Conspecific skin extracts elicit antipredator responses in juvenile rainbow trout (*Oncorhynchus mykiss*)

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Abstract: We investigated, under laboratory conditions, the presence of chemical alarm signals in juvenile rainbow trout (*Oncorhynchus mykiss*). In an initial experiment, we exposed trout to a whole-body extract from conspecifics or a distilled-water control. When exposed to whole-body extract, trout significantly (*i*) decreased time spent swimming, (*ii*) increased time taken to resume foraging, and (*iii*) decreased the number of food items eaten. These data indicate a significant chemically mediated antipredator response. A second experiment was conducted to determine (*i*) if this is a generalized response to injured fish or a specific response to injured conspecifics, and (*ii*) if the chemical signal is localized in the skin. We exposed juvenile trout to one of three chemical stimuli: (1) trout skin extract, (2) trout body extract, or (3) swordtail (*Xiphophorus helleri*) skin extract. Significant antipredator responses were observed in trout exposed to conspecific skin extract, but responses of those exposed to conspecific body extract or swordtail skin extract did not differ from those of distilled-water controls. These data strongly suggest that juvenile rainbow trout possess a chemical alarm signal, localized in the skin, that elicits antipredator behaviour when detected by conspecifics.

Résumé : Nous avons tenté de déterminer l'existence de signaux d'alarme chimiques chez les juvéniles de la Truite arc-en-ciel (*Oncorhynchus mykiss*), dans des conditions de laboratoire. Dans une première expérience, nous avons exposé les truites à un extrait de corps entier de truites conspécifiques ou à de l'eau distillée (témoin). L'exposition à l'extrait de corps entier a eu les résultats suivants : (*i*) les truites ont passé moins de temps à nager, (*ii*) elles ont mis plus de temps à se remettre à manger et (*iii*) elles ont réduit le nombre de leurs proies. Ces données indiquent une forte réaction anti-prédateur incitée par des médiateurs chimiques. Au cours d'une deuxième expérience, nous avons tenté de déterminer (*i*) s'il s'agit là d'une réaction généralisée à un poisson blessé ou d'une réaction spécifique à des poissons conspécifiques blessés et (*ii*) si le signal chimique est localisé dans la peau. Nous avons exposé des truites juvéniles à l'un des stimulus chimiques suivants : (1) extrait de peau de truite, (2) extrait de corps entier de truite, (3) extrait de peau du Queue d'épée (*Xiphophorus helleri*). Des réactions anti-prédateur marquées ont été observées chez les truites exposées à des extraits de peau de truite, mais les poissons exposés à des extraits de corps entiers de truite ou à des extraits de peau de *X. helleri* ont réagi de la même façon que les poissons témoins exposés à l'eau distillée. Ces résultats indiquent que les Truites arc-en-ciel juvéniles possèdent un signal d'alarme de nature chimique localisé dans la peau, signal qui déclenche un comportement anti-prédateur chez les poissons de la même espèce.
[Traduit par la Rédaction]

Introduction

Many fish species utilize chemical alarm signals to warn conspecifics of a predation threat (Pfeiffer 1982; Smith 1982*b*, 1992). The chemicals that elicit antipredator responses in nearby conspecifics are generally produced, or are contained, within the skin and are only released through mechanical damage (Smith 1992). Some species, such as the Iowa darter (*Etheostoma exile*), also possess disturbance pheromones that are released without mechanical damage to the individual and elicit antipredator behaviour in conspecifics (Wisenden et al. 1995*a*). For those species in which the alarm chemical is contained in the skin, it may be localized in special epidermal club cells (e.g., fathead minnow,

Pimephales promelas; Smith 1992), or the epidermis may lack specialized club cells (e.g., brook sticklebacks, *Culaea inconstans*; R.J.F. Smith, personal observation). Many of these signals elicit alarm responses (antipredator behaviours) in both conspecifics and heterospecifics (Verheijen 1956; Pfeiffer 1963; Smith 1982*b*; Smith and Smith 1989; Smith et al. 1991; Mathis and Smith 1993*c*; Chivers and Smith 1994*b*, 1994*c*; Brown et al. 1995*a*, 1995*b*; Mathis et al. 1996).

