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A COMPLEX, CROSS-TAXON, CHEMICAL RELEASER OF ANTIPREDATOR BEHAVIOR IN AMPHIBIANS

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Abstract—Prey species show diverse antipredator responses to chemical cues signaling predation threat. Among terrestrial vertebrates, the red-backed salamander, *Plethodon cinereus*, is an important species in the study of these chemical defenses. During the day and early evening, this species avoids rinses from garter snakes, *Thamnophis sirtalis*, independent of snake diet, but late at night, avoids only those rinses from garter snakes that have recently eaten *P. cinereus*. We tested whether the selective, late-night response requires the ingestion or injury of salamanders. In three experiments, we tested *P. cinereus* for their responses to separate or combined rinses from salamanders (undisturbed, distressed, and injured *P. cinereus*) and snakes (unfed, earthworm fed, and salamander-fed *T. sirtalis*). When paired against a water control, only rinses from salamander-fed snakes were avoided. When salamander treatments (undisturbed or distressed) were combined with the snake treatments (unfed or earthworm-fed) and tested against a water control, the combinations elicited avoidance. When selected treatments were paired against the standard rinse from salamander-fed snakes, only the combined rinses from salamanders and snakes nullified the avoidance response to the standard rinse. These data reveal a prey defense mechanism involving chemical elements from both the predator and prey that does not require injury or ingestion of the prey in the formation of the cue.

Key Words—Chemical cues, predation, scent, composition, alarm, synergism, defense, diet, salamander, *Thamnophis sirtalis*, *Plethodon cinereus*.

INTRODUCTION

Essentially all animals face predation threat at some point in their lives (Lima, 1998). Many of these species can significantly reduce predation risk by responding to chemosensory cues from predators or prey, with the cues varying from simple nitrogenous wastes to more specialized, damage-release chemicals stored in prey tissues (reviewed by Weldon, 1990; Kats and Dill, 1998). In some cases, combined chemical information from both the predator and prey appear to evoke a selective response in the prey species, suggesting either a complex chemical cue or multiple cues evoking a similar response. For example, prey individuals may vary their response depending on whether conspecifics occur in the predator's diet, or they may avoid both predator cues and damage-release cues (Chivers and Smith, 1998; Madison et al., 1999a,b; Murray and Jenkins, 1999; Grostal and Dicke, 2000; Chivers and Mirza, 2001). Chemosensory responses can also be context dependent, e.g., the responses vary at different times in the diel cycle (e.g., Madison et al., 1999b), with the size of the predator relative to that of the prey (Puttlitz et al., 1999), with refuge proximity (Sih and Kats, 1991), or seasonally between breeding and nonbreeding periods (Rohr et al., 2002). Despite the large number of species known to respond to chemical traces associated with predation, and despite the well-documented instances of damage-release cues particularly in aquatic species (reviewed by Chivers and Smith, 1998; Chivers and Mirza, 2001), surprisingly little is known about cue components and complexity, especially in terrestrial vertebrates (Kats and Dill, 1998).

The antipredator responses of plethodontid salamanders, especially *Plethodon* sp., have received considerable attention recently (e.g., Cupp, 1994; Lutterschmidt et al., 1994; Chivers et al., 1997; Madison 1999a,b; Murray and Jenkins, 1999; Graves and Quinn, 2000; Hucko and Cupp, 2001; Maerz et al., 2001; Sullivan et al., 2002), and this group offers a good opportunity for elucidating the source and complexity of the chemical cues signaling predation threat among terrestrial vertebrates. Their activity at night when higher relative humidity favors chemoreception (Madison, 1977), their elaborate chemosensory receptors and accessory structures (Madison, 1977), their behavioral sensitivity to a wide range of chemicals related to food, predation, social interactions, and interspecific recognition (Jaeger, 1986; Chivers et al., 1997), their diel shift from a chemically mediated daytime and early evening antipredator response to a late-night response (Madison et al., 1999b), their compromised foraging at night in the presence of predator chemical cues (Maerz et al., 2001), their avoidance of predator chemical cues in both laboratory and field studies (Sullivan et al., 2002); and their relative abundance and accessibility in many woodland habitats (Burton and Likens, 1975), collectively make them suitable for comprehensive studies of chemosensory defenses.

