PROCEEDINGS OF THE ROYAL SOCIETY B

Proc. R. Soc. B (2008) **274**, 101–105
doi:10.1098/rspb.2007.1283
Published online 24 October 2007

Scents and scents-ability: pollution disrupts chemical social recognition and shoaling in fish

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Chemical cues are of enormous importance in mediating the behaviour of animals, enabling them to navigate throughout their habitats, to detect the presence of predators or prey and for social recognition—identifying and discriminating between conspecifics. In many species of freshwater fish, social recognition is known to be based primarily on chemical cues. Such recognition mechanisms are vulnerable to disruption by the presence of anthropogenic contaminants in the aquatic environment. Here we show that acute exposure to low, environmentally relevant dosages of the ubiquitous contaminant, 4-nonylphenol, can seriously affect social recognition and ultimately social organization in fishes. A 1 hour 0.5 $\mu g\,l^{-1}$ dose was sufficient to alter the response of members of a shoaling fish species (juvenile banded killifish, *Fundulus diaphanus*) to conspecific chemical cues. Dosages of 1–2 $\mu g\,l^{-1}$ caused killifish to orient away from dosed conspecifics, in both a flow channel and an arena. Given the overall importance of shoaling as an adaptive strategy against predators and for locating food, it is likely that its disruption by anthropogenic contaminants would have serious implications for fishes' fitness.

Keywords: ecotoxicology; shoaling; recognition; chemosensory

1. INTRODUCTION

Contamination of the aquatic habitat by anthropogenic chemicals is a pernicious problem in many parts of the world. Lately, there has been a growing appreciation that contaminants can significantly affect organisms at concentrations far below those which might kill them (Fisher *et al.* 2006; Lurling & Scheffer 2007). These effects may include subtle behavioural or physiological effects which across populations may ultimately result in what researchers have termed 'ecological death' (Clotfelter *et al.* 2004; Scott & Sloman 2004).

For aquatic animals, chemical cues are of particular relevance owing to the properties of water as a solvent and a medium to disperse such cues, and owing to the limitations on vision at depth and in complex or turbid environments. Chemical cues are of enormous importance to shoaling fish, which are strongly attracted towards the smell of conspecifics (Keenleyside 1955). Recent findings suggest that chemical cues may be of greater relative importance than visual cues in this context especially for longer range detection (Ward *et al.* 2002). Chemical cues allow fish to discriminate between conspecifics with a high degree of specificity and play an important part in maintaining their patterns of social organization, from shoals to dominance hierarchies and territorial assemblages (Todd *et al.* 1967; Courtenay *et al.* 1997; Wyatt 2003; Ward *et al.* 2007).

Chemical contaminants in the aquatic environment have been implicated in disrupting the chemosensory abilities of fish. Bardach *et al.* (1965) reported that exposure to surfactants affected receptor function in catfish (*Ictalurus natalis*). Furthermore, Olsén & Höglund (1985) found that surfactants reduced chemoattraction of juvenile Arctic charr (*Salvelinus alpinus*) to conspecific chemical cues. Indeed, the near ubiquitous presence of surfactants in aquatic habitats associated with human settlements is of considerable concern, given the broad ranging ecotoxicological effects of these chemicals on aquatic life. 4-Nonylphenol (4-NP) is one of the most common contaminants in the aquatic environment and is employed extensively in industrial and sewage treatment processes, where it is used as a surfactant (Porter & Hayden 2002). As well as its documented oestrogenic effects in fish, 4-NP has been shown to have toxic effects on fish (Cardinali *et al.* 2004; Madsen *et al.* 2004; Ishibashi *et al.* 2006). Owing to its toxicity, 4-NP is subject to stringent regulations and in most industrialized countries, freshwater concentrations of 4-NP are typically low, yet near sewage outflows the concentrations range from 0.5 to 343 $\mu g\,l^{-1}$ (Helsinki Commission Report 2002; Ying *et al.* 2002; Jobling *et al.* 2003); few data exist for 4-NP contamination in developing countries, but in the absence of legislation, it is possible that concentrations in places may be even greater than this.

