

Brian D. Wisenden · Mike S. Pollock ·
Robyn J. Tremaine · Jeff M. Webb ·
Monique E. Wismer · Douglas P. Chivers

Synergistic interactions between chemical alarm cues and the presence of conspecific and heterospecific fish shoals

Received: 3 January 2003 / Revised: 14 March 2003 / Accepted: 4 June 2003 / Published online: 26 June 2003
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Abstract Chemical and visual sources of information are used by aquatic prey during risk assessment. Here, we test the behavioral response of littoral prey fish to combinations of chemical alarm cues (skin extract) and the visual presence of a fish shoal. We scented minnow traps with either alarm cues or water (control) placed inside the trap, a jar that contained either a fish shoal or nothing (control), and recorded the number and species of fish captured. We predicted that chemical alarm cues would reduce the number of fish captured and that a fish shoal would increase the number of fish captured. The predicted effect of chemical and visual cues combined depended on the nature of the interaction. We found that the lowest catch rate was for the combination of alarm cue + no shoal, but the highest catch rate occurred for the combination of alarm cue + shoal. Fish shoal + water had the second highest catch rate and no shoal + water had the second lowest catch rate. We conclude that chemical alarm cues induce area avoidance in the absence of a shoal, but a strong behavioral proclivity to increase shoal cohesion in the presence of a shoal. The presence of a shoal in the traps induced alarmed fish to shoal with them and thus, enter the traps. This occurred even though traps were the source of the alarm cue.

Keywords Chemical alarm cue · Minnow · Predation risk · Shoal behavior · Stickleback

Introduction

In aquatic environments, chemical cues reliably inform prey about the presence of predators (Kats and Dill 1998) or recently attacked conspecifics and ecologically similar heterospecifics (Chivers and Smith 1998). Other chemical cues can convey disturbance of nearby conspecifics (Hazlett 1990; Wisenden et al. 1997; Kiesecker et al. 1999; Zulandt Schneider and Moore 2001) and increase vigilance in those that detect these cues. Prey can detect conspecific alarm cues that leak from a predator's gut (Mathis and Smith 1993a; Chivers et al. 1996; Brown and Godin 1999a).

Past field research has shown that natural populations of prey fish avoid traps chemically labeled with alarm cues (skin extract) of conspecifics or ecologically similar heterospecifics (see Chivers and Smith 1998 for review). European minnows (*Phoxinus phoxinus*) in a natural fluvarium reduced feeding rate and increased shoal cohesion in response to injury-released chemical alarm cues (Irving and Magurran 1997).

Shoal membership provides several antipredator benefits, including statistical dilution of the risk of attack, confusion of the predator's ability to visually isolate individual prey, and use of fellow prey to shield oneself from direct attack (Pitcher 1986). Many fish species form permanent shoals because predation risk is constantly present. The visual presence of a shoal of conspecifics, or ecologically and morphologically similar heterospecifics, are attractive to prey species that typically shoal (Krause et al. 1998, 2000; Hoare et al. 2000a).

In ecological interactions, fish routinely encounter fish shoals (attractive) simultaneously with chemical alarm cues (aversive). How chemical and visual indicators of predation risk interact has not received detailed attention. Here, we examine the interaction between chemical and visual stimuli on antipredator behavior of natural populations of littoral freshwater fish, by combining presence or absence of chemical alarm cues with presence or absence of a fish shoal. The independent and interacting effects of chemical and visual risk indicators can be

Communicated by A. Mathis

B. D. Wisenden (✉)
Biology Department,
Minnesota State University Moorhead,
1104 7th Street S, Moorhead, MN 56563, USA
e-mail: wisenden@mnstate.edu

M. S. Pollock · R. J. Tremaine · J. M. Webb · M. E. Wismer ·
D. P. Chivers
Biology Department, University of Saskatchewan,
Saskatoon, Saskatchewan, S7N 5E2, Canada

Table 1 Predictions for the number of fish captured in traps with a chemically labeled sponge (alarm cue or water control) and a jar with or without a fish shoal. Three competing hypotheses for the interaction of visual and chemical produce different predicted outcomes

Experimental treatment		Additive Hypothesis	Competitive Hypothesis	Synergistic Hypothesis
Shoal	Cue			
Fish	Alarm	Second lowest	Lowest	Highest
None	Alarm	Lowest	Lowest	Lowest
Fish	Water control	Highest	Highest	Second highest
None	Water control	Second highest	Highest	Second lowest

measured by counting the number of fish caught in traps with these four treatment combinations.

