

CONDITIONED ALARM BEHAVIOR IN FATHEAD
MINNOWS (*Pimephales promelas*) RESULTING FROM
ASSOCIATION OF CHEMICAL ALARM PHEROMONE
WITH A NONBIOLOGICAL VISUAL STIMULUS

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Abstract—Fathead minnows (*Pimephales promelas*) adopt antipredator (alarm) behavior when they detect alarm pheromone released from an injured conspecific. This is an adaptive response since alarm pheromone is generally released only in the context of a predation event. Alarm reactions may also occur in response to chemical and visual stimuli that minnows learn to associate with release of alarm pheromone. Here, we tested if fathead minnows can learn to associate a nonbiological, visual stimulus with predation risk. Minnows were simultaneously exposed to red light and conspecific alarm pheromone, inducing an alarm reaction. When retested using red light alone, small shoals of minnows displayed an antipredator response: dashing movements and disorganized swimming followed by decreased height in the water column and increased shoal cohesion. This resulted from a single-trial exposure to the combined cues and demonstrates a robust ecological mechanism by which minnows learn to recognize indicators of predation risk that may vary in space and time. However, learning to associate risk with biologically irrelevant stimuli may be an ecological liability. How minnows discern between relevant and irrelevant stimuli in nature is not known.

Key Words—Fathead minnow, *Pimephales promelas*, alarm pheromone, Schreckstoff, learned recognition of predation risk, red light.

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INTRODUCTION

The fathead minnow (*Pimephales promelas*), like other members of the super-order Ostariophysi, possesses specialized epidermal club cells that contain alarm pheromone (Frisch, 1941; Pfeiffer, 1977; Smith, 1992; Chivers and Smith, 1998). When minnows are injured by a predator, these epidermal cells rupture, releasing alarm pheromone (*Schreckstoff*) into the water. This compound, reputedly hypoxanthine-3-(*N*)-oxide (Pfeiffer et al., 1985), serves to alert conspecifics to predation threat (Pfeiffer, 1963) and invokes a range of species-specific antipredator behaviors such as increased shoaling, seeking refuge, dashing, freezing, and area avoidance (Magurran, 1989; Mathis and Smith, 1992; Chivers and Smith, 1994a,b; Wisenden et al., 1995). Since ostariophysan fishes account for 28% of all known fish species and 64% of all freshwater species (Nelson, 1994), the *Schreckstoff* reaction system is extremely common and of great ecological importance.

Alarm pheromone release facilitates predator recognition in minnows (Magurran, 1989; Suboski et al., 1990). For instance, Göz (1941) found that blinded European minnows (*Phoxinus phoxinus*) displayed no initial response to pike (*Esox lucius*) odor. After being attacked, and thus exposed to pike odor and alarm pheromone simultaneously, the minnows reacted with alarm to pike odor alone. This paradigm for associated learning of novel chemical cues has subsequently been demonstrated for a number of species: fathead minnows (Mathis and Smith, 1993; Chivers and Smith, 1995), European minnows (Magurran, 1989), brook stickleback (Chivers et al., 1994), and damselfly larvae (Chivers et al., 1996; Wisenden et al., 1997). Only four previous fish studies have documented learned recognition of a visual stimulus by association with injury-released alarm hormone (Suboski et al., 1990; Chivers and Smith, 1994b; Hall and Suboski, 1995; Brown et al., 1997).

Although visual learning has been documented in fathead minnows in conjunction with the sight of pike (Chivers and Smith, 1994b; Brown et al., 1997) it has been suggested that minnows naive to the sight of pike may be genetically predisposed to avoid large fish as a consequence of previous generations of minnows occurring in similar habitats as pike. To determine if learning can occur in response to visual cues to which minnows cannot be genetically predisposed, a biologically irrelevant stimulus must be presented. The objective of this study was to determine if fathead minnows can learn to associate a nonbiological stimulus (a red light) with predation risk.

Depth and duration of vertical movement in the water column are reliable indicators of alarm in minnow species (Hall and Suboski, 1995; Chivers and Smith, 1998). These criteria were used to indicate alarm behavior in small groups of male fathead minnows exposed to alarm pheromone or red light stimulus alone. Both stimuli were applied simultaneously, and the response to light

stimulus alone was subsequently evaluated. We predicted that if fathead minnows can learn to associate a nonbiological visual stimulus with predation risk, the second application of red light alone should induce an antipredator response.

METHODS AND MATERIALS

Minnows. Adult male fathead minnows, 4–5 cm in length, were obtained from Hasse Lake, Alberta (53°20'N, 115°5'W) and maintained in a 300-liter flow-through aquarium at 15°C on a 16L : 8D photoperiod for at least two weeks prior to use. While in the holding tank, minnows were fed to satiation daily with Nutrafin fish flakes and frozen brine shrimp (*Artemia salina*). Feeding was restricted to the holding aquarium, so as to avoid the possibility that food odors might mask alarm pheromone released into the testing aquaria.

