



Aquatic flatworms use chemical cues from injured conspecifics to assess predation risk and to associate risk with novel cues

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A growing number of aquatic organisms have been shown to display antipredator behaviour in response to injury-released chemical cues from conspecifics. Here, we demonstrate a clear antipredator response in the form of avoidance behaviour by a free-living flatworm *Dugesia dorotocephala* to chemical cues from injured conspecifics. This is the first demonstration of a chemical alarm cue in a platyhelminth. In a second experiment, we exposed planaria to combined cues of sunfish odour and planaria alarm cue, or sunfish odour alone. Planaria avoided the sunfish+alarm cue but did not avoid the sunfish odour, indicating no prior aversion to sunfish odour. When these same planaria were subsequently retested 2 days later with sunfish odour only, planaria that had previously received sunfish odour+alarm cue avoided the cue but planaria that had previously received sunfish odour alone did not. These data indicate that planaria learned to recognize sunfish odour as an indicator of danger based on a single simultaneous exposure to conspecific alarm cue and the novel cue. This is the first demonstration of this phenomenon in a platyhelminth and the simplest nervous system known to be capable of learned risk association.

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Predation is a powerful selection force shaping morphology and behaviour (Sih 1987; Lima & Dill 1990). Animals that detect and respond to risk of predation have a higher probability of surviving to reproduce than animals that do not. Environmental indicators of predation risk can operate in any sensory modality. In aquatic environments, chemosensory cues are pervasive and the most ancestral (Hara 1992; Chivers & Smith 1998; Kats & Dill 1998; Sorensen & Caprio 1998; Wisenden 2000). Thus, the elaboration of sensory receptors and neural pathways to process chemical stimuli associated with risk might be expected in even the simplest of aquatic organisms.

Injury-released chemical cues are typically released during a predation event and thus, serve as a reliable indicator of predation risk. Consequently most aquatic taxa that have been tested display an antipredator response to chemical cues from injured conspecifics (Chivers & Smith 1998). Platyhelminthes represent a major phylum of aquatic organisms that have not received attention in this regard (Chivers & Smith 1998). This group is significant because of its early phylogenetic position in the evolution of life, with relatively simple neural organization for the detection and processing of

chemical information (Agata et al. 1998; Rieger 1998). The first objective of this study was to test for the existence of an antipredator response to injury-released chemical cues by the flatworm *Dugesia dorotocephala* (Platyhelminthes: Turbellaria, Planariidae). *Dugesia dorotocephala* are free-living flatworms found in springs and head waters (Pennak 1989). They form aggregations (Reynierse & Ellis 1967; Reynierse et al. 1969), use mucus to capture invertebrate prey (Ali 1996; Cash et al. 1996; Hart & Mertz 1998) and some members of the genus *Dugesia* are known to follow chemical cues to find food (Seaby et al. 1995). *Dugesia* themselves fall prey to other invertebrates (Gee et al. 1998) and presumably, any benthic omnivore. Although *Dugesia* possess photosensitive eye spots, they use their eyes primarily for negative phototaxis behaviour (Asano et al. 1998) and not for visual detection of approaching predators. Thus, risk assessment by *Dugesia*, if it occurs at all, probably relies largely upon chemosensory cues.

Chemical cues other than conspecific alarm cues can be important ecological indicators of risk. For example, predator odours (kairomones) are known to elicit antipredator responses for a range of aquatic animals (Kats & Dill 1998; Tollrian & Harvell 1999). In some cases recognition of predator odour is innate (Sih & Moore 1993; Storfer & Sih 1998) but for many it is not (Chivers & Smith 1998). In the case of the latter, naïve prey show no

