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ORIGINAL PAPER

Sound the alarm: learned association of predation risk with novel auditory stimuli by fathead minnows (*Pimephales promelas*) and glowlight tetras (*Hemigrammus erythrozonus*) after single simultaneous pairings with conspecific chemical alarm cues

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Abstract Fathead minnows, *Pimephales promelas*, and glowlight tetras, *Hemigrammus erythrozonus*, were tested for their ability to associate predation risk with novel auditory stimuli after auditory stimuli were presented simultaneously with chemical alarm cues. Minnows and tetras gave a fright response when exposed to skin extract (alarm cue) and an artificial auditory sound stimulus, but no response to water (control) and sound, indicating that they did not have a pre-existing aversion to the auditory stimulus. When retested with sound stimuli alone, minnows and glowlight tetras that had previously been conditioned with water and sound showed no response, but those that had been conditioned with alarm cues and sound exhibited antipredator behaviour (reduced activity) in response to the auditory cue. This is the first known demonstration of learned association of an auditory cue with predation risk, and raises questions about the role of sound in mediating predator-prey interactions in fishes.

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Introduction

Assessment of predation risk via chemical cues is widespread among aquatic organisms (Kats and Dill 1998; Wisenden 2003). In particular, fishes in the superorder Ostariophysi have been the focus of intense study (Smith 1992; Chivers and Smith 1998). Injury-released chemical alarm cues are released only in the context of a predation event, and thus, reliably indicate the presence of an actively foraging predator (Wisenden and Stacey 2005; Wisenden and Chivers 2006). Antipredator behaviour in response to these cues significantly reduces the probability of predation (Mathis and Smith 1993a).

Prey species use the presence of injury-released alarm cues to associate predation risk with stimuli correlated with the release of alarm cues (Chivers and Smith 1998; Brown and Chivers 2005). Remarkably, learned recognition of novel indicators of risk requires only a single opportunity for association; a phenomenon known as releaser-induced recognition learning (Suboski 1990). This learning mechanism allows minnows (and other fish groups) to learn to associate risk with kairomones (e.g. Chivers and Smith 1994a), dietary-based chemical cues emanating from the gut of a predator (e.g. Mathis and Smith 1993b; Chivers and Mirza 2001) or even water chemistry from a particular habitat (Chivers and Smith 1995). Acquired recognition and response to novel stimuli is not limited to chemical cues. Minnows associate risk with visual stimuli such as the presence of a predator (Chivers and Smith 1994b), the social behaviour of other frightened minnows (Suboski et al. 1990; Mathis et al. 1996) or even non-biological stimuli such as a flashing red light (Hall and Suboski 1995; Yunker et al. 1999) or moving objects (Wisenden and Harter 2001).

Fishes in the superorder Ostariophysi include minnows, characins, catfish, suckers and comprise some 64% of all freshwater fish species (Nelson 1994). Most Ostariophysans are also members of the series Otophysi, whose species possess a series of modified vertebrae (Weberian ossicles) that directly connect the inner ear to the swim bladder. The swim bladder serves as a resonating chamber and thus, these fishes are sensitive to a wide range of sound stimuli (50–2000 Hz) with low detection thresholds (Yan et al. 2000). The existence of specialised structures to detect auditory stimuli strongly suggests ecological and evolutionary significance of sound in aquatic habitats. Fish use sounds in intraspecific interactions in courtship and male-male territorial defence (Popper et al. 2003). It is not known if ostariophysans use auditory stimuli for the detection of predation risk or if they can learn to associate predation risk with sound.

Here, we report results from some simple tests for learned association of predation risk with sound stimuli, using fathead minnows, *Pimephales promelas*, and glowlight tetras, *Hemigrammus erythrozonus*, as test species.

Materials and methods

Fathead minnows

Thirty-six wild-caught fathead minnows (mean total length = 62.5 mm) were obtained from a commercial dealer and transported to the laboratory at the University of Minnesota's Itasca Biological Field Station in Itasca State Park, MN.

We used 24, 55 l plastic tubs (34 × 49.5 cm) with a transparent viewing pane at one end. All 24 tubs were filled with well water to a depth of 20 cm (water volume was approximately 34 l) and acclimatized to room temperature (22°C) before adding minnows. Fish were fed commercial flake food daily. The tubs were illuminated by natural sunlight. The photoperiod at the field station at the time of this experiment was 16 h:8 h L:D. The shallow depth, large surface area and low bioload allowed three minnows to be housed comfortably in each tub for several days without aeration or filtration. The sides of the tanks were covered with sheets of paper to prevent visual information from adjacent tubs potentially influencing fish behaviour. Grids of 24, 5 × 5 cm cells were drawn on the front (short) side of each tub to quantify minnow activity. Standard airline tubing ran into each tub through which chemical stimuli (alarm cue or water) could be injected surreptitiously. Two earbud headphones (Auriculares Esteruo, PL 1) were taped firmly against the external surface of the side walls of each tub. Adjacent tubs did not touch, to minimize the transmission of sound stimuli to adjacent tubs. To further reduce the chances of sound stimuli affecting the behaviour of fish in neighbouring tubs, fish were placed into every second tub so that a fishless (and soundless) tub separated all test tubs. Three minnows were placed into each of 12 tubs for the conditioning trials and transferred to the remaining 12 tanks for the test trials. The minnows were acclimatized to the test tubs for 3 days before testing began.

