Journal of Chemical Ecology, Vol. 26, No. 1, 2000

OSTARIOPHYSAN ALARM PHEROMONES: LABORATORY AND FIELD TESTS OF THE FUNCTIONAL SIGNIFICANCE OF NITROGEN OXIDES

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(Received April 7, 1998; accepted August 24, 1999)

Abstract—In laboratory and field-trapping studies, we exposed fathead minnows (*Pimephales promelas*) and finescale dace (*Chrosomus neogaeus*) to hypoxanthine-3-*N*-oxide and a suite of structurally and functionally similar compounds in order to determine if: (1) hypoxanthine-3-*N*-oxide functions as a chemical alarm signal in ostariophysan fishes and (2) the purine skeleton, a structural component, or the nitrogen oxide, a functional component, or both act as the molecular trigger in this chemical alarm signaling system. Minnows and dace exhibited significant antipredator responses when exposed to conspecific skin extract or hypoxanthine-3-*N*-oxide (increased shoaling and decreased area use) and the functionally similar pyridine-*N*-oxide (increased shoaling) but not to structurally similar molecules lacking a nitrogen oxide functional group or to a swordtail skin extract control. Field-trapping studies revealed similar results. Traps labeled with fathead minnows skin extract, hypoxanthine-3-*N*-oxide, or pyridine-*N*-oxide caught significantly fewer fish than did those labeled with distilled water. These data strongly suggest that the nitrogen oxide functional group acts as the chief molecular trigger in the Ostariophysan alarm pheromone system and that, contrary to previous research, hypoxanthine-3-*N*-oxide may be one of several possible molecules that function as a chemical alarm signal. Here we report the first example of a single functional group capable of eliciting a suite of behavioral responses.

Key Words—Ostariophysan fishes, chemical alarm signals, alarm pheromone, anti-predator behavior, hypoxanthine-3-*N*-oxide, nitrogen oxides.

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INTRODUCTION

Many fishes of the superorder Ostariophysi (and members of their prey guild) possess chemical alarm signals or alarm pheromones (Smith, 1992; Mathis and Smith, 1993a; Brown and Godin, 1997; Brown and Smith, 1997; Chivers and Smith, 1998). Although controversial (Magurran et al., 1996; Smith, 1997a; Brown and Godin, 1999b), there exists considerable evidence for the antipredator function of alarm pheromones for both signal senders and receivers in ostariophysan fishes (Smith, 1986, 1992, 1997b; Mathis and Smith, 1993b; Brown et al., 1995a,b; Mathis et al., 1995, 1996; Chivers et al., 1996; Brown and Godin, 1999a). When detected by nearby conspecifics (and sympatric heterospecifics), the alarm pheromone(s) elicits an alarm response, characterized by an increase in a variety of antipredator behaviors such as refuging, shoaling, and immobility (Lawrence and Smith, 1989; Mathis and Smith, 1993b; Brown et al., 1995a; Brown and Smith, 1997).

Pfeiffer et al. (1985) argued that hypoxanthine-3-*N*-oxide (H3NO) is the active component of the ostariophysan alarm pheromone system. Hypoxanthine-3-*N*-oxide is a relatively small molecule comprised of a purine skeleton with a nitrogen oxide functional group at the three position (Figure 1). Pfeiffer et al. (1985) presented individual black tetras (*Gymnocorymbus ternetzi*) with hypoxanthine-3-*N*-oxide and observed the change in dorsoventral orientation (an indicator of an alarm response). A significant behavioral response was reported, which did not differ from that elicited by conspecific skin extract.

There remain a number of unanswered questions regarding the chemical make-up of the ostariophysan alarm pheromone, however. First, it is unknown if hypoxanthine-3-*N*-oxide is the major component of the alarm pheromone or merely one of a suite of potential chemical signals. Given the diversity of the superorder Ostariophysi, which includes some 55 families (Moyle and Cech,

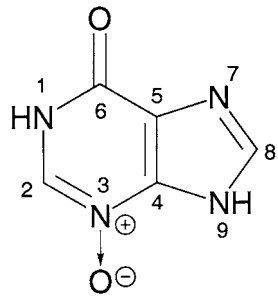


FIG. 1. Hypoxanthine-3-*N*-oxide, with standard purine numbering scheme shown.

1988) and almost 72% of all freshwater species (Nelson, 1984), it seems unlikely that a single compound would be common to all species. Second, hypoxanthine-3-*N*-oxide is a highly functionalized molecule, with a variety of functional groups, providing several potential molecular recognition points. It remains unknown if a single functional group or combination of functional groups organized by the purine skeleton acts as the molecular trigger. Finally, Pfeiffer et al. (1985) used a dorsal–ventral body position as an indicator of an antipredator response. Magurran et al. (1996) argued that the use of ecologically invalid measures of an antipredator response could result in false positive results under laboratory conditions. The use of ecologically valid laboratory and field tests are required for conclusive results (Magurran et al., 1996; Smith, 1997a; Brown and Godin, 1999b).

