

Fathead minnows, *Pimephales promelas*, acquire predator recognition when alarm substance is associated with the sight of unfamiliar fish

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Abstract. To determine whether fathead minnows can learn to recognize potential predators through releaser-induced recognition learning, predator-naive minnows were simultaneously exposed to a neutral visual stimulus, the sight of a northern pike, *Esox lucius*, or a goldfish, *Carassius auratus*, paired with either minnow alarm substance (Schreckstoff) or water. Two days after this initial conditioning trial the visual stimuli were presented alone and minnows previously conditioned with alarm substance exhibited an appropriate anti-predator response, while those conditioned with water did not. The conditioned minnows were tested approximately 2 months later and again the minnows that were previously conditioned with alarm substance showed an appropriate anti-predator response, while those conditioned with water did not. A comparison of the reaction of minnows conditioned to a natural predator (the pike) and those conditioned to a non-piscivorous exotic (the goldfish) revealed a similar response intensity when tested 2 days after the initial conditioning trial. However, approximately 2 months after the conditioning trial, the reaction of minnows conditioned to pike was stronger than that of minnows conditioned to goldfish, indicating that learning may be constrained to favour a response to the natural predator. Minnows that were initially conditioned to pike did not show an anti-predator response to goldfish nor did minnows that were conditioned to goldfish respond to pike, demonstrating that the learned response was specific to the species used in the conditioning trials and not to any large fish. These results extend the known benefits to alarm-signal receivers.

Failure to recognize predators may increase the risk of predation, and defensive responses to non-predators may conflict with activities such as foraging and reproduction (Edmunds 1974; Milinski 1986; Lima & Dill 1990). In this study we examine whether prey fish learn to recognize potential predators by releaser-induced recognition learning, i.e. simultaneous exposure to aversive ('releasing') stimuli and neutral stimuli causing learned aversion to the neutral stimuli (reviews in Suboski 1990, 1992a, b).

Members of the superorder Ostariophysi (including fathead minnows) possess alarm substance, or Schreckstoff, in distinctive epidermal club cells (review in Smith 1992). The alarm substance, possibly hypoxanthine-3(N)-oxide (Pfeiffer et al. 1985), is released only by mechanical damage to the club cells. Once released, the alarm substance can be detected by other ostariophysans which then perform defensive behaviour patterns (i.e. a species-specific fright reaction, Smith 1992).

Göz (1941) was the first to show that the alarm substance can be used to condition a fright response to previously neutral stimuli. Blinded European minnows, *Phoxinus phoxinus*, exhibited a fright reaction to chemical stimuli from pike, *Esox lucius*, only after the pike attacked conspecifics in their presence (Göz 1941). Göz suggested that alarm substance released during the attack conditioned the blind minnows to respond to chemical stimuli from the pike in later tests. Subsequently, Magurran (1989) demonstrated that naive European minnows were conditioned to respond to chemical stimuli from pike or tilapia, *Tilapia mariae*, by exposing the minnows to the odours of pike or tilapia in conjunction with alarm substance. The learned response was stronger to pike odour than to the odour of non-predatory tilapia, leading Magurran (1989) to suggest that learning was constrained to favour a response to the natural predator (the pike). Fathead minnows (Chivers & Smith 1994) and zebra danios, *Brachydanio rerio* (Suboski et al.

1990) also learned to show fright reactions to previously neutral chemical stimuli (pike odour or morpholine), that were paired with alarm substance.

We hypothesized that alarm substance can also be used to condition naive fish to respond to visual stimuli. If a fish sees a neutral stimulus, and at the same time smells alarm substance, it might learn to associate the visual stimulus with danger. To test this hypothesis, we exposed predator-naive fathead minnows to the sight of pike (a natural predator) or goldfish, *Carassius auratus* (an exotic non-predator) and introduced alarm substance into the tank. During subsequent exposures of the minnows to the 'predator' stimuli, we tested the following hypotheses: (1) fathead minnows will exhibit a fright response to previously neutral 'predator' stimuli 2 days after the conditioning trials, (2) fathead minnows conditioned to one 'predator' stimulus will not show a fright response to the other 'predator' stimulus and (3) fathead minnows will exhibit a fright response to previously neutral 'predator' stimuli 2 months after the conditioning trials.