Three anticipated outcomes were predicted from the combined effects of chemical alarm cues (avoidance) and fish shoals (attraction) (Table 1). First, if the effects are additive (e.g., Brown et al. 1997; Brown and Godin 1999a), then the attractive effect of a shoal should be partly offset by avoidance of chemical alarm cue. Depending on the relative strength of alarm cue avoidance and fish shoal attraction, the combination of fish shoal + alarm cue may be either second lowest (alarm > shoal) or second highest (shoal > alarm).

Second, if visual and chemical indicators compete in a hierarchical fashion (e.g., Magurran et al. 1996; Hartman and Abrahams 2000; Mathis and Vincent 2000), then fish should ignore the cue in the modality that is least salient or reliable. In that case, we would predict one modality of cue to prevail. We predict that chemical cues would prevail because while minnow shoals are constantly present, chemical alarm cues are released only in the context of a predation event in progress. Based on past field research using scented minnow traps (e.g., Mathis and Smith 1992) and alarm cue release (Irving and Magurran 1997; Wisenden et al. 2003), chemical alarm cues should therefore evoke a strong avoidance response that overrides behavioral responses to competing stimuli.

A third possibility is a synergistic interaction between sensory modalities, i.e., the effect of the combination is greater than or different from the sum of the individual components (e.g., Wisenden et al. 2003). Here, the behavioral response to the fish shoal may be intensified by exposure to the chemical cue. If alarm cues induce alarm behavior, and one component of alarm response is to increase shoal cohesion, then presence of alarm cue + fish shoal would catch the most fish, and alarm cue + no shoal would catch the least (Table 1).

Methods

Feedlot Pond experiment

The experiment was conducted at Feedlot Pond in October 2001. Feedlot Pond is a 1-ha pond located on the University of Saskatchewan campus, Saskatoon, Canada. It contains three fish species: fathead minnows (*Pimephales promelas*), brook stickleback (*Culaea inconstans*) and Iowa darters (*Etheostoma exile*). All of these fish breed in the spring and early summer.

Stimulus preparation

Chemical alarm cues were prepared from 24 adult stickleback (mean standard length + SE = 5.0+0.11 cm), sacrificed by a blow to the head (in accordance with the guidelines set by the Canadian Council on Animal Care). A skin fillet was removed from both sides of each fish to produce approximately 95.8 cm² of skin. The skin was added to 300 ml of distilled water and homogenized using a polytron homogenizer. The sample was filtered through glass wool to remove solid particles and diluted with an additional 658 ml of distilled water, for a total volume of 958 ml. Five milliliters (=0.5 cm² of skin) of the extract was infused into blocks of cellulose sponge (2×2×2 cm) and frozen at -20°C. A similar procedure using 22 fathead minnows (mean standard length + SE = 4.6+0.09 cm) was used to produce 103.5 cm² of skin, diluted to 1,035 ml with distilled water, infused into 2×2×2-cm blocks of cellulose sponge (5 ml, or 0.5 cm² of skin per sponge), and frozen at -20°C until needed.

Fishes used in the jar-shoals (see below), and alarm stimulus donors were collected from Feedlot Pond prior to the test day and separated by species. Individuals not used for stimulus preparation were held in 37 l tanks, fed commercial fish food and brine shrimp (*Artemia*), and subject to a 14L:10D photoperiod.