Apparatus. All experiments were conducted in aquaria with dimensions 40 × 19 × 19 cm. Each aquarium had three opaque sides painted light blue, a 1-cm layer of naturally colored gravel on the bottom, and was filled with 14.5 liters of dechlorinated, 21°C water. A single aeration stone and a 1-mm-diameter polyethylene tube (for the introduction of alarm pheromone) centered both horizontally and vertically were affixed to the back wall of each tank. Horizontal lines on the front wall of the aquarium divided the water column into four equal layers. The visual stimulus was a 9-V bulb covered by a transparent red filter, positioned external to and centered against the clear front pane of the aquarium. When used as a stimulus, the light was illuminated for 3 min (Hall and Suboski, 1995).

To collect alarm pheromone, two female minnows were killed via cervical dislocation. A scalpel was used to make 10 superficial epidermal cuts on each side of the fish. Alarm pheromone was extracted from females, rather than male minnows because the males were either sexually mature or only recently sexually regressed. Sexually mature male fathead minnows do not produce alarm pheromone (Smith, 1976). Ten milliliters of distilled deionized water (DDW) was washed over each fish. One-milliliter aliquots of this solution were kept frozen at –20°C until use. Aliquots were thawed as needed and diluted in 9 ml DDW.

Alarm pheromone exposure consisted of the addition of 5 ml of dilute extract solution. As a control, 5-ml samples of DDW not containing alarm pheromone were frozen and stored until needed. Introduction of water or alarm pheromone, light operation, and behavioral observation were performed from behind a black fabric blind. The pheromone solution injected into the treatment aquaria appeared clear; therefore, any behavioral response was likely not due to visual detection of a cloud of pheromone solution.

Procedure. Six minnows were used in each treatment group, following the

methods used by Hall and Suboski (1995). Six male minnows were subjected to five sequential experiments conducted in a single test session: (1) administration of water not containing alarm pheromone, (2) administration of alarm pheromone, (3) administration of a 3-min light stimulus without alarm substance, (4) administration of a light stimulus in conjunction with alarm substance, and (5) administration of light stimulus without alarm substance. The experimental series was repeated a few days later with a second group of six male minnows. The same minnows were not used in both sessions.

Six test minnows were transferred from a large holding tank to a test aquarium and left undisturbed for 24 hr. For each subsequent test (1–5 above), test fish were transferred to a new test aquarium containing fresh dechlorinated water. Transfers of fish between aquaria were conducted carefully so as not to release alarm pheromone or transport it from one tank to another. All six members of each group were placed in a 300-ml water-filled beaker to be rinsed free of any pheromone before being placed into the treatment aquarium. This was done by pouring off much of the water from the beaker and replacing it with fresh dechlorinated water. This procedure was repeated three times, after which the fish were released into the aquarium (Hall and Suboski, 1995). Curtains, lights, and remote administration tubes were prepared prior to transferring the minnows to the testing aquaria.

The schedule of pre- and poststimulus observations were based on that used by Suboski et al. (1990). Baseline observations were made at time zero (stimulus presentation) minus 9, 6.25, 4, 2.25, 1, and 0.25 min and at 0 min. Poststimulus observations were made at time zero plus 0.25, 1, 2.25, 4, 6.25, 9, 12.25, 16, 20.25, 25, 36, 49, 64, and 81 min.

Scoring. For all treatments, scan sampling performed by a single observer determined the vertical location of individual fish at each observation period. Each fish was assigned a score corresponding to its vertical location. A fish in the lowest level (level 1) was assigned a score of 0. Fish located within layers 2, 3, and 4 (layer 4 is the top) were assigned scores of 0.5, 1.5, and 2.5, respectively. In this manner, the mean score of the six fish at each time interval was determined. The means for each of the seven prestimulus intervals were averaged to give a single baseline mean. This baseline mean was used to convert the mean score of each of the 14 poststimulus time interval means into an index of vertical distribution. Each poststimulus observation mean was divided by the sum of itself plus the baseline mean. The resulting vertical distribution index could thus range from zero (all six fish in the lowest level) to 0.5 (the poststimulus mean is equal to the baseline mean) to 1.0 (the poststimulus mean is greater than baseline). In this type of design, the group of fish, instead of the individual fish, is the experimental unit.

Qualitative observations regarding individual fish posture, shoaling, swimming, and rate of fin flicking were also made at each observation period.

