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Fathead minnows avoid conspecific and heterospecific alarm pheromones in the faeces of northern pike

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Groups of fathead minnows *Pimephales promelas* were tested to determine if they avoided areas of a test tank labelled with the faeces of a predator (northern pike, *Esox lucius*) which had recently been fed minnows, brook sticklebacks *Culaea inconstans*, or swordtails *Xiphophorus helleri*. Minnows exhibited a fright reaction upon presentation of sponges labelled with faeces, when the pike had consumed minnows or sticklebacks, but not swordtails (which lack alarm pheromones). The fright reaction was characterized by increased shoal cohesiveness and increased dashing and freezing behaviour. Minnows avoided the area of the tank containing the faeces from pike on diets of minnows or sticklebacks, but not from pike fed a diet of swordtails. These data demonstrate that: (1) minnows actively avoid the faeces of pike fed minnows or brook sticklebacks, and (2) minnows exhibit a fright reaction to the faeces of a pike fed brook sticklebacks.

Key words: brook sticklebacks; alarm pheromone; alarm substance; Schreckstoff; localized defaecation; predator avoidance.

INTRODUCTION

Fathead minnows *Pimephales promelas* Rafinesque possess epidermal club cells which release an alarm substance or Schreckstoff (AS) when the minnow is mechanically damaged during an attack or capture by a predator (Smith, 1986, 1992). When detected by conspecifics [and some heterospecifics such as brook sticklebacks, *Culaea inconstans* (Kirtland); Mathis & Smith, 1993*a*], this pheromone elicits what can be considered as a stereotypic fright response, characterized by increased shelter use, freezing, dashing and/or shoaling (Heczko & Seghers, 1981; Lawrence & Smith, 1989; Mathis & Smith, 1993*b*).

Mathis & Smith (1993*b*, *c*) demonstrated that minnows from a population allopatric from northern pike *Esox lucius* L. respond to the chemical stimuli of a pike with a fright response if the pike had recently consumed minnows, but not swordtails *Xiphophorus helleri* Heckel or breeding male fathead minnows (which lack AS cells; Smith, 1973, 1976). Swordtails are non-ostariophysians, and hence lack AS-producing epidermal cells (Mathis & Smith, 1993*b*). The use of these two controls allowed Mathis & Smith (1993*c*) to conclude that minnows react to the presence of conspecific AS in the diet of the pike, rather than simply to the chemical cues of a fish predator. Brown *et al.* (1995) demonstrated that AS (or some metabolite of the alarm pheromone, hereafter referred to simply as alarm pheromone or AS) is contained within the faeces of pike which had fed on minnows and that it elicits a stereotypic fright reaction by pike-naive minnows. In addition, Brown *et al.* (1995) demonstrated that pike localize their defaecation

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away from their home/foraging areas, possibly allowing them to avoid labelling the area as a risky habitat to the minnows. If minnows actively avoid areas labelled with pike faeces, then this would suggest a possible adaptive value to the observed behaviour of pike localizing their defaecation. It remains unknown if fathead minnows actively avoid areas which have been ‘ labelled ’ with pike faeces.

This study was conducted to determine if fathead minnows actively avoid areas containing faeces of pike which have been fed minnows, brook sticklebacks or swordtails.

METHODS

TEST FISH

Fathead minnows and brook sticklebacks were collected from Marshy Creek, in southern Saskatchewan. Marshy Creek drains into Redberry Lake, a large saline evaporation basin, devoid of piscivorous fish species (Mathis & Smith, 1993*a*). Minnows were maintained in an 18 000 l, outdoor pool for a period of at least 4 weeks prior to testing. Sticklebacks were held in a 300-l stream channel at approximately 18° C and a 14 h : 10 h light : dark cycle for at least 2 weeks prior to use. Minnows were fed twice daily with Tetramin flakes. Sticklebacks were fed daily with frozen brine shrimp *Artemia franciscana* Kellogg. Swordtails, which were obtained commercially, were held in 37-l aquaria under a 14 : 10 light : dark cycle and fed daily with Tetramin flakes. Minnows ($n=96$) measured 4·28 ± 0·71 cm (mean ± S.D.) standard length at testing.