In the context of predation, the genus *Plethodon* appears to respond to four types of chemical cues that are often combined or confused in experimental studies:

(1) cues exclusively from predators (Madison et al., 1999a,b; McDarby et al., 1999; Murray and Jenkins, 1999), (2) “alarm” signals from wounded conspecifics and/or heterospecifics (Chivers et al., 1997; Hucko and Cupp, 2001), (3) “distress” chemicals from stressed, but not injured, conspecifics (Graves and Quinn, 2000; Mathis and Lancaster, 1998), and (4) cues from predators that have recently fed on conspecific prey (Madison et al., 1999a,b; Murray and Jenkins, 1999; Maerz et al., 2001; Sullivan et al., 2002). Cues in the fourth or “diet-specific” category are of interest because of the chemical involvement of organisms belonging to different taxa. These cues are also potentially problematic because they can easily be confused with alarm, distress, or other possible “labeling” substances transferred from prey to the predator that involve a chemical produced from just one source, in this case the prey.

In an effort to clarify the nature of the cue in the diet-specific category, single-source cues, such as alarm substances, must be shown not to be present or sufficient by themselves to elicit the response thought to result from complex, cross-taxon cues. If multi-species involvement in chemical cue production is demonstrated, we theorize three ways that true cross-taxon cues with both predator and prey involvement could be produced. The first requires the gastrointestinal digestion or alteration of a prey substance into a new product that is gradually secreted or excreted from the predator, eliciting avoidance when the chemical is encountered in the environment. A second possibility is that external integumentary or other bodily secretions from both the predator and prey chemically react during an encounter, forming a warning product that is rubbed off along the predator’s path. The third possibility is a compositional, nonreactive chemical mixture of chemical products from both prey and predator, where the products only elicit avoidance when they occur together in the same chemical trace.

One of the first steps in distinguishing among the “diet-specific,” cross-taxon pathways of cue production is to determine whether the ingestion of prey by the predator is required to generate the chemical cue that elicits a response among conspecific prey, i.e., is diet-specific antipredator behavior really “diet” specific? We approached this problem by collecting rinses from garter snakes and red-backed salamanders and then testing whether salamanders would avoid these rinses only when they were combined. We speculated that late at night when only rinse from salamander-fed snakes is avoided, *P. cinereus* would avoid a rinse of unfed or earthworm-fed snakes combined with undisturbed or noninjured salamanders, but would not avoid the snake or salamander rinses tested separately.

METHODS AND MATERIALS

A total of 416 adult salamanders [*P. cinereus*; snout/vent length (SVL) > 40 mm] and 8 adult garter snakes (*T. sirtalis*; SVL > 36 cm) were collected from

the Binghamton University Nature Preserve (Broome County, New York, USA). Salamanders and snakes were housed in separate rooms at 15°C (salamanders) and 25°C (snakes) on a natural photoperiod (15L:9D). Salamanders were housed individually in 15-cm diam Petri dishes lined with moistened paper towels. Each salamander was tested once within 10 d of capture and then released at the capture site. Snakes were housed individually in 38-l glass aquaria, provided with a heating block, given crumpled paper towels for cover, and maintained on a diet of *P. cinereus* or earthworms (*Lumbricus* sp.) before and during the experiment. They were released following the experiments.

Salamanders were subjected to distilled water rinses of different snake and salamander treatments or to distilled water only, in two-choice, substrate tests. The methods for preparing treatments and testing salamander avoidance are similar to those of Madison et al. (1999b), with the modifications described below.