METHODS AND RESULTS

Collection and Maintenance

We collected fathead minnows from Marshy Creek in south-central Saskatchewan. Marshy Creek is in an enclosed basin with no piscivorous fish species. Minnows from this site are inexperienced with predatory fish in general and specifically have no experience with northern pike or goldfish. Mathis et al. (1993) demonstrated that minnows from this population do not recognize pike as predators by visual or chemical cues. We maintained the minnows at approximately 15°C in a 300-litre holding tank on a 14:10 h light:dark cycle, and fed them daily with commercial fish food.

We collected northern pike from Eagle Creek, a tributary of the North Saskatchewan river in south-central Saskatchewan. We maintained the pike at approximately 15°C on an LD 14:10 h cycle in 150-litre holding tanks and fed them one to two fathead minnows every 5 days.

We obtained goldfish (gold morph) commercially and maintained them in a 100-litre tank at approximately 15°C on an LD 14:10 h cycle, and fed them daily with commercial fish food.

Alarm Substance Stimulus Preparation

We prepared the alarm substance stimulus from five female and 10 male fathead minnows ($\bar{X} \pm \text{SD}$ fork length = 5.15 ± 0.29 cm). After killing the donor by a blow to the head (anaesthetic was precluded because reduction in activity, such as might be induced by anaesthetic contamination, is one criterion of a fright reaction), we removed a skin fillet from both sides of each fish (total area of skin collected = approximately 20.4 cm²). Immediately upon removal, we placed the skin samples together in 50 ml of chilled glass-distilled water. We then homogenized the skin samples and filtered the homogenate through glass wool to remove any solid particles. We diluted the homogenate with 350 ml of glass-distilled water (total volume = 400 ml) and froze the extract in separate 5-ml containers. As a control stimulus, we also froze separate 5-ml containers of glass-distilled water.

EXPERIMENT 1: CONDITIONING OF A FRIGHT REACTION TO VISUAL STIMULI

We placed experimental observation tanks side by side (37 litre: 50 × 30 × 25 cm) so that two adjacent tanks were separated by a removable opaque barrier. We filled the tanks with dechlorinated tap water and aerated them with single airstones located at the back of each tank. The tanks contained no filtration system, but we covered the bottom of each tank with a shallow layer of sodium zeolite chips to remove ammonia from the water. One tank of each pair contained a central shelter, a ceramic tile (9.8 × 20.0 cm) mounted on three cylindrical glass legs (5.5 cm in length). We attached a plastic tube to the airline of each tank containing a shelter to introduce alarm substance or water during the conditioning trials.

We arbitrarily selected 52 fathead minnows ($\bar{X} \pm \text{SD}$ fork length = 5.62 ± 0.33 cm) from the holding tank and randomly placed them into separate experimental tanks that contained shelters (i.e. one fish per tank). We used 11 pike ($\bar{X} \pm \text{SD}$ fork length = 21.4 ± 1.51 cm) one to three times as visual stimuli. We randomly assigned one pike to each tank after maximizing the time span between tests using the same pike (\bar{X} time between tests = 14.4 days). We also randomly assigned six

goldfish ($\bar{X} \pm \text{SD}$ fork length = 14.7 ± 0.88 cm) to the tanks, each being used four or five times as visual stimuli (\bar{X} time between tests = 8.0 days). Although the pike were longer than the goldfish, the goldfish were wider, and in fact, were larger than the pike in total surface area (mean surface area, excluding fins: front view, pike = 4.4 cm^2 , goldfish = 10.9 cm^2 ; side view, pike = 42.9 cm^2 , goldfish = 51.9 cm^2). We conducted observations at a mean water temperature of 21°C (range = 19–23) on an LD 14:10 h cycle, after the minnows had remained in the observation tanks undisturbed, except for daily feedings, for 3 days.