It has been suggested that chemical alarm signals may be selected for through a process of kin selection (Smith 1982*b*). By warning nearby relatives of a potential predation risk, an individual's inclusive fitness may be maintained or increased. Rainbow trout (*Oncorhynchus mykiss*) are able to discriminate kin from non-kin and preferentially defend foraging territories near kin (Brown and Brown 1996*a*, 1996*b*). Since rainbow trout are able to recognize and preferentially assort with kin, this makes the species a likely user of chemical alarm signals.

In his review of the distribution of alarm pheromones in fishes, Pfeiffer (1977) suggested that rainbow trout lack chemical alarm signals. Conflicting results are presented by

Lebedeva et al. (1994). They suggest that rainbow trout release "chemical alarm signals" in response to a variety of stressors (including electric shock, handling, injection, and hypoxia). They recorded respiratory rates and blood glucose levels. Changes such as these would be expected as a result of any short-term stress (Mazeaud et al. 1977; Mazeaud and Mazeaud 1981; Woodward and Strange 1987; Barton and Iwama 1991). However, Lebedeva et al. (1994) did not record behavioural responses. As a result, these data do not demonstrate a fright response or antipredator behaviour in the presence of a chemical alarm signal. The goals of our study were to determine (i) if juvenile rainbow trout possess a chemical alarm signal, and (ii) if this chemical is localized in the skin, as in cyprinids (Smith 1992).

Experiment 1

Methods

Test fish

Juvenile rainbow trout were collected from Fort Qu'Appelle provincial hatchery, Fort Qu'Appelle, Saskatchewan, Canada. These trout were of a hatchery strain, originating from wild-caught broodstock collected from northern British Columbia. Trout were held in 300-L recirculating Living Stream tanks at approximately 15°C and exposed to a 14 h light (L) : 10 h dark (D) cycle. Fish were fed ad libitum daily with commercial trout food and given supplemental feedings three times per week with frozen adult brine shrimp (*Artemia franciscana*).

Test stimuli

Three juvenile trout (11.3 ± 0.6 cm (mean ± SD) standard length) were used to generate a whole-body extract. Trout were killed with a blow to the head (in accordance with the guidelines of the Canadian Council on Animal Care) and approximately equal amounts of skin and visceral tissue were collected and immediately placed in 50 mL of chilled glass-distilled water. A total of 3.02 g of tissue was collected. The tissue was homogenized and filtered through glass wool and we added glass-distilled water to bring it to a final volume of 400 mL. Whole-body extract was separated into 60-mL samples and frozen at −20°C until needed. As a control, we similarly froze 60-mL samples of glass-distilled water.

Test protocol

We tested 12 juvenile trout (11.06 ± 1.28 cm standard length) individually in 110-L aquaria at approximately 18°C. Each of the test tanks contained a cover object consisting of a ceramic tile (9.8 × 20 cm) mounted on three cylindrical glass legs (5.5 cm in height) and a silica sand substrate approximately 4 cm deep. A single airstone was mounted in the centre of an end wall and a second length of airline tubing terminated near the airstone. This tube was used to introduce chemical stimuli. An additional length of airline tubing was placed in the centre of the opposite end wall and was used to deliver food items to the test tank. The test tank was divided into two sections (front and back) with markings on the exterior of the tank.

Trials consisted of a 30-min prestimulus and a 30-min poststimulus observation period. Trout were placed individually in the tanks 72 h prior to testing and fed daily with adult brine shrimp ad libitum during the acclimation periods. During both the pre- and post-stimulus observation periods we recorded (i) the amount of time spent under the cover object, (ii) the amount of time spent swimming in the front half of the tank (near the feeder) and the back half of the tank (near the stimulus-introduction tube), (iii) the time elapsed until the first food item was taken, and (iv) the total number

of food items consumed. All observations were made live from behind a black plastic viewing blind. Food items were likewise introduced into the test tanks from behind the viewing blind.

