Chemoecology 16: 93–96 (2006)
0937-7409/06/020093-4

DOI 10.1007/s00049-005-0333-5

CHEMOECOLOGY

Can the ratio of aromatic skeletons explain cross-species responses within evolutionarily conserved Ostariophysan alarm cues?: testing the purine-ratio hypothesis

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Summary. While the response to damage-released chemical alarm cues within the superorder Ostariophysi appears to be highly conserved across species, it is generally observed that the intensity of response to heterospecific alarm cues decreases with increasing phylogenetic distance. Recent studies have demonstrated that purine-*N*-oxides function as chemical alarm cues within Ostariophyian fishes and that the nitrogen-oxide functional group is conserved as the chief molecular trigger. According to the purine-ratio hypothesis, these cross-species differences may be due to the relative proportion of different carrier compounds associated with the nitrogen-oxide molecular trigger. To test this hypothesis, we exposed glowlight tetras (*Hemigrammus erythrozonus*, Characidae, Ostariophysi) to one of five synthetic stimuli (hypoxanthine-3-*N*-oxide (H3NO), pyridine-*N*-oxide (PNO) or mixed stimuli of 75 % H3NO-25 % PNO, 50 % H3NO-50 % PNO, or 25 % H3NO-75 % PNO), natural conspecific chemical alarm cue or a distilled water control. We quantified changes in shoal cohesion and vertical area use as species typical indicators of an antipredator response. As predicted, response intensity decreased as the ratio of hypoxanthine-3-*N*-oxide to pyridine-*N*-oxide decreased and the strongest response was to natural alarm cue. These results suggest that species-specific carrier compounds may account for the well-documented cross-species differences in the response to heterospecific alarm cues within phylogenetically related taxa.

Key words. Ostariophysan fishes – chemical alarm cue – predation – prey guilds – *Hemigrammus erythrozonus* – cross-species responses

Introduction

A wide range of taxonomically diverse prey fishes rely on damage released chemical alarm cues to detect and avoid potential predators (Chivers & Smith 1998, Smith 1999, Brown 2003). These alarm cues are typically sequestered in the epidermis and released following mechanical damage to the skin, as would occur during a predation event (Smith 1992, 1999). When released into the water column, these chemical alarm cues can elicit dramatic, short-term increases in species typical antipredator behaviours in nearby conspecifics and some heterospecifics (Chivers & Smith 1998, Smith 1999), leading to increased survival benefits to alarm cue receivers (Mathis & Smith 1993, Mirza & Chivers 2001a).

Several authors have demonstrated that within taxonomically related groups, the response to heterospecific chemical alarm cues can be evolutionarily conserved (Brown *et al.* 2001a, 2003, Mirza & Chivers 2001b, Mirza *et al.* 2001, Leduc *et al.*, 2003). The general trend, however, is that the intensity of behavioural response to heterospecific alarm cues decreases as the phylogenetic distance between donor and receiver species increases (Shutz 1956, Mirza & Chivers 2001b, Mirza *et al.* 2001). Within prey guilds, there should exist strong selection pressures to learn to recognize heterospecific alarm cues as reliable information sources for local predation risk assessment (Brown 2003).

The superorder Ostariophysi is a diverse, species rich taxonomic group of primarily freshwater fishes, accounting for approximately 65 % of all freshwater species (Nelson 1994) and includes over 6500 described species (Moyle & Cech 1996). Hypoxanthine-3-*N*-oxide (H3NO, Figure 1), characterized by a purine skeleton with a N-O functional group at the three position, has been suggested as an active component of the Ostariophysan chemical alarm cue system (Argentini 1976, Pfeiffer *et al.* 1985, Brown *et al.* 2000). However, recent work has suggested that the Ostariophysan alarm cue may consist of a suite of aromatic compounds related through a common N-O functional group and that purine compounds lacking a N-O functional group do not elicit any behavioural response (Brown *et al.* 2000, 2001a, 2003). Moreover, the response to compounds containing a N-O functional group appears to be highly conserved within at least three orders of Ostariophysan fishes (Cyprinids, Brown *et al.* 2000; Characins, Brown *et al.* 2001a; Silurids, Brown *et al.* 2003).

Brown *et al.* (2000, 2003) suggested that the Ostariophysan alarm cue system may be comprised of a suite of purine compounds sharing a common N-O functional group. Species differences in the ratio of specific purines (and possibly associated proteins/peptides, Kasumyan &

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Ponomarev 1987) would be expected to be more similar among closely related species compared to more distantly related species (Brown *et al.* 2000). The 'purine-ratio' hypothesis (Brown *et al.* 2000) argues that even though the N-O functional group appears to be highly conserved, species-specific differences in associated compounds might account for the well-documented decline in response intensity with increasing phylogenetic distance within Ostariophysan fishes. To date, no tests of this model have been conducted.