Test stimuli

Conspecific alarm cue was made from skin extract of six adult fathead minnows. We avoided any minnows that showed any outward sign of being male (nuptial tubercles on snout, dorsal mucus pad, banded coloration) because adult males lose their epidermal club cells during the breeding season (Smith 1976). Club cells are thought to contain an olfactorally conspicuous compound(s) that serves as the alarm cue (Pfeiffer 1977; Smith 1992; Chivers and Smith 1998; Wisenden and Chivers 2006). Minnows were killed by cervical dislocation with a razor blade (University of

Minnesota IACUC protocol 0304A46087). Skin extract (alarm cue) was obtained by making ten superficial cuts to the flank on each side, rinsing the minnow with 20 ml of well water into a petri dish, and collecting the solution in a syringe. Skin extract was prepared immediately before testing began and used within the hour to ensure cue potency.

A tone of 21.83 Hz (F note) was recorded from an electronic piano to a compact disc and played back on a Sony®, TCM-50DV tape recorder at volume setting seven into two earbud headphones taped to the external surface of the tub.

Experimental protocol

There were two treatment groups. Fish in the experimental group ($n = 6$) were conditioned by exposing them to the sound stimulus and alarm cues, and then later re-tested with the sound stimulus only. Fish in the control group ($n = 6$) were conditioned with the sound stimulus and water, and then re-tested with the sound stimulus only. We used reduction in activity as our measure of antipredator response (Lawrence and Smith 1989). Activity was recorded as the frequency with which the three fish crossed grid lines on the front viewing pane. After 8 min of pre-stimulus data, the sound stimulus was played for 30 s while simultaneously introducing 20 ml of water (control trials) or 20 ml of alarm cue (experimental trials) through the stimulus injection tube at a rate of 1 ml s^{-1}. A second 60 ml syringe filled with well water was used to flush the test cue from the injection tube into the test tub. Alarm cue dispersed in the tub by diffusion without aid of aerators or pumps. Starting at the beginning of the ninth minute, post-stimulus activity was recorded for 8 min. Within several hours of each conditioning trial, test fish were transferred to a fresh tub that did not contain any chemical alarm cue. The protocol for test trials (conducted the day after the conditioning trial) was the same as for the conditioning trials except that all groups received the sound stimulus and well water.

Glowlight tetras

Experimental set-up

Glow light tetras were obtained from a commercial supplier and housed at the MSUM aquatic research facility in a 185 l aquarium at 26–27°C and a 12 h:12 h L:D cycle. The tank had continuously filtered, dechlorinated tap water with a gravel substrate and the fish were fed daily with commercial flake food.

Two glowlight tetras were added to each of 12, 37 l glass test tanks. Test tanks had a gravel substrate and were filled with dechlorinated tap water and maintained at 26°C on a 12 h:12 h L:D cycle. A grid of 25 (5 × 5 cm squares) was drawn on the small pane of each test tank. Air-powered sponge filters were used to filter tank water and disperse chemical stimuli. A second length of plastic airline tubing, 2.5 m in length was wedged into the lift tube of the sponge filter for the purpose of stimulus injection.

Test stimuli

Skin extract was prepared before each conditioning trial for the experimental group using one glowlight tetra per trial. They were humanely sacrificed by cervical dislocation (MSUM IACUC protocol 04-T/R-Biol-01150N-R-C) and lightly sliced 5 times on each flank. The carcass was then placed in 30 ml of dechlorinated tap water and swirled for 30 s. This alarm cue was used within 30 min of preparation to ensure cue potency.

The auditory stimulus was a 400 Hz tone produced with a function generator (Goodwill Instrument Co., Ltd; GFG-8250A) and earbud headphones. One earbud headphone was taped to the external surface of the side wall on the lower right side of the tank in each group's trial.