Experimental protocol

The experiments were conducted using Gee's Improved Minnow Traps, which are roughly cylindrical wire enclosures (43 cm long × 22 cm wide) with an inverted funnel located at each end. Each trap contained a 500-ml mason jar (with a screen lid) containing either five fathead minnows, five brook stickleback, or nothing (control). Jar-shoal fish were adults of a size typically vulnerable to the trapping gear. Sex was not noted and assumed to be unimportant outside of the breeding season, and in any case, fish were dispersed arbitrarily among sponge treatments.

For each experiment, the traps were set in trios at about 2 m from shore at a depth of about 1 m, with each trap 10 m from the next. The sponges were attached to the traps with stainless steel wire. One sponge was placed within 5 cm of each entrance to each trap. Trap triplicates were set at 4-min intervals, and removed at 4-min intervals 2.5 h later. Field populations avoid areas chemically labeled with alarm cues for two to four hours (Wisenden et al. 1995). All fish were removed, identified to species, counted, and returned to the pond. The experiment was repeated 9 times. On the first 3 days, 10 triplicates of each jar-shoal treatment (30 traps) were set each day using the injured stickleback sponge stimulus for all traps. On days 4, 5 and 6, we set 10 triplicates each day, this time using the injured minnow cue for all traps. On the 7th, 8th, and 9th days, 10 triplicates of each jar type were set each day, all with sponges of blank cue.

Data analysis

We did a ln(x+1) data transformation on the number of fish caught of each species to conform to the assumptions of normality. We did one-way ANOVAs on the number of fish caught to compare the effect of jar type (minnow shoal, stickleback shoal, empty jar) within each cue type. We did not test for the effect of cue, nor the

interaction between cue type and jar type. Because each cue was presented on different days, we could not distinguish between differences attributable to cue from differences in fish activity due to daily variation in cloud cover, temperature, wind direction, etc.

Deming Lake experiment

Deming Lake is a small (5.5 ha) meromictic lake located in Itasca State Park, in central Minnesota (47°11'N, 95°09'W). It is devoid of piscivorous fish species. It contains fathead minnows, brook stickleback, redbelly dace (*Phoxinus eos*), blacknose shiners (*Notropis heterolepis*), golden shiners (*Notemigonus crysoleucas*), pumpkinseed sunfish (*Lepomis gibbosus*), and johnny darters (*Etheostoma nigrum*).

Stimulus preparation

In June 2000, skin extract was made by killing five female fathead minnows [mean±SE total length (TL) = 63.8±1.36 mm] to collect 36 cm² of skin. We used female minnows only because male fathead minnows lose their alarm substance cells during the breeding season (May and June) (Smith 1976). Skin fillets were placed in a beaker of well water resting on a bed of crushed ice. The skin was blended with a conventional hand blender to rupture epidermal alarm substance cells and release Ostariophysan alarm cue. The solution was filtered through polyester fiber and diluted with well water to a total volume of 144 ml. Blank well water, to serve as a control sponge stimulus, was similarly passed through polyester fiber. Cellulose sponge of dimensions 2×2×2 cm each received 4 ml of cue (alarm cue or water), and frozen at -20°C until needed. Therefore, each block represented 1 cm² of minnow skin. In June 2001, we used six female fathead minnows (mean±SE TL = 61.0±3.14 mm) to collect 37.2 cm² of skin. The skin fillets were chilled, blended, filtered, and diluted to a final volume of 148 ml. Thus, 4 ml of skin extract solution represented about the amount of alarm cue in 1 cm² of skin. Control water sponges were prepared as in 2000. Sponge blocks were slightly larger this time: 2.5×2.5×2 cm, but the amount of cue, 4 ml per block of alarm substance or water, was the same as that used for the 2000 data. Although the Deming Lake sponges contained twice the concentration of alarm cue as sponges used in Feedlot Pond, the Feedlot Pond experiment used two sponges per trap whereas the Deming Lake experiment used only one sponge per trap.