In a previous study (Ward *et al.* 2006), we detected effects of exposure to 4-NP on the behaviour of the rainbow trout (*Oncorhynchus mykiss*), that potentially implicated it in the disruption of chemical communication in these fish, although this was not specifically tested. In this study, we explicitly tested whether a brief exposure to low concentrations of 4-NP would affect chemical communication in a shoaling fish, the banded killifish (*Fundulus diaphanus*).

2. MATERIAL AND METHODS

(a) *Study animals*

Juvenile, young-of-the-year, banded killifish were collected for use in the experiments from Morice Lake (near Sackville,

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Received 18 September 2007
Accepted 8 October 2007

New Brunswick, Canada; 45°55′ N, 64°21′ W). Banded killifish are a common shoaling fish found in the littoral zone of many North American lakes. All fish used in the experiments were screened visually for parasites and only uninfected fish measuring 30 ± 5 mm were used in the experiments.

(b) *Experimental equipment and protocol*

(i) *Detection of conspecific chemical cues in a flow channel*

In order to test the effects of 4-NP on banded killifish social recognition by chemical cues, we studied the behaviour of test fish in a flow channel. The flow channel was made of Perspex and measured $71 \times 38 \times 20$ cm ($L \times W \times D$). Mesh barriers were used to create a central compartment measuring $34 \times 38 \times 20$ cm ($L \times W \times D$) within the flow channel itself. We distributed a thin layer of sand as a substrate in this central compartment. Water flowed into the channel at two points, the top left and the top right corners, from two 15 l reservoir buckets at a rate of 380 ± 20 ml min^{-1} at each point. The water drained out of the flow channel at the bottom left and the bottom right corners through holes drilled into the walls. This had the effect of creating two parallel currents within the channel. Baffles placed at right angles to the current served to constrain the two streams. We repeatedly tested the flow patterns using water-borne dye and found that the dye streams remained separate for the full length of the flow channel with only a small amount of mixing in the centre of the flow channel. We used this information to define three equally sized areas within the flow channel, with one neutral zone in the centre and a zone for each stream. We marked these zones by placing thin strips of plastic in the sand substrate. The water used in the flow channel was not recirculated.

Prior to the start of the trials, we added 10 stimulus fish to one of the two reservoir buckets. To avoid transferring excess chemicals to the stimulus fish, the fish were dipped briefly into a tank of clean water at the same temperature before being added to the bucket. Based on our earlier dye experiments, we then allowed 30 min to elapse so that the cues provided by the stimulus fish would have ample time to form a chemical plume in the flow channel. Once this 30 min period had elapsed, a single focal fish was introduced to the centre of the central compartment in a mesh cylinder of 10 cm diameter and was left to acclimatize for 5 min before the cylinder was raised by a pulley and the focal fish was released. We then observed the focal fish for the next 5 min and recorded the amount of time that it spent in each of the three zones. We then removed the focal fish and replaced the stimulus fish. We then waited 30 min to allow the water to flow through the system to allow chemical cues from the previous trial to dissipate. We then added a new batch of stimulus fish into the other reservoir bucket and proceeded as before. We conducted 20 replicates per treatment; none of the fish were re-used.

(ii) *Treatments and dosing protocol*

All the fish that were used as part of the experiment were dosed using the same dosing protocol. For dosing, the fish were added to a 12 l all-glass dosing aquarium with a sand substrate and then left to acclimatize for 1 hour. Gentle aeration was provided by an air stone. After 1 hour, the chemical dosage was added in 10 ml of a 10% ethanol solution. The exposure period was 1 hour, following which the fish were removed and used in the experiments. None of the fish showed any obvious signs of stress during or after their exposure.

We conducted three separate experiments: which are as follows.