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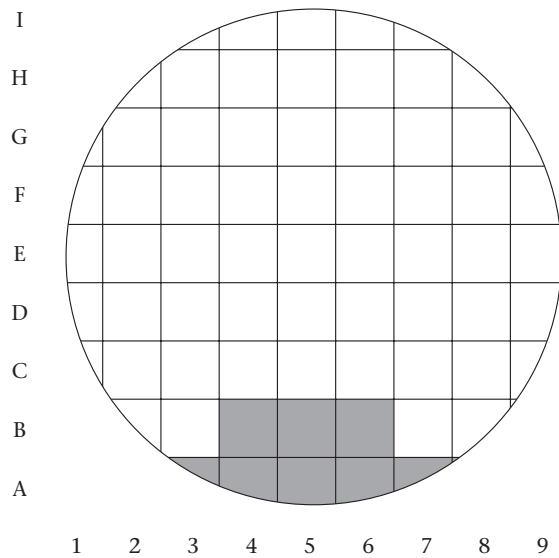


Figure 1. Diagram of the grid pattern etched on the underside of a standard petri dish used as the test arena. Test stimuli were introduced into cell A5. The shaded region indicated the 'risky area'.

response when first presented with a novel predator odour. However, naïve prey that are presented with a novel stimulus simultaneously with conspecific alarm cues learn to associate risk with the novel stimulus. After a single exposure to both cues, the formerly novel cue alone elicits a full suite of antipredator behaviour (e.g. Göz 1941; Magurran 1989; Chivers & Smith 1994a).

Thus far, learned recognition of novel stimuli as dangerous has been demonstrated for several fish species and one insect (Chivers & Smith 1998). Planarians, however, represent the most primitive living animals with an organized central nervous system (Agata et al. 1998) comprising two cephalic ganglia and two longitudinal commissures. The second objective of this study was to test whether naïve *Dugesia* can associate risk with a neutral stimulus after simultaneous exposure to the novel cue and conspecific alarm cues.

METHODS

We obtained adult *D. dorotocephala* from a commercial supplier, who in turn collected them from natural populations in Wisconsin, U.S.A. Flatworms were held in 18-litre aquaria filled with dechlorinated tap water, filtered by an air-driven sponge filter. Water temperature was 20 °C and light schedule was 10:14 h light:dark cycle. We fed the flatworms a diet of beef heart. Tank water was changed periodically.

Test for the Presence of an Alarm Cue

We placed individual flatworms into standard plastic laboratory petri dishes (diameter=8.5 cm) with 30 ml of tank water. A grid of squares 1 × 1 cm was etched on the underside of the petri dish so that the position and movement of each flatworm could be recorded (Fig. 1).

Adult flatworms were used in this experiment, measuring approximately 10 mm in length. A different flatworm was used for each trial.

Room lights were turned off during testing because *Dugesia* are negatively phototactic (Reynierse 1966; Asano et al. 1998). A small desk lamp in a distant corner of the room was the only illumination. Each flatworm was given 5 min to acclimate to the test arena before data collection began. During the prestimulus period, we recorded the flatworm's position for 10 min at intervals of 10 s. We then carefully introduced 1 ml of either tank water (control) or 1 ml of tank water containing injury-released chemical alarm cues from a conspecific planaria. The water treatment controlled for the effect, if any, of stimulus injection on flatworm behaviour. We prepared injury-released chemical alarm cues by reducing one adult flatworm to a thin pulp using a mortar and pestle and suspending the pulp in 1 ml of tank water.

Stimulus injection lasted 30 s and was gently added next to one edge of the petri dish (cell A5). The point location of stimulus introduction and adjacent grid cells (A3, A4, A5, A6, A7, B4, B5, B6) was designated as the 'risky' area. We then resumed recording the position of the flatworm at 10-s intervals for 10 min. This is referred to as the poststimulus period. Petri dishes were thoroughly rinsed and wiped clean between test trials to remove any trace of chemical stimuli from previous trials. We conducted 15 alarm cue trials and 16 control trials.