During both pre- and post-stimulus observation periods, we introduced 1 brine shrimp into the tank every 2 min (a total of 30 shrimp per trial). At the end of the prestimulus period, we removed and discarded 60 mL of water from the stimulus-injection tube. We then removed and retained an additional 60 mL of water. We injected 15 mL of stimulus (glass-distilled water for control trials and 15 mL of whole-body extract for experimental trials) and then flushed the stimulus into the test tank with the retained water. Control trials began at approximately 8:30 a.m. and experimental trials began at approximately 1:00 p.m. Control trials were always conducted in the morning, since any response to the experimental stimulus may have masked a response to the control stimulus (Smith 1982a).

Statistical analysis

For all behavioural measures we calculated, for each fish, the difference between the pre- and post-stimulus observation periods and then compared these differences between the control and experimental trials using a Mann−Whitney U test (Siegel 1956).

Results

When presented with whole-trout body extract, individual trout exhibited a significant increase in the time taken to capture the first food item ($Z = -2.25, p < 0.02$; Fig. 1A) and the total number of food items captured was significantly reduced ($Z = -3.41, p < 0.001$; Fig. 1B).

Trout significantly reduced the amount of time spent swimming in both the front half of the test tank ($Z = -2.02$, $p < 0.03$; Fig. 2A) and back half of the test tank ($Z = -1.94, p < 0.03$; Fig. 2A). No significant difference was found in the amounts of time trout spent under the cover object between the control and experimental trials ($Z = -0.78, p = 0.22$; Fig. 2B).

Experiment 2

The results of experiment 1 strongly suggest that juvenile rainbow trout possess a chemical alarm signal. The data, however, do not allow us to determine whether (i) the observed changes in behaviour represents a generalized response to injured fish or (ii) the putative alarm signal is localized in the skin, like the alarm pheromone system of cyprinid fishes (Smith 1992).

Methods

Test fish

Juvenile rainbow trout were obtained from Mainstream Hatcheries, Vanscoy, Saskatchewan, Canada. These trout were also of a hatchery strain but originated from wild-caught broodstock collected from Washington State. Trout were housed and fed as in experiment 1. Swordtails (*Xiphophorus helleri*) were obtained commercially, held in a 37-L aquarium on a 14 h L : 10 h D cycle, and fed daily with commercial tropical fish food ad libitum.

Test stimuli

We tested individual trout in one of three conditions: (1) trout skin extract (TSE), (2) trout body extract (TBE), and (3) swordtail skin extract (SSE). Swordtails were used because they lack known alarm pheromones (Mathis and Smith 1993a) and are phylogenetically distant and allopatric from trout. We used 14 juvenile rainbow trout

