

Acquired Recognition of Chemical Stimuli from an Unfamiliar Predator: Associative Learning by Adult Newts, *Notophthalmus viridescens*

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Many vertebrates recognize potential predators using only chemical cues (Weldon, 1990). Chemical detection of predators is particularly important at night, in dark or murky habitats, in areas with dense vegetation, or with cryptic or ambush predators, all conditions that are common in aquatic environments (Dodson et al., 1994). Chemical recognition of some common predators may be innate (e.g., Elliot et al., 1993). Natural selection also may favor the ability to learn to associate unfamiliar predators with danger (e.g., Maloney and McLean, 1995; Chivers et al., 1996). Previous attempts to demonstrate associative learning by amphibians have been relatively unsuccessful (Suboski, 1992).

Chemical alarm signals play an important role in the antipredator behavior of many aquatic vertebrates (e.g., Hews and Blaustein, 1985; Smith, 1992; Mathis and Smith, 1993a). For amphibians, response to chemical alarm signals has been documented for some anuran tadpoles (Kulzer, 1954; Hews, 1988; Petranksa, 1989) and for several species of salamanders (Lutterschmidt et al., 1994), including the newt *Notophthalmus viridescens* (*N. v. viridescens*; Marvin and Hutchison, 1995; *N. v. louisianensis*; Woody and Mathis, 1997). In this paper, we use Wilson's (1975) definition of the terms "pheromone" and "signal," which requires only that the chemicals be used to convey information during communication.

In several species of fishes, chemical alarm signals play an important role in acquired predator recognition. Chivers et al. (1995) used skin extracts containing alarm pheromones as the unconditioned stimulus in an associative learning experiment with naive brook stickleback, *Culaea inconstans*. The sticklebacks exhibited a fright response when exposed to chemical cues from a northern pike, *Esox lucius*, only when these cues had been previously paired with the alarm pheromone. Similar experiments have demonstrated acquired recognition of predatory stimuli through association with alarm signals for fathead minnows, *Pimephales promelas* (Chivers and Smith, 1994; Mathis and Smith, 1993b), and European minnows, *Phoxinus phoxinus* (Maggurran, 1989).

Our study tested the hypothesis that central newts, *N. v. louisianensis*, can learn to recognize

unfamiliar potential predators through association of predator cues with chemical alarm signals. We exposed adult newts to chemical cues from an unfamiliar predator (smallmouth bass, *Micropterus dolomieu*) paired with either newt skin extract (NSE), terrestrial salamander (*Plethodon serratus*) skin extract (PSE), or dechlorinated water. Marvin and Hutchison (1995) reported that *N. v. viridescens* do not avoid skin extracts from *P. serratus*. We predicted that newts previously exposed to NSE plus fish stimuli would exhibit antipredator avoidance behavior when subsequently exposed to chemical stimuli containing only bass chemical cues. The newts previously exposed to PSE plus fish stimuli or water plus fish stimuli should not exhibit avoidance behavior.

We performed a second experiment to determine whether acquired avoidance responses were specific to the training stimulus or were general conditioned responses to disturbance (e.g., Mathis and Smith, 1993b). In this experiment, we conditioned all of the newts with water from an unfamiliar aquatic salamander (*Siren intermedia*) plus NSE. In subsequent trials, we predicted that newts would avoid chemical stimuli from *Siren* but would not avoid chemical stimuli from an unfamiliar source (bass).

MATERIALS AND METHODS

Care and maintenance of animals.—We collected adult central newts, *N. v. louisianensis*, from several farm ponds in Webster County, Missouri. Because these ponds were fishless, the newts probably did not have experience with fish predators. *Siren* were collected from Stoddard County, Missouri, and also were unfamiliar stimuli because they do not coexist with the population of newts used in our study. Newts were housed for at least one week prior to testing in opaque plastic containers (10 × 10 × 10 cm) filled with dechlorinated water and covered with glass lids. Room temperature was 20–25 C, and the light:dark cycle was 14:10. We fed the newts frozen brine shrimp (*Artemia* sp.) every other day. Bass were fed weekly with small fishes (primarily *Luxilus pilsbryi*), and *Siren* were fed weekly with commercial pellets.