For each trial, we calculated the change in the use of the risky area after the introduction of the test stimulus (water or alarm cue). We used Wilcoxon matched-pairs signed-ranks tests to determine a consistent change in area use (Siegel & Castellan 1988). Sample size, *N*, refers to the number of nontied comparisons used in each test. We then compared the magnitude of the change in response to alarm cues to the magnitude of the change in response to water using a Wilcoxon two-sample test (Siegel & Castellan 1988). Area avoidance would indicate an antipredator response. If planaria have chemical alarm cues, then we predicted avoidance of the risky area following the introduction of the alarm cue but not in response to water.

Test for Learned Association of Alarm Cues with Novel Stimuli

We repeated the first experiment using the same methodology as described above, except that instead of tank water from the planaria holding tank, we used tank water from a 37-litre aquarium containing five juvenile bluegill sunfish, *Lepomis macrochirus*. Therefore, test stimuli comprised (1) one adult *D. dorotocephala* crushed to a fine pulp with mortar and pestle and diluted in 1 ml of sunfish odour, or (2) 1 ml of sunfish odour (control). The sunfish were placed in a clean aquarium, maintained on a diet of commercial flake food for 5 days. Sunfish odour was collected in large quantity and frozen at -20 °C in 10-ml aliquots until needed.

We tested behavioural responses to the test stimuli in small petri dishes as before (i.e. 10-min prestimulus observation followed by 30 s stimulus injection followed by a

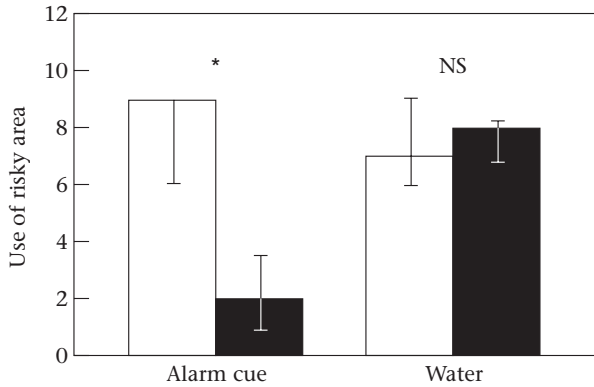


Figure 2. Use of the risky area expressed as the median (± 25 percentiles) number of times planaria were observed in the danger zone before (\square) and after (\blacksquare) the addition of injury-released chemical cues from conspecific flatworms (experimental trials) or water (control trials). * $P < 0.05$.

10-min poststimulus observation). In addition to scoring use of the risky area, we recorded cell location at each 10-s interval. We used cell location to calculate distance travelled. In many species, antipredator behaviour includes a reduction in activity (Lima & Dill 1990).

After testing we transferred each flatworm from the petri dish to a separate holding container. The following day, we placed each flatworm in a clean petri dish and retested it with 1 ml of sunfish odour. We conducted 15 experimental trials (sunfish odour+alarm cue, retested with sunfish odour) and 15 control trials (sunfish odour, retested with sunfish odour).

As before, we reduced the 60 data points from each prestimulus and poststimulus observation period to a single statistically independent score for that individual. We tested for a consistent change in use of the risky area using a matched-pairs signed-ranks test to compare behaviour before versus after stimulus introduction. We compared control versus experimental trials in the behavioural response to test stimuli using Wilcoxon two-sample tests. For each 10-s interval, we calculated the Euclidian distance travelled and summed them to calculate the total distance travelled over the 10-min observation period. We tested for the effect of treatment on distance travelled using Wilcoxon two-sample tests. All tests report two-tailed probabilities.

RESULTS

Test for the Presence of an Alarm Cue

Introduction of injury-released chemical cues from conspecifics resulted in significant avoidance of the risky area (Wilcoxon matched-pairs-signed ranks test: $T = 120$, $N = 15$, $P < 0.001$; Fig. 2). Introduction of tank water had no significant effect on flatworm use of the risky area ($T = 54.5$, $N = 14$, $P = 0.942$). The change in use of the risky area following introduction of the alarm cue was significantly different from the change in response to water (Wilcoxon two-sample test: $W = 133.5$, $N = 31$, $P < 0.001$).