Alarm Status and Analysis. A group of fish was considered alarmed if the first six of the 14 poststimulus means were below baseline (Hall and Suboski, 1995). Binomial statistical tests were used to determine the P value for the number of observations below the prestimulus mean for each treatment (Hall and Suboski, 1995). The probability of six sequential vertical indices randomly occurring below the prestimulus mean is $P = 0.0078$ (binomial test).

RESULTS

Treatment 1—Water with No Alarm Substance. Injection of DDW via the 1-mm-diameter polyethylene tube resulted in no observable change in vertical distribution of either test group (binomial test, group 1 $P = 0.4240$, group 2 $P = 1.0000$, binomial test; Figure 1). No changes in the distribution or behavior of the minnows was observed. The result of this control treatment eliminates the possibility that water currents or temperature changes caused by injection of a solution resulted in any behavioral response in the minnows.

Treatment 2—Alarm Substance Alone. Injection of alarm substance provoked an alarm response. For both groups, injection of alarm substance resulted in vertical distributions lower than the pretreatment mean for all 14 of the post-treatment observation periods (binomial test, both groups $P = 0.0001$; Figure 2). Alarm substance was, thus, effective in inducing a strong alarm response.

Administration of alarm substance corresponded with an increase in short, rapid swimming or darting motions followed by increased shoaling, increased frequency and rapidity of pectoral fin motion, and cessation of substrate probing.

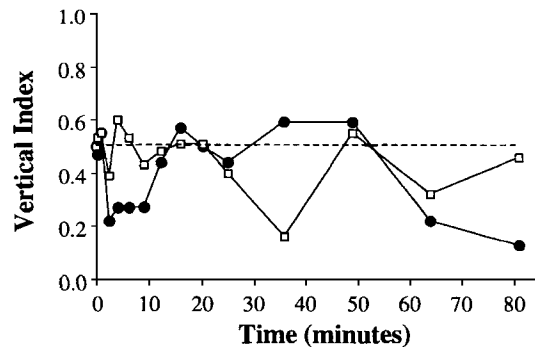


FIG. 1. Vertical distribution of a shoal of six fathead minnows over 81 min following the introduction of distilled deionized water (at time zero). Vertical distributions are relative to the baseline mean established during the 9 min prior to stimulation. Each line represents a replicate experiment. Group 1, circles; group 2, squares.

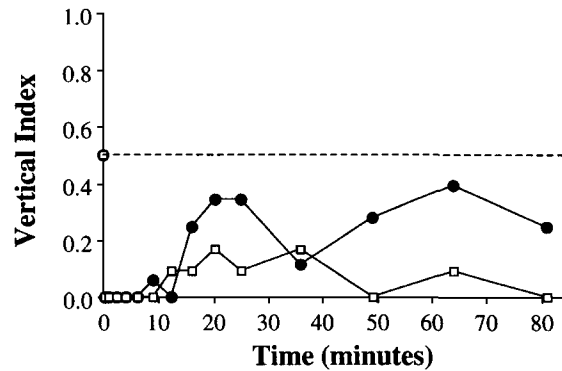


FIG. 2. Vertical distribution of a shoal of six fathead minnows over 81 min following the introduction of distilled deionized water containing alarm pheromone (at time zero). Vertical distributions are relative to the baseline mean established during the 9 min prior to stimulation. Each line represents a replicate experiment. Group 1, circles; group 2, squares.

Treatment 3—Light Stimulus Alone. This treatment was necessary to demonstrate that red light was initially an irrelevant stimulus that would not induce alarm behavior. Neither group displayed alarm behavior in response to the light (binomial test, group 1 $P = 0.1796$, group 2 $P = 1.0000$; Figure 3). Initially, minnows congregated near the light in loose shoal formation for the first

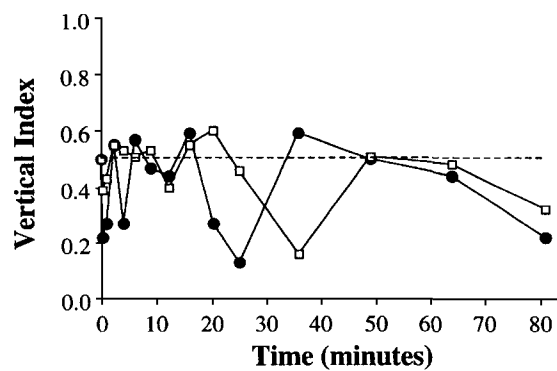


FIG. 3. Vertical distribution of a shoal of six fathead minnows over 81 min following illumination of a red light for 3 min (at time zero). Vertical distributions are relative to the baseline mean established during the 9 min prior to stimulation. Each line represents a replicate experiment. Group 1, circles; group 2, squares.