(i) *Mechanism*. To investigate whether, and how, social recognition was being disrupted by the exposure to 4-NP, we dosed focal and stimulus fish separately. In the first treatment, neither focal nor stimulus fish were dosed with 4-NP, although each was subjected to a sham dosing wherein 10 ml of water was added to the dosing aquarium. In the second treatment, only the focal fish were dosed with 2 μg l^{-1} 4-NP as described previously. In the third treatment, only the stimulus fish were dosed with 2 μg l^{-1} 4-NP.

(ii) *Dosage level*. Following the analysis of the first experiment, we conducted seven further treatments consisting of three controls and four dosage levels of 4-NP. In each case, we dosed only the stimulus fish. The fish were dosed at 1, 0.5 and 0. 25 μg l^{-1} 4-NP. We also applied two further controls: an ethanol control, where the stimulus fish were dosed solely with 10 ml of 10% ethanol, and a positive control using 100 ng l^{-1} oestrogen, since 4-NP is known to be an oestrogen mimic.

(iii) *Duration of the effect*. We dosed stimulus fish at 1 and 2 μg l^{-1} 4-NP for 1 hour before returning the fish to their holding aquaria. Trials were conducted at 3 and 6 hour post-dosing with the 1 μg l^{-1} 4-NP-dosed stimulus fish and at 6 and 12 hour post-dosing with the 2 μg l^{-1} 4-NP-dosed stimulus fish.

(ii) *Shoaling in an arena*

Following completion of the flow channel work, we tested the effects of exposure to 4-NP on the shoaling behaviour of killifish in an arena. To do this, we divided 120 killifish into 20 groups of 6 fish and placed each group in a 30 l aquarium supplied with a sand substrate and aeration. Two days after this, we added a dose of 1 μg l^{-1} 4-NP or 10 ml of 10% ethanol as a control to each aquarium. After an hour had elapsed, each group was transferred to an arena measuring $1 \text{ m} \times 1$ m with a water depth of 15 cm. The arena contained food (defrosted frozen bloodworms) concealed in a 5 cm section of pipe with a diameter of 2 cm. To minimize stress to the fish, the temperature of the water in the arena matched that of the holding aquaria and the sides of the arena were screened with black plastic above the waterline. The fish were filmed for 30 min following their introduction to the arena. To assess shoal cohesion, we recorded the mean distance between the fish after 20, 25 and 30 min. We then calculated the overall mean of these three. We also recorded the swimming speed of the fish and their feeding behaviour, measured as the time taken to locate the food and the total number of fish that fed during the trial. Once 30 min had elapsed, we caught and removed the fish to their original holding aquarium, which in each case had been scrubbed and the water replaced during the 30 min of the experiment. Five days later, each group of fish was dosed again with either 1 μg l^{-1} 4-NP or 10 ml of 10% ethanol and the procedure was repeated. Groups 1–10 were dosed with ethanol on day 2 and 4-NP on day 7; groups 11–20 were dosed with 4-NP on day 2 and ethanol on day 7.

(c) *Data analysis*

The data satisfied the requirements for parametric testing. To analyse the flow channel data, we subtracted the time spent in

