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Skin-penetrating parasites and the release of alarm substances in juvenile rainbow trout

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Juvenile trout *Oncorhynchus mykiss* did not react to the odours of *Diplostomum* sp. cercariae alone, indicating that they were incapable of detecting the parasites directly. However, they increased the number of random darts as well as the amount of time spent motionless when exposed to the odours of a conspecific that was being infected by *Diplostomum* cercariae. These results suggest that even the minor damage inflicted by the cercariae to the fish's skin was enough to cause the release of alarm substances. The effectiveness of the fish's response with respect to the avoidance of parasites remains to be demonstrated.

Key words: alarm substances; *Diplostomum*; *Oncorhynchus mykiss*; parasite avoidance; trematode cercariae.

INTRODUCTION

Many strategies are available to animals to oppose parasites; avoiding infection has advantages over other strategies initiated after infection (Hart, 1994). By avoiding infection a host can avoid its consequences at a relatively low cost. Three prerequisites are necessary for a host to use this strategy. First, the cost of infection must exceed the cost of avoiding parasites. Second, the host must be able in some way to detect the presence of parasites in its immediate surroundings, in its conspecifics, or in its food. Third, the host must be able to use this information to avoid infection actively, either by moving away from a source of parasites or discriminating against infected prey. Few host–parasite systems meet all of these criteria.

In the case of fish, skin-penetrating parasites may trigger a chemical alarm system originally evolved as a defence against predators. Fish from more than a dozen families are known to release alarm substances when injured or captured by a predator (Smith, 1992; Chivers & Smith, 1998). These alarm substances elicit clear-cut antipredator responses in conspecific fish, that include moving away from the odour source, freezing, seeking shelter and changing colour. Alarm substances are released by fragile, easily ruptured club cells in the

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epidermis. Even a very dilute concentration of alarm substances in the water can trigger a response (Smith, 1992; Chivers & Smith, 1998). The content of a single club cell is enough to create an active detection volume of several dozen litres (Lawrence & Smith, 1989). Just 1 cm^2 of fish skin may contain hundreds of club cells (Lawrence & Smith, 1989). Direct penetration of fish by the cercariae of trematode parasites causes injury to the epidermis, and haemorrhage and inflammation in the subcutaneous tissue (Ratanarat-Brockelman, 1974). Conceivably such parasites that damage the skin of fish could cause the discharge of a few club cells thereby eliciting a response in fish within moderate distance from the conspecific being infected. There have been no studies investigating this possible phenomenon. It has been shown that female mosquitoes use chemical cues to avoid ovipositing in water bodies containing mosquito larvae parasitized by trematodes. They apparently detect substances released by parasitized larvae (Lowenberger & Rau, 1994; Zahiri *et al.*, 1997). Similarly, fish could use alarm substances from conspecifics in the process of being infected as cues that a particular area should be avoided.

Here, it was investigated whether rainbow trout *Oncorhynchus mykiss* (Walbaum) can detect and respond to substances released by a conspecific being infected by cercariae of the trematode *Diplostomum* sp. It was also investigated whether trout can use direct chemical detection of free-swimming cercariae of *Diplostomum* sp. prior to their contact with fish. Rainbow trout possess an alarm substance system located in their skin (Brown & Smith, 1997, 1998). Typically, trout increase the time they spend motionless and decrease the time they spend foraging when they detect conspecific alarm substances. *Diplostomum* sp. adults infect piscivorous birds; their eggs pass out in the faeces of their hosts and larval parasites use freshwater snails as first intermediate hosts, in which they multiply asexually. The asexually produced cercariae emerge from snails and penetrate various fish species, most *Diplostomum* species establishing in the eyes of fish. Infections with eyeflukes cause emaciation, deformities, blindness and death in fish (Shariff *et al.*, 1980; Chappell, 1995). Small fishes may also die during the invasion and migration process when exposed to large numbers of cercariae (Hoffman & Hundley, 1957; Berrie, 1960), and cercarial penetration increases swimming activity, heart rate and ventilation rate (Laitinen *et al.*, 1996). This host–parasite system is thus ideal for a test of chemical detection of infective larvae by hosts because fish would benefit by avoiding infection.