All fathead minnows used for the jar shoal stimulus were captured from Deming Lake a few days prior to the experiment and held in outdoor 900-l holding tanks at the University of Minnesota Lake Itasca Biological Field Station. Pumps supplied a continuous flow of fresh lake water to maintain water quality. Fish were fed commercial flake food during captivity once daily. All fish were returned to Deming Lake immediately after the experiment was concluded for that season. Total time in captivity was 5 days. Fish used for the jar shoals were adults of unknown sex except that mature males (denoted by the presence of tubercles) were not used.

Experimental protocol

Data were collected on two separate occasions separated by about a year. The experimental protocol was similar to that of the Feedlot Pond experiment. On each test day, we set 36 Gee's improved minnow traps representing nine traps for each of four treatments. The treatments were: (1) sponge with skin extract of fathead minnows + five fathead minnows in a wide-mouth quart (925 ml) mason jar with a window-screen lid, (2) skin extract of fathead minnows + empty jar, (3) sponge with water + five fathead minnows in a jar, and (4) sponge with water + empty jar. Wire was used to attach a single sponge to the trap center, midway between the two trap entrances on either end.

The field protocol was identical for the 2000 and 2001 experiments. We placed the traps around the perimeter of Deming

Lake spaced about 8–10 m apart. All traps were left in the water for 2 h. Traps were set four at a time (with each combination of cue and shoal treatment represented) at intervals of 5 min between each group of four traps. This allowed 5 min per set of four traps in which to retrieve and process the catch at the end of 2 h in order to keep fishing time constant for all traps.

Data analysis

Data for the two seasons were pooled. Overall, fewer fish were captured in the second season (2001), but the trends were not qualitatively different between them (year effect: $F_{1,68}=0.16$, $P=0.691$ for total cyprinids caught). We did a $\ln(x+1)$ data transformation on the number of fish caught of each species to conform to the assumptions of normality. Because treatments were not confounded by other differences that may have existed between the test days, we were able to perform a 2×2 ANOVA on the $\ln(x+1)$ -transformed data. A separate ANOVA was computed for each fish species.

Results

Feedlot Pond experiment

The catch comprised two fish species: 1,885 brook stickleback (Fig. 1) and 199 fathead minnows (Fig. 2). When the water sponge was placed in the traps, the jar-shoal treatment significantly affected the number of stickleback captured ($F_{2,87}=5.698$, $P=0.005$). Newman-Keuls post hoc comparisons showed that traps with stickleback jars caught more than traps with empty jars ($P=0.003$). A similar result was found for traps with minnow jars ($P=0.060$). Traps with stickleback jars did not differ from traps with minnow jars ($P=0.148$) (Fig. 1).

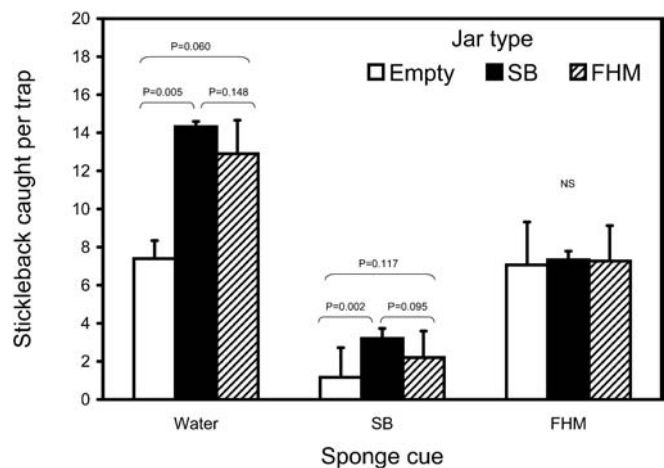


Fig. 1 Mean±SE number of brook stickleback (*Culaea inconstans*) captured per trap in Feedlot Pond, SK. Traps were labeled with one of three chemical stimuli: water (control), skin extract of conspecific stickleback (SB), or fathead minnows (*Pimephales promelas*) (FHM). In each trap was a jar containing one of three shoal treatments: no shoal (open bars), stickleback shoal (solid bars) or a minnow shoal (hatched bars). P -values above bars indicate the result of Newman-Keuls post hoc pair-wise comparisons within each sponge cue. NS no significant differences among any of the shoal types