As a standard testing protocol we recorded the amount of time that each minnow spent beneath the shelter for 8 min prior to the stimulus presentation. After injection of a chemical stimulus (i.e. water or alarm substance) into each minnow tank, we immediately removed the barriers between the minnow tanks and the adjacent tanks containing a pike or goldfish (dye trials, conducted separately, indicated that it took 16 s for the stimulus to become distributed within the tanks). We observed each minnow for an additional 8 min to determine the amount of time it spent beneath the cover. During the post-stimulus period, we also recorded the presence or absence of dashing, a sudden burst of very rapid, apparently disoriented swimming (Mathis et al. 1993). Increased shelter use and the presence of dashing have been interpreted as components of the fright reaction in fathead minnows (e.g. Lawrence & Smith 1989; Mathis et al. 1993). The barriers between the tanks were replaced at the end of the trial.

On day 1 of the experiment, 26 minnow tanks received 5 ml of alarm substance and 26 minnow tanks received 5 ml of control water. Within each of the stimulus treatment groups, minnows in 13 of the tanks were exposed to pike and 13 were exposed to goldfish.

We repeated observations 2 days later (day 3) with the same protocol as above except that we did not inject chemical stimuli into the tanks. The minnows were exposed to the sight of the same fish as on day 1. Because a neutral chemical stimulus (i.e. water) that is presented in the same manner as a fright stimulus (chemical stimuli from pike which had eaten fathead minnows) may result in a fright reaction in fathead minnows (Mathis & Smith 1993), we removed the barrier between the tanks on day 5, exposing the minnows

to an empty tank. This treatment was a control for possible conditioning to barrier removal.

We used the Wilcoxon–Mann–Whitney test (Siegel & Castellan 1988; Mann–Whitney *U*-test, Siegel 1956) to compare cover use between the alarm substance and water treatment groups and a Fisher's exact probability test (Siegel & Castellan 1988) to compare the frequency of dashing between the alarm substance and water treatment groups. As there was a predicted direction of difference in all statistical comparisons, one-tailed statistical tests were performed, with $\alpha = 0.05$.

EXPERIMENT 1

Results

Day 1: conditioning trials (initial response to alarm substance)

Minnows simultaneously exposed to alarm substance and pike stimuli during conditioning trials significantly increased their use of cover ($W_x = 103$, $m = 13$, $N = 13$, $P < 0.001$) (Fig. 1) and exhibited significantly more dashing behaviour (Table I) than those exposed to water and pike stimuli. Similarly, minnows simultaneously exposed to alarm substance and goldfish stimuli during conditioning trials significantly increased their use of cover ($W_x = 113$, $m = 13$, $N = 13$, $P < 0.001$) (Fig. 2) and exhibited significantly more dashing (Table I) than those exposed to water and goldfish stimuli. Therefore, minnows exposed to alarm substance exhibited a fright response, while those exposed to water did not, regardless of whether the minnows were exposed to pike or goldfish stimuli.

Day 3: conditioned responses to pike and goldfish

During exposure to pike, minnows that had been conditioned to pike with alarm substance exhibited significantly greater increases in their cover use ($W_x = 136$, $m = 13$, $N = 13$, $P = 0.023$) (Fig. 1) and greater (but non-significant) increases (Table I) in their frequency of dashing than minnows conditioned with water. Similarly, the minnows that were conditioned to goldfish with alarm substance also showed significantly greater increases in cover use ($W_x = 134$, $m = 13$, $N = 13$, $P = 0.018$) (Fig. 2), and greater (but non-significant) increases (Table I) in their frequency of dashing than minnows that were conditioned with water.

