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Aaron M. Sullivan

John C. Maerz

Dale M. Madison

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DECLINE IN AVOIDANCE OF PREDATOR CHEMICAL CUES: HABITUATION OR BIORHYTHM SHIFT?

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

1. INTRODUCTION

Studies of the behavior of captive animals are widespread in the literature. Such practices may occur because animals are not seasonally available, do not occur near the home institution of the researcher, or because it is much easier to control for multiple variables in the laboratory. In addition, because of animal rarity or inaccessibility, repeat testing procedures are often used on small numbers of animals, potentially causing pseudoreplication problems (Ramirez et al., 2000). It is reasonable to expect that the laboratory environment at some point will alter the natural responsiveness of animals to particular stimuli, or result in behavior that may be an artifact of stress, housing, diet, and the testing apparatus (Hennig and Dunlap, 1978; Jarvi, 1990). Through differential mortality, captivity can also create laboratory populations of atypical animals most tolerant of captive conditions (Navas and Gomes, 2001). Even when captivity effects are detected, there are few opportunities or rewards for investigators to report shortcomings in their own methodology or in correcting long-standing methods widely in use. Field validation of results is one way of detecting possible laboratory artifact (e.g., Sullivan et al., 2002). However, field studies may not be possible, may not be directly comparable to laboratory studies, and may introduce other experimental effects (Rohr and Madison, 2001; Rohr et al., 2002). An alternative approach to detecting captivity effects is to record data on changes in behavior of captive animals through time (Rohr et al., 2003).

In seven years of studies, we have documented a highly predictable avoidance response in the red-backed salamander, *Plethodon cinereus*, to body rinses from garter snakes, *Thamnophis sirtalis*, that have been feeding on *P. cinereus* (standard TS_{PC} stimulus; Madison et al., 1999a,b; McDarby et al., 1999; Maerz et al., 2001; Madison et al., 2002; Sullivan et al., 2002, 2003). While we always get avoidance of TS_{PC}, no such avoidance occurs in response to body rinses from garter snakes feeding on earthworms,

* Dale M. Madison and Aaron M. Sullivan, Department of Biological Sciences, State University of New York at Binghamton, Binghamton, NY, 13902 USA. John C. Maerz, Cornell University, Ithaca, NY 14853 USA

goldfish, or other salamander species when these tests are conducted late at night (Madison et al., 1999a,b; Sullivan et al., 2003), so avoidance is not simply to nitrogenous wastes. The response to TS_{PC} occurs regardless of the individual adult garter snake used to collect the rinse (Madison et al., 1999b; Madison et al., 2002), and we have validated this response under field conditions (Sullivan et al., 2002). Even in occasional testing situations where salamanders were held captive for up to a month and were used in repeat-testing experimental designs, *P. cinereus* continued to selectively avoid TS_{PC}. Because of these observations, because *P. cinereus* are routinely held captive for 3 to 9 months during all seasons prior to behavioral studies involving chemical cues (e.g., Jaeger et al., 1982; Mathis, 1990; Mathis and Simons, 1994; Simons et al., 1994; Gabor and Jaeger, 1995; Jaeger et al., 1995; Mathis and Lancaster, 1998), and because the studies above have generally been field validated (e.g., Mathis, 1989), we expected captive *P. cinereus* to avoid TS_{PC} throughout winter in our studies beginning Fall 1999. We did not expect that repeat testing at intervals greater than a week apart would affect the avoidance response, because habituation to predator stimuli rarely, if ever, occurs in animals (Blanchard et al., 1998). Instead of recording avoidance to TS_{PC} throughout winter, we recorded a progressive weakening and loss of avoidance. This paper documents our findings in a long series of experiments before, during, and after the winter in question, and attempts to explain the cause of the winter decline.

2. MATERIALS AND METHODS

2.1. Collection and Maintenance of Animals

For our behavioral studies, adult *P. cinereus* (snout/vent length > 40 mm) were collected from the Binghamton University Nature Preserve (Broome Co., New York): 132 for multiple experiments throughout the summer and fall of 1999, 130 in late October and early November for our winter studies, and 48 animals in April 2000 to see if newly captured animals would respond to the TS_{PC} used throughout the winter. Two adult garter snakes (*T. sirtalis*; snout/vent length > 36 cm) were collected from the Nature Preserve during the summer of 1999 to use in preparing TS_{PC} for all the trials.

