# The effects of reduced pH on chemical alarm signalling in ostariophysan fishes

**Grant E. Brown, James C. Adrian, Jr., Michael G. Lewis, and Jon M. Tower**

**Abstract**: Under laboratory conditions, we examined the effects of acute exposure to weakly acidic conditions (pH 6.0) on the ability of fathead minnows (*Pimephales promelas*) and finescale dace (*Phoxinus neogaeus*) to detect and respond to conspecific and artificial alarm pheromones. Initially, minnows and dace exhibited normal antipredator responses when exposed to conspecific alarm pheromones under normal (pH 8.0) conditions. When retested at pH 6.0, we observed no significant antipredator response. However, when returned to normal pH conditions, both exhibited normal antipredator responses. Minnows exposed to the putative ostariophysan alarm pheromone (hypoxanthine-3-*N*-oxide) exhibited a similar trend in behavioural response. Finally, we manipulated the pH of minnow skin extract and hypoxanthine-3-*N*-oxide to determine the chemical mechanism responsible for this observed loss of response. Minnows exhibited significant antipredator responses to natural and artificial alarm pheromones at normal pH conditions, but did not respond to either stimulus once they had been buffered to pH 6.0 or acidified and rebuffered to pH 7.5. These data suggest that the ability of minnows and dace to detect and respond to alarm pheromones is impaired under weakly acidic conditions and that this loss of response is due to a nonreversible covalent change to the alarm pheromone molecule itself.

**Résumé** : Nous avons examiné en laboratoire les effets d'une exposition aiguë à des conditions de faible acidité (pH 6,0) sur la capacité de têtes-de-boule (*Pimephales promelas*) et de ventres citron (*Phoxinus neogaeus*) de détecter les phéromones d'alerte de leur espèce et les phéromones artificielles et d'y réagir. Au départ, les deux espèces ont des comportements anti-prédateurs normaux à l'exposition à leurs phéromones d'alerte spécifiques dans des conditions normales (pH 8,0). À pH 6,0, elles n'ont pas de comportement anti-prédateurs significatif. Au retour aux conditions normales de pH, les deux espèces retrouvent leurs réactions anti-prédateurs normales. Les têtes-de-boule exposées à l'hormone d'alerte putative des ostariophyses (oxyde-3-*N* d'hypoxanthine) ont des comportements similaires. Enfin, nous avons manipulé le pH d'extraits de peau de tête-de-boule et de l'oxyde-3-*N* d'hypoxanthine afin de déceler le mécanisme chimique responsable de la perte de réaction observée. Les têtes-de-boule ont une réaction anti-prédateurs significative à l'hormone d'alerte naturelle ou artificielle aux conditions normales de pH; ils n'ont cependant aucune réaction à ces stimulus lorsque le pH a été modifié à 6,0 à l'aide d'un tampon ou lorsque le milieu a été acidifié de nouveau et fixé à pH 7,5 à l'aide d'un tampon. Ces données nous amènent à croire que la capacité des têtes-de-boule et des ventres citron de détecter les phéromones d'alerte et d'y réagir est affaiblie dans des conditions légèrement acides et que cette perte de réaction est due à un changement irréversible de covalent dans la molécule même de la phéromone d'alerte.

[Traduit par la Rédaction]

## Introduction

Many ostariophysan fishes possess specialized epidermal club cells that, when mechanically damaged, release a chemical alarm signal (alarm pheromone) into the water column (Smith 1992; Chivers and Smith 1998). When detected by nearby conspecifics and some sympatric heterospecifics, this chemical alarm signal elicits a dramatic, short-term increase in species-specific antipredator behaviours (Smith 1992; Chivers and Smith 1998). Although controversial (Magurran et al. 1996; Smith 1997), there exists considerable laboratory and field evidence for the antipredator function of these alarm

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pheromones to both signal senders and receivers (e.g., Smith 1997; Chivers and Smith 1998; Brown and Godin 1999).

