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Motion, not Shape, Facilitates Association of Predation Risk with Novel Objects by Fathead Minnows (*Pimephales promelas*)

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Abstract

Injury-released chemical cues are reliable indicators of predation risk among many aquatic taxa. When a novel, neutral stimulus is presented in tandem with chemical cues from an injured conspecific, an association is formed between the novel stimulus and apparent risk. Learned recognition of predation risk is well documented for fathead minnows, *Pimephales promelas*. When minnows detect alarm cues in nature they are also potentially exposed to multiple environmental stimuli, few of which are likely to be relevant indicators of risk. How do minnows discern among candidate stimuli potentially associated with predation risk? Two possibilities are shape and motion. In this study, individual piscivore-naïve minnows were presented simultaneously with conspecific chemical alarm cues and two stimulus objects. One object was a darkened tube with its long axis in the horizontal plane (fish-like). The second object was a black disk. Following introduction of chemical alarm cues, one of the objects was raised and lowered repeatedly. After a single conditioning trial, minnows associated risk significantly more with the previously moving object than the previously stationary object, as indicated by reduced activity. Object shape had no significant effect on response intensity in test trials. Our data suggest that minnows have been selected to form aversive responses to moving objects at a site of recent predation because movement is a more predictable indicator of predator identity than shape.

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Introduction

Predation is a major agent of selection shaping the evolution of life history and behavioral traits (Lima & Dill 1990). Effective anti-predator behavior accrues

high fitness benefits. Environmental cues signal the appropriate context and timing for maximal effectiveness of anti-predator behavior. In aquatic environments, publicly available chemical cues released from prey during predation events serve as indicators of predation risk (Wisenden 2000). A reliable indicator of risk is injury-released chemical cues from conspecific prey (Smith 1992; Chivers & Smith 1998). These cues indicate the presence of an actively foraging predator of conspecifics.

When prey detect chemical alarm cues they adopt anti-predator behaviors that reduce their probability of predation, thereby improving their chance of surviving to reproduce (Hews 1988; Mathis & Smith 1993; Stewart et al. 1999; Wisenden et al. 1999). Typical anti-predator responses include reduced locomotion, movement to the substrate, and seeking refuge (Chivers & Smith 1998). Close encounters with predators permit prey to learn to associate novel cues with predation risk (Brown & Godin 1999). Prey learn to associate risk with novel olfactory or visual stimuli when the novel stimulus is presented simultaneously with injury-released chemical cues (see Chivers & Smith 1998 for review).

Learned recognition of predation risk typically occurs in a single trial. Goldfish (*Carassius auratus*), an arbitrary water sample and a non-biological stimulus such as a red light or morpholine, can become associated with risk (Suboski et al. 1990; Chivers & Smith 1994, 1995; Hall & Suboski 1995; Yunker et al. 1999). The ease with which minnows learn to associate risk with novel stimuli raises the question of how they avoid forming aversive responses to irrelevant stimuli. The formation of anti-predator responses to irrelevant stimuli would carry fitness costs in terms of unnecessary reductions in foraging and reproduction (Lima & Dill 1990).

In the current study, we investigated learned association of predation risk by fathead minnows (*Pimephales promelas*) in response to two novel objects presented simultaneously with chemical alarm cues. We manipulated two aspects of the novel objects: motion and shape. Object motion may serve to trigger a learned association with risk because predators generally move during an attack and subsequent handling of prey. Minnows may more readily form an association of risk with objects elongated in the horizontal plane because piscivorous fish, particularly northern pike (*Esox lucius*), an important minnow predator, have this general shape.

Materials and Methods

We collected fathead minnows in late fall from a small pond near Moorhead, MN (46°48' N, 96°15' W) using conventional minnow traps. Minnows spawn in the spring and early summer and therefore were not in reproductive condition. The pond does not contain predatory fish. The minnows were maintained at approximately 17°C in 190-l aquaria maintained on a 12 h light : 12 h dark illumination cycle. They were fed twice daily with commercial flake food.