Salamanders and snakes were kept in separate rooms at 15 °C (salamanders) and 25 °C (snakes) on a natural photoperiod (14L:10D). Salamanders were housed individually in 15-cm diameter petri dishes lined with moistened paper towels. Long-term captive salamanders were fed 5-10, 5 mm-long cricket nymphs once per week, and the towel lining was changed once every 3 wks. 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 earthworms, *Lumbricus* sp., until 3 weeks before the collection of the TS_{PC} rinse used in our trials. After all tests were completed, we released all *P. cinereus* and *T. sirtalis* at their original capture locations.

2.2. Collection of TS_{PC}

The methods for preparing TS_{PC} and testing salamander avoidance are similar to the laboratory techniques reported by Sullivan et al. (2002). Briefly, we obtained TS_{PC} by collecting a 200 ml rinse of an adult garter snake that had been maintained on a diet

of 4 *P. cinereus* (2 male, 2 female) per week for two weeks prior to sample collection. Immediately after the snake ate the last salamander, we transferred the snake to a 4-L beaker, covered the beaker with 8 layers of cheesecloth secured by rubber bands, and then placed a heating pad (26°C) under half the beaker. After 72 h, we removed the snake from the container. We then added 200 ml of distilled water to the beaker, swirled it for 10 min, and transferred it to a polypropylene container. We filtered the rinse through HPLC-grade nylon filters (0.45µm) to remove any solid materials, and then divided the filtered solution into smaller aliquots for rapid freezing in liquid nitrogen and freezer storage. Previous research has shown that salamanders still avoid TS_{PC} that has been frozen using this method, and that individual differences in *T. sirtalis* secretions or excretions do not affect salamander avoidance of TS_{PC} (Madison et al., 2002; Sullivan et al., 2002). All tests were conducted with samples that had just been removed from the freezer and thawed, so aging of thawed samples was not a factor in our studies.

2.3. Experimental Protocol

For testing TS_{PC}, we placed two filter paper semi-circles with a 3-mm gap between them into 15-cm petri dishes, inoculated the semi-circles with 1.5 ml of TS_{PC} or distilled water, and then randomly placed these test dishes (position and orientation) on a 6 x 8 grid on a foam board on the floor of the test room. Salamanders were moved to the test room one hour prior to the experiments, which were conducted between 1030 and 1315 h. We transferred each salamander from its home dish to the test dish with a cotton swab, and then placed a 15-mm collar of brown paper around the covered dish to visually isolate the salamanders from each other during the transfer process. After all salamanders were distributed (~10 min), the lights were turned off and the trial was recorded in complete darkness using an infrared video camera (SONY TRV66) suspended 3 m above the dishes. From videotapes, we scored which semicircle the salamander occupied at 3-min intervals for one hour beginning a time 0, which gave 21 positions per salamander. When salamanders straddled the 3 mm gap, two criteria were used to judge side position. First, 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 was scored.

For those *P. cinereus* tested two or more times, salamanders were selected at random from the stock population no sooner than one week (including feeding and a change of toweling) after previous testing. Between 24 and 80 animals were given a choice of the TS_{PC} vs. distilled water substrates during each trial day. We used a chi-square goodness-of-fit test (Sheskin, 2000) to compare the number of salamanders that spent more than 50% of their time on the treatment vs. the water side of the test dish. Over ninety percent of the salamanders spent at least 70 % of their time on one side or the other, so choices were very clear for most animals (see Sullivan et al., 2003). We could not use parametric statistics (e.g. *t*-tests, regression analyses on winter trends) to analyze the avoidance trials because *P. cinereus* most often showed scores that were near the response extremes, 0 or 21, and hence were bimodal. A 2 x 2 chi-square contingency test corrected for continuity was used to look for differences between trials (Siegel and Castellan, 1988).

To measure activity among salamanders, the number of times an animal crossed from one side of the petri dish to the other in 60 min was recorded. The activity data were not bimodal, and a *t*-test for two independent samples was used to test for differences in activity between samples, following the assumptions and guidelines of Sheskin (2000).

3. RESULTS

Except for one animal that died from unknown causes in January, all captive salamanders survived and consumed some of the crickets offered each week. The few animals that didn't feed weekly were not used in the behavioral trials. In total, 305 of the 310 original animals fed regularly and were used in the experiments.