Fig. 1. Mean ($\pm$ SE) changes in time taken to capture the first food item (A) and in number of food items consumed (B). Shaded bars represent the distilled-water control (DW) and open bars trout extract (WTE). An asterisk denotes a significant difference at $p < 0.05$, Mann–Whitney U test (see the text for details).
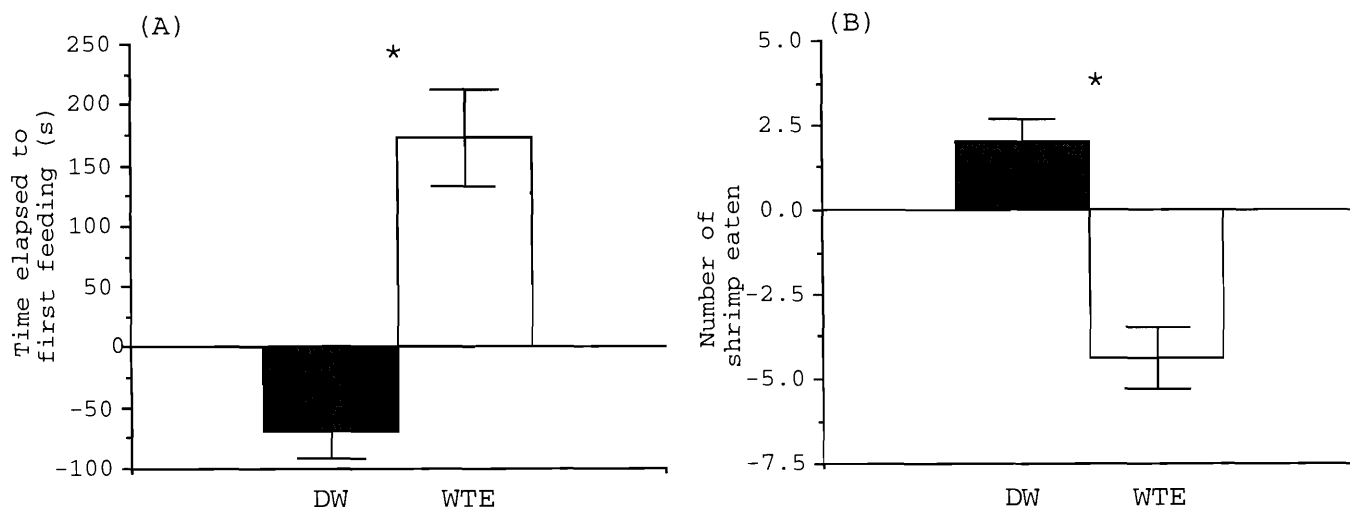
Fig. 2. Mean ($\pm$ SE) changes in time spent swimming in the back half (near the stimulus injection tube) and front half (near food delivery tube) of the tank (A) and in time spent under cover (B). Shaded bars represent the distilled-water control (DW) and open bars trout extract (WTE). An asterisk denotes a significant difference at $p < 0.05$, Mann–Whitney U test (see the text for details).
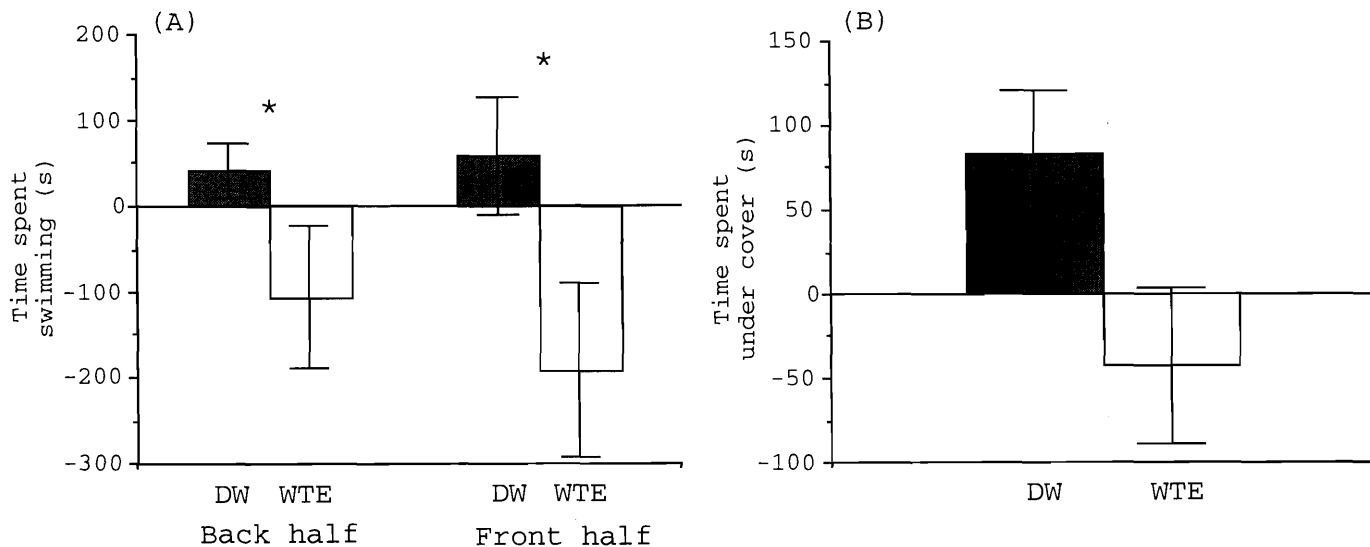
(5.06 $\pm$ 0.40 cm standard length) to generate the TSE and TBE stimuli. Trout were killed with a blow to the head and the skin was removed from both sides of the body, rinsed in glass-distilled water (to remove any unwanted tissue), and placed in 50 mL of chilled glass-distilled water. A total of 49.18 cm² of skin was sampled. This is equivalent to 2.69 g (wet mass). For the TBE stimuli, we sampled 2.81 g of visceral tissue, ensuring that no skin was present, rinsed the tissue in glass-distilled water, and placed it in 50 mL of chilled glass-distilled water.