Experimental observation aquaria (38 l, 51 × 26 × 31 cm) were filled with dechlorinated tap water and aerated with single airstones. A second airline tube to

be used for stimulus injection was attached to the airstone such that water currents from the airstone quickly dispersed test stimuli. A thin layer of washed granite gravel lined the bottom of the tanks. Ceramic flowerpots were placed in each tank to provide a shelter. A grid of lines (cell dimensions 5×5 cm) was drawn on the end face of each test aquarium (26×31 cm) to be used for scoring activity. Test aquaria were visually isolated from each other by opaque partitions.

Juxtaposed to the long side of each test aquarium was a stimulus display aquarium filled with water and a thin layer of granite gravel. Each stimulus display aquarium contained two stimulus objects, which were used to test for the role of motion and shape in learned risk recognition. One object was a circular disk (5 cm diameter) made from a black plastic bottle cap. The second object was a clear plastic tube (10 cm long \times 1 cm diameter) with a blue cap, suspended horizontally, that contained red/black gravel (granite). The objects were tied to monofilament fishing line, which extended approximately 2 m from the tank, allowing objects to be manipulated without disturbance. By presenting test objects in a separate aquarium we controlled for any olfactory cues associated with each test object.

Individual adult minnows (5–7.5 cm, total length) were selected from holding aquaria and placed arbitrarily in separate experimental aquaria. Test conditions were identical to holding conditions (17°C, 12 h light : 12 h dark). Minnows were left undisturbed in the test aquaria, except for feeding times, for 2 d prior to testing.

Injury-released chemical cues used for alarm stimuli were prepared by humanely killing 11 adult fathead minnows ($\bar{x} \pm SE = 51.2 \pm 1.6$ mm) by cervical dislocation and carefully removing the skin from each side of the body. We collected a total of 51.8 cm² of skin. We placed skin fillets in chilled dechlorinated tap water, homogenized them to rupture alarm substance cells contained in the skin (Pfeiffer 1977; Smith 1992), filtered the resulting suspension through polyester floss to remove particulate tissue and then diluted the filtrate with dechlorinated water to a total volume of 518 ml. Thus, each 10-ml aliquot contained the amount of alarm substance in 1.0 cm² of minnow skin. The alarm stimulus was frozen in 10-ml aliquots at -20°C until needed. Freezing does not affect the potency of minnow alarm cues (Lawrence & Smith 1989).

The experiment consisted of paired condition and test trials, separated by 2 d. In the condition trials, we recorded activity as the number of times the test minnow crossed one of the grid lines, for 5 min prior to the presentation of stimuli (pre-stimulus period). Activity is a sensitive measure of anti-predator behavior (Lawrence & Smith 1989; Lima & Dill 1990).

Following the 5 min of pre-stimulus recording, we slowly injected 10 ml of minnow skin extract into the test aquarium over a 1-min period. The disk and tube in the stimulus display aquarium were at rest on the bottom of the display aquarium, in view of the test fish, during the pre-stimulus period. After injection of skin extract we raised them simultaneously into the middle of the water column. One object, determined at random, remained stationary in mid water, whilst the other object was gently raised and lowered 10 times (vertical range about 20 cm) by pulling and releasing its monofilament line. The stimulus objects

were allowed to gently settle on the bottom after the motion presentation was completed and remained in view of the test fish for the duration of the trial. Post-stimulus recording of activity continued for 5 min immediately after stimulus presentation.

Observations were repeated 2 d later in a test trial using the same protocol as described above, with the following exceptions: (i) no skin extract was injected into the test aquarium, and (ii) only one of the objects was presented – either the object that was previously moved or the object that was previously stationary. The object presented in each test trial remained at rest on the tank bottom during the pre-stimulus period in view of the test fish. For 1 min between observation periods the stimulus object was raised to mid column and held stationary, that is, it was not raised and lowered. After the stimulus presentation was completed, the object was gently lowered to the bottom of the display aquarium and remained in view of the test fish for the duration of the trial. Individual minnows were used for only one condition–test sequence.