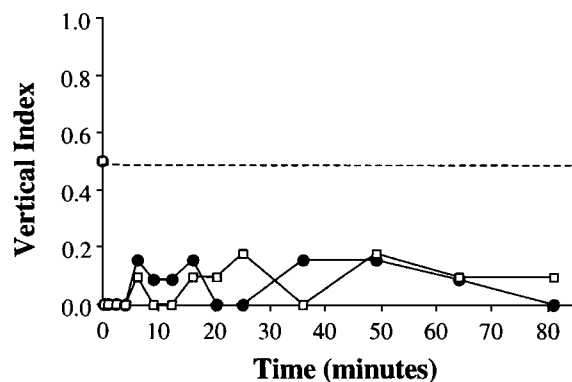


FIG. 4. Vertical distribution of a shoal of six fathead minnows over 81 min following introduction of distilled deionized water containing alarm pheromone plus illumination of a red light for 3 min (at time zero). Vertical distributions are relative to the baseline mean established during the 9 min prior to stimulation. Each line represents a replicate experiment. Group 1, circles; group 2, squares.

three observation periods before dispersing to a more even distribution. The light however, remained illuminated for the first six observation periods, indicating that after the initial visual inspection, the minnows perceived no threat from the novel stimulus.

Treatment 4—Alarm Substance and Light Stimulus. This treatment was required to demonstrate that alarm behavior occurred at the time of conditioning. As with treatment 2, alarm behavior was noted in both aquaria (binomial test, both groups $P = 0.0001$; Figure 4). All four qualitative behaviors in response to alarm pheromone alone occurred in response to the combined stimulus of red light plus alarm pheromone.

Treatment 5—Light Stimulus Alone. This treatment tested if a single tandem exposure to red light and alarm pheromone resulted in learned recognition of red light as an indicator of predation risk. Group 1 showed vertical indices below the prestimulus mean for all observation periods (binomial test, $P = 0.0001$; Figure 5). Group 2 displayed alarm behavior for the first eight observation periods (binomial test, $P = 0.0129$; Figure 5). This red light treatment resulted in the same spatial organization of minnows as when pheromone was added (treatments 2 and 4), except the shoaling response was delayed.

As with treatments 2 and 4, increased rates of fin flicking and increased shoal cohesion were observed. However, the intensity and duration of these behaviors were less than the responses in treatments 2 and 4. In addition, no minnows were observed to cross in front of the light while it was illuminated.

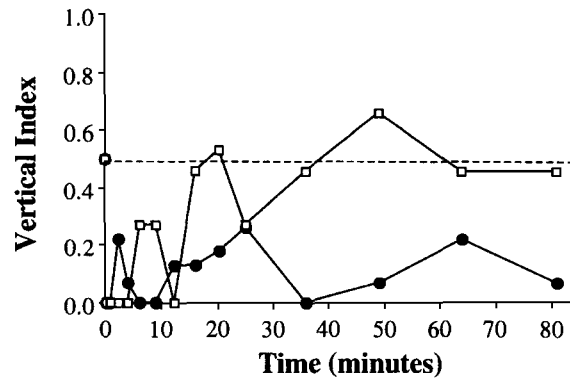


FIG. 5. Vertical distribution of a shoal of six alarm pheromone plus red light preconditioned fathead minnows over 81 min following illumination of a red light for 3 min (at time zero). Vertical distributions are relative to the baseline mean established during the 9 min prior to stimulation. Each line represents a replicate experiment. Group 1, circles; group 2, squares.

DISCUSSION

This study shows that a single simultaneous exposure to alarm pheromone and a novel stimulus conditions an alarm response in fathead minnows (Chivers and Smith, 1998). This is the first demonstration of a learned response to non-biological visual stimulus in fathead minnows and an important confirmation of a similar finding in zebra danios, *Brachydanio rerio* (Suboski et al., 1990; Hall and Suboski, 1995).

This study and others (Suboski et al., 1990; Chivers and Smith, 1994b, 1995; Hall and Suboski, 1995) underscore the flexibility of this type of learning. The learning mechanism confers upon individual minnows the ability to learn to recognize indicators of predation risk across a broad range of spatial and temporal scales where predator diversity and abundance can be expected to vary widely. This occurs after a single simultaneous encounter with alarm substance and a novel chemical or visual stimulus. Although it is possible to invoke a number of scenarios where this learning paradigm would be maintained by natural selection, if minnows can be easily tricked into learning to respond to nonbiological or irrelevant stimuli, then this learning paradigm may represent a fitness liability. In nature, a response to irrelevant stimuli would carry significant cost. This leads to the intriguing question of how minnows assign a hierarchy of salience to visual or chemical stimuli that may be present at the time of alarm pheromone release.

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