STIMULUS PREPARATION

To collect faeces stimulus solutions, three pike (19·5, 18·8 and 18·2 cm S.L.) were fed approximately 4 to 4·5 ml (volume measured by volumetric displacement in water) of fathead minnows, brook sticklebacks or swordtails once every 4 days for a total of three feedings.

One hour after the final feeding, pike were removed from their holding tanks and rinsed in dechlorinated tap water (to remove any remaining scales or skin) and returned to identical holding tanks containing fresh dechlorinated tap water. Eleven hours later (12 h after final feeding), they were transferred to plexiglas stimulus collection chambers (described in Mathis & Smith, 1993*b*, *c*) measuring 26 × 8 × 8 (h) cm and filled with 1200 ml of dechlorinated tap water. The stimulus collection chambers were aerated but not filtered.

Thirty-six hours after placing the pike in the stimulus chambers, they were removed and all faeces in the chambers were collected with a siphon and vacuum filtered through 20 μm filter paper. All material left on the filter was suspended in 100 ml of glass-distilled water.

Cellulose sponge, cut into 2 cm cubes, was threaded on to pieces of stainless steel wire 8 cm in length. Sponges were saturated in one of three stimulus solutions: pike fed minnows (FHM-diet), sticklebacks (STB-diet), or swordtails (SWT-diet) and frozen in plastic bags at approximately − 20° C until testing. Additional control sponges were prepared by saturating sponges in glass-distilled water and were also frozen until testing.

TEST TANK

Three identical 500-l glass aquaria (183 × 49 × 56 cm) were used as test tanks. The floor of each tank was covered with a 4 cm deep layer of silica sand. The tanks were divided into three equal sections with grease-pencil marks on the exterior of the tanks.

A 2 m bar was used to suspend three, 55 cm lengths of 0·5 cm diameter rods. The rods were positioned at each end (1 cm from the end walls) and in the centre of the tank.

Sponges were tied to the ends of the rods with stainless steel wire such that they were positioned approximately 0·5 cm above the substrate.

EXPERIMENTAL PROTOCOL

Each trial lasted 3 days. Four minnows, matched for size, were placed in the test tank and allowed a 48 h acclimation period (days 1 and 2). On day 3, the behavioural data were recorded. Four, 10-min observation periods (pre 1 to 4) were conducted 1 h apart, beginning at 08.00 hours. Immediately after the fourth observation (pre 4), the stimulus sponges were placed in the tank. Three sponges were presented simultaneously, one sponge with faeces from pike fed minnows, sticklebacks or swordtails and two sponges saturated in glass-distilled water. The faeces sponge was always presented at an end section of the tank since minnows were observed typically in either end section, rarely in the central section. The faeces sponge was placed in the end section of the tank where the minnows had spent the majority of their time (i.e. $>60\%$). In the event that the minnows spent nearly equal amounts of time in either end section over the four pre-stimulus observation periods, the location of the faeces sponge was chosen randomly.

Three minutes after the introduction of the sponges, the post-stimulus observations began. The duration of the observation periods and the data recorded were the same as the pre-stimulus observations. Four post-stimulus observations were recorded, 1 h apart.

During all observations, area use and a shoaling index were recorded every 30 s. Area use was recorded as the number of minnows in each of the three sections of the test tank, giving a score out of 80 for each observation period (four minnows $\times$ 20 intervals per observation period). The shoaling index (further described in Mathis & Smith, 1993*d*) ranged from 1 to 4 and was assigned as follows: (1) no minnow within 1 body length of another; (2) two minnows within 1 body length of each other and the other two more than 1 body length from their nearest neighbour; (3) three minnows within 1 body length or two groups of two minnows within 1 body length of each other but the groups separated by at least 1 body length; or (4) a single group with all four minnows within 1 body length of another minnow. In addition, the presence or absence of two components of a stereotypic fright response in fathead minnows were also recorded: (1) dashing—very rapid, apparently disorientated swimming and, (2) freezing—the cessation of movement where the fish drops to the substrate and remains immobile for a minimum of 30 s. Since individual minnows were difficult to identify, the presence of either dashing or freezing was recorded if it was performed by any one of the four minnows in the group.