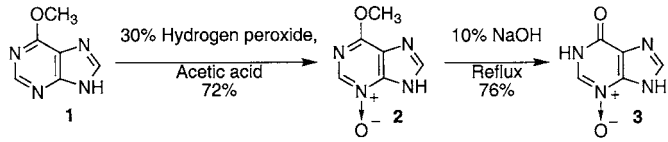
Here, we report the results of a study designed to determine whether the nitrogen oxide functional group, purine skeleton, or an organized combination acts as the main signaling agent. Fathead minnows and finescale dace were exposed to conspecific skin extracts, swordtail skin extract [which lacks any known ostariophysan alarm pheromone (Pfeiffer, 1977; Mathis and Smith, 1993a], synthesized hypoxanthine-3-*N*-oxide, and a suite of seven structurally similar molecules. We also report the first complete spectroscopic characterization of hypoxanthine-3-*N*-oxide.

METHODS AND MATERIALS

Preparation of Hypoxanthine-3-N-oxide and Chemical Characterization

The preparation of hypoxanthine-3-*N*-oxide (**3**) was accomplished in two steps in an overall 55% yield according to the method of Kawashima and Kumashiro (1969) (Scheme 1). The commercially available 6-methoxypurine (**1**) was oxidized exclusively at the three position by the action of hydrogen peroxide in glacial acetic acid to afford the 6-methoxypurine-3-*N*-oxide (**2**). The oxide **2** was then converted to the desired oxide **3** by alkaline hydrolysis of the methoxy group.

General. Melting points were determined on a Mel-Temp II capillary melting point apparatus and are uncorrected. Elemental analyses were performed by Atlantic Microlabs of Norcross, Georgia. Low- and high-resolution mass spec-



SCHEME 1.

tra were performed by the Mass Spectrometry Service of the University of Illinois. Infrared spectra were recorded on a Mattson Genesis II FTIR spectrometer. Ultraviolet–visible spectra were recorded on a Hewlett Packard 8452A diode array spectrophotometer. ^{1}H NMR spectra were determined at 200 MHz; ^{13}C NMR spectra were determined at 50 MHz and were obtained on a Varian Gemini 200 MHz spectrometer. Chemical shifts are expressed in parts per million (δ units). All starting materials and solvents were purchased from Sigma or Aldrich and were used as received. In vacuo usually refers to concentration on a Rotavapor (25 mm Hg, 37–40°) followed by high vacuum (≤0.1 mm Hg).

6-Methoxypurine-3-N-*oxide* (***2***). To a stirred solution of 5.00 g (26.7 mmol) of 6-methoxypurine (**1**) in 100 ml of glacial acetic acid was added 50 ml of 30% aqueous hydrogen peroxide solution, and the reaction mixture was protected from light. After 14 days the reaction mixture was concentrated in vacuo to afford a yellow oil. Triteration of the oil with ≈50 ml of cold 95% ethanol precipitated the oxide **2** as a white powder, which was isolated by filtration, washed twice with ≈50-ml portions of hot 95% ethanol, and air dried to afford 3.18 g (72%), which was used in the next step without further purification. An analytical sample was recrystallized from water and dried in vacuo at 80°C; mp 205–210°C with decomposition [Lit. 216–218 (d) °C; (Scheinfeld et al., 1969) the sample explodes if inserted above 220°C]; IR (KBr) 3350, 3142, 2951, 1629, 1488, 1447, 1407, 1323, 1254, 1168, 1065, 943, 892, 773, 663, 616 cm^{-1}; 200 MHz ^{1}H NMR (D_2O, 0.05 M phosphate, pD = 7.20) δ 8.54 (s, 1H), 8.26 (s, 1H), 4.17 (s, 3H); 50 MHz ^{13}C NMR (DCl-D_2O, pD = 0.09) δ 156.1, 150.4, 145.8, 142.7, 129.2, 55.1; HR-MS (EI), m/z calcd for $C_6H_6N_4O_2$ (M^+) 166.0491, measured 166.0495; UV $\lambda_{max}^{H_2O}$ (pH ≤ 1) 208; $\lambda_{max}^{H_2O}$ (pH = 6.76) 226; Anal. calcd for $C_6H_6N_4O_2 \cdot 0.5\ H_2O$: C, 41.15; H, 4.03; N, 31.99. Found: C, 41.11; H, 4.03; N, 31.89.