Experimental protocol

Fish were observed twice, as in the first experiment. We conducted 15 pairs of trials using skin extract as the conditioning stimulus and 15 pairs of trials using water as the conditioning stimulus. Fish were allowed to acclimate in the test tanks for four days before we collected data. Conditioning trials comprised a 5 min prestimulus period, a 2 min stimulus introduction period in which 30 ml of glowlight skin extract or de-chlorinated tap water was injected. The tone was played through the earbud headphones during the entire 2 min stimulus presentation period. A 5 min post-stimulus period began immediately after the cessation of the 2 min stimulus presentation period. Test trials were conducted the same way except only the tone (but not any chemical cue) was introduced during the stimulus-presentation period.

Activity and vertical distribution were recorded in response to the tone. Antipredator responses involve reduction in activity and movement to the bottom (Lawrence and Smith 1989; Wisenden et al. 2004). Activity was scored as the number of times both fish crossed grid lines before and after the presentation of the tone and chemical stimulus (conditioning trials) or tone alone (test trials). Vertical distribution was scored as the horizontal row occupied by each fish every 10 s for 5 min before and after stimulus presentation. Fish in the horizontal row at the bottom were scored as a ‘1’, those in the next row as ‘2’, etc, and those in the surface row were scored as ‘5’. Vertical distribution score therefore ranged from 60 (both fish in the bottom row for all observations) to 300 (both fish in the surface row for all observations).

Data analysis

If test fish use chemical alarm cue to associate predation risk with sound stimuli, then we predicted that fish in the alarm cue treatment would respond to the tone stimulus in the test trials with antipredator behaviour (reduced activity, movement toward the bottom), whereas fish from the water treatment would not. We used Wilcoxon Mann–Whitney tests (Siegel and Castellan 1988) to compare the magnitude of the behavioural change between control and experimental treatment groups. All test statistics were interpreted using two-tailed probability distributions.

Results

Fathead minnows

Fathead minnows generally reduced activity when the sound stimulus accompanied the introduction of skin extract but not when the sound was accompanied by water (Fig. 1). However, one fish in the alarm treatment exhibited dashing behaviour, thereby greatly increasing its post-stimulus activity score. Consequently, the magnitude of the before-after reduction in activity in alarm trials was not significantly different from control trials (Wilcoxon Mann–Whitney test: $z = 1.44$, $p = 0.150$). Nevertheless, those fish that had received skin extract when they heard the sound the first time responded with reduced activity relative to control trials when the sound stimulus was replayed the following day ($z = 2.002$, $p = 0.046$, Fig. 1).

Glowlight tetras

In conditioning trials, glowlight tetras in alarm cue trials significantly reduced activity relative to those in control trials (Wilcoxon Mann–Whitney test: $z = 2.34$, $p = 0.010$, Fig. 2) and spent significantly more time near the bottom than fish in control trials ($z = 1.76$, $p = 0.039$, Fig. 3).

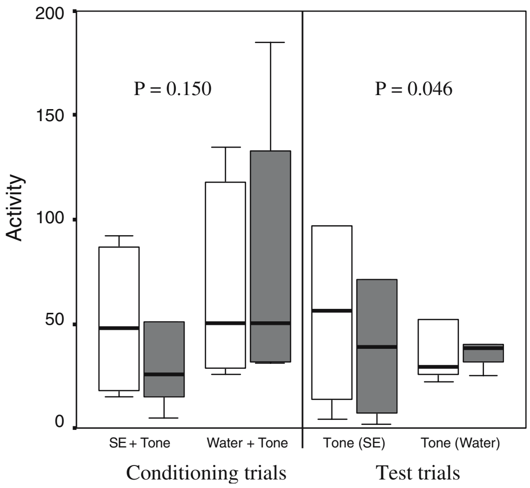

Fig. 1 Median (± quartiles) activity of fathead minnows before (open) and after (hatched) introduction of test stimuli. Labels for stimuli: SE + Tone = Conspecific skin extract and electronic tone; Water + Tone = water and electronic tone; Tone (SE) = Tone stimulus alone played to fish that previously experienced skin extract and the tone; Tone (Water) = Tone stimulus alone played to fish that previously experienced water and the tone. *P* values above bars are from Wilcoxon Mann–Whitney tests comparing the magnitude of the change in trials using SE versus trials using water

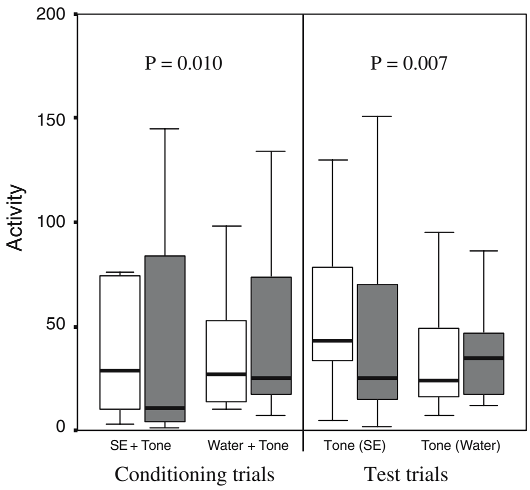

Fig. 2 Median (± quartiles) activity of glowlight tetras before (open) and after (hatched) introduction of test stimuli. Labels for stimuli and p values are described in the legend for Fig. 1

