidance

We examined the laboratory responses of red-backed salamanders to two predator diet treatments at two different time periods to determine whether the chemical “label” that identifies individual garter snakes as an elevated risk is conserved within the Plethodontidae, and to examine a possible temporal shift in red-backed salamander antipredator behavior. The “early” trials were conducted between 2100 and 2230 hours, and the “late” trials were conducted between 2330 and 0100 hours. In each of these trial periods, test salamanders were given the choice between *T. sirtalis* fed *P. cinereus* (TS_{Pc}) or *E. bislineata* (TS_{Eb}) versus distilled water. To assess the relative strength of each snake diet treatment, test salamanders also were given the choice between TS_{Pc} and TS_{Eb} .

We used the behavioral assay described in Sullivan et al. (2003) to examine salamander responses to predation-related chemical cues. In brief, we placed two filter paper semicircles on opposite sides of 15 cm petri dishes while maintaining a 3 mm gap between each semicircle. We added 1.5 ml of treatment solution (TS_{Pc} , TS_{Eb} , or distilled water) to each semicircle using a 10 ml tuberculin syringe, and randomly distributed treatment dishes on an 8 \times 7 grid on the floor of our experimental room. 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 visually isolate each salamander within test dishes during the set-up period. After all salamanders were distributed (~15 min), the lights were turned off and the trial was recorded for 60 min in complete darkness with a video camera sensitive to infrared light.

We recorded the side occupied by each salamander every three minutes for one hour. Since red-backed salamanders tend to be relatively inactive and their behavioral response is bimodally distributed (Madison et al., 1999a; Sullivan et al., 2003), we used binomial and Chi-square goodness-of-fit tests to analyze salamander avoidance responses. We considered salamanders located on the treatment substrate ≤ 6 out of 21 observations as avoiding the treatment because this ratio is below an alpha value of 0.05 using a binomial test (Siegel & Castellan, 1988), and those located on the treatment side ≥ 15 out of 21 observations as being attracted to the treatment. Individuals on the treatment substrate for > 6 and < 15 observations were considered indifferent to the treatment, and were excluded from the analyses. We used Chi-square tests to determine whether the number of salamanders avoiding the treatment differed significantly from random expectation.

2.4. Experiment 2: Field Study of Predator Cue Avoidance

We conducted a field experiment to examine whether a temporal shift in antipredator responses to predators based on the predator’s diet would be observed in a more natural context. We performed the “early” experiment (conducted between 2030 and 2330 hours) and “late” experiment (conducted between 2330 and 0230 hours) on a single night in July 2003 following a day of rain so that leaf litter was wet and air was saturated with moisture. Plethodontid activity is generally restricted to moist conditions within a few

days of rain (Grover, 1998; Feder, 1983), and the movement of salamanders among shelters at this site is positively correlated with precipitation (Maerz and Madison, 2000). Therefore, salamander movement into and out of shelters was not restricted by low moisture during the trials.

We exposed salamanders found under rocks to TS_{Pc}, TS_{Eb}, or distilled water during early and late experiments. If a salamander was present beneath the rock, we removed it by hand and recorded distinguishing characteristics including sex (males were identified by the enlargement of the mental gland and swollen cloacal vent), distinctive patterns in stripe, and tail autotomy. While salamander characteristics were being recorded, the rock was numbered with flagging and the soil beneath the rock was uniformly sprayed with 2 ml of snake rinse or water. We returned the rock to its original position, and then released the salamander under the rock edge. We re-examined the cover rocks for the presence of salamanders beginning two hours after initiating the experiment. We examined the rocks in the same sequence, but were “blind” to treatment during our search. For the early trial, we exposed thirty salamanders to each of the three treatments, and for the late trial, we exposed twenty salamanders to each treatment. We recorded whether the original or new salamander was under each test rock.

We examined the effects and possible interactions between snake diet treatment and salamander sex using a binomial regression with a log-log canonical link in Statistica’s (StatSoft, Inc., 2001) Generalized Linear Model (GZLM). We tested the full factorial model, which examined the effects of treatment (TS_{Pc}, TS_{Eb}, distilled water) and sex of the test salamander on salamander responses. We tested for significant effects using the Wald statistic (analogous to least-squares estimates).