When flatworms encountered injury-released chemical cues from conspecifics they engaged in 'flipping' behaviour, whereby they abruptly lifted their anterior end off of the substrate and doubled back on themselves (180° turn) causing them to travel away from the cue. Flipping occurred 2.8 ± 0.7 times per 10-min poststimulus period of alarm cue trials. It always occurred at the boundary of the danger zone and never occurred in distal regions of the petri dish nor during prestimulus observation periods. Flipping never occurred during control trials. Flatworms clearly reacted aversely to the cue. These data are consistent with an antipredator response to an alarm cue.

Test for Learned Association of Alarm Cues with Novel Stimuli

Planaria showed no detectable response to the odour of sunfish in terms of area use (Wilcoxon matched-pairs signed-ranks test: $T = 45$, $N = 11$, $P = 0.320$) or distance travelled ($T = 92$, $N = 15$, $P = 0.073$), indicating no pre-existing recognition of, or aversion to, sunfish odour (Figs 3, 4). As in the first experiment, when chemical cues from injured conspecifics were present, planaria responded with area avoidance behaviour (Wilcoxon matched-pairs signed-ranks test: $T = 120$, $N = 15$, $P < 0.001$) but, contrary to expectations, there was no change in distance travelled ($T = 91$, $N = 15$, $P = 0.083$). The magnitude of change in area use in experimental trials was significantly greater than the magnitude of change in control trials (Wilcoxon two-sample test: $W = 124.5$, $N = 30$, $P < 0.001$). There was no difference in the change in distance travelled between experimental and control trials ($W = 229$, $N = 30$, $P = 0.904$).

When the same planaria were retested the following day with sunfish odour only, those planaria that had been previously exposed to sunfish odour+alarm cue now actively avoided the area with sunfish odour (Wilcoxon matched-pairs signed-ranks test: $T = 120$, $N = 15$, $P < 0.001$; Fig. 3). Activity level, measured as distance travelled, was not affected ($T = 91$, $N = 15$, $P = 0.083$; Fig. 4). Thus, a single simultaneous exposure to a neutral, novel stimulus and conspecific alarm cues was sufficient for *D. dorotocephala* to associate predation risk with the novel cue. Planaria previously exposed to sunfish odour alone showed no response to sunfish odour upon subsequent exposure in terms of area avoidance (Wilcoxon matched-pairs signed-ranks test: $T = 28$, $N = 9$, $P = 0.570$) or distance travelled ($T = 36$, $N = 11$, $P = 0.831$). The magnitude of the change in avoidance behaviour for each treatment differed significantly (Wilcoxon two-sample test: $W = 125$, $N = 30$, $P < 0.001$). Change in distance travelled did not differ between experimental and control trials ($W = 167$, $N = 26$, $P = 0.322$).

DISCUSSION

These data show, for the first time (Chivers & Smith 1998), the presence of a chemical alarm cue in a member of the platyhelminthes. When exposed to injury-released