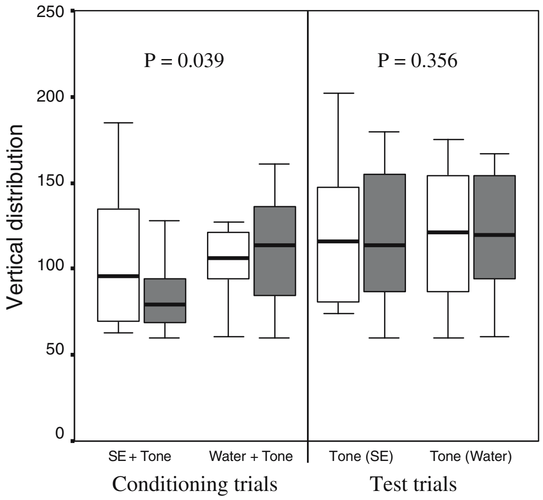

Fig. 3 Median (± quartiles) of vertical distribution of glowlight tetras before (open) and after (hatched) introduction of test stimuli. Labels for stimuli and p values are described in the legend for Fig. 1

Therefore, the sound stimulus by itself was not alarming to glowlight tetras. When retested with the sound stimulus only, glowlight tetras in the alarm-conditioned treatment group responded with significantly reduced activity relative to control fish (z = 2.41, p = 0.007, Fig. 2) but there was no significant change in time near the bottom attributable to conditioning stimulus (z = 0.373, p = 0.356, Fig. 3). Therefore, glowlight tetras learned to associate the sound with predation risk (Fig. 2) but the learned response was less intense than the response to the conditioning stimulus (Fig. 3).

Discussion

These simple experiments provide evidence that Ostariophysan fish associate novel sound stimuli with predation risk after a single simultaneous exposure to sound and conspecific chemical alarm cue. This occurred under two different experimental conditions, using different frequencies of sound stimuli and different test species. The response was manifested as a reduction in activity, a component of antipredator behaviour known to reduce the probability of predation (Mathis and Smith 1993a; Mirza and Chivers 2002; Chivers et al. 2002). This study is novel in two ways. First, it extends the known range of sensory modalities with which fish assess predation risk. Second, it extends the known range of sensory modalities with which fish can use chemical alarm cues to associate novel stimuli with predation risk. Studies demonstrating single-event acquired recognition of novel indicators of risk are testimony to the ecological and evolutionary benefits of early and rapid attendance to public information (Wisenden and Stacey 2005; Wisenden and Chivers 2006).

Sound is used by many different groups of fishes, including some ostariophysans, typically in direct agonistic interactions over food resources, or accompanying territoriality and/or courtship behaviour (e.g. Pruzsinszky and Ladich 1998; Johnston and Johnson 2000; see Popper et al. 2003 for review). The role of sound in the context of predator-prey interactions is less common in the fish literature. Juvenile Atlantic salmon avoid low frequency sounds (Knudsen et al. 1992) and herring detect and avoid echolocation pulses of cetacean predators (Wilson and Dill 2002). When firmly grasped (as if by a predator) *Cynoscion regalis* weakfish (Connaughton et al. 2000), centrarchid sunfishes (Gerald 1971), *Ictalurus punctatus* catfish (Fine et al. 1997), and *Archocentrus* *nigrofasciatus* convict cichlids (Wisenden personal observation) emit sounds that may serve the same function as alarm calls in birds (Högstedt 1983). Fish hearing these cries can localize their source (Fay and Edds-Walton 2000) and may acquire information about predator identity. Moreover, the swimbladders of large predators scatter ambient noise (wave action for example) potentially allowing nearby otophysan prey to detect and identify nearby predators (Rogers 1986; Lewis and Rogers 1996).

Clearly, more information is needed on the types of sound stimuli generated by predators or startled prey to more fully explore the role of audition in mediating predator-prey interactions. In this study, the conditioning stimulus was highly artificial, being an arbitrary electronic tone sustained for an unnatural length of time. Future studies should quantify frequencies and durations of sounds produced during predation events so that ecologically realistic experiments can be developed. Regardless of the ecological role of sound in the assessment of predation risk, the results of this study show that releaser-induced recognition learning for acquiring new ways to recognize the presence of predation risk is sufficiently plastic to accommodate sensory input from auditory stimuli, in addition to olfactory and visual sensory modalities.

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