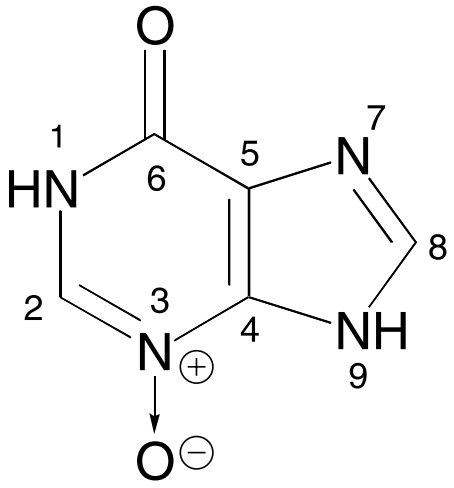
Although much is known regarding the effects of heavily acidic conditions on aquatic communities (e.g., Somers and Harvey 1984; Haines and Baker 1986; Baker et al. 1990), relatively little is known regarding the potential effects of weakly acidic conditions on individual behavioural responses. In particular, little attention has been paid to the effects of weakly acidic conditions on chemical communication within prey fish communities. Conflicting reports exist regarding the detection of chemical cues by fathead minnows. Lemly and Smith (1985, 1987) demonstrated that minnows are unable to detect foraging cues in water at pH 6.0. Smith and Lawrence (1988), however, demonstrate that minnows from a high predation pressure population are able to detect conspecific skin extracts (alarm pheromones) as low as pH 5.0, though their data suggests a reduced response intensity at lower pH.

The putative ostariophysan alarm pheromone has been identified as hypoxanthine-3-*N*-oxide (H3NO; Pfeiffer et al. 1985). H3NO is characterized by a purine skeleton and a nitrogen oxide functional group at the three position (Fig. 1). However, recent work suggests that the alarm pheromone system of the superorder Ostariophysi may actually consist of a suite of aromatic compounds with a nitrogen oxide functional group (Brown et al. 2000, 2001*a*). Two cyprinid species (fathead minnows, *Pimephales promelas*, and finescale dace, *Phoxinus neogaeus*) and two characin species (glowlight tetras, *Hemigrammus erythrozonus*, and neon tetras, *Paracheirodon innes*) significantly increase their antipredator responses when exposed to conspecific skin extracts (natural alarm pheromones), H3NO, or pyridine-*N*-oxide, a structurally dissimilar compound. No significant response was found following exposure to a suite of structurally similar compounds lacking a nitrogen oxide functional group (Brown et al. 2000, 2001*a*). Similar results have been shown for channel catfish (*Ictalurus punctatus*, Siluriformes; G.E. Brown, J.C. Adrian, Jr., and N.T. Naderi, unpublished data). In addition, replacing the double-bonded oxygen in the six position (Fig. 1) with progressively larger alkoxy functional groups appears to have no effect on the ability of the purine-3-*N*-oxide family to elicit a significant alarm response (G.E. Brown, J.C. Adrian, Jr., and J.M. Tower, unpublished data).

These data suggest that the N–O functional group acts as the "molecular trigger", eliciting the overt behavioural response. Compounds lacking the N–O functional group do not elicit any behavioural response. Previous reports observed that, under acidic conditions, H3NO is converted to 6,8-dioxypurine with a loss of the 3-*N*-oxide functional group (Kawashima and Kumashiro 1969; Wölcke and Brown 1969). This suggests that acute exposures to acidic conditions might result in the nonreversible loss of the N–O functional group, rendering the alarm pheromone nonfunctional (Brown et al. 2000).

We conducted the current series of experiments to determine if a reduction in pH would result in an inability of two ostariophysan fishes (fathead minnows and finescale dace) to detect and respond to natural (experiment 1) or artificial alarm (experiment 2) pheromones. Our third experiment probes the chemical mechanism that might account for any loss of function of the cyprinid alarm pheromone under weakly acidic conditions.

**Fig. 1.** Hypoxanthine-3-*N*-oxide, with standard purine numbering scheme shown.




## Methods

### Experiment 1: response to conspecific skin extract

#### *Test fish and stimulus collection*

Fathead minnows were collected from an outlet pond at State University of New York at Cobbleskill, Cobbleskill, N.Y. Finescale dace were collected from Lock 7 Kill, a small tributary stream of the Mohawk River near Schenectady, N.Y. Minnows and dace were held in 60-L aquaria, filled with continuously filtered, dechlorinated tap water (pH ~8.0 owing to a high concentration of, presumably, calcium carbonate, 18°C) on a 12 h light : 12 h dark cycle and were fed ad libitum, twice daily with commercial flake food. Mean (± standard error, SE) standard length (SL) at time of testing was 4.97 ± 0.29 and 5.22 ± 0.38 cm, minnows and dace, respectively.