Experiment 1. Trials with Distressed Salamanders and Earthworm-Fed Snakes. Four treatment rinses were prepared: salamander-fed snake, earthworm-fed snake, distressed salamander, and a combination of the latter two. The salamander-fed snake treatment is a standard treatment used in all our experiments to provide a measure of whether recently captured salamanders are behaving similarly to previously tested salamanders. The rinses from the earthworm-fed snake and the distressed salamander were tested singly and then together against distilled water to determine whether the combination rinse, but not the independent rinses, would elicit avoidance.

The salamander-fed snake rinse consisted of a 200-ml rinse of an adult garter snakes (snake 1) that had been maintained on a diet of 4 *P. cinereus*/w for 2 w prior to sample collection, with 4 of these salamanders (2 male, 2 female) being fed to the source snake just prior to sample collection. Although individual snakes may differ at least in some of their chemical secretions (Oldak, 1976), previous research has shown that the response of individual *P. cinereus* to snake chemical cues does not noticeably vary with the snake used to collect the test rinses (Madison et al., 1999b). In addition, rinses from 18 *T. sirtalis* that have been fed *P. cinereus* over the past 7 yr have never failed to elicit avoidance in *P. cinereus* (Madison, Sullivan, Maerz, McDarby, personal observation). Collection of the snake rinses entailed transferring the snakes to individual 4-l beakers, covering them with 8 layers of cheesecloth secured by rubber bands, placing a heating pad (26°C) under half the beaker base, waiting 72 hr, carefully removing the snake, and then collecting a 200-ml distilled water rinse of all smears, secretions, and excretions deposited in the beaker.

The earthworm-fed snake treatment rinse was identical to the above, except that earthworms equivalent in mass to the salamanders were fed to an adult garter snake (snake 2). The distressed salamander treatment rinse consisted of a 200-ml sample of distilled water in which 4 adult *P. cinereus* (2 male, 2 female) had been held by blunt forceps and agitated for 2 min each until defensive

secretions were released from their tails and body (but where no wounding had occurred).

We filtered all rinses through a 0.45- μm filter to remove solid materials, and divided the filtrate into four 50-ml aliquots. The fourth treatment (combination treatment) was prepared by combining 50-ml aliquots of the earthworm-fed snake and the distressed salamander rinses. The resulting combination rinse, therefore, had half the concentration of each of the two separate components. All four treatments were rapidly frozen in liquid nitrogen, stored in a freezer, and thawed at room temperature just prior to use. Sullivan et al. (2002) showed that salamanders still avoid salamander-fed snake rinse that has been frozen using this method.

For testing (Madison et al., 1999a,b; Sullivan et al. 2002), we placed two filter paper semicircles with a 3-mm gap between them into 15-cm Petri dishes, inoculated the semicircles with 1.5 ml of treatment rinse or distilled water, and randomly placed these test dishes (position and orientation) in an 8×7 pattern on the floor of the test room. A total of 168 salamanders were tested once beginning just after midnight over three consecutive nights. On each night, 14 replicate dishes were prepared for each treatment comparison, giving 14 replicates for each of the four comparisons per night. Thus, 56 salamander dishes were distributed at random and tested in a 8×7 pattern each night. Salamanders were moved to the test room 1 hr prior to the experiments, which began between 24:00 and 01:00 hr. We transferred each salamander under dim red light from the home dish to the test dish with a cotton swab, and placed a 15-mm collar of brown paper around the covered dish to isolate each salamander visually during the transfer process. After all salamanders were distributed (~ 10 min), the lights were turned off and the trial was recorded with an infrared, 0-lux, video camera (Sony TRV66) suspended 3 m above the dishes. We scored which semicircle the salamander occupied at 3-min intervals for 1 hr beginning a time 0, which gave 21 positions per salamander. A score of 10 or less for a treatment was recorded as avoidance. Where salamanders straddled the 3-mm gap, two criteria were used to judge side position. If the salamander was moving, the side into which the salamander was moving was scored. If the salamander was not moving, the side occupied by more than half the body, measured from the midpoint of the trunk, was scored. All scoring was performed by an observer blind to treatment type and right/left side in the test dishes. We used a chi-square goodness-of-fit test to determine whether salamander responses to treatments departed significantly from random expectation (Siegel and Castellan, 1988).