STATISTICAL ANALYSIS

Area use for the section of the test tank containing the faeces sponge was compared for the pre 4 and post 1 observation periods (i.e. immediately before and after the sponges were introduced; ‘ immediate response ’) using a Mann–Whitney U test (Siegel, 1956). In addition, the overall use of the section of the test tank containing the faeces sponge was examined by comparing the total use for the first three pre-stimulus observations (pre 1 to 3) *v.* the use for the final three post-stimulus observations (post 2 to 4; ‘overall response’) using a Mann–Whitney U test. Similar comparisons were made for the shoaling index data. For the overall shoaling response, the median of the first three pre-stimulus observations was compared to the median of the final three post-stimulus observation periods. The occurrence of dashing and freezing was compared using a Fisher’s exact probability test (Siegel & Castellan, 1988). Each group of minnows was tested in only one trial, with each group being considered a single replicate. A total of eight groups of four minnows were tested for each treatment condition ($n=96$ minnows).

RESULTS

Minnows avoided the area of the test tank labelled with the faeces of a pike which had been fed fathead minnows or brook sticklebacks. For the immediate

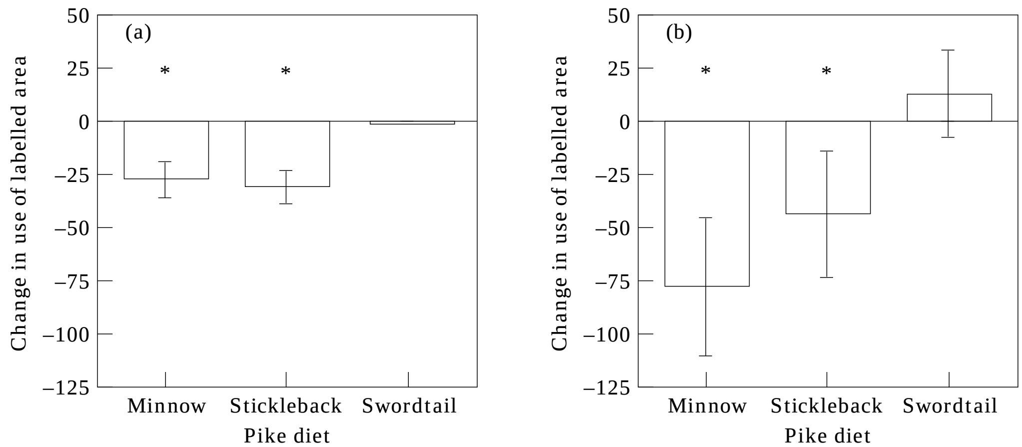

FIG. 1. Mean (± 1 S.E.) change in use of the labelled area for each of the three treatment conditions for the immediate (a) and overall (b) responses. n = eight replicates of four minnows each per treatment condition. *Significant differences at $P \leq 0{\cdot}05$. Negative scores indicate area avoidance. Means ± S.E. are used to illustrate the trends in the data only; data were analysed using non-parametric tests, see text for details.

response, minnows exhibited a significant decrease in the use of the section containing the minnow (FHM) or stickleback (STB) stimulus sponges (Mann–Whitney U, FHM-diet $Z = -2{\cdot}05$, $P \leq 0{\cdot}02$; STB-diet $Z = -3{\cdot}24$, $P \leq 0{\cdot}0001$), but not the swordtail (SWT) stimulus sponges [$Z = -0{\cdot}11$, $P = 0{\cdot}46$; Fig. 1(a)]. Minnows continued to avoid areas labelled with the FHM-diet and STB-diet sponges over the remaining observation periods (FHM-diet $Z = -2{\cdot}21$, $P \leq 0{\cdot}02$; STB-diet $Z = -1{\cdot}73$, $P \leq 0{\cdot}04$) but not for the SWT-diet condition [$Z = 1{\cdot}15$, $P = 0{\cdot}12$; Fig. 1(b)]. No difference was found between the decrease in area use for FHM-diet and STB-diet conditions in immediate ($Z = 0{\cdot}74$, $P = 0{\cdot}23$) or the overall comparisons ($Z = -0{\cdot}37$, $P = 0{\cdot}36$).