Fathead minnow and finescale dace skin extracts (natural alarm pheromone) were harvested from seven minnow and six dace donors. Donor fish were killed with a blow to the head (in accordance with Union College IACUC protocol 2-27-98) and skin was removed from either side and immediately placed into chilled, glass-distilled water. Skin samples were then homogenized, filtered through glass wool and the final volume adjusted by adding glass-distilled water. A total of 24.97 $cm^2$ of minnow skin (in 310 mL of distilled water) and 30.60 $cm^2$ of dace skin (in 380 mL of distilled water) were collected. This final concentration was similar to that used by Lawrence and Smith (1989). Conspecific skin extracts were frozen in 15-mL aliquots at –20°C until needed. As a control, we also froze 15-mL aliquots of distilled water at –20°C.

#### *Experimental protocol*

Test tanks consisted of 37-L glass aquaria, filled with 35 L of dechlorinated tap water, a gravel substratum and a single air stone, mounted along the back wall of the tank. An additional length of airline tubing was attached to the air-

stone, allowing for the introduction of the chemical stimuli from behind a black plastic viewing curtain, from a distance of approximately 3 m (so as not to disturb the test fish). Test tanks were illuminated, on a 12 h light : 12 h dark cycle with 25-W Sunglow™ fluorescent lamps.

Shoals of four fathead minnows or finescale dace were placed into test tanks and allowed a 24-h acclimation period. Each minnow or dace was tested a total of three times; under normal pH (pH 8.0, pre-acid), under acidic conditions (pH 6.0, acid), and again under normal pH (8.0, post-acid). Following the initial test in the pre-acid treatment, test fish were transferred to a 60-L holding tank (as described above) that had been buffered to pH 6.0 by titration of concentrated sulfuric acid. Test fish were held for three days and then transferred back to the test tanks, which had been likewise buffered to pH 6.0, and allowed an additional 24-h acclimation period. After being tested under the acid treatment, test fish were transferred back to the holding tank (pH 8.0) and held for three days, returned to the testing tanks and retested again under the post-acid condition (following an additional 24-h acclimation period). Minnows and dace exhibited no visible signs of stress and actively fed during the acclimation periods at all pH levels.

Trials consisted of paired control and experimental observations. Control and experimental observations consisted of a 10-min pre-stimulus and a 10-min post-stimulus observation. During both the pre- and post-stimulus observation periods, we recorded area use and shoaling index every 15 s. Area use was recorded as the position of each fish (1, bottom third of the test tank; 3, top third of the test tank), yielding scores ranging from 4 (all fish near substrate) to 12 (all fish near surface). Shoaling index (modified from Mathis and Smith 1993) ranged from 1 (no fish within one body length of each other) to 4 (all fish within one body length of each other).

Following the pre-stimulus observation period, we withdrew and discarded 60 mL of tank water (to remove any stagnant water in the stimulus injection tube) and then withdrew and retained an additional 60 mL. We then injected 5 mL of conspecific skin extract (experimental trials) or distilled water (control trials) and slowly flushed it into the test tank using the retained tank water. Dye tests demonstrate that this method results in an even distribution of the stimulus throughout the tank in about 15 s.

### *Statistical analysis*

We calculated the mean area use and shoaling index scores for the pre- and post-stimulus observation periods and calculated the difference between them. We then compared these difference scores between the distilled water controls and experimental trials using Mann–Whitney *U* tests. To test for significant overall differences in baseline activity, pre-stimulus observation scores were compared between the pre-acid, acid, and post-acid treatments using a Friedman's nonparametric analysis of variance (ANOVA).

### Experiment 2: response to hypoxanthine-3-*N*-oxide

Fathead minnows were collected and housed as in experiment 1. The experimental test protocol was as described above, with the exception that instead of conspecific skin extract, minnows were exposed to H3NO. Mean (± SE) SL at time of testing was 4.82 ± 0.19 cm. Hypoxanthine-3-*N*-oxide was synthesized according to Brown et al. (2000). A stock solution was generated by stirring 0.002 g of H3NO into 200 mL of glass-distilled water for 15 min. H3NO and distilled water controls were frozen in 15-mL aliquots at –20°C until used. Five millilitres of this stock solution was used as the experimental stimulus, yielding a final tank concentration of 0.4 nM (5 mL into 35 L). This is the minimum behavioural response threshold concentration of H3NO for this population of fathead minnows (Brown et al. 2001*b*, 2001*c*). The data were analyzed as in experiment 1.