Experiment II. Trials with Distressed Salamanders and Unfed Snakes. In the previous experiment, it is possible that any avoidance elicited by the combined rinse of earthworm-fed snake and distressed salamander might have nothing to do with an interaction of snake and salamander chemicals, and instead be a response to an interaction between chemicals from earthworms and salamanders. Experiment II repeats experiment Experiment I, except rinses from unfed snakes replace those

from earthworm-fed snakes, and two snakes are used for each snake rinse. If only the standard and combined rinses elicit avoidance, the results would more clearly indicate that neither ingestion nor injury of the salamander is required to elicit avoidance, and that chemicals from both noninjured salamanders and snakes are required.

Seven treatments were prepared and tested. Treatments A and B consisted of a 200-ml rinse from two different adult garter snakes (snakes 3 and 4) that had not been fed for 3 wk prior to sample collection, a period sufficient to negate a diet effect (Madison et al., 1999b). The use of different snake sources for treatments A and B, and two snake sources for other treatments mentioned below, was an effort to provide an additional test of possible snake source effects, as referred to in experiment 1. Treatment C consisted of rinse from distressed *P. cinereus*, collected in the same manner as in experiment I. Treatments D and E consisted of salamander-fed snake rinse from two different adult garter snakes (snakes 5 and 6) that had been treated identically to the comparable treatment in experiment 1. Treatments F and G were combination treatments made up of equal portions of treatments A and C and of B and C, respectively. The combined treatments were prepared as in experiment 1.

Testing was performed as previously stated. A total of 168 salamanders were tested once just after midnight over three consecutive days. On each night, 7 replicate dishes were prepared for each treatment, except for treatment C (distressed salamander), which was represented by 14 replicated dishes (giving 14 replicates for each of the four main treatments per night). Thus, 56 salamander dishes were distributed at random and tested each night, as in experiment 1.

We used a χ^2 goodness-of-fit test to determine whether salamander responses to treatments departed significantly from random expectation, and then a 2×2 χ^2 contingency test to look for differences between snake sources and between treatments (Siegel and Castellan, 1988). Interactions between *T. sirtalis* and *P. cinereus* can only be inferred using chi-square tests, and since this experiment with unfed snakes was the clearest demonstration of salamander and snake component effects, we used binomial regression with a log–log canonical link in Statistica's (Statsoft, Inc., 1998 version 5.5A) Generalized Linear model (GLZM) to quantitatively test for the predicted snake by salamander interaction. We chose to test for significance of this interaction using the Wald statistic, rather than likelihood ratios, due to its ease and efficiency of computation (Dobson, 1990).

Experiment III. Selected Individual and Combination Treatments Tested Against Rinses from Salamander-Fed Snakes. The previous experiments left unresolved several questions: (1) whether always giving salamanders a choice of moving to nonsoiled substrates would affect their response to treatment substrates, (2) whether salamanders would discriminate between the substrates of two snakes on different diets, and (3) whether the distressed salamanders may have elicited a subtle but nonsignificant avoidance effect that would still act as a prey label in

the combined tests. In a single attempt to address these questions, we paired our standard salamander-fed snake rinse against four treatment rinses: earthworm-fed snake, injured salamander, earthworm-fed snake + undisturbed salamander, and earthworm-fed snake + injured salamander.