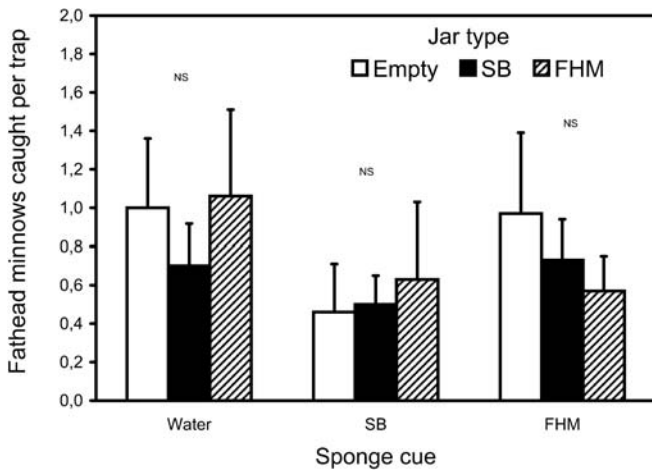


Fig. 2 Mean+SE number of fathead minnows captured per trap in Feedlot Pond, SK. Graph features as described for Fig. 1

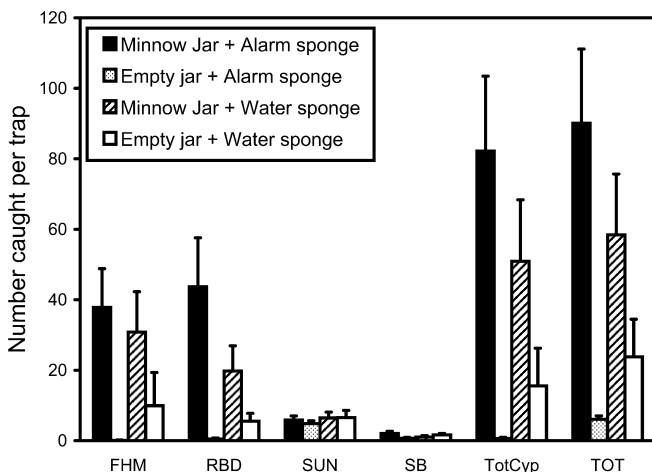


Fig. 3 Mean+SE number of fish caught per trap in Deming Lake, MN. Traps were chemically labeled with fathead minnow alarm cue or water control and contained either a shoal of fathead minnows or no shoal. *FHM* fathead minnow; *RBD* redbelly dace (*Phoxinus eos*); *SUN* pumpkinseed sunfish (*Lepomis gibbosus*); *SB* brook stickleback; *TotCyp* total cyprinids (fathead minnows + redbelly dace); *TOT* total of all fish captured

When stickleback alarm cue was placed in the traps, the jar-shoal treatment again significantly affected the number of stickleback captured ($F_{2,87}=5.341$, $P=0.006$). Newman-Keuls post hoc comparisons show that traps with stickleback jars caught more than traps with empty jars ($P=0.002$). Traps with stickleback jars did not catch more than traps with minnow jars ($P=0.095$), and traps with empty jars did not differ from traps with minnow jars ($P=0.117$) (Fig. 1). When fathead minnow alarm cue was placed in the traps, the jar-shoal treatment did not significantly affect the number of stickleback captured ($F_{2,87}=0.888$, $P=0.415$) (Fig. 1).

Fathead minnow catch numbers were very low and probably do not provide a rigorous test of the effect of chemical cue and shoal treatments. The number of fathead

Table 2 ANOVA results from analyses of Deming Lake catch records testing the effects of a shoal in a jar and chemical alarm cue of fathead minnows. Species abbreviations: *RBD* redbelly dace (*Phoxinus eos*), *FHM* fathead minnow (*Pimephales promelas*), *SB* brook stickleback (*Culaea inconstans*), *CYP* the combined cyprinid catch of *RBD* + *FHM*, *PKS* pumpkinseed sunfish (*Lepomis gibbosus*)