Swordtail skin samples were collected from 20 adult swordtails (4.46 $\pm$ 0.35 cm standard length). A total of 46.4 cm² of skin was collected (= 2.74 g wet mass) and immediately rinsed and placed in 50 mL of glass-distilled water.

Each of the three tissue samples was homogenized and filtered through glass wool. Glass-distilled water was added to bring the final volume of each stimulus to 400 mL. Stimuli were frozen in 30-mL samples at $-20°C$ until required. Thirty-millilitre samples of glass-distilled water were also frozen as a control stimulus.

Test protocol

Juvenile rainbow trout were tested individually in a series of 37-L aquaria at approximately 18°C. Each tank was equipped with a single airstone located along the centre of the back wall. A stimulus-injection tube and a food-delivery tube were present, as described in experiment 1. The tank contained a single cover object and a silica sand substrate as described in experiment 1. The test tanks were divided into three equal compartments with markings on the exterior of the tank. Trout were placed individually in the test tanks 72 h prior to testing and fed daily with brine shrimp ad libitum during the acclimation periods.

Trials consisted of a 10-min pre- and a 10-min post-stimulus injection observation period. During both the pre- and post-stimulus observation periods, we recorded (*i*) area use (1, back third of the tank, near the cover object; 2, central third of the tank; 3, front third of the tank, near the food-delivery tube) every 15 s, (*ii*) time elapsed until the first food item was captured, (*iii*) number of shrimp eaten, and (*iv*) time spent under the cover object. In addition, we also