Hypoxanthine-3-N-*oxide* (***3***). A stirred solution of 1.01 g (6.08 mmol) of **2** in 50 ml of 10% NaOH was heated at reflux. After 4 hr, the reaction solution was cooled with an ice bath and concentrated HCl was added to pH 9.0, decolorized with charcoal, and filterd through Celite. The solution was then made acidic (pH ≤ 1) by the addition of concentrated HCl thus precipitating **3** as a light yellow solid, which was isolated by filtration. The precipitate was stirred in ≈10 ml of water and 10% NaOH was added dropwise until the solid was dissolved. The resulting solution was decolorized with charcoal a second time and filtered through Celite. The filtrate was cooled in an ice bath and glacial acetic acid was added with gentle stirring until precipitation commenced (pH ≈ 4). The reaction mixture was then cooled overnight in a refrigerator. The resulting pale yellow crystals were isolated by filtration and washed successively with anhydrous ethanol and diethyl ether affording 0.70 g (76%); mp: slow decomposition from 210–300°C [Lit. 210–300 (d) °C, (Kawashima and Kumashiro, 1969)]; IR (KBr) 3474, 3120, 3064, 1703, 1609, 1454, 1408, 1221, 1099, 903, 797, 684, 624, 560 cm^{-1}; 200 MHz ^{1}H NMR (D_2O, 0.05 M phosphate, pD = 7.20) δ 8.29 (s, 1H), 8.21 (s, 1H); 50 MHz ^{13}C NMR

(NaOD-D_2O, pD = 11.54) δ 162.6, 149.2, 147.8, 138.2, 123.9; HR-MS (EI), m/z calcd for $C_5H_4N_4O_2$ (M^+) 152.0334, measured 152.0335; UV $\lambda_{max}^{H_2O}$ (pH $\leq$ 1) 210; $\lambda_{max}^{H_2O}$ (pH = 6.76) 220; Anal. calcd for $C_5H_4N_4O_2 \cdot 1H_2O$: C, 35.30; H, 3.56; N, 32.93. Found: C, 35.61; H, 3.51; N, 32.72.

Experimental Stimuli Preparation

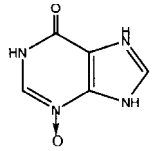
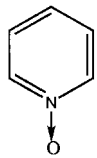
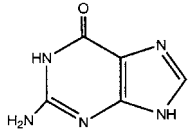
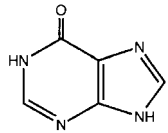
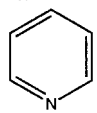
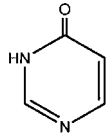
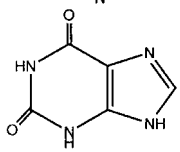
Fathead minnows (*Pimephales promelas*) were collected from an outlet pond at SUNY Cobleskill, Cobleskill, New York. Finescale dace (*Chrosomus neogaeus*) were collected from Lock 7 Kill, a small tributary stream to the Mohawk River near Schenectady, New York. Minnows and dace were held in 60-liter aquaria, filled with continuously filtered, dechlorinated tap water (pH 8.0, 18°C) on a 12L : 12D cycle. Swordtails were obtained commercially and held under similar conditions, except the water temperature was maintained at approximately 24°C. Fish were fed ad libitum, twice daily with commercial flake food. Mean (± SE) standard length at time of testing was 5.32 ± 0.18 cm for minnows and 4.98 ± 0.14 cm for dace.

To generate natural skin extracts, donor fish were killed with a blow to the head (in accordance with Union College Institutional Animal Care and Use Protocol #2-27-98). The skin was removed from either side of the donor fish and immediately placed in 50 ml of chilled glass distilled water. Skin samples were then homogenized, filtered through glass wool, and the final volumes adjusted by adding glass distilled water. A total of 27.02 cm^2 (in 325 ml), 26.21 cm^2 (in 300 ml), and 29.2 cm^2 (in 360 ml) was collected for minnows, dace, and swordtails, respectively. Our final concentrations were similar to those used by Lawrence and Smith (1989). All skin extracts were frozen in 15-ml aliquots at −20°C until needed. As a control, we also froze glass-distilled water in 15-ml aliquots at −20°C.

In addition to the hypoxanthine-3-*N*-oxide, synthesized in the laboratory (as described above), six other chemicals were obtained from commercial suppliers (Table 1). Hypoxanthine, xanthine, and guanine are structurally similar purine compounds. The purine ring system contains a fused six-member pyrimidine ring and five-membered imidazole ring which share a common side. The six membered 4(3H)-pyrimidone is almost identically to the pyrimidine moiety of hypoxanthine-3-*N*-oxide. The final two compounds, pyridine (PN) and pyridine *N*-oxide (PNO) are both six-membered heterocycles incorporating a single nitrogen. We chose these compounds because of their structural or functional similarities to hypoxanthine-3-*N*-oxide. It is important to note that hypoxanthine-3-*N*-oxide and pyridine *N*-oxide are related only by the nitrogen oxide functional group that is absent in the remaining compounds.