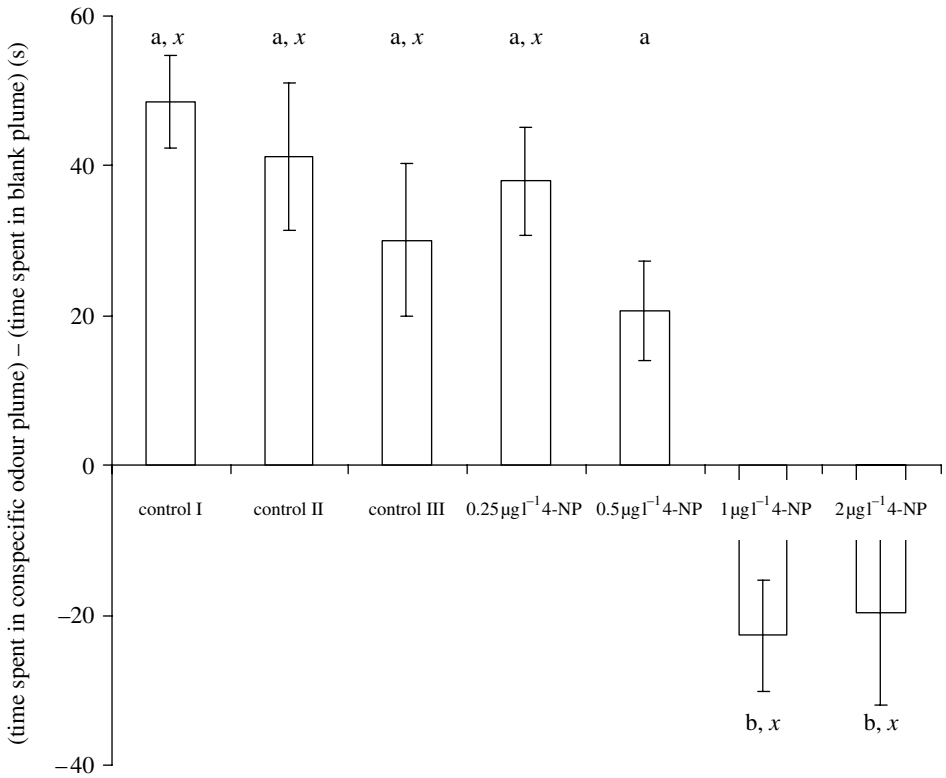

Figure 1. The behaviour of focal fish in response to conspecific stimulus fish in a flow channel, following exposure of the latter to 4-nonylphenol. Control I used undosed stimulus fish, in control II stimulus fish were dosed using 10 ml of 10% ethanol, in control III stimulus fish were dosed with 100 ng l^{-1} oestrogen. Letters denote firstly the results of Tukey *post hoc* tests: 'a' treatments were significantly different from 'b' treatments; secondly, *x* denotes a significant departure from a null expectation of 0 when time spent in a blank odour plume was subtracted from time spent in a conspecific odour plume and the result compared with the null using a one-sample *t*-test, and sequential Bonferroni procedures were used ($n=20$ throughout).

plume B from the time spent in plume A and compared the resultant values with the null expectation of zero using a one-sample *t*-test. Where we performed multiple tests within a treatment, we applied a sequential Bonferroni technique to recalculate significance levels (Holm 1979; Rice 1989). We analysed the dose–response data using ANOVA with Tukey's HSD *post hoc* tests. The arena data were analysed using a paired sample *t*-test.

3. RESULTS

(a) *Detection of conspecific chemical cues in a flow channel*

4-NP affected the behaviour of undosed fish with respect to dosed conspecifics, but not vice versa: the behaviour of focal fish did not change when they, and not the stimulus fish, were dosed at 2 µg l^{-1} (independent samples *t*-test: $t_{1,19}=1.2$, $p=0.27$). However, the behaviour of focal fish did change when stimulus fish were dosed with 4-NP as a function of the dosage level experienced by the stimulus fish (ANOVA: $F_{6,133}=10.9$, $p<0.001$). The response of the focal fish to the stimulus fish dosed at 1 or 2 µg l^{-1} was significantly different from other stimulus fish treatments (figure 1). Furthermore, the results of the experiments carried out at dosage levels of 1 and 2 µg l^{-1} 4-NP showed a significant departure from a null expectation of zero, indicating that focal fish oriented away from the odour plume of dosed stimulus fish. In all three controls, and at a dosage level of 0.25 µg l^{-1}, our results also showed a significant departure from the null expectation of zero; in these cases showing that focal fish oriented towards the odour plume of conspecifics (figure 1). At a dosage level of 0.5 µg l^{-1} 4-NP, the orientation of focal fish was not influenced by the presence of conspecific odour cues.