Preparation of chemical stimuli.—We prepared skin extracts from both *N. v. louisianensis* and *P.*

serratus to use as the unconditioned stimulus. We decapitated three newts (SVL: 42 mm, 40 mm, and 39 mm) and removed the skin from the head, neck, torso, and tail. This tissue consisted mostly of skin, but included some muscle. We homogenized 0.8 g of newt tissue with 100 mL of dechlorinated water, using an Omni tissue homogenizer. The mixture was then filtered through glass wool, and its volume was increased to 533 mL by the addition of dechlorinated water. We prepared the terrestrial salamander skin extract (control) in the same manner, but eight *P. serratus* (mean SVL = 40.9 mm, range 37–45 mm) were required to equal the concentration (0.8 g of tissue in 533 mL of dechlorinated water) of the NSE. We divided both stimulus solutions into 10-mL portions in plastic snap-cap tubes and immediately placed them in a freezer at -20°C . Dechlorinated water was similarly frozen. We collected fish chemical cues from smallmouth bass, *Micropterus dolomieu*, to use as the conditioned stimulus. We placed bass (11.1 g and 7.7 g) individually into two 8-L glass aquaria each containing 2 L of dechlorinated tap water. After five days, we removed the water and placed it in plastic sample bags in 50-mL aliquots. The bags were immediately placed in a freezer at -20°C . For the second experiment, we collected stimuli from *Siren* ($n = 7$, mean mass = 47.1 g, range 31–64 g) using the same methods as were used for the bass. Because the *Siren* were about five times the mass of the bass, they were kept in aquaria containing 10 L of water so that the stimulus strength would be roughly equivalent for the two stimuli.

We removed all stimulus solutions from the freezer on the day of the experiment and allowed them to thaw at room temperature. We mixed the stimulus solutions from the two bass, so that any possible effects of individual differences were minimized. Identity of individual stimulus predators does not affect responses by prey (Mathis and Smith, 1993b, 1993c; Gelowitz et al., 1993).

Testing chambers.—We performed both experiments in 38-L glass aquaria with cardboard placed around the four sides to minimize external visual cues. A glass partition (length = 36 cm) partially divided each tank lengthwise, leaving an undivided neutral space (26×14 cm) on one end (total length of tank = 50 cm). A removable plastic divider placed crosswise in each tank separated the neutral area from the two stimulus chambers. Numerous holes drilled in the divider allowed free flow of water between the neutral area and the stimulus chambers. We filled tanks to a depth of 10 cm with

dechlorinated tap water. Stimulus solutions were injected into both stimulus chambers at the end of the tank opposite the neutral chamber, using 60-mL plastic syringes. Each syringe was attached to 30 cm of clear plastic tubing, which was secured to the back wall of the tank. The tubing ended 1 to 2 cm above the water level. Separate stimulus injection tubes were attached to each of the two chambers so that 60 mL of stimulus water could be added to one side and 60 mL of dechlorinated tap water could be added to the opposite chamber.

Protocol for experiment 1.—On the first day of the experiment, newts ($n = 12$ per treatment group; mean SVL = 40.6 mm, range 33–50 mm) were exposed to a training trial of the fish stimulus plus one of the three randomly chosen extracts. We placed individual newts in the neutral area with the plastic divider in place and allowed them to habituate for 5 min. This short habituation period was chosen because our previous observations indicated that newts almost immediately resumed apparently normal swimming activity following introduction into a new aquarium. At the end of the habituation period, we injected 60 mL of stimulus water into a randomly selected right or left chamber at a rate of about 2 mL/sec, and simultaneously injected 60 mL of dechlorinated tap water (= blank) into the opposite chamber at the same rate. The stimulus water consisted of 50 mL fish stimulus plus 10 mL of one of the following: newt skin extract (NSE), *Plethodon* skin extract (PSE), or dechlorinated water. In preliminary trials with dye, we determined that it took about 1.5 min for fluid to diffuse through the chamber to the plastic divider. Therefore, we removed the divider 1.5 min after injection of the stimulus water, allowing the newt to either enter one of the two chambers or remain in the neutral area. The trial ended 10 min after the divider was removed. At the end of each trial, we removed the newt from the aquarium and returned it to its housing chamber. We videotaped the trials from above, and recorded the data from the video images. The observer did not know the identity of the stimulus being presented and did not know which side received the experimental stimulus and which received the "blank" stimulus (water). Responses were quantified as the amount of time spent in each of the two chambers. One-half of the newt's body had to cross the dividing line into the chamber for it to be scored as "in" the chamber.