We prepared our experimental stimuli by dissolving 0.002 g of the compound of interest in 200 ml of glass-distilled water with stirring for 15 min. The

TABLE 1. CHEMICAL STRUCTURES OF SYNTHETIC STIMULI USED IN BOTH LABORATORY AND FIELD TRIALS

	Hypoxanthine-3-*N*-oxide
	Pyridine-*N*-oxide
	Guanine
	Hypoxanthine
	Pyridine
	4(3H)-Pyrimidone
	Xanthine

resulting solutions were frozen in 15-ml aliquots at −20°C until required. These concentrations are similar to those used by Pfeiffer et al. (1985) and Mathis and Smith (unpublished data).

Experimental Protocol

Laboratory Trials. Test tanks consisted of 37-liter aquaria, filled with dechlorinated tap water (pH 8.0) and containing a gravel substrate and a single

airstone, mounted along the back wall of the tank. An additional length of tubing was attached to the airstone, which allowed us to introduce a chemical stimulus from behind a black viewing curtain, at a distance of about 3 m (without disturbing the test fish). Test tanks were illuminated, on a 12L : 12D cycle, with 25-W Sunglow fluorescent lamps. Shoals of four minnows or dace (matched for size) were placed in the test tanks and allowed a 24-hr acclimation period (N = 10 shoals for each treatment condition). Control and experimental trials consisted of a 10-min prestimulus and a 10 min poststimulus observation period. Prior to the prestimulus period, 60 ml of tank water was withdrawn from the stimulus injection tube and discarded (to remove any stagnant water in the tube). An additional 60 ml was withdrawn and retained. At the beginning of the poststimulus period, 5 ml of distilled water (control trials) or 5 ml of conspecific skin extract (CSE), swordtail skin extract (SWT), or one of the seven synthetic stimuli (Table 1) was injected and slowly flushed into the tank using the retained 60 ml of tank water. Dye tests demonstrated that this technique results in the distribution of chemical stimuli throughout the tank in about 16 sec. Control trials were conducted between 08 : 00 and 11 : 00 hr daily, and experimental trials were conducted between 13 : 00 and 16 : 00 hr. Control trials were conducted before experimental trials because any response to the experimental stimuli may have masked any response to the control stimuli (cf. Lawrence and Smith, 1989; Hazlett, 1997).

During both the pre- and poststimulus observation periods, we recorded the position of the fish as "area use" and "shoaling index," every 15 sec. Area use was recorded as the position of each fish (1 = bottom third of the test tank, 3 = top third of the test tank) giving scores ranging from 4 (all fish near the bottom) to 12 (all fish near the surface). Shoaling index (modified from Mathis and Smith, 1993b) ranged from 1 (no fish within one body length of each other) to 4 (all fish within one body length of each other). A decrease in area use and an increase in shoaling index are indicative of an antipredator response under laboratory conditions (Mathis and Smith, 1993b; Brown et al., 1995a,b).

For both behavioral measures, we calculated the difference between the pre- and poststimulus observation periods for the control and experimental trials and compared them using the Mann-Whitney U test. We also compared the intensity of behavioral responses between the two experimental conditions using the Mann-Whitney U test.

Field Trials. We conducted a series of field trapping studies at Lincoln's Pond and Lake Myocotis, E. N. Huyck Preserve, Rensselaerville, New York, during July and August 1998, to ensure that the laboratory results were not simply artifacts (sensu Magurran et al., 1996). For each trapping study, we set Gee's Improved minnow traps in trios consisting of one trap labeled with a distilled water control and two traps labeled with separate experimental stimuli. A total of eight experimental stimuli were tested in four trapping studies (Table 2).

TABLE 2. PAIRINGS OF EXPERIMENTAL STIMULI AND DISTILLED WATER CONTROLS USED IN FIELD TRIALS

Distilled water	Distilled water	Distilled water	Distilled water
Hypoxanthine-3-*N*-oxide	Pyridine	Xanthine	Hypoxanthine
Fathead minnow skin extract	Pyridine-*N*-oxide	Guanine	4(3H)-pyrimidone

The pairings of experimental stimuli were randomly assigned. For each study, a total of 10 trios were set (except the PNO/PN+ study, were only nine trios could be set).

We labeled the traps by attaching two cellulose sponges ($2 \times 2 \times 2$ cm) to the funnel end of the trap using stainless steel wire. Sponges were infused with 20 ml of distilled water or the experimental stimulus. Traps were set no less than 15 m apart along the shoreline at a depth of approximately 50 cm, and the order of stimuli within each trio was randomly assigned. Care was taken to ensure that each trap within a trio was set on similar substrate. Traps were set for exactly 2 hr, beginning at 8 : 30 AM each day. Wisenden et al. (1995) have demonstrated that fathead minnows actively avoid areas labeled with conspecific skin extract for periods of at least 2 hr. This observation suggests that the sponges used in this study should remain active for at least the 2-hr trapping period. After the 2-hr period, we removed the traps and recorded the number and species of all fish caught. Because of daily variability in capture rates, each trap study included a distilled water control and was analyzed independently.