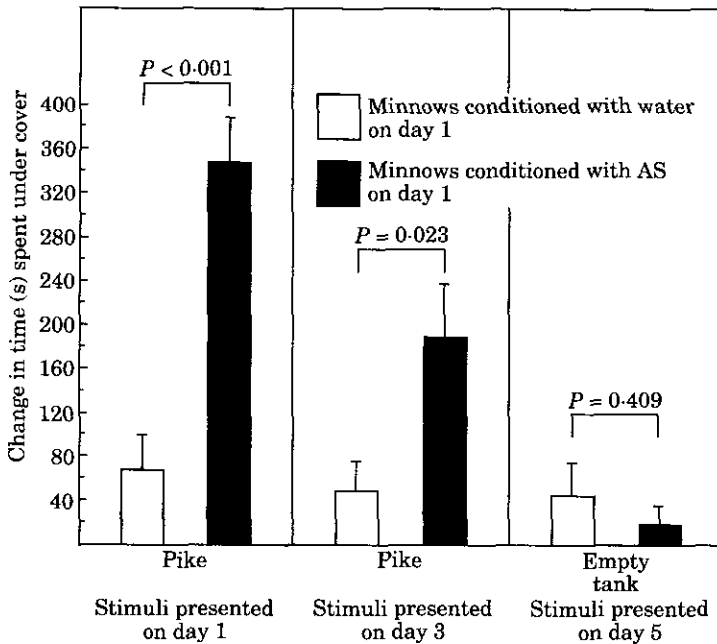


Figure 1. Mean (+SE) increase in time (s) spent under cover by minnows in experiment 1 initially exposed to the sight of a pike associated with control water or alarm substance (AS) on day 1 and subsequently exposed to the sight of a pike without chemical stimuli on day 3 or the sight of an empty tank on day 5.

Table 1. Number of minnows exhibiting dashing behaviour during initial conditioning trials (day 1), and during subsequent exposures (without chemical stimuli) to the sight of a pike or goldfish (day 3) or the sight of an empty tank (day 5)

Day	Stimulus used in conditioning	Stimulus presented subsequently	Number of minnows dashing	Number of minnows not dashing	P
Day 1	AS+Pike		9	4	0.024
	Water+pike		3	10	
Initial response to AS	AS+goldfish		10	3	0.024
	Water+goldfish		4	9	
Day 3	AS+pike	Pike	7	6	0.113
	Water+Pike	Pike	3	10	
	AS+goldfish	Goldfish	8	5	0.055
	Water+goldfish	Goldfish	3	10	
Day 5	AS+pike	Empty tank	1	12	0.297
	Water+Pike	Empty tank	3	10	
	AS+goldfish	Empty tank	2	11	0.703
	Water+goldfish	Empty tank	2	11	

AS: Alarm substance.

The use of cover by minnows that were conditioned to pike was not significantly greater than that of minnows conditioned to goldfish ($W_x = 163.5$, $m = 13$, $N = 13$, $P = 0.278$).

Day 5: conditioned responses to barrier removal?

Exposure to an empty tank did not lead to any significant differences in responses between