### Experiment 3: chemical mechanism

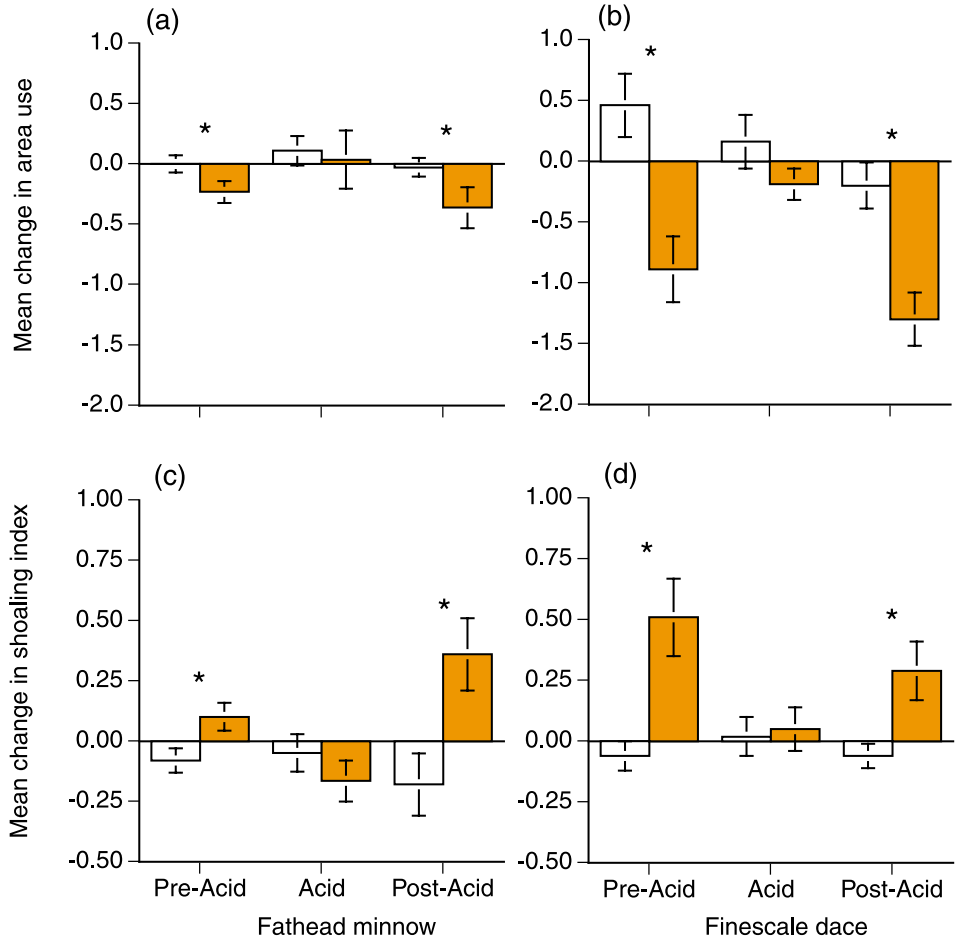
We conducted this experiment to determine which of two possible chemical mechanisms might account for the observed loss of response under weakly acidic conditions. The most likely mechanism is the nonreversible covalent bond change mechanism (cf. Introduction). Alternatively, a decrease in pH would shift the acid–base equilibrium from H3NO (the active form) to the conjugate acid 3-hydroxyhypoxanthine (the inactive form; Brown et al. 2000), resulting in a reversible change to the alarm pheromone molecule. Whereas this acid–base mechanism is extremely unlikely to account for any observed loss of function (see below), the concentrations of H3NO employed in experiment 2 are at (or near) the minimum behavioural response threshold. As such, a slight decrease in functional concentrations may account for an observed loss of function. We conducted this final experiment to determine if the effect of acidifying the alarm signal itself is a reversible mechanism (acid–base) or a nonreversible mechanism (covalent change).

Fathead minnows were collected and housed as described for experiment 1. Mean (± SE) SL at time of testing was 4.67 ± 0.29 cm. The experimental protocol and statistical analysis were conducted as described above, with two exceptions. First, minnows were only tested once. Second, the pH of the experimental stimuli was buffered rather than the pH of the test tank water. Minnows were exposed to conspecific skin extract or H3NO, which had been untreated, buffered to pH 6.0, or acidified and rebuffered to pH 7.5.