For each study, we compared the number of fish caught per trap between the distilled water control and the two experimental treatments using the Kruskal-Wallis test. Individual comparisons were made, if required, using the Mann-Whitney U test. A χ^2 test was used to detect any effect of the chemical stimuli on species composition of fish caught.

RESULTS

Laboratory Trials

We observed significant differences in area use scores (i.e., individuals remained closer to the substrate) in both minnows and dace when exposed to conspecific skin extract or H3NO (compared to distilled water controls; Figure 2 and 3; Tables 3 and 4). No significant effects were found with the remaining experimental stimuli (Figure 2 and 3; Tables 3 and 4). In addition, no significant difference was observed in the area use response to conspecific skin extract or H3NO for fathead minnows (Mann-Whitney U, $Z = -0.15$, $P = 0.88$) or in the area use response of finescale dace to conspecific skin extract, H3NO or PNO (Kruskal-Wallis, $H = 0.30$, $P = 0.86$).

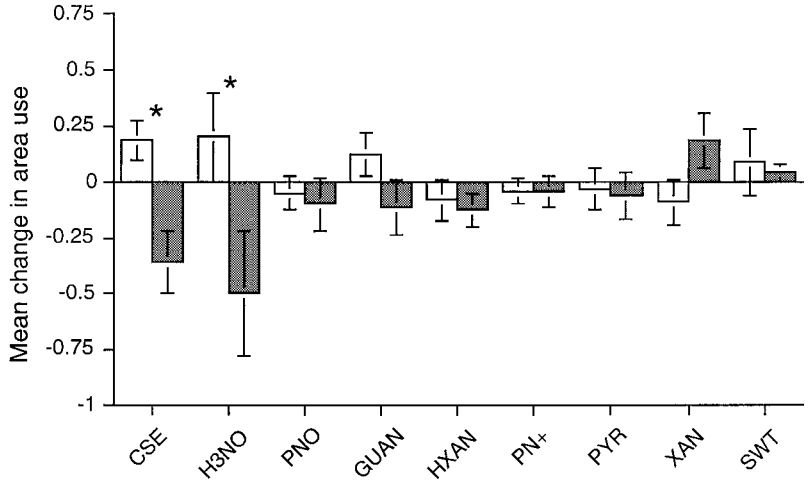


FIG. 2. Mean (± SE) change in area use scores by fathead minnows exposed to distilled water controls (open bars) and experimental stimuli (solid bars). CSE = conspecific skin extract, H3NO = hypoxanthine-3-*N*-oxide, PNO = pyridine-*N*-oxide, GUAN = guanine, HXAN = hypoxanthine, PN+ = pyridine, PYR = 4(3H)-pyrimidone, XAN = xanthine, SWT = swordtail skin extract. *Significant differences at $P < 0.05$. Probabilities based on Mann-Whitney U test. See text and Table 3 for details.

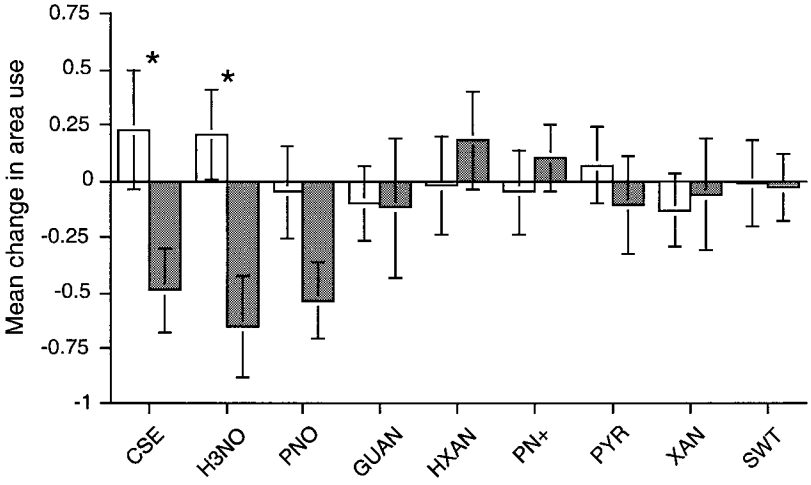


FIG. 3. Mean (± SE) change in area use scores by finescale dace exposed to distilled water (open bars) and experimental stimuli (solid bars). Abbreviations as in Figure 2. *Significant differences at $P < 0.05$. Probabilities based on Mann-Whitney U test. See text and Table 4 for details.