3. RESULTS

3.1. Results of Laboratory Experiments

During “early” night trials, red-backed salamanders significantly avoided both TS_{Pc} ($\chi^2= 8.0, P<0.01$) and TS_{Eb} ($\chi^2=6.12, P=0.01$). When the two snake diet treatments were paired against one another to test for their relative effect, we found no significant avoidance of TS_{Pc} or TS_{Eb} ($\chi^2=0.14, P=0.71$) (Table 1). During “late” night trials, salamanders still significantly avoided TS_{Pc} ($\chi^2=12.5, P<0.01$), but not TS_{Eb} ($\chi^2=0.36, P=0.54$). When the two snake treatments were paired against one another, TS_{Pc} was significantly avoided relative to TS_{Eb} ($\chi^2=4.5, P=0.03$) (Table 2).

Table 1. The number of salamanders that avoided, were attracted to, or showed no choice to chemical treatments during early night trials.

Treatment:		No. of salamanders choosing:			χ^2	P
A	B	A	B	No choice		
TS _{Pc}	Vs dH ₂ O	7	22	3	7.76	0.005
TS _{Eb}	Vs dH ₂ O	8	22	2	6.53	0.011
TS _{Pc}	Vs TS _{Eb}	13	15	4	0.14	0.705

Table 2. The number of salamanders that avoided, were attracted to, or showed no choice to chemical treatments during late night trials.

Treatment:		No. of salamanders choosing:			χ^2	P
A	B	A	B	No choice		
TS _{Pc}	Vs dH ₂ O	5	19	8	8.17	0.004
TS _{Eb}	Vs dH ₂ O	11	14	7	0.36	0.589
TS _{Pc}	Vs TS _{Eb}	8	19	5	4.48	0.034

3.2. Results of Field Experiments

During the “early” field trials, the binomial regression on salamander response by treatment (TS_{Pc}, TS_{Eb}, or distilled water) and salamander sex showed a significant treatment effect (Table 3). Red-backed salamanders exposed to TS_{Pc} were more likely to remain in refuges than individuals exposed to TS_{Eb} or distilled water (Table 4). During the “late” field trials, the binomial regression on salamander response by treatment (TS_{Pc}, TS_{Eb}, or distilled water) and salamander sex showed no significant treatment, sex, or interaction effect (Table 3). During the “late” trial, red-backed salamanders tended to leave refuges regardless of treatment (Table 4).

Table 3. The results of the binomial regression of salamander sex and diet treatment on salamander response in early and late night field trials.

Effect	Early night			Late night		
	Df	Wald	P	df	Wald	P
Sex	1	0.43	0.514	1	3.09	0.079
Treatment	2	6.02	0.049	2	0.34	0.819
Treatment × sex	2	5.56	0.062	2	1.09	0.581

Table 4. The number of salamanders that remained under rock refuges or left refuges when exposed to H₂O, TS_{Eb}, or TS_{Pc} in early and late night field trials.

Treatment	Early night		Late night	
	No. remaining	No. leaving	No. remaining	No. leaving
H ₂ O	11	19	5	15
TS _{Eb}	7	23	7	13
TS _{Pc}	16	14	6	14

4. DISCUSSION

These results generally corroborate previous studies of red-backed salamanders, demonstrating that this species can distinguish among individual predators based on diet-related cues, but that the response is contingent on the diel cycle of the test salamanders (Madison et al., 1999a, b). In the current study, test salamanders discriminated among garter snakes fed phylogenetically related (confamilial and conspecific) prey in both laboratory and field settings. During the day and early evening, the risk imposed by garter snakes, regardless of their recent diet, dominates the chemosensory behavior of red-backed salamanders. Since garter snakes forage on *P. cinereus* and other salamander species (Hamilton, 1951; Carpenter, 1952; Moreno, 1989), and since these snakes can extend their diurnal activity into warm evenings during summer (Reichenbach and Dalrymple, 1986), the avoidance by red-backed salamanders of all snake chemical traces seems like an adaptive response to predator cues.