Fig. 3. Mean (± SE) changes in time spent under a cover object (A) and in area use (B). Shaded bars represent the distilled-water control and open bars the experimental stimulus. TSE, trout skin extract; TBE, trout body extract; SSE, swordtail skin extract. An asterisk denotes a significant difference at $p < 0.05$, Mann–Whitney U test (see the text for details).
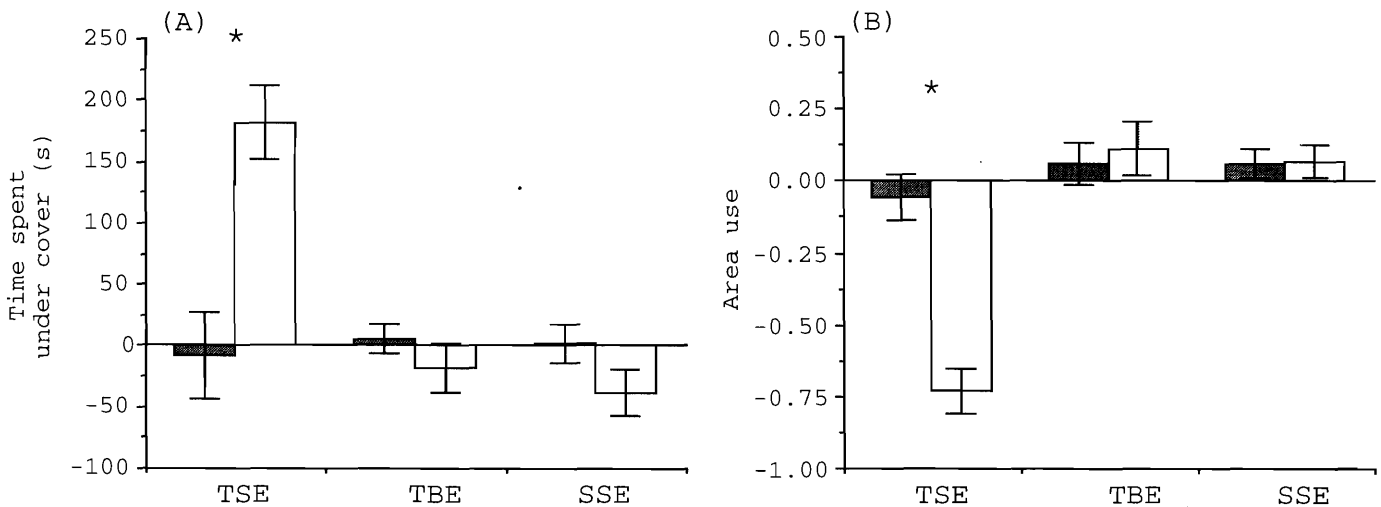
recorded the presence of any freezing behaviour. Trout were considered to freeze when they ceased all movement, settled on the substrate, and remained motionless for at least 30 s. Observations and food introduction were made as in experiment 1.

Ten juvenile trout were tested in each of the three treatments. The standard length (mean ± SD) of trout in each treatment was 4.91 ± 0.62 (TSE), 5.15 ± 0.64 (TBE), and 5.34 ± 0.75 (SSE) cm. For each treatment, control trials began at approximately 8:30 a.m. and experimental trials began at approximately 1:00 p.m. Ten millilitres of glass-distilled water was used as a control stimulus and 10 mL of one of the three treatment stimuli was used for the experimental treatments. Individuals were tested in only one of the three treatments.

Statistical analysis

Time spent under cover, time elapsed to first feeding, and number of shrimp consumed were analyzed as in experiment 1. We calculated a mean area-use index for both pre- and post-stimulus injection observation periods for each test fish and a mean difference for both control and experimental trials and compared these differences using a Mann–Whitney U test (Siegel 1956). Frequencies of occurrence of freezing behaviour were compared using Fisher's exact probability test (Siegel and Castellan 1988).

Results

We observed a significant increase in time spent under cover objects between control and experimental trials in the TSE treatment ($Z = -3.29, p < 0.001$; Fig. 3A). No significant differences were found in either the TBE or the SSE treatment (TBE: $Z = -0.61, p = 0.54$; SSE: $Z = -1.39, p = 0.16$; Fig. 3A). A similar trend was observed in the area-use measure, a significant decrease being observed in the TSE treatment ($Z = -3.52, p < 0.0004$; Fig. 3B) and no significant differences in either the TBE or SSE treatment (TBE: $Z = -0.53, p = 0.60$; SSE: $Z = -0.15, p = 0.88$; Fig. 3B).

Trout in the TSE treatment exhibited a significant increase in the time required to capture the first food item in the experimental versus the control trials ($Z = -3.74, p < 0.0002$; Fig. 4A). No such differences were seen in either the TBE or SSE treatment (TBE: $Z = -1.59, p = 0.11$;

Table 1. Occurrence of freezing behaviour.

	Yes	No	P^*
Trout skin extract			
Control	1	9	
Experimental	7	3	<0.001
Trout body extract			
Control	0	10	
Experimental	1	9	>0.05
Swordtail skin extract			
Control	0	10	
Experimental	0	10	>0.05

*Fisher's exact probability test (Siegel 1956).