Treatment rinses were collected as before, except that two new treatments were prepared: undisturbed salamander and injured salamander. Undisturbed-salamander rinse was collected by housing two salamanders (one male and one female) in separate Petri dishes on moistened filter paper for the 72 hr during which time snake chemical cues were accumulating for the snake rinses. After 72 hr, the filter paper was removed from both Petri dishes and placed in 200 ml of distilled water. The injured salamander rinse was collected using the two undisturbed salamanders above in a two-step procedure. First, they each were immersed in the same 200 ml of distilled water during which they were physically disturbed, identical to the collection of distressed salamander rinse previously described. Second, they were removed, decapitated, and homogenized in the same 200 ml of distilled water with the distress substances. Both rinses were passed through glass wool before use in preparing the test dishes. All tests were performed beginning at midnight immediately after treatment preparation, and all observations were performed and analyzed as in previous studies (Madison, 1999a,b). We used a chi-square goodness-of-fit test to determine whether salamander responses to the different treatments departed significantly from random expectation (Siegel and Castellan, 1988).

RESULTS

The first experiment showed that *P. cinereus* did not avoid rinses from earthworm-fed snake or the distressed salamander when these were tested separately against distilled water in late-night trials. However, salamanders significantly avoided the standard salamander-fed snake rinse ($\chi^2 = 13.71$, $df = 1$, $P < 0.001$). The combined rinse of earthworm-fed snake and distressed salamander was also avoided ($\chi^2 = 7.71$, $df = 1$, $P = 0.005$), despite the concentrations of the components in the combined rinse being half those in the separate rinses (Table 1).

In the second experiment, we first looked for differences in response to the two unfed snakes when tested alone (treatments A and B) or in combination with salamander rinse (treatments F and G). No difference occurred between treatments A and B ($\chi^2 = 0.86$, $df = 1$, $P = 0.35$; Table 2) or between F and G ($\chi^2 = 0.12$, $df = 1$, $P = 0.73$). In addition, no difference in response was given to the two snakes that were fed salamanders (treatments D and E; $\chi^2 = 0.00$, $df = 1$, $P = 1.00$). Thus, the results for each snake source within each treatment were combined for further analyses.

TABLE 1. NUMBER OF SALAMANDERS PREFERRING SUBSTRATES WITH DISTILLED WATER VS. SUBSTRATES WITH DISTILLED WATER RINSES^a

Trial type	Salamanders preferring (<i>N</i>):			χ^2b	<i>P</i>
	Treatment	Water			
T _{SpC} vs. water	9	33		13.71	<0.001
T _{Sw} vs. water	17	25		1.52	0.217
P _{C_D} vs. water	20	22		0.10	0.758
T _{Sw} + P _{C_D} vs. water	12	30		7.71	0.005

^a Rinses were of *T. sirtalis*-fed *P. cinereus* (T_{SpC}), *T. sirtalis*-fed earthworms (T_{Sw}), distressed *P. cinereus* (P_{C_D}), or T_{Sw} and P_{C_D} Rinses Combined (T_{Sw} + P_{C_D})

^b *df* = 1.

The unfed snake treatments (A + B) elicited no avoidance behavior in salamanders during the late-night trials ($\chi^2 = 0.00$, *df* = 1, *P* = 1.00; Table 2), nor did the distressed salamander rinse ($\chi^2 = 0.38$, *df* = 1, *P* > 0.50). However, the salamander-fed snake treatments (D + E) elicited significant avoidance ($\chi^2 = 16.10$, *P* < 0.001), as did the combination treatments (F + G; $\chi^2 = 9.52$, *df* = 1, *P* < 0.01). These results corroborate the results for those treatments that were equivalent in experiment 1 and suggest that natural body secretions or excretions from undisturbed salamanders are sufficient to elicit avoidance when combined with rinses from recently foraging (on earthworms) or nonforaging snakes.