In a series of laboratory trials, we exposed glowlight tetras (*Hemigrammus erythrozonus*, Characidae, Ostariophysi) to a combined stimulus of hypoxanthine-3-*N*-oxide and pyridine-*N*-oxide (PNO, Fig. 1). Glowlight tetras exhibit strong species typical antipredator responses to H3NO and significant, but weaker, responses to similar concentrations of PNO (Brown *et al.* 2001a). The objective of this study was to assess the potential effects of varying the ratio of hypoxanthine versus pyridine skeletons, while holding the absolute concentration of the nitrogen-oxide functional group constant. According to the 'purine ratio' hypothesis, we predict a decreasing response intensity as the proportion of PNO increased (and H3NO decreased).

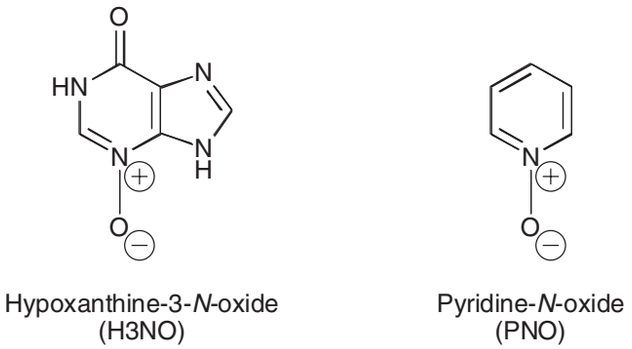

Fig. 1 Chemical structure of hypoxanthine-3-*N*-oxide and pyridine-*N*-oxide

Methods

Test fish: Glowlight tetras were obtained from a commercial supplier and held in 110 L glass aquaria, filled with continuously filtered, dechlorinated tap water (pH 7.2, 26 °C, 12:12 Light:Dark cycle) and a gravel substrate. Tetras were fed *ad libitum*, twice daily with commercial flake food and brine shrimp (*Artemia* spp.).

Experimental stimuli: Hypoxanthine-3-*N*-oxide (molecular weight = 170.13 g mol^{-1}) was synthesized as described in Brown *et al.* (2000). Pyridine-*N*-oxide (molecular weight = 95.10 g mol^{-1}) was obtained from Aldrich Chemical and was used as shipped. We prepared stock solutions of H3NO and PNO by dissolving 4.30 x 10^{-3} g and 2.40 x 10^{-3} g respectively, into 250 ml of glass-distilled water and stirring for at least 30 min. Stock solutions were then frozen at –20 °C in 15 ml aliquots until required. The use of these initial stock solutions ensured that the absolute concentration of N-O functional groups was consistent across all synthetic treatments (see below).

Natural skin extracts were collected from five donors (mean ± S.E. = 3.02 ± 0.07 cm). Donors were humanely killed with a blow to the head, in accordance with Concordia University Animal Care Protocol AC-2002-BROW and skin filets were immediately removed from either side and placed into chilled distilled water. Skin fillets were then homogenized and filtered through polyester floss and diluted to final volume with distilled water. We collected a total of 11.80 cm^2 of skin in a final volume of 140.60 ml. The final concentration was similar to that used by Brown *et al.* (2001a). Skin extracts were frozen in 15 ml aliquots at –20 °C until required.

Experimental protocol: Trials were conducted in a series of 37 l glass aquaria, filled with 35 l of dechlorinated tap water (pH 7.0-7.2, 25-26 °C), and wrapped on three sides with brown paper to prevent visual communication between test tanks. Each tank contained a single airstone anchored along the back wall. We attached an additional 2 m length of silicon tubing to the airstone to allow for the injection of stimuli from behind a black plastic viewing blind.

We placed shoals of four tetras (matched for size, N = 15 for each treatment) and allowed a 24 hour acclimation period prior to testing. Individual tetras were only tested once. Trials consisted of a 10 min pre-stimulus injection and a 10 min post stimulus injection observation period. Prior to the pre-stimulus observation period, we withdrew and discarded 60 ml of tank water through the stimulus injection tube and then withdrew and retained an additional 60 ml. Following the pre-stimulus observation period, we injected one of the six experimental stimuli or the distilled water control stimulus. Experimental stimuli included 5 ml of tetra skin extract or 2.8 ml of the synthetic stimuli in one of 5 treatments: 1) 100 % H3NO, 2) 75 % H3NO-25 % PNO, 3) 50 % H3NO-50 % PNO, 4) 25 % H3NO-75 % PNO or 5) 100 % PNO. The control stimulus consisted of 5 ml of glass-distilled water. We slowly flushed the experimental or control stimuli into the test tank using the retained 60 ml of tank water and began the post-stimulus observation period once the stimulus was fully introduced. All trials were videotaped for later behavioral analyses.