The effects of the stimulus fish dosage on the focal fish behaviour decreased over time (ANOVA: 1 µg l^{-1} 4-NP, $F_{2,57}=4.3$, $p=0.02$; 2 µg l^{-1} 4-NP, $F_{2,57}=5.1$, $p=0.01$). *Post hoc* tests showed no difference between the control and dosage responses after 6 hours for 1 4-NP and after 12 hours for 2 4-NP.

(b) *Shoaling in an arena*

Groups of fish exposed to 1 µg l^{-1} 4-NP had significantly greater mean nearest-neighbour distances than the control treatment of 10 ml of 10% ethanol (paired sample *t*-test: $t_{1,19}=3$, $p=0.007$). Neither mean swimming speed ($t_{1,19}=0.84$, $p=0.41$) nor feeding behaviour (time taken by the first fish to locate food: $t_{1,19}=1.7$, $p=0.11$; number of fish feeding: $t_{1,19}=1.75$, $p=0.1$) varied between treatments; the fact that the fish swam and fed normally in both treatments suggests that the observed effects were not induced by stress.

4. DISCUSSION

This study is the first to show the effects of exposure to very low, environmentally relevant levels of chemical contamination on the behaviour of shoaling fish. At the dosage levels of 1 and of 2 µg l^{-1} 4-NP, which correspond with levels recorded in aquatic habitats near outflows across the world, shoaling killifish actually avoided conspecifics, in both the flow channel and arena trials,

where they were also able to use visual cues. Such effects on the fish's social behaviour are likely to have major fitness implications, even in the short term, as shoaling is an adaptive response to predation (Krause & Ruxton 2002) and provides fish with an effective means of defence. It could also impact upon fitness in more subtle ways, such as by interfering with the transmission of social information (Reader *et al.* 2003), or by negating some of the foraging advantages of group living (Pitcher *et al.* 1982).

Our results suggest that exposure to 4-NP does not affect the ability of fish to detect chemical cues in the environment, but rather that it affects some property of the signal, or the signaller itself, potentially obscuring the chemical signature of the fish, a key component in social recognition (Christensen & Sorensen 1996; Sorensen & Caprio 1998). For example, variations in habitat or diet are known to affect the chemical cues that fish emit (Ward *et al.* 2004). These cues influence social recognition and social attraction in shoaling fish (Ward *et al.* 2007), such that fish that smell differently from one another tend to form less cohesive shoals (Webster *et al.* 2007). Contact with lipophilic chemicals, like nonylphenol, in the environment is likely to produce rapid and profound changes in the chemical profiles of fish and, in turn, affect social recognition.

While nonylphenol appears to affect signallers, other anthropogenic contaminants are known to affect signal receivers. Metals, such as cadmium, damage the olfactory epithelium of fish (Baker & Montgomery 2001; Beyers & Farmer 2001; Carreau & Pyle 2001; Scott *et al.* 2003; Sloman 2007) and therefore damage their ability to detect chemical cues. Similarly, many pesticides are implicated in a loss of receptor function in fish (Tierney *et al.* 2007). In the real world, aquatic environments may often be contaminated simultaneously by more than one, and often several, different chemicals. The possibility exists therefore that different contaminants, each disrupting a separate element of the chemical communication pathway, may act in concert to seriously impair or to knock out the chemosensory abilities of fish at levels below those currently considered to be a problem based on single-chemical ecotoxicological studies.

The results are relatively short-lived, lasting for under 6 hours for a 1 $\mu g\,l^{-1}$ and under 12 hours for a 2 $\mu g\,l^{-1}$ dose. However, these effect durations relate to a dosage period of only 1 hour. Nonylphenol levels are likely to vary both temporally and spatially in the environment, and it is possible that fish could be exposed repeatedly over their lifetimes to these levels of nonylphenol, with concomitant impacts on fitness.

All experimental work was approved by the animal ethics committee at Mount Allison University.

The authors would like to thank the Fisheries Society of the British Isles and the Natural Sciences and Engineering Research Council of Canada for their financial support of this work. The authors would also like to thank Wayne Anderson for his help in the field.

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