TABLE 2. NUMBER OF SALAMANDERS PREFERRING SUBSTRATES WITH DISTILLED WATER VS. SUBSTRATES WITH DISTILLED WATER RINSES^a

Trial type	Treatment	Salamanders preferring (<i>N</i>):		χ^2	<i>P</i>
		Treatment	Water		
Ts vs. water	(A) Ts ₁	12	9	0.00	1.00
	(B) Ts ₂	9	12		
		21	21		
P _{C_D} vs. water	(C) P _{C_D}	19	23	0.38	>0.50
T _{SpC} vs. water	(D) Ts _{3P_C}	4	17	16.10	<0.001
	(E) Ts _{4P_C}	4	17		
		8	34		
Ts + P _{C_D} vs. water	(F) Ts ₁ + P _{C_D}	6	15	9.52	<0.01
	(G) Ts ₂ + P _{C_D}	5	16		
		11	31		

^a Rinses were of unfed *T. sirtalis* (Ts), distressed *P. cinereus* (P_{C_D}), *T. sirtalis*-fed *P. cinereus* (T_{SpC}), or Ts and P_{C_D} combined (Ts + P_{C_D}). Numbers following Ts refer to the different snakes used. Chi-square results are given for within-trial comparisons (*df* = 1 for each comparison); no differences occurred between snakes within trial types.

Comparing the results of the four main treatments, there was no difference between unfed snake and distressed salamander treatments (χ^2 2×2 contingency test, $\chi^2 = 0.19$, $df = 1$, $P = 0.66$), but there was a difference between rinses from unfed snakes vs. salamander-fed snakes ($\chi^2 = 8.90$, $df = 1$, $P = 0.003$) and between unfed snakes vs. the combination of unfed snakes and distressed salamanders ($\chi^2 = 5.10$, $df = 1$, $P = 0.03$). There was a significant difference between distressed salamanders and the salamander-fed snake standard ($\chi^2 = 6.60$, $df = 1$, $P = 0.01$), but a marginally insignificant difference between distressed salamanders and the combined unfed snake + distressed salamander treatment ($\chi^2 = 3.30$, $df = 1$, $P = 0.068$). Finally, there was no difference between the standard rinse and the combined unfed snake + distressed salamander ($\chi^2 = 0.61$, $df = 1$, $P = 0.43$).

Since there was no independent avoidance of unfed snake rinse or distressed salamander rinse, but a significant avoidance of these treatments combined, a snake \times salamander interaction was suggested. We combined the data for the standard rinse and the combination rinse of distressed salamander + unfed snake because both contained chemicals from stressed salamander and snake, and then we compared these against the independent results for snakes (treatments A and B) and distressed salamanders (treatment C). An unfed snake \times distressed salamander interaction was confirmed using binomial regression ($Wald_{1,206} = 5.42$, $P = 0.02$).

Finally, in experiment 3, the results of the dual treatment tests gave results consistent with previous predictions and findings for individual treatment tests against a distilled water control. Rinses from standard salamander-fed snake treatments were avoided relative to earthworm-fed snake ($\chi^2 = 5.00$, $df = 1$, $P = 0.03$) and to injured (homogenized) salamander treatments ($\chi^2 = 7.20$, $df = 1$, $P < 0.01$), indicating that neither earthworm-fed snake rinses nor injured salamander rinses were as aversive as the standard rinse (Table 3). The lack of a difference between the combined samples and the standard salamander-fed snake rinse suggests that *P. cinereus* finds these treatments equally undesirable.

DISCUSSION

The results corroborate previous findings that rinses from *P. cinereus*-fed *T. sirtalis* consistently elicit avoidance in laboratory trials when compared to water controls (e.g., Madison et al. 1999a,b; Maerz et al., 2001; Sullivan et al., 2002), regardless of different source snakes being used, and that earthworm-fed snake rinse and unfed snake rinse fail to elicit avoidance in late-night tests when salamander-fed snake rinse continues to elicit avoidance (Madison et al., 1999a,b). The results also show that, under the conditions in our study, rinse from distressed *P. cinereus* repeatedly fails to elicit avoidance. When the combined rinses from distressed

TABLE 3. NUMBER OF SALAMANDERS PREFERRING SUBSTRATES WITH DISTILLED WATER RINSES OF *T. sirtalis*-FED *P. cinereus* (T_{SpC}) VS. DISTILLED WATER RINSES OF *T. sirtalis*-FED EARTHWORMS (T_{SW}), HOMOGENIZED *P. cinereus* (P_{CH}), T_{SW} COMBINED WITH UNDISTURBED *P. cinereus* (PC) (T_{SW} + PC), AND T_{SW} COMBINED WITH P_{CH} (T_{SW} + P_{CH}).