The responses of 24 "captive" *P. cinereus*, previously tested 4 to 5 times with TS_{PC} and held captive for 5 months from June 18 to November 9, 1999, were compared to the responses of 24 "fresh" salamanders captured on November 1, 1999 and tested for the first time on Nov 9, 1999. Both groups avoided the same TS_{PC} rinse ("captive", $\chi^2 = 4.17$, $P < 0.05$; "fresh", $\chi^2 = 8.17$, $P < 0.01$), and there was no significant difference between the responses of the captive and fresh animals ($\chi^2 = 0.11$, $P > 0.70$) (Figure 1). There was also no difference in the activity levels of the two groups (captive mean \pm SD = 3.17 ± 2.35 ; fresh mean = 3.25 ± 2.40 ; $t = 0.121$, $P > 0.80$, $df = 46$). We therefore conducted trials on salamanders captured in late October and early November through the winter, expecting continued avoidance of TS_{PC}.

Trials were conducted on 11 experimental days between November 1999 and April 2000, and the results for these trials showed a steady decline in the percent of the salamanders tested that avoided TS_{PC} (Figure 1). Whereas significant avoidance was recorded in each of the first 5 trials up to January 26, trials after that failed to showed significant avoidance behavior. In no case were the data for adjacent testing days significantly different from each other, although there was a significant difference in preference scores of captive salamanders between the first and last trial days, November 9 and April 7 ($\chi^2 = 5.15$, $P < 0.05$). The activity levels for these two groups of salamanders also differed (Nov mean \pm SD = 3.17 ± 2.35 ; Apr mean = 0.81 ± 1.38 ; $t = 5.33$, $P < 0.001$, $df = 70$).

To determine whether loss of TS_{PC} potency or salamander non-responsiveness was responsible for the decline in behavioral avoidance during late winter, the responses of 48 "captive" *P. cinereus* were compared to those from 48 newly captured ("fresh") salamanders to the same TS_{PC} rinse on 10 April 2000. The captive animals had been used in multiple TS_{PC} tests throughout winter (Mean \pm SD = 5.77 ± 1.19 , Range 3 – 8). Unlike the captive salamanders tested in November after 5 months in summer/fall captivity, these over-winter captives in early April failed to avoid TS_{PC} ($\chi^2 = 0.00$, $P = 1.00$; Figure 1), but the newly-captured salamanders tested with the same rinse avoided TS_{PC} ($\chi^2 = 12.0$, $P < 0.001$), and the difference in responses between the two groups was significant ($\chi^2 = 5.38$, $P < 0.05$). The activity levels also differed between the two groups of salamanders (captive mean \pm SD = 0.81 ± 1.381 , fresh mean = 3.96 ± 3.07 ; $t = 6.48$, $P < 0.001$, $df = 94$).