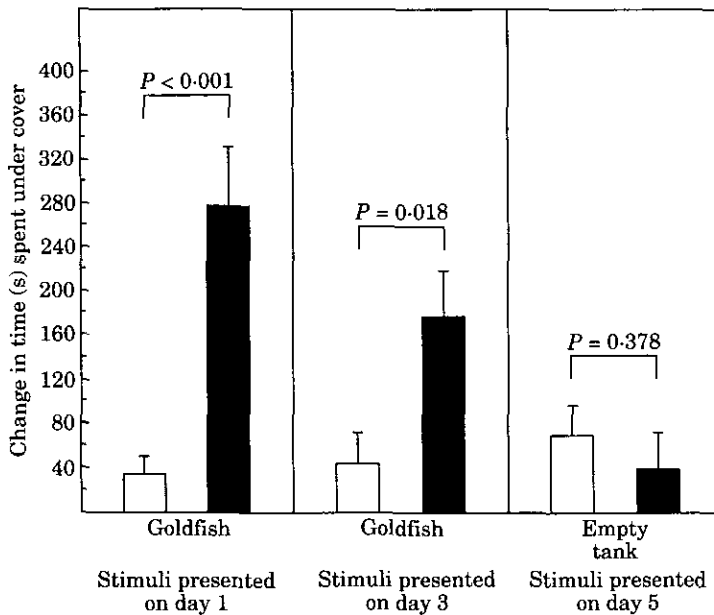


Figure 2. Mean (+SE) increase in time (s) spent under cover by minnows in experiment 1 initially exposed to the sight of a goldfish associated with control water or alarm substance on day 1 and subsequently exposed to the sight of a goldfish without chemical stimuli on day 3 or the sight of an empty tank on day 5. See Fig. 1 for symbol designations.

alarm-substance and water-conditioned minnows (Figs 1, 2, Table I).

EXPERIMENT 2: THE SPECIFICITY AND RETENTION OF THE CONDITIONED FRIGHT REACTION

We maintained the groups of minnows that had been tested in experiment 1 at approximately 15°C on an LD 14:10 h cycle in two 200-litre tanks for approximately 2 months ($\bar{X} \pm SD = 69 \pm 5.5$ days). Each tank contained a barrier between groups that was permeable to water and all groups shared water through a common filter.

We conducted tests on day 1 to determine whether minnows conditioned with pike in experiment 1 would show a fright reaction when exposed to the sight of a goldfish, and vice versa. On day 1, we exposed minnows that had been conditioned to pike in experiment 1 to a goldfish, and exposed minnows conditioned to goldfish to a pike. We conducted tests on day 3 to determine whether the conditioned fright

reaction observed in experiment 1 would be retained for an extended period (i.e. approximately 2 months). On day 3, we exposed minnows that had been conditioned to pike in experiment 1 to a pike, and exposed minnows conditioned to goldfish to a goldfish.

In experiment 2 we used six pike from experiment 1 as visual stimuli two to five times. We randomly assigned the pike to the tanks after maximizing the time span between tests using the same pike (\bar{X} time between tests = 8.0 days). We also randomly assigned the six goldfish used in experiment 1 to each of the tanks, with each being used three or four times as visual stimuli (\bar{X} time between tests = 13.1 days). We had too few tanks to house the 52 minnows individually, and therefore, we do not know whether the minnows were exposed to the same individual stimulus fish in both experiments. However, as we randomly assigned the pike and goldfish to the tanks, the chance that a minnow was exposed to the same individual fish in both experiments was equal across fish.

Experimental protocol and statistical comparisons were as in experiment 1.

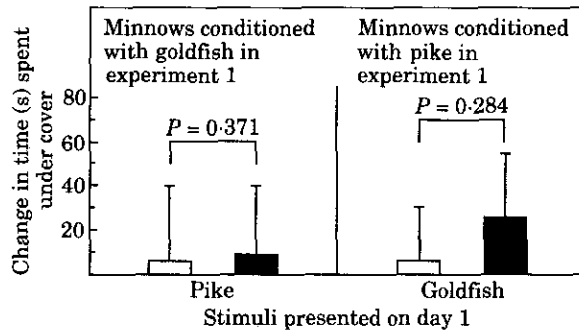


Figure 3. Mean (+SE) increase in time (s) spent under cover by minnows in experiment 2 that were exposed to the sight of a pike or goldfish during initial conditioning trials and subsequently exposed to the sight of the fish species that was not seen in the initial conditioning trials. See Fig. 1 for symbol designations.