The results of our laboratory experiments suggest that the discriminatory abilities of red-backed salamanders are quite refined. During the early night trials, red-backed salamanders avoided chemical cues from garter snakes fed red-backed and two-lined salamanders when paired against distilled water, but did not avoid either when paired against each other. These results show that at the time of study, the two predator stimuli were probably considered equally repulsive by test salamanders, suggesting that the chemical cue “labeling” the predator as an increased risk is conserved within the Plethodontidae. However, during the late-night laboratory trials, red-backed salamanders avoided chemical cues from garter snakes fed red-backed salamanders, but not from snakes fed two-lined salamanders. In addition, when given the choice between the two predator stimuli, salamanders chose to occupy the substrate labeled with TS_{Eb} , suggesting that late at night, TS_{Pc} is considered more aversive. These results imply either refined discrimination of predator diet below the family level, and/or greater familiarity with conspecifics by red-backed salamanders.

The lack of response to TS_{Eb} late at night negates the phylogenetic relatedness hypothesis of chemical cue homology between red-backed and two-lined salamanders. This result is surprising since two-lined salamanders are preyed on by garter snakes (Ducey and Brodie, 1983; Dowdey and Brodie, 1989), are similar in size to adult red-backed salamanders (Petranka, 1998), belong to the Plethodontidae, and are seasonally syntopic with red-backed salamanders (Stewart, 1956; MacColloch and Bider, 1975). However, two-lined salamanders are not syntopic to red-backed salamanders where our test *P. cinereus* were collected. Thus, the individual red-backed salamanders tested may not have been familiar with *E. bislineata*, nor been exposed to chemical cues from garter snakes foraging on two-lined salamanders. It is possible that test salamanders did not recognize or interpret TS_{Eb} as a risk equivalent to TS_{Pc} late at night.

The results of our field trials, expressed as a reduction in activity and a tendency to remain beneath rock refuges when exposed to aversive chemical cues (Sullivan et al., 2002), confirm the diet discrimination seen in the laboratory. However, the cues that elicited this discrimination in the field were unexpected. In the field, salamanders did not respond to TS_{Eb} in the early night trial (despite laboratory avoidance), and did not respond to either TS_{Pc} or TS_{Eb} late at night. We hypothesize that cues from invertebrate prey were abundant in the field, but not in the laboratory, so salamanders may have disregarded all except the most threatening predator stimuli. The activity of the invertebrate prey consumed by red-backed salamander tends to be highest in the leaf litter

at night (Jaeger, 1978; Holomuzki, 1980), so salamanders that remain under refuges when exposed to chemical cues may miss out on important foraging opportunities and incur a non-lethal cost of predation risk (Lima, 1998a,b). In addition, the threat of snake predation declines after sunset, especially late at night, when garter snakes tend to be relatively inactive (Reichenbach and Dalrymple, 1986; Madison et al., 1999).

In conclusion, our data show the ability of red-backed salamanders to discriminate among chemical cues from predators according to predator diet in both laboratory and field settings. This cue discrimination shows nocturnal variation that may be related to the fluctuation in predation risk due to the activity period of the predator, or the conflicting demands of foraging and antipredator responses. Furthermore, our findings seem to reject the hypothesis that phylogenetic relatedness among prey species contributes to the diet cue discrimination (Chivers and Mirza, 2001) by salamanders in our system, at least at the level of Plethodontidae. Rather, our data suggest that the discriminatory ability of red-backed salamanders may be related to the ecological relatedness among prey species as demonstrated by Chivers et al. (1997) who show that being ecologically familiar with the chemical traces of recent prey in a snake's diet, rather than chemically recognizing a phylogenetic relationship with these prey, may elevate the perceived risk of predation in salamanders in an ecological community. Further studies examining the mechanisms by which diet cue discrimination occurs should yield valuable insight into studies of chemically-mediated predator assessment.

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