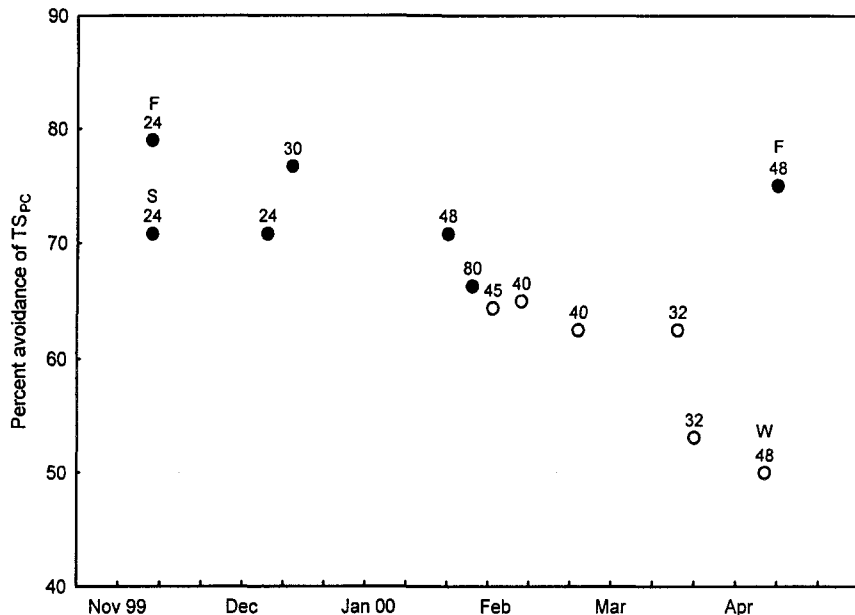


Figure 1. The percent of salamanders tested that avoided (had fewer recorded locations on) the TS_{PC} side of the test dish during trials from November 1999 to April 2000. The number of salamanders tested on each date is indicated above each circle. Unlabelled circles represent results from salamanders that had previously been exposed to TS_{PC}, with the number of exposures increasing with time through the winter. Salamanders that had just been captured and were tested for the first time are designated as fresh (F) salamanders. These fresh salamanders were tested with the same test samples at similar times as captive salamanders repeatedly exposed to TS_{PC} for 5 months during summer (S) or winter (W). Significant statistical outcomes ($P < 0.05$) from random expectation (at 50 %) are indicated by solid circles; non-significant results, by open circles.

4. DISCUSSION

There was a clear, progressive loss of responsiveness to snake predator cues (TS_{PC}) in *P. cinereus* held over winter. It is not clear what caused the decline in responsiveness. The animals actively fed and appeared in good health throughout the winter. One explanation is habituation to the predator stimulus, since habituation is a widespread response to repetitive stimulation in the lower vertebrates (Goodman and Weinberger, 1973). However, we do not believe that habituation, at least by itself, was the cause of the decline for several reasons. First, the frequency of exposure to TS_{PC} and the maintenance conditions of the summer and winter captives were comparable, and the summer animals did not show a decline in avoidance and activity. Second, the interval (1 week +) between exposures to TS_{PC} is relatively long to induce habituation. Habituation typically occurs following continuous or frequent stimulation over shorter periods from several minutes or hours to a few days at most (Goodman and Weinberger, 1973). Third, prey species generally do not rapidly habituate, or even habituate at all, to predator stimuli,

even following chronic exposure over many days (e.g., Sullivan et al., 1985; Magnhagen and Vestergaard, 1991; Epple et al., 1993; Holomuzki and Hatchett, 1994; Blanchard et al., 1998), and some prey may even become sensitized to predator stimuli following repeat exposure (Ducey et al., 1991). Finally, it seems unlikely that a salamander would habituate to predator cues in the wild when these are encountered less often than once per week.

We also considered whether the decline in responsiveness was a natural decrease in sensitivity to cues from a predator that is not a threat during winter, at least at our geographic location. Biorhythms have been reported for amphibians (e.g., Madison et al. 1999a,b; Maerz et al., 2001), and it is not unreasonable to expect that sensitivity to snake predators might also be linked to a circannual rhythm (e.g., Pancak and Taylor, 1983). Indeed, Hileman and Brodie (1994) showed that plethodontid responses to predators vary seasonally with changes in encounter rates with predators. Our original maintenance plan was to hold salamanders on a late summer photoperiod of 14L:10D and a temperature of 15 °C to keep them under environmental conditions where snakes would still be a natural threat. Inadvertently, we may have slowed but not arrested the seasonal onsets of winter (to early February) and spring (until after April 10) of the salamander's circannual rhythm, and hence prolonged predator avoidance until February and delayed seasonal resumption of predator avoidance until after early April. Some sensitivity of circannual rhythms to local external conditions has been reported in amphibians (Harri and Koskela, 1977), and circannual shifts in amphibian hormones are known to occur despite unchanging photoperiods (Pancak and Taylor, 1983). On a strict circannual cycle, both winter-captive and fresh-captured salamander groups should have responded similarly in April, but they didn't, and this is preliminary evidence that the two groups may have had circannual rhythms out of phase with each other. Testing two groups of captive salamanders throughout winter, one on a seasonally adjusted photoperiodic cycle, and a second on a constant late summer photoperiodic cycle, would be one way to test the delayed circannual rhythm hypothesis.

Whatever the reason is for the observed reduction in avoidance behavior in *P. cinereus* during winter, we hope our experience alerts other investigators to the possibility of seasonal or captivity and repeat-testing effects on the natural response patterns of amphibians, and that one way to detect possible effects is to record the same behavioral response through time with and without previous exposure to test stimuli. Further research is necessary concerning the influence of circannual rhythms on amphibian behavior in winter.

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