When the FHM-diet and STB-diet stimulus sponges were introduced to the tank, minnows exhibited a stereotypic fright response. The change in the shoaling index was significantly higher for the immediate response for both the FHM-diet and STB-diet conditions [Mann–Whitney U, FHM-diet $Z = -1{\cdot}68$, $P \leq 0{\cdot}04$; STB-diet $Z = -2{\cdot}52$, $P \leq 0{\cdot}006$, Fig. 2(a)]. No significant change in shoaling index was observed for the SWT-diet condition [Mann–Whitney U, $Z = -0{\cdot}79$, $P = 0{\cdot}21$, Fig. 2(a)]. For the overall comparison, no significant increases in the shoaling index were observed for any of the treatment conditions [$Z = -0{\cdot}32$, $-0{\cdot}53$ and $-0{\cdot}16$, $P > 0{\cdot}05$, FHM-diet, STB-diet and SWT-diet respectively, Fig. 2(b)].

Significantly more dashing was observed in the FHM-diet and STB-diet treatments than in the SWT-diet treatment (Table I). Significantly more freezing was observed in the STB-diet *v.* the SWT-diet conditions, but not in the FHM-diet *v.* SWT-diet comparison. While no significant difference was observed for freezing behaviour in the FHM-diet condition, the trend was in the predicted direction (Table I). No occurrences of dashing or freezing were observed outside of the first post-stimulus observation period.

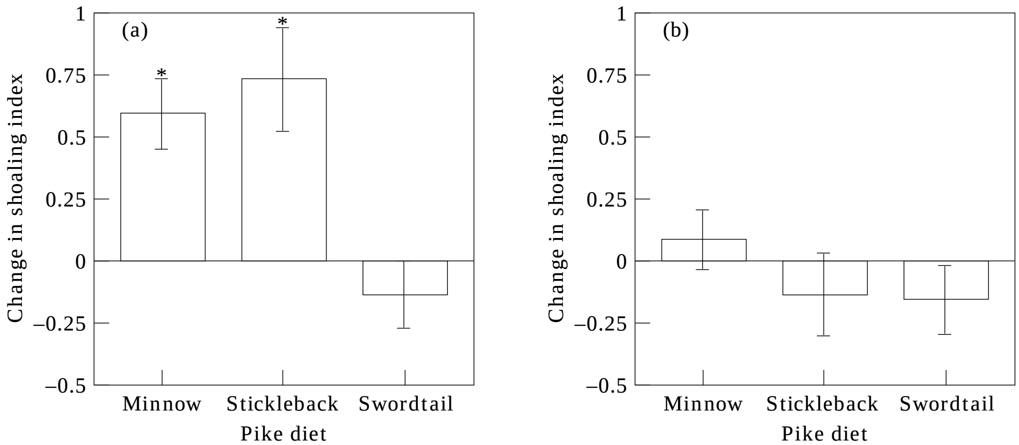

FIG. 2. Mean (±1 S.E.) change in shoaling index for each of the three treatment conditions for the immediate (a) and overall (b) responses. n = eight replicates of four minnows each per treatment condition. *Significant differences at $P \leq 0{\cdot}05$. Increased shoaling indicates a fright response, means ± S.E. are used to illustrate the trends in the data only; data were analysed using non-parametric tests, see text for details.