Learning trials were conducted on the third day of the experiment. We repeated the experimental protocol as above for each newt tested

on day 1. However, in this trial the stimulus was the same for all newts: 50 mL of fish stimulus plus 10 mL of dechlorinated tap water. Newts that remained in the neutral chamber on both days were not included in the statistical analyses.

Protocol for experiment 2.—The newts used in the first experiment were not used in the second experiment. On the first day of the experiment, newts ($n = 10$; mean SVL = 39.4 mm, range 37–41 mm) were transferred individually to new plastic chambers ($31 \times 17 \times 8$ cm) that contained 1000 mL of dechlorinated tap water. Each newt was exposed to 50 mL of the *Siren* stimulus plus 10 mL of the NSE solution. After one hour, newts were replaced in their individual holding chambers.

The design of this experiment required each newt to be tested in two learning trials. We exposed each newt to chemical stimuli from *Siren* (the conditioned stimulus) in one trial and to chemical stimuli from an unfamiliar stimulus (smallmouth bass) in the other. The order of stimulus presentation was determined randomly, with exposure to the first stimulus on day 3 of the experiment and exposure to the second stimulus on day 4. The testing protocol was the same as on day 3 of the first experiment.

Data collection and analyses.—For each newt, we calculated an index of avoidance as the difference between the amount of time spent on the stimulus side and the amount of time spent on the "blank" side. The indices were compared for the three treatments using a Kruskal-Wallis nonparametric ANOVA followed by nonparametric multiple comparison (NMC) tests (Zar, 1984) in the first experiment and by a Wilcoxon matched-pairs signed-ranks test in the second experiment (Siegel, 1956).

RESULTS

Experiment 1.—For the training trials (day 1), there was an overall significant difference among avoidance responses to the three stimuli (Kruskal-Wallis: $H = 6.13$, $P < 0.05$, Fig. 1A). The response to the PSE treatment did not differ significantly from the response to the water controls (NMC: $q = 1.05$, $P > 0.50$, Fig. 1A). In contrast, the response to the NSE treatment differed significantly from the response to the water controls (NMC: $q = 3.39$, $P < 0.05$, Fig. 1A). The positive-response indices for the PSE and water treatments and the negative-response index for the NSE treatment (Fig. 1A) indicate that the newts were not initially repelled by ei-

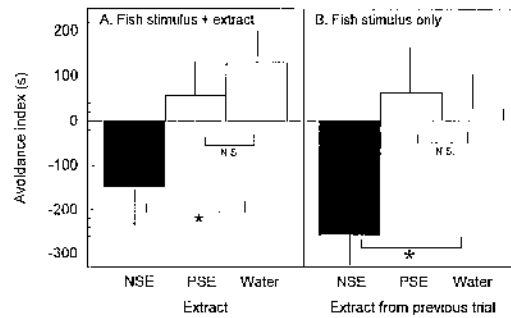


Fig. 1. Avoidance indices ($\bar{x} \pm 1$ SE) for newts exposed to (A) 50 mL of fish stimulus plus 10 mL of either newt skin extract (NSE), *Plethodon* skin extract (PSE), or water, and (B) 50 mL of fish stimulus plus 10 mL of water. Newts were trained on day 1 (A) and were retested on day 3 (B). The fish stimulus was water from tanks containing smallmouth bass. Indices were calculated as amount of time spent on the stimulus side of the tank versus time spent on the control (blank) side of the tank; positive numbers indicate overall attraction, and negative numbers indicate overall avoidance of the stimulus side of the tank. Kruskal-Wallis ANOVA followed by nonparametric multiple comparisons: *, $P < 0.05$; N.S., $P > 0.50$.

ther the fish stimulus or PSE but were initially repelled by the NSE.