We mixed a stock solution of 0.006 g of H3NO in 600 mL of glass-distilled water, yielding stimulus concentrations of H3NO identical to that used in experiment 2. Four hundred millilitres of this stock solution were buffered to pH 6.0 by titration with concentrated sulfuric acid, and stirred for 1 h. Finally, 200 mL of the acidified H3NO solution was buffered back to pH 7.5 by titration with a Borax buffer, and stirred for one hour. This gave us three experimental stimuli: H3NO, H3NO buffered to pH 6.0 (H3NO + a), and H3NO rebuffered to pH 7.5 (H3NO + a + b). Additional controls were created by buffering 200 mL of tank water (TW) to pH 6.0 by titration with sulfuric acid (TW + a). An additional 200 mL of tank water was buffered to pH 6.0 and then back to pH 7.5 using a Borax buffer (TW + a + b). Experimental and control stimuli were frozen in 15-mL aliquots at –20°C until required.

We collected conspecific skin extract (CSE) as described in experiment 1. We collected a total of 47.95 $cm^2$ (in 600 mL of glass-distilled water). The skin extract solution was then

**Fig. 2.** Mean (±1 standard error) change in area use (*a*, fathead minnows, *Pimephales promelas*; *b*, finescale dace, *Phoxinus neogaeus*) and shoaling index (*c*, fathead minnows; *d*, finescale dace) in response to distilled water (open bars) and conspecific skin extract (shaded bars) under the pre-acid (pH 8.0), acid (pH 6.0), and post-acid (pH 8.0) treatments. $N$ = 10 shoals per treatment, *, significant differences between control and experimental stimuli at $P < 0.05$, based on Mann–Whitney $U$ tests.




buffered to pH 6.0 and acidified and rebuffered to pH 7.5 as described above. Stimuli were frozen in 15-mL aliquots at –20°C until required.

We predicted that if a reversible acid–base equilibrium was operating (cf. Introduction), the untreated H3NO and CSE and the H3NO and CSE buffered back to pH 7.5 should elicit a significant antipredator response. However, if a non-reversible covalent bond change occurred (cf. Introduction), then only the untreated H3NO and CSE should elicit any response.

## Results

### Experiment 1: response to conspecific skin extracts

When tested in the pre-acid condition, both fathead minnows and finescale dace exhibited significant increases in shoaling index scores and significant decreases in area use scores (Fig. 2; Table 1). When tested four days later in the acid treatment, we found no significant difference in either behavioural measure, relative to the distilled water controls (Fig. 2; Table 1). Finally, when retested four days later, the significant antipredator response (increased shoaling and decreased area use) returned (Fig. 2; Table 1). No significant differences in baseline activity were observed (shoaling index: minnows $\chi^2 = 0.91$, dace $\chi^2 = 1.95$, df = 2, $P > 0.05$; area use: minnows $\chi^2 = 1.05$, dace $\chi^2 = 1.55$, df = 2, $P > 0.05$).

### Experiment 2: response to hypoxanthine-3-*N*-oxide

We observed a similar response pattern to that seen in experiment 1, with minnows exhibiting a significant antipredator response in the pre-acid treatment (Fig. 3; Table 2). When retested four days later under the acid treatment, no significant differences in shoaling index or area use scores (relative to distilled water controls) were observed (Fig. 3; Table 2). As in experiment 1, when retested four days later under the post-acid treatment, we saw a return of the significant antipredator response (Fig. 3; Table 2). As with conspecific skin extracts, we found no significant differences in baseline activity among the three treatments (shoaling index: $\chi^2 = 0.35$, df = 2, $P > 0.05$; area use: $\chi^2 = 2.40$, df = 2, $P > 0.05$).

### Experiment 3: chemical mechanism

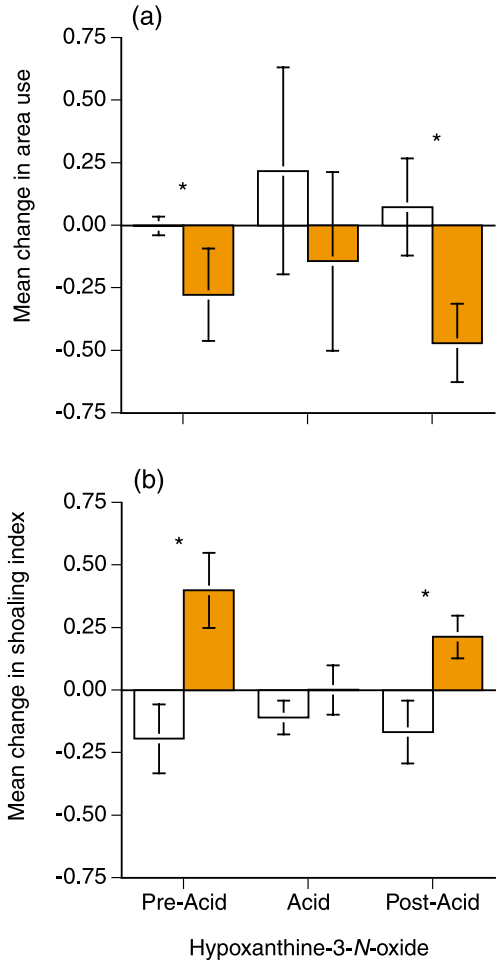
We found a significant increase in antipredator behaviour (increased shoaling and decreased area use) in the untreated