MATERIALS AND METHODS

Juvenile rainbow trout were taken at random from a large number of fish originally acquired from a hatchery and maintained for several months at 15° C in a large indoor tank on a diet of trout pellets. *Diplostomum* cercariae were obtained from laboratory-infected snails *Stagnicola elodes* (Say) maintained at room temperature on a diet of lettuce. Infection of snails was achieved by exposing uninfected, laboratory-reared snails to miracidia hatched from eggs obtained from laboratory-raised ring-billed gulls *Larus delawarensis* Ord that had been infected with metacercariae from the eyes of Atlantic tomcod *Microgadus tomcod* (Walbaum). Infected snails were isolated in small containers under constant light for 24 h prior to a series of experiments; cercariae were pipetted at random from the large numbers shed by isolated snails. Identification of species in the genus *Diplostomum* is problematic since the taxonomy of the genus is in great need of resolution (Chappell, 1995; Gibson, 1996). Accordingly, parasites here are referred to as *Diplostomum* sp.

Experiments were performed in large aquaria (90 × 30 × 40 cm high), filled to a depth of 15 cm. Two 1-l plastic containers, fixed to the bottom at each end of the tank, served as sources of odours. These containers were perfectly opaque so that no visual stimuli, for example, a fish silhouette or motion, could be received by the test fish (see below). The lid of each container consisted of a window covered with 100-μm mesh netting. The mesh size was too small to allow the passage of cercariae, and only allowed limited diffusion of water from inside the container to the outside. Preliminary trials with dyes showed that the turbulence created by an airstone inside the containers was sufficient to ensure a steady diffusion toward the outside. Therefore all containers were equipped with an airstone connected to a pump via a tube passing through a small opening on the side of the container. The light above the experimental aquaria was arranged in order to avoid any gradients in illumination within aquaria. All experiments were performed at 15° C. Experimental aquaria and containers were all rinsed thoroughly after each trial.

A trial began by placing the sources of odours in the containers (either water, fish, and/or cercariae), and a test fish under an upside-down 1-l glass container exactly in the middle of the tank. Sources of odours were allocated to each container at random, as determined by a coin toss. After a 20-min acclimation period, during which infection of fish by cercariae also took place in the containers, the air pumps connected to airstones in the containers were turned on, and the glass jar holding the test fish was lifted gently. The test fish was then observed for 5 min; all observations were made live from behind a dark plastic blind. The following behaviours were recorded: (i) time spent by the fish in each half of the tank; (ii) time spent motionless by the fish, i.e. either resting on the bottom of the tank, or being stationary in the water column (similar to the freezing behaviour of Brown & Smith, 1997); and (iii) number of quick darting bursts made by the fish, where a dart is a sudden displacement of at least five body lengths occurring in a fraction of second and in an apparently random direction. At the end of a trial, all fish were measured [total length (L_T), nearest mm]. A number of fish exposed to cercariae in containers were kept alive for >24 h and then killed by decapitation and dissected to verify that infection had taken place.

To determine whether rainbow trout can detect, and respond to, the presence of cercariae using chemical stimuli, trials were performed ($n=15$) in which one plastic container held water and *c.* 50–60 cercariae, and the other held only water. Since test fish may not perceive the gradient in odour but simply the presence of an odour, each test fish was used in a second (control) trial in which both containers held only water. Paired comparisons between treatment and control trials would allow a more generalized response to cercarial odour to be detected. Both trials using the same test fish were performed on the same day, in random order, and separated by about 1 h to allow the test fish to recover in a separate tank.