TABLE I. Comparison of the occurrence of dashing and freezing behaviours for FHM-diet and STB-diet trials, compared to the SWT-diet control

	Pike diet	Yes	No	Probability
Dashing	Minnow	6	2	$P \leq 0{\cdot}004$
	Swordtail	0	8	
Freezing	Minnow	4	4	$P \leq 0{\cdot}14$
	Swordtail	1	7	
Dashing	Stickleback	7	1	$P \leq 0{\cdot}001$
	Swordtail	0	8	
Freezing	Stickleback	6	2	$P \leq 0{\cdot}02$
	Swordtail	1	7	

Fisher's exact probability, see text for details.

DISCUSSION

Fathead minnows actively avoided areas labelled with the faeces of pike which had recently consumed minnows or brook sticklebacks, but not swordtails which lack an alarm pheromone. By responding to the alarm pheromone (or its metabolites) in the faeces of a predator, minnows may have another means by which to reduce their risk of predation. By avoiding high risk areas (areas containing alarm pheromone, Chivers & Smith, 1995) individual minnows can increase their fitness potential by (1) decreasing predation risk and (2) increasing time available for foraging and/or reproduction.

Minnows exhibited a stereotypic fright response only during the first post-stimulus observation period, as indicated by increased shoaling indices and increased occurrences of dashing and freezing behaviour. While minnows continued to avoid the area labelled with FHM-diet and STB-diet faeces during the remaining observation periods, the shoaling index and the occurrence of dashing and freezing behaviour did not differ from the SWT-diet control. This suggests that even though minnows no longer exhibited a fright reaction, they were still exhibiting area avoidance to the FHM-diet and STB-diet faeces. Two, non-mutually exclusive alternative explanations are possible. Initially, minnows could learn the location of the sponges, and avoid the risky area of the test tank. Alternatively, minnows could simply be habituating to the presence of the alarm pheromone. The latter explanation is unlikely, since a continued avoidance of the labelled area would not be predicted if the minnows habituated to the presence of alarm pheromone. Fathead minnows can learn the identity of water from a particular microhabitat when it is paired with alarm substance (Chivers & Smith, 1995), demonstrating that minnows are able to learn and retain locational cues.

Cross-species responses to alarm pheromones are common for closely related fishes (e.g. Pfeiffer, 1963; Smith, 1982; Smith *et al.*, 1991). Few studies however, have demonstrated cross-superorder responses. Chivers & Smith (1994) have shown that finescale dace [*Phoxinus neogaeus* (Cope) superorder Ostariophysi] respond to brook stickleback alarm pheromone. Mathis & Smith (1993*a*) have shown that brook sticklebacks (superorder Acanthopterygii) respond to fathead minnow AS. Fathead minnows and brook sticklebacks commonly share micro-habitats and frequently experience predation by the same predators (Whitaker, 1968; Becker, 1983). As such, we predict that fathead minnows should respond to brook stickleback alarm pheromone.

Mathis & Smith (1993*a*) did not detect a fright response by solitary fathead minnows to stickleback skin extract in a laboratory study. Chivers & Smith (1994), in a field experiment, found that significantly fewer fathead minnows were captured in experimental traps marked with stickleback skin extract than in control traps marked with distilled water, contradicting the results of Mathis & Smith (1993*a*). Chivers & Smith (1994) could not, however, rule out the confounding effects of interspecific social facilitation. The fathead minnows may have been attracted to the large number of sticklebacks in the control traps, as opposed to avoiding stickleback skin extract in the experimental traps. These data clearly demonstrate a cross-superorder response to heterospecific skin extract (i.e. brook stickleback of the superorder Acanthopterygii, fathead minnow of the superorder Ostariophysi).

The current study demonstrates that upon initial exposure to the stickleback alarm pheromone, minnows exhibit a stereotypic fright response, as indicated by area avoidance, increased shoal cohesiveness and the occurrence of dashing and freezing. By responding to heterospecific alarm pheromones, minnows can increase their predator detection and avoidance abilities, hence obtaining some of the same benefits as responding to conspecific alarm pheromones.

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