SSE: $Z = -1.39, p = 0.16$; Fig. 4A). Along with an increase in time required to capture the initial food item, we observed a significant decrease in the number of shrimp taken by trout in the TSE experimental trials ($-3.82, p < 0.0001$; Fig. 4B). When presented with either TBE or SSE, trout significantly increased the number of shrimp taken (TBE: $Z = -2.51, p < 0.02$; SSE: $Z = -2.19, p < 0.03$; Fig. 4B). The increase in the number of shrimp consumed may be considered a response to a feeding stimulus.

We observed a significant difference in the occurrence of freezing behaviour in the TSE treatment but no difference in either the TBE or the SSE treatment (Table 1).

Discussion

Our results clearly demonstrate for the first time that juvenile rainbow trout show significant increases in antipredator behaviours (fright response, as in von Frisch 1938) when presented with conspecific skin extract. By reducing time spent swimming, freezing, and increasing time taken to resume feeding in response to conspecific skin extract, individual trout would likely increase their probability of survival by reducing the risk of detection and capture by predators (Donnelly and Whoriskey 1993; Gotceitas and Godin 1993).

Fig. 4. Mean (± SE) changes in time required to capture the first food item (A) and in numbers of shrimp eaten (B). Shaded bars represent the distilled-water control and open bars the experimental stimulus. TSE, trout skin extract; TBE, trout body extract; SSE, swordtail skin extract. An asterisk denotes a significant difference at $p < 0.05$, Mann–Whitney U test (see the text for details).
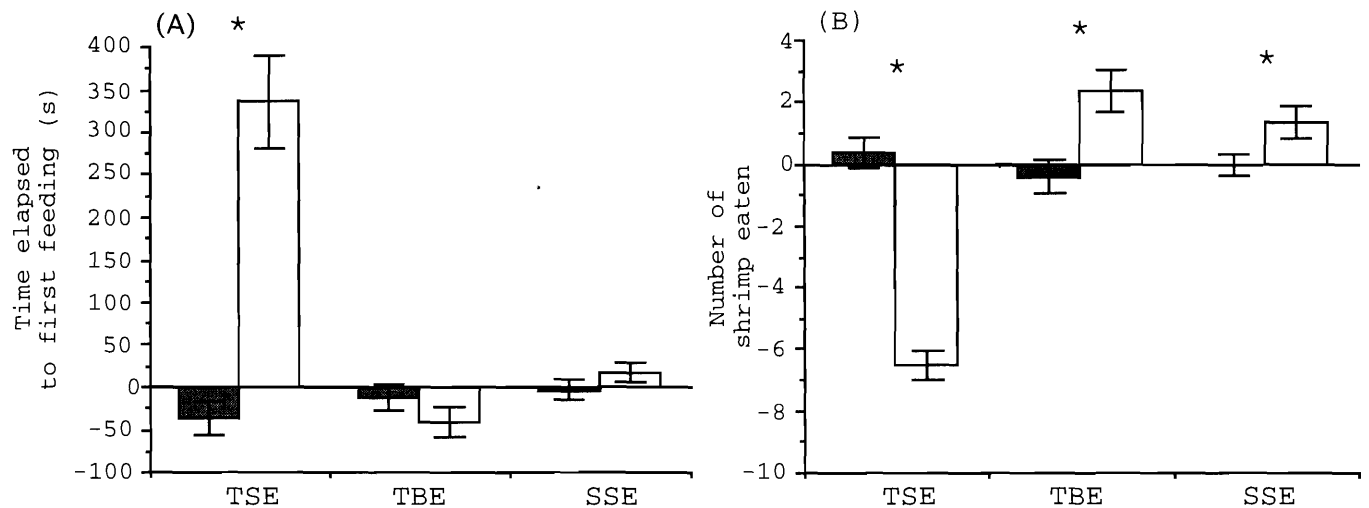
Chemical alarm signals have been shown to benefit both the sender and the receiver. Mathis and Smith (1993a) have demonstrated that a fathead minnow's probability of surviving an encounter with northern pike (*Esox lucius*) is significantly increased if minnow alarm pheromone is present. Alarm pheromone increase a minnow's chance of survival by eliciting antipredator responses (Mathis and Smith 1993a, 1993c) or possibly by increasing vigilance against the visual threat of predation (Brown and Smith 1996).