To determine if fish can detect and respond to alarm substances released by a conspecific that is being infected by cercariae, trials were performed ($n=15$) in which one container held a fish and *c.* 50–60 cercariae, and the other held only a fish in water. In each trial, the two stimulus fish were matched for size as closely as possible. Again, test fish may not perceive the gradient in alarm substance but only that the substance is present. Therefore, each test fish was used in a second (control) trial in which both containers held only a fish and no cercariae. As before, a more generalized reaction to alarm substances can be detected by paired comparisons between treatment and control trials, which were carried out on the same day, in random order, and separated by 1 h.

The relative avoidance of the aquarium half from which cercarial odour or alarm substances originated was calculated for each trial as the difference between the time spent in that half and time in the other half. Comparisons of relative avoidance, number of darts, and time spent motionless were made between control and treatment trials using paired *t*-tests, so that each fish served as its own control; all tests were two-tailed. Data are reported as means ± S.E.

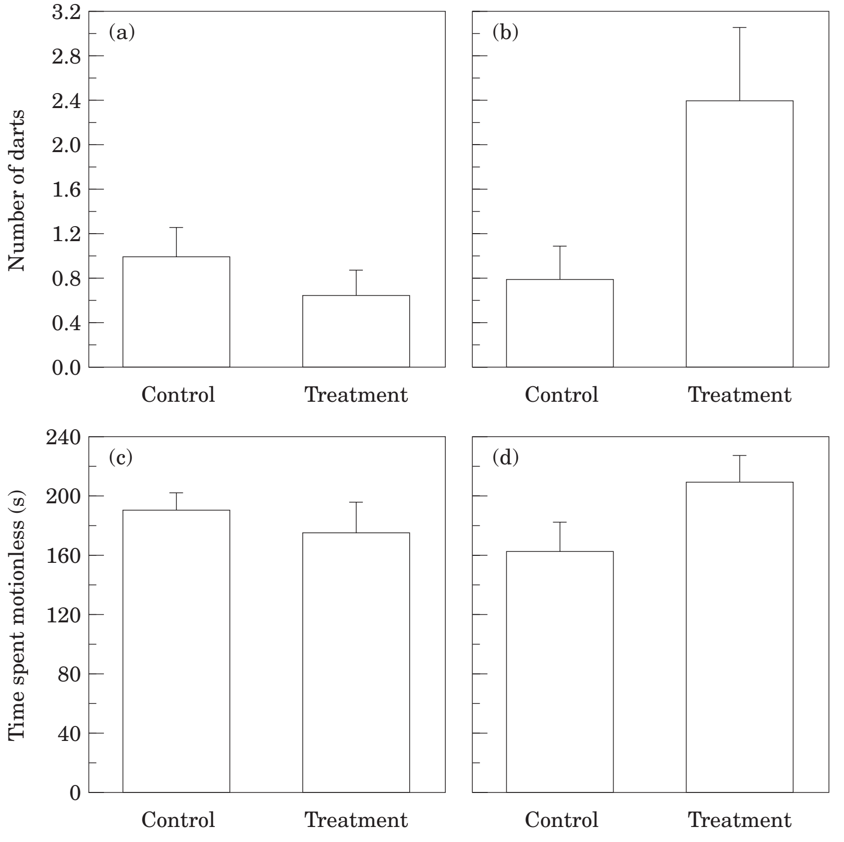

Fig. 1. Behavioural responses (mean ± s.e.) of juvenile rainbow trout: (a) and (c) during exposure to water only (control) and when exposed to chemical cues from *Diplostomum* cercariae (treatment); and (b) and (d) during exposure to chemical cues from two conspecifics only (control) and when exposed to chemical cues from one conspecific being infected by *Diplostomum* cercariae and one uninfected conspecific (treatment).

RESULTS

In the experiments testing whether rainbow trout can detect directly the presence of cercariae using chemical stimuli, the mean length of test fish was 95·4 ± 2·4 mm. The relative avoidance shown by fish for the aquarium half from which putative cercarial odours originated did not differ between control and treatment trials (t=0·711, P=0·489), indicating no actual avoidance of the odour source. There was no difference between control and treatment trials in either the number of darts (t=1·435, P=0·173) or in time spent motionless (t=0·828, P=0·421) by test fish [Fig. 1(a) and (c)].