TABLE 3. INDIVIDUAL COMPARISONS FOR FATHEAD MINNOW RESPONSE TO EACH EXPERIMENTAL STIMULUS VERSUS DISTILLED WATER CONTROL BASED ON MANN-WHITNEY U TESTS

	Area use		Shoaling index	
Experimental stimulus	*Z*	*P*[a]	*Z*	*P*[a]
Fathead minnow skin extract	−2.91	<0.05	−2.31	<0.05
Hypoxanthine-3-*N*-oxide	−2.21	<0.05	−2.42	<0.05
Pyridine-*N*-oxide	−0.38	NS	−3.06	<0.05
Guanine	−1.21	NS	−0.64	NS
Hypoxanthine	−0.34	NS	−0.19	NS
Pyridine	−1.02	NS	−0.11	NS
4(3H)-Pyrimidone	−0.11	NS	−0.38	NS
Xanthine	−0.72	NS	−0.57	NS
Swordtail skin extract	−0.72	NS	−0.23	NS

[a]Probabilities corrected for multiple comparisons with modified Bonferonni test.

We observed similar tends in shoaling index scores. Both minnows and dace significantly increased shoal cohesion in response to conspecifics skin extract, H3NO and PNO (Figures 4 and 5; Tables 3 and 4) but not to the remaining experimental stimuli (Figures 4 and 5; Tables 3 and 4). Again, there were no significant differences in the magnitude of the shoaling response between conspecific skin extract, H3NO or PNO for minnows ($H = 0.88$, $P = 0.64$) or dace ($H = 1.94$, $P = 0.38$).

TABLE 4. INDIVIDUAL COMPARISONS FOR FINESCALE DACE RESPONSE TO EACH EXPERIMENTAL STIMULUS VERSUS DISTILLED WATER CONTROL BASED ON MANN-WHITNEY U TESTS

	Area use		Shoaling index	
Experimental stimulus	*Z*	*P*[a]	*Z*	*P*[a]
Finescale dace skin extract	−2.27	<0.05	−2.91	<0.05
Hypoxanthine-3-*N*-oxide	−2.19	<0.05	−2.26	<0.05
Pyridine-*N*-oxide	−2.01	NS[b]	−2.29	<0.05
Guanine	−0.15	NS	−0.19	NS
Hypoxanthine	−0.11	NS	−0.79	NS
Pyridine	−0.78	NS	−0.91	NS
4(3H)-Pyrimidone	−0.64	NS	−0.04	NS
Xanthine	−0.11	NS	−1.10	NS
Swordtail skin extract	−0.23	NS	−0.84	NS

[a]Probabilities corrected for multiple comparisons with modified Bonferonni test.
[b]Significantly different prior to Bonferonni correction.

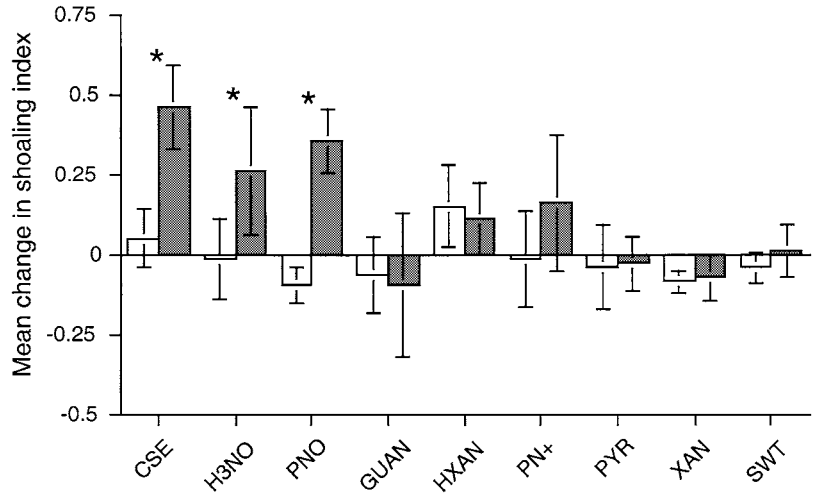


FIG. 4. Mean (± SE) change in shoaling index scores by fathead minnows exposed to distilled water (open bars) and experimental stimuli (solid bars). Abbreviations as in Figure 2. *Significant differences at $P < 0.05$. Probabilities based on Mann-Whitney U test. See text and Table 3 for details.

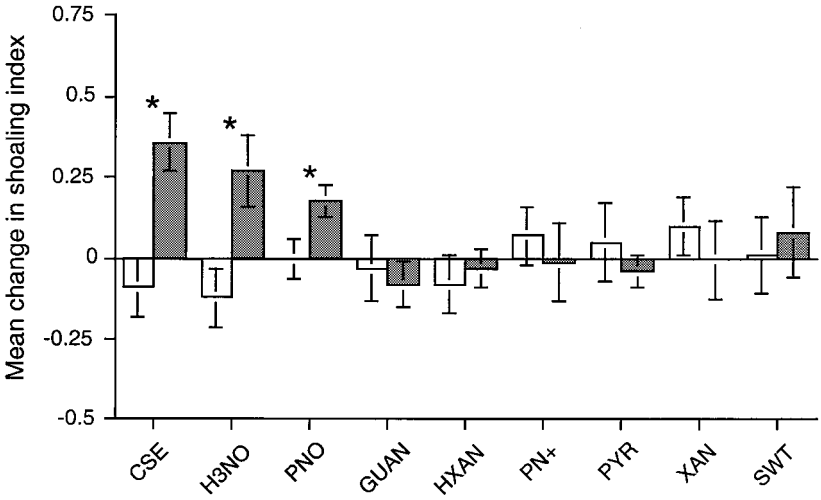


FIG. 5. Mean (± SE) change in shoaling index scores by finescale dace exposed to distilled water (open bars) and experimental stimuli (hatched bars). Abbreviations as in Figure 2. *Significant differences at $P < 0.05$. Probabilities based on Mann-Whitney U test. See text and Table 4 for details.