**Table 1.** Individual comparisons for fathead minnow and finescale dace response to conspecific skin extract versus distilled water control under pre-acid (pH 8.0), acid (pH 6.0), and post-acid (pH 8.0) treatments.

| | | Area use | | Shoaling index | |
|---|---|---|---|---|---|
| Species | Treatment | *Z* | *P* | *Z* | *P* |
| Fathead minnow | Pre-acid | –2.04 | <0.04 | –2.01 | <0.05 |
| | Acid | –0.08 | NS | –1.06 | NS |
| | Post-acid | –2.31 | <0.02 | –2.27 | <0.02 |
| Finescale dace | Pre-acid | –2.00 | <0.04 | –3.25 | <0.001 |
| | Acid | –1.93 | NS | –0.15 | NS |
| | Post-acid | –2.12 | <0.03 | –2.23 | <0.03 |

**Note:** Probabilities based on Mann–Whitney *U* tests. NS, not significant.

**Fig. 3.** Mean (±1 standard error) change in (*a*) area use and (*b*) shoaling index in response to distilled water (open bars) and hypoxanthine-3-*N*-oxide (shaded bars) by fathead minnows (*Pimephales promelas*) under the pre-acid, acid, and post-acid treatments. *N* = 10 shoals per treatment, *, significant differences between control and experimental stimuli at $P < 0.05$, based on Mann–Whitney *U* tests.




CSE and H3NO conditions only (Fig. 4; Table 3). We observed no significant change in antipredator behaviour to either experimental stimuli that had been acidified or rebuffered back to pH 7.5 (Fig. 4; Table 3). In addition, we found no significant effect of either the acid or Borax buffers (Table 3).

**Table 2.** Individual comparisons for fathead minnow response to hypoxanthine-3-*N*-oxide versus distilled water control under pre-acid (pH 8.0), acid (pH 6.0), and post-acid (pH 8.0) treatments.

| | Area use | | Shoaling index | |
|---|---|---|---|---|
| Treatment | *Z* | *P* | *Z* | *P* |
| Pre-acid | –2.31 | <0.02 | –1.99 | <0.05 |
| Acid | –0.72 | NS | –0.23 | NS |
| Post-acid | –2.16 | <0.03 | –2.42 | <0.02 |

**Note:** Probabilities based on Mann–Whitney *U* tests. NS, not significant.

## Discussion

The results of experiments 1 and 2 clearly demonstrate that the ability of fathead minnows and finescale dace to detect and respond to conspecific alarm pheromones and H3NO (the putative active component of the ostariophysan alarm signalling system; Pfeiffer et al. 1985; Brown et al. 2000) is significantly impaired under weakly acidic conditions. When exposed to a chemical alarm signal (both natural and synthetic), minnows and dace significantly increased shoaling and decreased area use at normal pH levels. However, when the pH was reduced to 6.0 (weakly acidic), the response disappeared. When retested under normal pH, the response returned to the pre-acid levels.