Table II. Number of minnows conditioned to pike or goldfish that exhibited dashing behaviour when exposed to the sight of the species not used in the initial conditioning trials (day 1), and when exposed to the same species used in the initial conditioning trials (day 3). (Initial conditioning trials were conducted approximately 2 months earlier)

Day	Stimulus used in conditioning in experiment 1	Stimulus presented	Number of minnows dashing	Number of minnows not dashing	<i>P</i>
Day 1 Specificity of the conditioned response	AS+goldfish	Pike	2	9	0.466
	Water+goldfish	Pike	1	11	
	AS+pike	Goldfish	2	11	0.260
	Water+goldfish	Goldfish	0	12	
Day 3 Conditioned response to pike and goldfish (2 months after initial conditioning)	AS+pike	Pike	6	7	0.046
	Water+pike	Pike	1	11	0.003
	AS+goldfish	Goldfish	6	4	
	Water+goldfish	Goldfish	0	12	

AS: Alarm substance.

EXPERIMENT 2

Results

Day 1: specificity of the conditioned response

Exposure to the species to which the minnows had not previously been exposed did not lead to any significant differences between the alarm-substance and water-conditioned minnows (Fig. 3, Table II).

Day 3: retention of the conditioned response

During exposure to pike, minnows that had previously been conditioned to pike with alarm

substance exhibited significantly greater increases in both use of cover ($W_x=107$, $m=12$, $N=13$, $P=0.004$) (Fig. 4) and frequency of dashing (Table II) than minnows conditioned with water. Similarly, when minnows conditioned to goldfish were subsequently exposed to goldfish, the minnows conditioned with alarm substance exhibited significantly greater increases in both use of cover ($W_x=108.5$, $m=12$, $N=10$, $P=0.028$) (Fig. 4) and frequency of dashing (Table II) than minnows conditioned with water. Therefore, fathead minnows show appropriate anti-predator behaviour for at least 2 months following one exposure to alarm substance paired with the sight of a potential predator.

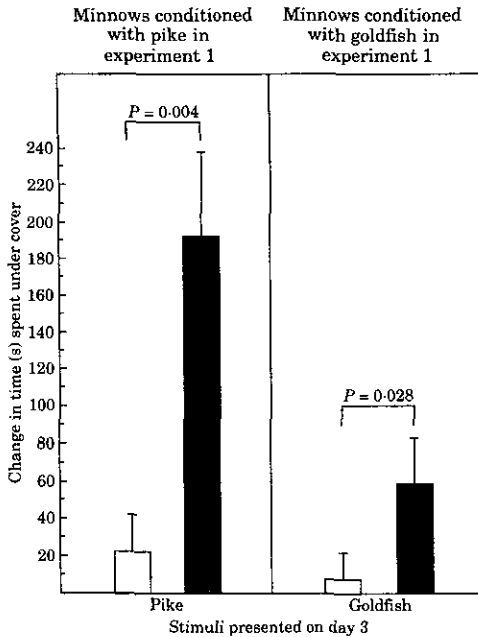


Figure 4. Mean (+SE) increase in time (s) spent under cover by minnows in experiment 2 that were exposed to the sight of a pike or goldfish during initial conditioning trials and subsequently exposed to the sight of the fish species that was seen in the initial conditioning trials. See Fig. 1 for symbol designations.

The use of cover by minnows conditioned to pike and subsequently exposed to pike was significantly greater ($W_x=92$, $m=10$, $N=13$, $P=0.044$) than for minnows conditioned to goldfish and subsequently exposed to goldfish, indicating that learning may be constrained to favour a response to the natural predator.

DISCUSSION

The results of this study demonstrate that a single exposure to minnow alarm substance can condition a fright response to previously neutral visual stimuli, the sight of a natural predator, the pike, or a non-piscivorous exotic, the goldfish. To our knowledge this is the first study to demonstrate that alarm substance can be used to condition a fright reaction to natural visual stimuli. Previous conditioning studies with alarm substance (Göz 1941; Magurran 1989; Suboski 1990; Chivers & Smith 1994) have used chemical stimuli

(i.e. fish odours or morpholine). Similar releaser-induced recognition learning has been reported for other animals (reviews in Suboski 1990, 1992a, b). For example, Lorenz's (1952) observations of jackdaws, *Corvus monedula*, suggested that the young birds learned to recognize predators by observing the mobbing response of adults. Similarly, in experimental studies, juvenile blackbirds, *Turdus merula*, learned to recognize predatory little owls, *Athene noctua*, by observing adult mobbing (Curio et al. 1978), and juvenile squirrel monkeys, *Saimiri sciureus*, learned to avoid stuffed toy animals (a snake or tiger) when alarm calls were given in association with the stuffed toy (Herzog & Hopf 1984).