TABLE 5. OVERALL AND INDIVIDUAL COMPARISONS OF MEAN NUMBER OF FISH CAUGHT IN TRAPS DURING FIELD TRIALS

	Fish caught (mean ± SE)	Overall comparison (H)[a]	Individual comparisons vs. distilled water control traps (Z)[b]
Trial 1			
Distilled water	10.7 ± 2.2		
Fathead minnow skin extract	0.7 ± 0.7	$H = 13.71$	$Z = -2.87$
		$P < 0.001$	$P < 0.002$
Hypoxanthine-3-*N*-oxide	0.0 ± 0.0		$Z = -3.02$
			$P < 0.001$
Trial 2			
Distilled water	9.2 ± 2.0		
Pyridine	5.4 ± 1.8	$H = 9.20$	$Z = -1.37$
		$P < 0.004$	$P = 0.16$
Pyridine-*N*-oxide	0.0 ± 0.0		$Z = -2.78$
			$P < 0.002$
Trial 3			
Distilled water	4.7 ± 0.7		
Xanthine	3.1 ± 1.1	$H = 0.16$	N/A
		$P = 0.92$	
Guanine	5.3 ± 1.2		N/A
Trial 4			
Distilled water	6.0 ± 1.7		
Hypoxanthine	3.9 ± 0.9	$H = 4.51$	N/A
		$P = 0.12$	
4(3H)-pyrimidone	5.1 ± 1.4		N/A

[a]Kruskal-Wallis nonparametric ANOVA.
[b]Mann-Whitney U test.

Field Trials

Five species were commonly caught during the field trapping studies: green sunfish (*Lepomis cyanellus*), yellow perch (*Perca flavescens*), emerald shiners (*Notropis atherinoides*), finescale dace, and fathead minnows. The results of our trappings studies confirm those of our laboratory studies. Traps with fathead minnow skin extract, hypoxanthine-3-*N*-oxide, and pyridine-*N*-oxide caught significantly fewer fish than did their paired distilled water control traps (Table 5). There were no significant effects of guanine, hypoxanthine, pyridine, 4(3H)-pyrimidone, or xanthine on the number of fish caught (relative to the paired distilled water controls; Table 5) or on the distribution of species caught (relative to the paired distilled water controls; $\chi^2 = 7.01, 7.02, 1.65, 6.89, 3.57$, $P > 0.05$ for all).

DISCUSSION

Our laboratory and field data strongly suggest that the nitrogen oxide functional group is a significant component of the ostariophysan chemical alarm signal system. In the laboratory studies, we observed significant increases in antipredator behavior in both fathead minnows and finescale dace when they were exposed to conspecific skin extract, hypoxanthine-3-*N*-oxide, and pyridine-*N*-oxide, but not to guanine, hypoxanthine, xanthine, 4(3H)-pyrimidone, pyridine, or swordtail skin extract. If the purine skeleton were the critical arrangement, we should have seen an increased antipredator response to any of the purine compounds (i.e., hypoxanthine, guanine, xanthine), but not to the monocyclic 4(3H)-pyrimidone, pyridine, or pyridine-*N*-oxide. If a combination of the purine skeleton and the nitrogen oxide functional group were important, we would predict a significant antipredator response to hypoxanthine-3-*N*-oxide and the conspecific skin extract only. If, however, the presence or absence of the nitrogen oxide functional group were the molecular trigger, only those compounds containing that functional group (hypoxanthine-3-*N*-oxide and pyridine-*N*-oxide) should elicit a significant behavioral response.

We obtained similar results from the field-trapping experiments, supporting our laboratory results. Significantly fewer fish were caught in traps labeled with fathead minnow skin extract, hypoxanthine-3-*N*-oxide, and pyridine-*N*-oxide compared with the distilled water control traps. Captures in traps labeled with guanine, hypoxanthine, pyridine, or 4(3H)-pyrimidone were not significantly different from those in traps labeled with distilled water. Lincoln's Pond and Lake Myocotis contain a diverse prey guild (prey species utilizing similar microhabitats and subject to the same predation pressures), consisting of both ostariophysan and nonostariophysan species. The observation that nonostariophysan species (sunfish and perch) avoided traps labeled with minnow skin extract, hypoxanthine-3-*N*-oxide, and pyridine-*N*-oxide suggests that they may also rely on nitrogen oxides as an alarm signaling agent. Yellow perch and green sunfish have recently been shown to possess analogous chemical alarm signals (Reehan Mirza, personal communication, personal observations). It is possible that nitrogen oxides may act as a common signaling agent in a variety of prey fishes, including nonostariophysans. This hypothesis remains to be tested.