Species	Shoal		Alarm cue		Shoal × Alarm cue	
	$F_{1,68}$	P	$F_{1,68}$	P	$F_{1,68}$	P
RBD	16.99	0.001	0.02	0.888	4.50	0.038
BS	0.242	0.625	0.17	0.681	4.74	0.033
FHM	18.59	0.001	0.03	0.856	1.92	0.170
CYP	20.45	0.001	0.10	0.753	5.31	0.024
PKS	0.072	0.789	0.001	0.983	0.128	0.722

minnows captured was not significantly influenced by the jar-shoal treatment on the day the water cue was used ($F_{2,87}=1.126$, $P=0.329$), the stickleback alarm cue was used ($F_{2,87}=0.318$, $P=0.728$) or the day the fathead minnow alarm cue was used ($F_{2,87}=0.245$, $P=0.783$). The lack of an avoidance response to the minnow cue indicates the poor information content of the minnow catch data in the Feedlot Pond experiment.

Deming Lake experiment

The behavioral responses of redbelly dace, fathead minnows and brook stickleback are consistent with a synergistic interaction between the chemical alarm cue and the jar shoal treatment (Fig. 3). The interaction term was significant for redbelly dace and brook stickleback, but not for fathead minnows (Table 2). Low catch numbers of fathead minnows in 2001 created high variances and compromised statistical power. However, the fathead minnow patterns mirrored those of redbelly dace, and when the minnow and dace catches are combined, the interaction between chemical cue and shoal is significant (Table 2). Although catch rates were high, pumpkinseed sunfish showed no response to either the cue or the shoal treatments (Table 2). Other fish species, golden shiners, bluntnose shiners and johnny darters, were caught only sporadically and were excluded from analyses. In the 2 years combined, we caught only one golden shiner, 18 bluntnose shiners and no darters. In contrast, we caught 1,417 fathead minnows, 1,251 redbelly dace, 427 sunfish, and 98 stickleback.

Discussion

The presence of chemical alarm cues without a shoal caused prey to avoid traps. The presence of a fish shoal without chemical alarm cues attracted fish into traps. When chemical alarm cues were combined with the presence of a shoal, alarm cues significantly increased the attractiveness of the trap above the effect of the shoal

alone. This is a synergistic interaction between these two sensory modalities (Table 1).

Antipredator behavior may take several forms depending on species-specific behavioral patterns, and social context (Lawrence and Smith 1989; Lima and Dill 1990; Magenhagen 1992; Kerby and Kats 1998). In field studies, small fishes that detect chemical alarm cues engage in area avoidance (Mathis and Smith 1992; Chivers and Smith 1994; Chivers et al. 1995; Wisenden et al. 1995; Brown and Godin 1999b) and increased shoal cohesion (Irving and Magurran 1997). These responses contribute to decreased predation risk in the laboratory (Mathis and Smith 1993b; Hossain et al. 2002) and field (Mirza and Chivers 2000). We found that the presence of chemical alarm cue significantly increased the attractiveness of the shoal because the chemical cue enhanced the perceived antipredator benefit of shoal membership.

The Deming Lake data show a clear synergistic interaction between chemical and visual stimuli. The rank of catch abundance in descending order was: (1) alarm cue + fish shoal, (2) water + fish shoal, (3) water + no shoal, (4) alarm cue + no shoal. This rank order is consistent with the predicted sequence for a synergistic interaction (Table 1). The most plausible explanation for these data is that chemical alarm cues induce an avoidance response in the absence of a shoal, but a strong shoaling response in the presence of a shoal.

Dace and minnows are both small, schooling cyprinids. They share similar habitat and predators and both possess epidermal chemical alarm substance cells characteristic of the superorder Ostariophysi (Frisch 1938; Pfeiffer 1977; Smith 1992). Chemical alarm cues of either species indicate predation risk to both. The antipredator benefits that shoaling behavior provides extend to mixed species shoals, particularly if the two species are similar in form and swimming behavior (Krause et al. 1998, 2000; Hoare et al. 2000a, 2000b). Cross-species reactions occur between species with shared phylogenies (Smith 1982; Brown and Godin 1997; Commens and Mathis 1999; Mirza and Chivers 2001; Mirza et al. 2001) and shared ecology (Mathis and Smith 1993c; Chivers et al. 1995; Wisenden et al. 1995; Mathis et al. 1996; Brown et al. 2001). Paradoxically, redbelly dace showed a clearer behavioral synergism to the fathead minnow alarm cue than fathead minnows did.