We conducted 40 pairs of trials (paired conditioning and test trials) balanced equally among the four treatment combinations, that is, a 2×2 factorial design with two levels of conditioning stimulus (moving tube or moving disk) and two levels of test stimulus (tube or disk). Our response variable was the change in activity in response to stimulus presentation, that is, the difference between post-stimulus and pre-stimulus activity levels. Because this type of data typically deviates from normality, we used a non-parametric two-factor analysis of variance (Zar 1984) to evaluate the independent effects of the main factors in the model and their interaction. This type of ANOVA produces an H statistic, which is closely approximated by χ^2 . In addition, we used Wilcoxon Mann–Whitney tests (Siegel & Castellan 1988) to compare the effect of object shape on activity in conditioning and test trials. All test statistics report two-tailed probabilities.

Results

In the conditioning trials, fish decreased their activity level after the introduction of skin extract and presentation of either of the two stimulus objects (Fig. 1). There was no effect of the shape of the moving object on response intensity (Wilcoxon Mann–Whitney test $Z = 0.419$, $p = 0.67$). Response intensity by fish that would later be tested with the tube alone did not differ during conditioning trials from those that would later be tested with the disk alone (ANOVA, $H = 0.036$, $p > 0.75$), nor was there an interaction between shape and motion ($H = 0.059$, $p > 0.75$). In other words, differences in responses during the second exposure to the stimulus objects were not present during conditioning trials.

In subsequent re-testing with one stimulus object alone, minnows responded with an intense anti-predator response (decrease in activity) if the shape of the object matched the one that moved during the previous conditioning trial, independently of its shape (Fig. 2). The effect of previous motion on response intensity was significant ($H = 4.683$, $p < 0.05$), but the effect of shape on

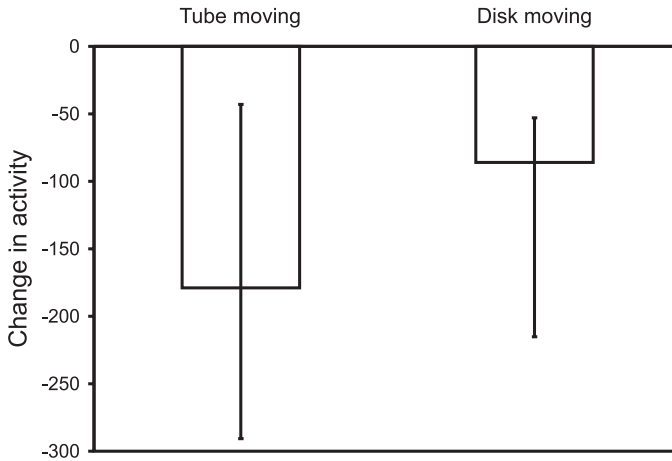


Fig. 1: Median (and quartile) change in activity (number of grid lines crossed per 5 min) in conditioning trials in response to chemical alarm cues present in minnow skin extract, a darkened horizontal tube and a black disk in an adjacent tank, one of which was in motion

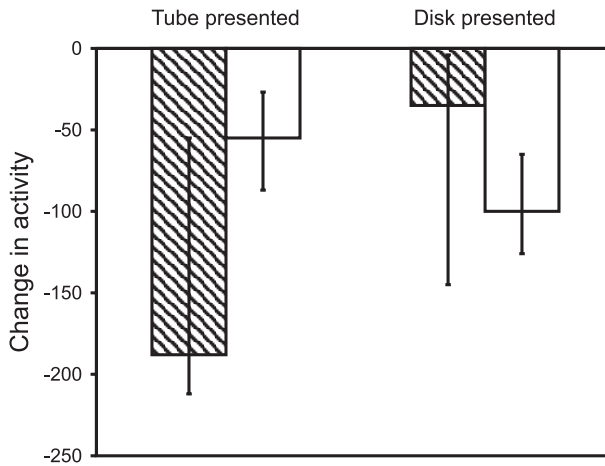


Fig. 2: Median (and quartile) change in activity (number of lines crossed in 5 min) in subsequent exposure to a stationary stimulus object (either tube or disk). Hatched bar denotes the response of fish presented with a moving tube and stationary disk in prior conditioning trials. Open bar denotes the response of fish presented with a moving disk and stationary tube in prior conditioning trials

response intensity was not ($H = 0.457$, $p = 0.50$). The interaction between motion and shape was not significant ($H = 0.124$, $p > 0.50$). Minnows learned to associate apparent risk with either the tube or disk with equal effectiveness, as long as the object was in motion during the introduction of alarm cues in the previous conditioning trial.