Trial type	Salamanders preferring (<i>N</i>):		Ties	χ^{2a}	<i>P</i>
	Treatment	T _{SpC}			
T _{SW} vs. T _{SpC}	15	5	0	5.00	0.03
P _{CH} vs. T _{SpC}	16	4	0	7.20	<0.01
T _{SW} + Pc vs. T _{SpC}	10	9	1	0.05	0.82
T _{SW} + P _{CH} vs. T _{SpC}	7	13	0	1.80	0.18

^a *df* = 1.

P. cinereus and unfed or earthworm-fed *T. sirtalis* elicited avoidance late at night, two things became clear. First, the cue eliciting avoidance behavior must require chemical involvement of both the predator and the prey. Second, neither ingestion by the predator nor distress or alarm substances from disturbed or injured prey is necessary to elicit late-night avoidance in *P. cinereus*.

There are two possible explanations for the above findings: (1) substances from the snake and salamander remained active in the separate rinses and chemically reacted to produce a novel “warning” substance when the rinses were combined, or (2) chemicals from snakes and salamanders only evoke a response when they occur as a mixture in the same scent trace, and in this case, the cue would be compositional rather than being a novel chemical product. It is difficult to determine which of these two hypotheses is more likely. In support of both is that during snake attack, *P. cinereus* usually deposits milky tail secretions directly to the snake’s head, and that after ingestion or prey escape, the film remains and the snake goes through head rubbing on the substrate for a few minutes. This behavior has also been reported for *T. sirtalis* in response to the adhesive mucus of earthworms (Cunningham and Burghardt, 1999). Not only is there potential mixing of substances from salamander and snake, but the subsequent effort by the snake to free its integument (eyes, scales) of the gluey matrix would facilitate the deposit of substances in this matrix on the nearby landscape.

The above scenario for a novel chemical cue or a composite cue used by *P. cinereus* late at night does not preclude the concurrent existence of multiple chemical mechanisms involved in antipredator behavior in this species. In fact, the avoidance of any *T. sirtalis* trace during the day or early evening, but only of *P. cinereus*-fed *T. sirtalis* rinses late at night, indicates two mechanisms with temporal dependence (Madison et al., 1999a,b). In addition, other data show a third mechanism in *P. cinereus*: the late-night avoidance of damage-release “alarm” substances (Sullivan, Madison and Rohr, unpublished results). Damage-release

cues, by themselves, are probably not responsible for the avoidance of the standard salamander-fed snake rinses late at night, since in the present study these alarm cues failed to cancel the effect of the standard rinses in eliciting avoidance. Thus, the new chemical mechanism, which does not require tissue injury, could be the basis for the avoidance of both the combination rinses and the standard rinse from salamander-fed snakes. Whatever the ultimate explanation is, at least four different chemical mechanisms are used by *P. cinereus* in the context of predator avoidance, with the fourth or “dietary” mechanism requiring chemicals from both the predator and prey and being either a compositional mixture or a novel cue formed by a non-ingestive chemical reaction between superficial rinse products from both predator and prey.

Our study is the first demonstration of antipredator behavior being released by a cue where substances from both the predator and prey are required to elicit a response. How common such complex, “cross-taxon” cues may be in nature is unknown, but our findings strongly suggest that future studies should not restrict the search for chemical cues that elicit antipredator behavior to substances released from just the prey or predator.

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