In the experiments testing whether rainbow trout can detect alarm substances released by conspecifics being infected by cercariae, the mean length of test fish was 81·1 ± 3·1 mm, whereas the size of fish placed in containers serving as sources of odour stimuli was 90·1 ± 3·8 mm. Pairs of stimulus fish used in the same trials did not differ in size, either in control (t=0·048, P=0·962) or treatment (t=0·703, P=0·494) trials. Of 12 stimulus fish kept for dissection, four died in less than 24 h. No metacercariae were recovered from their eyes, probably because their migration was interrupted by host death. Five of the remaining eight fish were infected with metacercariae in the eyes (range 11–56 per fish). This suggests that fish were being penetrated by cercariae during the trials.

The relative avoidance shown by fish for the aquarium half from which alarm substances were released by infected conspecifics did not differ between control and treatment trials (t=0·608, P=0·553), indicating no actual avoidance of the odour source. There were, however, differences between control and treatment trials in the number of darts (t=2·779, P=0·015) and in the time spent motionless (t=3·139, P=0·007) by test fish. Fish consistently made more darts and spent more time motionless during the treatment trials, in which conspecifics were exposed to 50–60 cercariae, than in control trials [Fig. 1(b) and (d)].

DISCUSSION

The harmful effects of *Diplostomum* infection on fish have been well documented (Shariff *et al.*, 1980; Chappell, 1995). Any fish capable of detecting the presence of *Diplostomum* cercariae and avoiding them would obtain substantial fitness benefits at relatively low cost. The present experiments showed that juvenile rainbow trout can detect and respond to substances released by a conspecific being penetrated by cercariae, but not the cercariae themselves. Whether these substances were the same as the alarm substances postulated by Brown & Smith (1997, 1998) was not determined, although this would be the most parsimonious explanation. There is some doubt, however, regarding how effective the fish's response would be at avoiding cercariae. It may well be that the penetration of cercariae in one fish coincidentally triggers in other nearby fish a response evolved specifically for predator avoidance, not parasite avoidance.

Juvenile rainbow trout increased the number of darts they made as well as the time they spent motionless when they detected chemical cues coming from a conspecific exposed to *Diplostomum* cercariae. The latter response agrees with the results of Brown & Smith (1997), who observed that juvenile rainbow trout exposed to skin extracts from conspecifics spent more time freezing, i.e. motionless. Both darting away from the area where alarm substances have been detected and/or remaining immobile may help the fish escape predation. Can these responses also serve to avoid cercariae? Only experiments in large tanks could provide a definitive answer to this question. In experiments here, test fish appeared unable to determine where the odour came from; this may not matter against a mobile predator but it does when the danger comes from a slowly dispersing, almost stationary swarm of cercariae. In still water, remaining motionless near a conspecific that has encountered cercariae may increase the likelihood of contacting parasites, whereas darting away could take the fish to a cercaria-free area. In running water, though, neither response is likely to have an effect on the probability of infection since cercariae are being swept downstream continuously.

The distinction between the likely usefulness of fish responses in still and running water raises questions about their adaptiveness in nature. The phenomenon of alarm substances and the fright responses they trigger, documented mostly in laboratory studies, may not operate in natural systems (Magurran *et al.*, 1996; Irving & Magurran, 1997). Whether or not fish alarm substances play an important role in nature, they exist in a wide range of fish taxa and can be detected by fish at least in a confined volume (Smith, 1997). The results indicate that the alarm substance system of rainbow trout is so sensitive that even cercarial penetration can activate it or a similar system. Given that a single club cell in minnows *Pimephales promelas* (Rafinesque) can release enough alarm substance to trigger a response in nearby conspecifics (Lawrence & Smith, 1989), and that a wriggling cercaria penetrating the skin of its host may rupture more than one of these cells, this result is not surprising. Further experiments will be necessary to determine if it is of benefit to the fish, or a coincidental side-effect.

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