Discussion

Learned recognition of risk confers tremendous fitness benefits by allowing prey to rapidly adapt to temporal and spatial variation in predation risk. This form of associative learning is termed releaser-induced recognition learning (Suboski 1990). The rapidity (single-trial learning) and effectiveness of releaser-induced recognition learning is testimony to the importance of predation as a selection agent (Lima & Dill 1990).

Laboratory experiments have clearly demonstrated that prey, including minnows, learn to associate risk with a novel stimulus when the novel stimulus is presented simultaneously with chemical alarm cues (reviewed by Chivers & Smith 1998). However, when minnows detect chemical alarm cues in nature they are concurrently exposed to a plethora of other stimuli in the olfactory, visual, and other sensory modalities. Although any of these other stimuli could potentially be associated with risk, few of them are likely to be relevant to predation risk. The formation of aversive behavior to innocuous commonplace objects would be maladaptive because animals that hide in times of low predation risk will forage less and ultimately reproduce less than animals that respond only to relevant indicators of predation risk (Lima & Dill 1990). However, laboratory experiments demonstrate that the perception of predation risk can be associated with irrelevant stimuli (Magurran 1989; Suboski et al. 1990; Chivers & Smith 1994; Hall & Suboski 1995; Yunker et al. 1999). How do minnows in nature discern ecologically relevant indicators of risk from irrelevant ones?

We presented alarmed minnows with a fish-like tube and a disk. Even though the horizontal tube object used in this study was similar in outline to that of a potentially piscivorous fish, we detected no difference in intensity in response to each of these shapes in the conditioning trials. Minnow response in test trials to previously moving objects was not due to bias in conditioning trials. Based on a tube and a disk, we found no evidence for an internal inherent template for shapes representative of fish predators. We conclude that motion is a more reliable indicator of predator identity than shape. Minnow predators come in many shapes and sizes. One thing that they all have in common is that they move during acts of predation. Therefore, when chemical alarm cues are released, learned associations with predation risk are triggered by motion.

Anti-predator behavior has both environmental and genetic underpinnings (Magurran & Pitcher 1987). In past studies, the interaction between genetic and environmental influences has been portrayed as a learning constraint, where the recognition or learning of novel indicators of risk is less effective or does not persist for novel stimuli that are non-natural or allopatric from the test fish. For example, European minnows (*Phoxinus phoxinus*) can be conditioned to fear the odor of a natural predator, northern pike, or the odor of an allopatric non-predator, the cichlid *Tilapia mariae*, by pairing these odors with minnow skin extract Magurran (1989). Although minnows learned to respond aversely to the odor of both fish species, their response to pike odor was more pronounced.

Chivers & Smith (1994) conditioned fathead minnows to fear the sight of either northern pike or common goldfish by pairing the sight of either pike or goldfish with alarm substance. Minnows learned to associate risk with either species with equal effectiveness when re-tested 2 d later. When tested again 2 mo after the conditioning trials, the magnitude of the response to pike remained unchanged but the response to goldfish, while still present, had diminished significantly compared to that elicited by pike. During conditioning trials, the stimulus objects (live goldfish and live pike) were both in motion but differed in shape. In this case, motion may have facilitated learned recognition of risk to both species, whereas shape differences affected long-term retention of risk association. The relative roles of shape and motion in determining learned risk recognition are not well explored and likely involve internal sensory biases towards shapes historically correlated with predator species. The results of Chivers & Smith (1994) would lead us to predict that retention of the response to the tube object may ultimately have been greater than that for the disk object.

To eat, predators must approach, grasp, handle and swallow prey. Even predators that remain stationary while in ambush must engage in motion during a predation event. Motion, and not shape per se, is thus a predictable and reliable component of predation and may serve as an immediate releaser of learned risk association.

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