During the learning trials (day 3), the three treatment groups had significantly different indices of avoidance to fish cues alone (Kruskal-Wallis: $H = 9.01$, $P < 0.05$, Fig. 1B). The trials in which the newts had previously been exposed to PSE plus the fish stimulus did not differ from the control trials (NMC: $q = 0.40$, $P > 0.50$, Fig. 1B). The trials in which the newts had previously been exposed to NSE plus fish cues differed significantly from those where the newt had previously been exposed to the control of water plus fish cues (NMC: $q = 3.45$, $P < 0.05$, Fig. 1B). Newts that had been previously trained with the NSE continued to be repelled (negative avoidance index: Fig. 1B) by the fish stimulus, whereas newts trained with either PSE or water showed an overall attraction to the fish stimulus (positive avoidance indices: Fig. 1B).

Experiment 2.—In the learning trials, eight of 10 newts "avoided" (spent more time in the blank side of the chamber) the conditioned stimulus (*Siren*; Avoidance Index: $\bar{x} \pm 1$ SE = -109.9 ± 106.74). In contrast, only four of 10 newts "avoided" the unfamiliar stimulus (bass; Avoidance Index: $\bar{x} \pm 1$ SE = 90.9 ± 1.156). The conditioned *Siren* stimulus elicited a significantly stronger avoidance response than did the unfamiliar bass treatment ($t = 11$, $P < 0.05$).

DISCUSSION

The results of this study support the hypothesis that *N. v. louisianensis* can become conditioned to recognize a novel chemical cue as dangerous through associations of this cue with a familiar fright stimulus (conspecific skin extract). This response was specific to the conditioned stimulus and was not merely a general conditioned response to disturbance. Suboski (1992) suggested that some previous attempts to demonstrate associative learning in amphibians may have been unsuccessful due to the use of both response variables and stimuli (such as electric shock) that were inappropriate for the species (Suboski, 1992). The success of our associative learning experiment may have been facilitated by the use of a natural unconditioned stimulus (conspecific alarm pheromone), an appropriate conditioned stimulus (chemical stimuli from an unfamiliar heterospecific), and a response variable (avoidance behavior) that has been documented to be a common response to chemical alarm signals by amphibians (Hews and Blaustein, 1985; Petranka, 1989; Marvin and Hutchison, 1995).

The response of newts to chemical alarm signals was only described recently (Marvin and Hutchison, 1995), and details of the process are not understood very well. For example, it is not known whether the chemical stimulus is a glandular product or whether mechanical damage to the skin is required for its release (as in ostariophysan system of fishes). Many salamanders secrete noxious skin products when they are attacked by a predator (Arnold, 1982; Evans and Brodie, 1994; Williams, 1994). These types of secretions are not damaged-released in the strictest sense, because mechanical damage is not always necessary for release. Products of the granular glands can be released when pressure is applied to the skin or when the skin is actually damaged (Noble, 1931). Both of these situations occur when a predator comes into physical contact with the prey.

Association of unfamiliar chemical cues with alarm signals is an efficient way for prey to learn to recognize novel predators. The presence of macerated tissue of a conspecific is a reliable indicator that a predation event has occurred and thus makes an ideal "unconditioned" stimulus for associative learning. The ability to acquire new information concerning predator recognition may be of particular importance to animals such as newts that may experience a wide range of predators (e.g., invertebrates, fish, snakes, birds) that vary in abundance seasonally.

Marvin and Hutchison (1995) and Woody and Mathis (1997) showed that newts avoid areas marked by chemical stimuli from injured conspecifics. Our study extends the known benefits to receivers; not only can they recognize that a conspecific has been injured, but they are able to learn the chemical signature of the predator that caused harm to that conspecific. This study is the first to demonstrate acquired recognition of predators through associative learning for amphibians.

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