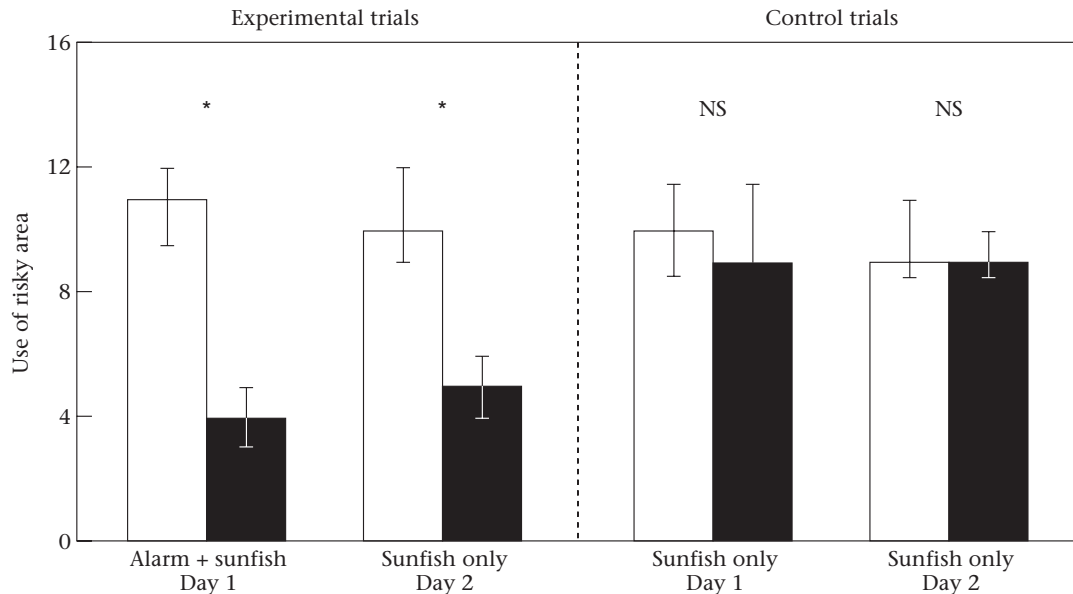


Figure 3. Use of the risky area expressed as the median (± 25 percentiles) number of times planaria were observed in the danger zone before (\square) and after (\blacksquare) the addition of test stimuli. On day 1: flatworms received sunfish odour plus chemical cues from injured conspecific flatworms (experimental trials) or sunfish odour alone (control trials). All flatworms were retested on day 2 with sunfish odour alone. * $P < 0.05$.

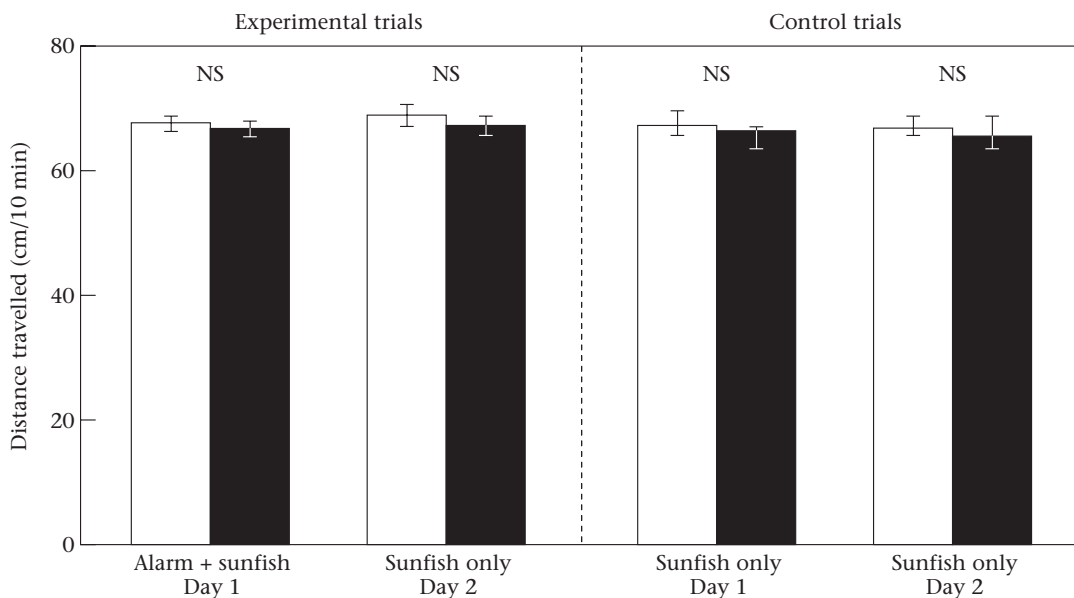


Figure 4. Median (± 25 percentiles) distance travelled (cm) during the prestimulus (\square) and poststimulus (\blacksquare) observation periods. On day 1: flatworms received sunfish odour plus chemical cues from injured conspecific flatworms (experimental trials) or sunfish odour alone (control trials). All flatworms were retested on day 2 with sunfish odour alone.