Stickleback in the Feedlot Pond experiment did not show any effect of the jar-shoal treatment when minnow alarm cue was used. This may reflect a relatively weak aversion to a heterospecific alarm cue compared to conspecific alarm cue (Mirza and Chivers 2001). Stickleback response to fathead minnow alarm cue in the Deming Lake experiment mirrored the synergistic response observed for the cyprinids. The large number of dace and minnows drawn into the alarm cue + shoal traps, and not the jar-shoal treatment per se, could have contributed to the effect of jar treatment on stickleback response. The absence of a response by sunfish is puzzling because centrarchids syntopic with cyprinids are known to respond to cyprinid alarm cue (Brown et al.

2001). The sunfish population in Deming Lake is stunted and many minnow-sized sunfish were captured in the traps. Unlike cyprinids, sunfish have spiny median fins and a laterally-compressed body shape. Dissimilar body morphology may make sunfish less inclined to shoal with cyprinids or identify with minnow chemical alarm cue (Hoare et al. 2000a).

Why then did these fishes choose to shoal with the jar-shoal fish at the source of the cue rather than flee the area and join a free-swimming shoal at a distance from the source of the chemical alarm cue? Antipredator response to chemical alarm cues may be a two-stepped behavior. First, seek immediate safety by increased shoal cohesion, then flee the area as a group. If fish traveling in small shoals encounter the trap, then increase in shoal cohesion (with the jar shoal) would take precedence over area avoidance causing them to enter the trap.

These data add to the evidence refuting the hypothesis that avoidance of alarm cue in scented traps is an artifact of enclosed spaces (Magurran et al. 1996) because the highest catches of all were in traps issuing alarm cue. In another field study, alarmed fathead minnows significantly increased their entry rate into unscented traps located 30 m from the location of the minnow's encounter with chemical alarm cues (Wisenden et al. 1995). In the absence of traps or any enclosed structures, free-swimming minnows avoid areas where alarm cues has been released (Wisenden et al. 2003). In the same experiment (Wisenden et al. 2003), antipredator response of free-swimming minnows to an approaching model predator (visual cue) was significantly intensified if the minnows were first exposed to chemical alarm cues, again producing a synergistic interaction between visual and chemical indicators of predation risk.

A synergistic interaction between chemical alarm cues and presence of a shoal indicates that assessment of predation risk is not completely species stereotypical, but rather, it is influenced by social environment and context. Risk assessment occurs continuously while animals balance the activities of life with the risk of death. All forms of information potentially contribute to these behavioral trade-offs, and the behavioral responses to these interactions are context-dependent.

Acknowledgements Field assistance at Deming Lake was provided by (2000): Waima Azizi, Daniel Budge, Jennifer Dehn, Delaina Hartung, Mary Kosuth, Doug Ludemann, Dana Peltier, and Lizbeth Zaro, and (2001): Nick Altringer, Alina Baum, Annie Felix, Katie Haggblom, Kim Harmon, Erica Hatfield, Katie Johnson, Alison La Pean, Carlee Olson, and Summer Smith. Logistical support was provided by Jon Ross of the Itasca Biological Field Station. This research was funded by an NSERC operating grant to Douglas Chivers and a faculty research grant to Brian Wisenden from the Minnesota State University Moorhead College of Social and Natural Sciences. IACUC protocol number for work in Feedlot Pond was 19920077, issued to Douglas Chivers. IACUC protocol number from the University of Minnesota to Brian Wisenden for use at the Itasca Biological Field Station was 0005A51841, issued to Brian Wisenden.

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