These data also suggest that, contrary to Pfeiffer et al. (1985), hypoxanthine-3-*N*-oxide may not be the sole active molecule in the ostariophysan alarm signaling system. Rather, as our data suggest, any compound with a nitrogen-oxide functional group may act as a potential signaling agent. Pfeiffer et al. (1985) found no response by black tetras to purported "hypoxanthine-1-*N*-oxide" (**4**). In their studies the analyte samples were prepared by desolusion in dilute HCl with a final acid concentration of 0.01 M (pH 2). Under these conditions it has been shown that "hypoxanthine-1-*N*-oxide" exists as the neutral 1-hydroxyhypo-

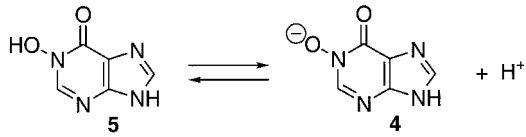


FIG. 6. Acid-Base equilibrium between 1-hydroxyhypoxanthine (**5**) and hypoxanthine-1-*N*-oxide (**4**).

xanthine (**5**, Figure 6) with $pK_a = 5.68 \pm 0.06$ for the hydroxy proton (Parham et al., 1966). If the final pH of the test sample were never significantly above the pK_a of **5**, the test hypoxanthine would remain in the neutral-protonated form, providing additional, indirect support for our assertion that the nitrogen oxide functional group serves as the primary molecular trigger.

A comparison of the nitrogen–oxygen bond of pyridine-*N*-oxide and 1-hydroxyhypoxanthine is also illustrative and further supports our hypothesis. Beyond the fact that these bonds incorporate the same atoms, nitrogen and oxygen, they are not similar; the nitrogens in both hypoxanthine-3-*N*-oxide and pyridine-*N*-oxide bare a formal positive charge whereas the nitrogen in 1-hydroxyhypoxanthine does not. This difference will lead to a significant decrease in the overall bond polarization between the nitrogen and oxygen. Thus, even the deprotonated form of 1-hydroxyhypoxanthine would not be predicted to function as signaling agent. Analytical work is ongoing to investigate this hypothesis.

These data also suggest that the alarm signaling system of ostariophysan fishes may be affected by acidification of natural waterways. Fathead minnow and finescale dace held at pH 6.0 do not show a significant response to conspecific skin extract, but do display a normal antipredator response to the same chemical alarm signals when held at pH greater than 7.5 (Brown, Adrian, and Lewis, unpublished data). This may result from one of at least three nonmutually exclusive factors. Smith and Lawrence (1989) report an overall decrease in activity in fathead minnows held at lower pH. A reduced level of activity would make it difficult to detect a significant alarm response under laboratory conditions. Chronic exposure to acidified waters may result in damage to the olfactory epithelium (Lemly and Smith, 1985, 1987), reducing an individual's ability to detect chemical signals. Finally, reduced pH of natural waterbodies may result in a structural change to the chemical alarm signals, reducing its efficiency as an alarm signal. It is likely that reduced pH levels (e.g., in acidified waterbodies) result in the protonation of some functional groups, most notably the nitrogen oxide group.

The acidity constant of the conjugate acid of the *N*-oxide functionality of **3** (structure **6** in Figure 7) has been measured: $pK_a = 1.2 \pm 0.1$ (Scheinfeld et al., 1969); as the pH of the medium decreases, the equilibrium will shift in favor of the hydroxy form **6** and thus decrease the relative concentration of the depro-

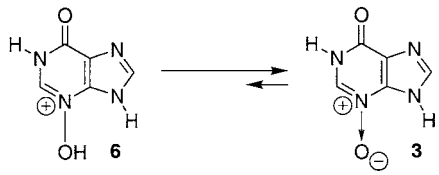


FIG. 7. Acid-Base equilibrium between 3-hydroxyhypoxanthine (**6**) and hypoxanthine-1-*N*-oxide (**3**).

tonated active form of the chemical signal. It would be expected that such a chemical change would affect this compound's interaction within the binding site, thus precipitating a change in the observed behavioral response. Experiments are ongoing to address this question.

Acknowledgments—The authors wish to thank Brian Wisenden and Leo Fleishman for their comments on earlier versions of the manuscript. Financial support was provided by Union College to G.E.B. and J.C.A. and by a Merck Summer Scholarship to H.L. The authors wish to dedicate this manuscript to the memory of Professor R. Jan F. Smith.

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