chemical cues from conspecifics, *D. dorotocephala* actively avoided the area. Because *D. dorotocephala* aggregate in nature (Reynierse & Ellis 1967), predator activity would quickly alert remaining members of a group to the presence of predation risk. This behavioural response would serve to reduce the probability of encounter with a predator and thus, reduce risk of predation (Hews 1988; Mathis & Smith 1993; Wisenden et al. 1999). These data fill a conspicuous gap in the survey of aquatic taxa (Chivers & Smith 1998) that display antipredator

behaviour in response to injury-released chemical cues of conspecifics. As is the case for most other aquatic animals, injury-released chemical cues are not necessarily evolved signals per se but passively released chemical cues that inform and benefit nearby conspecific (and perhaps heterospecific) prey of the predator without any benefit accruing to the individual releasing the cue.

Fish, and in particular, members of the superorder Ostariophysi such as the fathead minnow, *Pimephales promelas*, and the zebra fish, *Danio rerio*, are well known

for their ability to acquire recognition of novel stimuli as dangerous. A single, simultaneous exposure to conspecific alarm cues and a novel stimulus transfers risk to the novel stimulus whether it is a novel chemical cue (e.g. Göz 1941; Chivers & Smith 1994a, 1995; Suboski et al. 1990; Hall & Suboski 1995; Korpi & Wisenden 2001) or a novel visual cue (Magurran 1989; Suboski et al. 1990; Chivers & Smith 1994b; Hall & Suboski 1995; Yunker et al. 1999; Wisenden & Harter 2001).

Dugesia dorotocephala learned to recognize and avoid sunfish odour after a single simultaneous encounter with sunfish odour and conspecific alarm cues. This is the first demonstration of recognition learning by a platyhelminth, and the first such demonstration in an animal whose nervous system is so modestly endowed. The next simplest animal known to be capable of recognition learning is an aquatic insect (Chivers et al. 1996; Wisenden et al. 1997).

The central nervous system of planaria comprises two cerebral ganglia connected to two ventral nerve cords that run the length of the animal. *Dugesia japonica* are used as a model for the study of the evolution of the central nervous system because of organizational similarities with the central nervous system of vertebrate embryos (Agata et al. 1998) and conserved homeobox genes for rostral brain development shared with vertebrates (Umesono et al. 1999). *Dugesia japonica* also use the same melatonin-based mechanism for regulating circadian behaviour as found in vertebrates (Itoh et al. 1999), reinforcing the idea that some basic behavioural repertoires either arose independently in response to pervasive selection pressures, or arose very early in metazoan evolution. The proximate mechanisms observed in vertebrates for the behavioural response to conspecific alarm cues and subsequent association of risk with correlated stimuli may have had an ancient evolutionary origin.

Our data do not provide any evidence for or against the presence of an alarm pheromone, alarm substance, or any type of specialized chemical signal that communicates alarm. Injured planaria probably release a unique bouquet of proteins, lipids and carbohydrates to which planaria have been selected to avoid. What the data show is the presence of an alarm response to conspecific injury-released cues. Natural selection should strongly select individuals that respond aversely to these cues and do so without prior experience. To perform this response, planaria would require sensory receptors capable of detecting injury-released cues and the requisite associative neural pathways to interpret these stimuli and initiate an appropriate behavioural response. Water is the universal solvent and an ideal medium for the solution and dispersal of chemical information. The data from this study indicate that ancient and steep selection pressure has long been acting on individuals to successfully exploit chemical sources of environmental information to avoid predation risk.

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