From the videotapes, we recorded shoaling index and area use scores every 15 sec. Shoaling index, a measure of shoal cohesion, ranged from 1 (no fish within one body length of each other) to 4 (all fish within one body length of each other). Area use was recorded as the position of each fish within the test tank. Scores ranged from 4 (all fish near the substrate) to 12 (all near the surface).

For both shoaling and area use scores, we calculated the difference between the mean pre-stimulus and mean post-stimulus observation periods (post-stimulus – pre-stimulus) and used these difference scores as dependant variables in all analyses. We tested for any overall effects of stimulus using one-way ANOVAs. Individual post-hoc comparisons were made using Fisher's Protected Least Squared differences. We used Pearson's product moment correlations to determine if the response intensities were related to the H3NO-PNO ratio.

Results

For both shoaling and area use measures, we found significant overall treatment effects (shoaling: $F_{(6, 98)} = 8.22$, $P < 0.0001$; area use: $F_{(6, 98)} = 4.92$, $P = 0.0002$; Fig. 2). We found significant increases in shoaling index and significant decreases in area use for tetras exposed to the experimental stimuli versus the distilled water control (Fig. 2A, 2B). Moreover, highest intensity response was seen for those tetras exposed to the natural skin extract and the 100 % H3NO treatments. As the proportion of PNO increased, the intensity of the behavioural response decreased, resulting in a significant negative correlation for shoaling index ($r = –0.56$, $P < 0.0001$) and a significant positive correlation for area use ($r = 0.45$, $P < 0.0001$). Given the strong response towards natural skin extracts, we repeated the correlation comparisons, omitting the natural skin extract treatment (i.e. including the five synthetic stimuli and the distilled water control). We still find significant correlations for shoaling index ($r = –0.47$, $P < 0.0001$) and area use ($r = 0.36$, $P = 0.0004$), demonstrating that as the ratio of

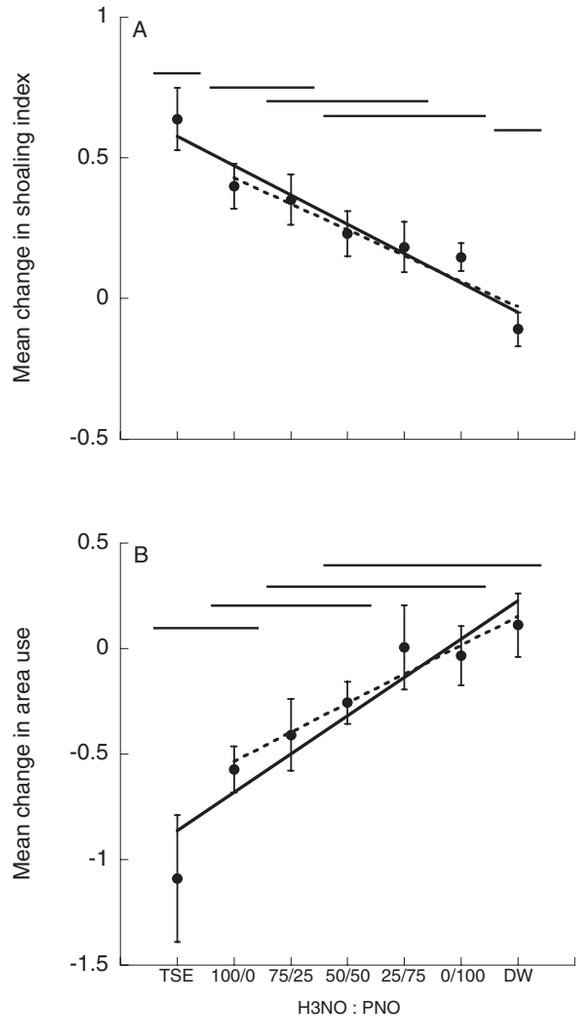

Fig. 2 Mean (± SE) change in shoaling index (A) and area use (B) for shoals of glowlight tetras exposed to conspecific skin extract (TSE), hypoxanthine-3-*N*-oxide and pyridine-*N*-oxide at ratios of 100 % H3NO (100/0), 75 % H3NO, 25 % PNO (75/25), 50 % H3NO, 50 % PNO (50/50), 25 % H3NO, 75 % PNO, 100 % PNO (0/100) or a distilled water control (DW). Treatments under different bars are significantly different ($P < 0.05$, based on Fisher's Protected Least Square differences, $N = 15$ shoals for each treatment)

H3NO to PNO decreases, the intensity of the antipredator response is likewise reduced.