Our results agree, in part, with previous findings on the effects of weakly acidic conditions on the alarm signalling system of fathead minnows. Smith and Lawrence (1988) demonstrated that solitary fathead minnows exhibited an increase in behaviour patterns indicative of an alarm response at acidity levels as low as pH 5.0. However, their results suggest a reduction in response intensity at pH 6.0 and below. The differing results may be due to experimental and (or) population differences. Initially, Smith and Lawrence (1988) tested solitary minnows in an automated tracking system. It is likely that a solitary minnow would perceive a weak chemical signal (low stimulus concentration) as a predation threat, since they would lack the antipredator benefits of a larger social aggregation (Smith 1992; Chivers et al. 1995). Minnows in a larger social aggregation, however, might not show an overt behavioural response at similar concentrations (sensu Brown and Smith 1996; Smith 1999), as they would gain some survival benefits associated with shoaling (Magurran 1990). Secondly, the minnows tested by Smith and Lawrence (1988) originated from a population exposed to relatively high predation pressures (D.P. Chivers, Department of Biology, 112 Science Place, University of Saskatchewan, Saskatoon, SK, S7N 5E2, Canada, personal communication). Ambient predation pressure has been shown to have significant effects on the minimum stimulus response threshold to artificial alarm pheromones in fathead minnows (Brown et al. 2001*c*). Finally, it is unknown what the normal seasonal range of pH is for the population of minnows tested by Smith and Lawrence (1988). The populations tested in the current study are exposed to relatively little seasonal variation in ambient pH (G.E. Brown, personal

**Fig. 4.** Mean (±1 standard error) change in area use (*a*, conspecific skin extract (CSE); *b*, hypoxanthine-3-*N*-oxide (H3NO)) and shoaling index (*c*, CSE; *d*, H3NO) at normal pH (CSE, H3NO), buffered to pH 6.0 (CSE + a, H3NO + a), or acidified and buffered back to pH 7.5 (CSE + a + b, H3NO + a + b). Open bars, distilled water controls; shaded bars, experimental stimuli. $N$ = 10 shoals per treatment, *, significant differences between control and experimental stimuli at $P < 0.05$, based on Mann–Whitney $U$ tests.