We have demonstrated that the conditioned fright response of fathead minnows to visual stimuli is retained for at least 69 days. This length of time is substantial considering that the life span of fathead minnows is rarely over 2–3 years (Scott & Crossman 1979). Little is known concerning the retention of learned responses in fish. Bluntnose minnows, *Pimephales notatus*, can retain a learned attraction or avoidance response to stream odours for 6 weeks, even with weekly extinction testing (Hasler & Wisby 1951). Similarly, paradise fish, *Macropodus opercularis*, exposed to goldfish for a single 5-min period can recognize them after 3 months (Csányi et al. 1989). Although we do not know whether the learned response in our study is permanent, in nature fish may have the opportunity for continual reinforcement as they may frequently be exposed to alarm substance in association with the appearance of predators.

Our results also demonstrate that the conditioned fright response is specific to the species used in conditioning and not a general response to any large fish. This distinction is important because falsely recognizing a large fish as a predator may decrease time and opportunities for activities such as foraging and reproduction.

When tested 2 days after the conditioning trials there was no evidence that a natural predator (a pike) induced a stronger fright reaction than a non-piscivorous exotic (a goldfish). These results do not suggest an immediate constraint on learning as reported by Magurran (1989) for European minnows. In her study, the conditioned fright reaction of European minnows to chemical stimuli from tilapia (a non-piscivorous exotic) was less than the conditioned reaction of minnows to chemical stimuli from a pike (a natural predator)

when tested 2 days after the conditioning trials. However, when we tested fathead minnows approximately 2 months after the conditioning trials, we also found a constraint on learning: the reaction of minnows conditioned with goldfish was less than the reaction of minnows conditioned with pike.

Constraints on learning are well known (reviews in Domjan 1983; Shettleworth 1984). Animals appear to have a predisposition to acquire those skills that have survival value. The different constraints exhibited by fathead minnows and European minnows raise the question as to whether these differences result from species differences or differences in the sensory modality (i.e. visual versus olfactory) used to condition the response (or possibly something else altogether). Shettleworth (1972) has demonstrated that the sensory modality used in conditioning may account for constraints on learning. In her study, domestic chicks, *Gallus gallus*, learned to avoid quinine flavoured water more easily when visual stimuli were used to condition the response than when auditory stimuli were used.

By demonstrating that minnow alarm substance can condition a fright response to previously neutral visual stimuli, our study extends the known mechanisms through which alarm substance may benefit the receivers of an alarm signal. An individual that has acquired the ability to recognize predators through alarm-substance conditioning may show a fright response upon subsequent encounters with the predator. Such a result may increase the survival of the individual. The fright response of the individual may also act to warn other prey in the vicinity (Verheijen 1956), and thereby increase the inclusive fitness of the individual through the process of warning close relatives (Sherman 1977). Warning other conspecifics may also benefit the signaller or its kin through reducing predator success, and thus discouraging future hunting in the signaller's home range (Trivers 1971). In addition, the response of the individual may act directly upon the predator to inform it that its presence has been detected and that an attack is unlikely to succeed (Högstedt 1983).

Through releaser-induced recognition learning, fathead minnows learn to show an appropriate anti-predator response to previously neutral visual stimuli (i.e. the sight of pike or goldfish) after the neutral stimuli are paired with minnow

alarm substance. Only a single exposure to alarm substance and the sight of a pike or goldfish is required to condition the fright response. Furthermore, this learned aversion to predatory stimuli is retained for at least 2 months and is a specific response to the predator used in the conditioning trials. Acquired recognition of predators may influence many future predator-prey interactions.

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