Discussion

Our current results demonstrate a significant relationship between the intensity of antipredator behaviour and the relative proportion of H3NO versus PNO presented. We found that as the proportion of H3NO decreased, while holding the absolute concentration of the N-O molecular trigger constant, glowlight tetras significantly reduced the intensity of both shoaling and area use. We found the highest intensity response by shoals exposed to the natural alarm cue and to the 100 % H3NO treatments. As we increased the proportion of pyridine vs. purine skeleton, we found a concomitant decrease in response intensity. The response to the 100 % PNO treatment, though less intense than that elicited by the 100 % H3NO treatment, was still significantly different from the distilled water control. As such, our current results provide initial support for the 'purine-ratio' hypothesis and suggest that species specific differences in the ratio of highly conserved components of alarm cues may account for the well-documented cross-species response patterns within phylogenetically related species.

There should exist strong selection pressures for prey to recognize the alarm cues of heterospecific prey guild members (Smith 1999; Chivers *et al.* 2002). Such recognition may result from learning in the case of taxonomically distant prey guild members (Chivers *et al.* 1995, Pollock *et al.* 2003) or via conservation of some recognizable component of the alarm cue within taxonomically related groups (Smith 1999, Mirza & Chivers 2001b, Brown *et al.* 2003). Conservation of the alarm cue would allow for related species to respond to heterospecific alarm cues, independent of prior experience (Smith 1999). This would be potentially beneficial for phylogenetically related prey guild members (Smith 1999).

Damage-released chemical alarm cues are argued to be derived from metabolic byproducts (Brown *et al.* 2001a) and, in the case of Ostariophysan fishes, sequestered into specialized epidermal club cells (Chapman & Johnson 1997). Specific purine compounds, proteins and/or peptides associated with the highly conserved N-O trigger are likely to be more similar among closely related species (for example, two cyprinid species should show higher similarity than a cyprinid and a characin species). The most parsimonious scenario is that species specificity is derived not from the actual 'molecular trigger' (Brown *et al.* 2000) but rather from species-specific carrier compounds (sensu Wyatt 2003). Differences in the purine ratio and in associated carrier proteins could influence the species-specific binding potentials with receptor proteins, altering the molecular recognition of the cue (Mezler *et al.* 2001). Species specific differences in carrier compounds may therefore influence the binding efficiency with species specific receptor proteins.

Alternatively, the observed results may be due to a simple concentration gradient. The relative concentration of alarm cue detected should be directly related to proximity to a predation event, and as such, prey fishes should be able to assess local predation risk based on the concentration of alarm cue detected (Lawrence & Smith 1989, Dupuch *et al.* 2004). Prey capable of adjusting the intensity of their antipredator response should be at a selective advantage, as they would be able to optimize the threat-sensitive trade-off between predator avoidance and foraging benefits (Helfman 1989). This is unlikely for two reasons. Initially, in all treatments with synthetic stimuli, the absolute concentration of the N-O functional group was constant. Secondly, Brown *et al.* (2001b) have previously shown that fathead minnows (*Pimephales promelas*, Cyprinidae, Ostariophysi) exhibit an all-or-nothing response pattern when exposed to varying concentrations of hypoxanthine-3-*N*-oxide. Minnows exposed to H3NO at concentrations between 0.4 and 6.7 nM exhibited strong antipredator responses of similar intensities, while those exposed to concentrations below 0.4 nM were

not significantly different from a distilled water control (Brown *et al.* 2001b).

Nitrogen-oxides may have been selected and/or conserved as an active component of the Ostariophysan alarm cue system for a number of reasons: 1) they are metabolically inexpensive, 2) would allow for reliable cross-species responses among taxonomically related species, independent of prior experience and 3) are stable and active under normal environmental conditions (Brown *et al.* 2001a; Brown *et al.* 2002). Clearly, additional work on the isolation and further characterization of the alarm cue of Ostariophysan fishes is required, but these data do demonstrate that the purine-ratio hypothesis is a potential mechanism accounting for the well-documented cross-species response patterns. In fact, Smith (1999) commented that one of the major deficiencies in our current understanding of the evolution of damage released chemical alarm cues in Ostrariophysan fishes is the lack of detailed chemical analysis.

Acknowledgements

We thank James Grant and Douglas Chivers for comments and Isabelle Désormeaux, Harrison Brown and Antoine Leduc for assistance in the laboratory. Financial support was provided by Concordia University and NSERC of Canada to G.E.B.

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Received 19 September 2005; accepted 11 December 2005
Published Online First 7 March 2006.