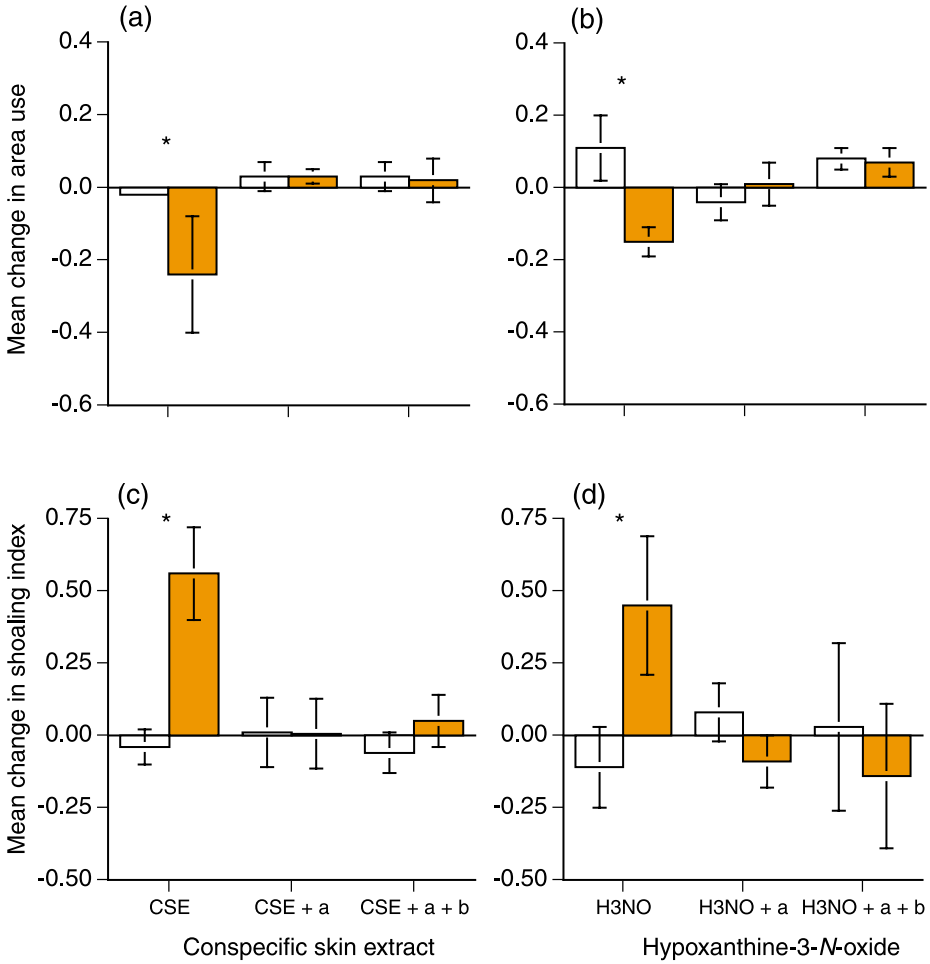



**Table 3.** Individual comparisons for fathead minnow response to experimental stimuli versus distilled water controls.

| | Area use | | Shoaling index | |
|---|---|---|---|---|
| Treatment | *Z* | *P* | *Z* | *P* |
| CSE | –3.79 | <0.001 | –3.24 | <0.001 |
| CSE + a | –0.68 | NS | –0.79 | NS |
| CSE + a + b | –0.05 | NS | –1.52 | NS |
| H3NO | 2.89 | <0.004 | –2.24 | <0.03 |
| H3NO + a | –0.81 | NS | –1.21 | NS |
| H3NO + a + b | –0.82 | NS | –0.64 | NS |
| TW + a | –1.51 | NS | –0.45 | NS |
| TW + a + b | –0.31 | NS | –0.08 | NS |

**Note:** CSE, conspecific skin extract; H3NO, hypoxanthine-3-*N*-oxide; TW, tank water; + a, stimulus buffered to pH 6.0; + a + b, stimulus buffered to pH 6.0 and rebuffered to pH 7.0. Probabilities based on Mann–Whitney $U$ tests. NS, not significant.

observations). It is possible that populations exposed to seasonal variation (due to spring thaws or extended periods of drought) might be selected to respond at lower concentrations or possess a greater density of receptors. Current studies are ongoing to examine these factors.

The observed loss of response could be due to one of four possible mechanisms. First, the overall level of baseline activity of the test fish may have been reduced (Lemly and Smith 1985, 1987; Smith and Lawrence 1988). Such a reduction in baseline activity would have made it difficult to detect a significant difference between the distilled water controls and the experimental stimuli. However, when we statistically tested for this, we found no evidence for a reduction in baseline activity levels among the treatment conditions. As such, this mechanism is unlikely to account for the observed loss of response.

Second, the reduced pH level in the acid condition may have caused tissue damage to the olfactory epithelium, rendering individuals unable to detect the alarm pheromone (sensu Lemly and Smith 1985, 1987). This mechanism is also unlikely to account for our observed results. As the pre-acid, acid, and post-acid conditions were only separated by four days, it is unlikely that tissue lost owing to acute exposure to the weakly acidic conditions could regenerate. Also, Lemly and Smith (1987) found no significant morphological damage to olfactory epithelial tissue following acute exposures to weakly acidic water (pH 6.0) in fathead minnows.

Third, the loss of response could be due to the acid–base equilibrium. As the pH decreases, the acid–base equilibrium should favour a shift from the functional form of H3NO to the nonfunctional hydroxy form. However, the acidity constant (pKa) of the conjugate acid of the nitrogen oxide functionality of H3NO has been measured at a pKa = 1.2 ± 0.1 (Scheinfeld et al. 1969). This means that at a pH of 6.0, the concentration will change by a factor of only 0.0001%; effectively, the concentration does not change, thus it is unlikely that this mechanism could account for our observed loss of response.

Finally, the observed loss of the alarm pheromone activity is most likely due to a nonreversible covalent bond change to the alarm pheromone molecule itself, similar to that outlined in the Introduction. The data from experiment 3 strongly supports this model. We observed a significant increase in antipredator behaviour only when minnows were exposed to the untreated H3NO or conspecific skin extract. If the acid–base equilibrium were operating, untreated and rebuffered H3NO and conspecific skin extract should have elicited an alarm response. However, both H3NO and conspecific skin extract acidified to 6.0 and then buffered from 6.0 back to 7.5 did not elicit any significant change in behaviour relative to the distilled water controls, implicating an irreversible covalent change in the alarm pheromone. Experiments are underway to investigate the chemical fate and degradation rate of H3NO under weakly acidic conditions.

These data suggest the possibility of significant community level effects due to the loss of functionality of the ostariophysan alarm pheromone. These chemical alarm signals are used by a variety of heterospecific prey guild members to detect and respond to potential predators (Chivers and Smith 1998; Brown et al. 2000, 2001*d*). In addition, a variety of predators use these chemical cues to localize potential prey (Mathis et al. 1995; Brown et al. 2001*d*). As such, the loss of function of these alarm pheromones under weakly acidic conditions, due to the loss of the nitrogen oxide molecular trigger, is likely to have potentially significant recruitment and survival consequences for both prey and predator species. Experiments to determine the exact nature of the chemical change to the alarm pheromone, H3NO, are ongoing and will be reported in due course.

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