Chemical alarm signals may benefit prey species in other ways as well. Fathead minnow alarm substance is attractive to at least two predators (diving beetles (family Dytiscidae) and northern pike; Mathis et al. 1995). Once attracted, secondary predators may interfere with a primary predator, resulting in longer handling time by the primary predator and increased opportunity for escape by the prey fish (Chivers et al. 1996). The role of salmonid chemical alarm signals in predator recognition and (or) attraction remains to be tested.

The presence of a fright response within a social group may have significant fitness benefits for its members. One of the potential benefits which accrue is that nearby related conspecifics are warned of a potential predation risk. Rainbow trout are able to discriminate siblings from unrelated conspecifics on the basis of waterborne chemosensory cues (Brown and Brown 1992; Brown et al. 1993). Juvenile rainbow trout (and Atlantic salmon, *Salmo salar*) preferentially defend foraging territories near kin versus non-kin (Brown and Brown 1993a, 1993b, 1996a). By warning nearby kin of a perceived risk of predation, individuals could gain significant inclusive fitness benefits (Smith 1982b).

Several investigators have previously attempted to demonstrate the presence of chemical alarm signals in salmonids (reviewed in Pfeiffer 1977). Their inability to demonstrate this phenomenon in salmonid fishes may be due to one (or more) of several factors. Initially, the fright response of rainbow trout in response to the chemical alarm signal is relatively subtle compared with that of ostariophysian fishes. The fathead minnow and the European minnow (*Phoxinus phoxinus*) are probably two of the most common species studied in this context. In response to con-

specific alarm pheromone, minnows engage in a variety of dramatic antipredator behaviours, including dashing (or skittering), freezing, more time spent under cover, increased area avoidance, and more shoaling (Heczko and Seghers 1981; Lawrence and Smith 1989; Krause 1993; Mathis and Smith 1993b; Brown et al. 1995b, 1996; Wisenden et al. 1995b). Other species that were previously thought not to possess chemical alarm signals have recently been shown to respond to conspecific signals with increases in antipredator behaviours. These include brook sticklebacks (Mathis and Smith 1993c) and threespine sticklebacks (*Gasterosteus aculeatus*; Brown and Godin 1997). The response of these fishes, while much less dramatic than that of ostariophysian fishes, is reliably quantifiable in both laboratory (Mathis and Smith 1993c; Brown and Godin 1997) and field (Mathis and Smith 1993c; Wisenden et al. 1995b) studies. These results highlight the need to rely on quantitative rather than qualitative measures of behaviour (Mathis and Smith 1993c).

Related to this is the possible confounding factor of the ecological relevance of the measures used to determine the presence of a fright response. The behavioural measures recorded must have some relevance to the natural antipredator behaviour of the study species. Thus, using dashing or skittering behaviour (like fathead minnows in Lawrence and Smith 1989) as a measure for trout would give a false negative result, whereas using time required to resume foraging does provide a measure of fright response (as in the current study).

There may also exist significant population differences in an individual's response to a chemical alarm signal. It is not known if rainbow trout from other populations would show a fright response. Fathead minnows from at least one population have been shown not to respond to conspecific alarm pheromone (Reehan Mirza, personal communication). Mathis et al. (1993) demonstrated population differences in fathead minnows' response to the visual and chemical cues from a potential predator. In this context, the use of two different hatchery strains of trout in this study adds to the external validity of our results.

Finally, an individual's hunger level may have significant

effects on its "motivation" to respond to a chemical alarm signal. Iowa darters (Smith 1981) and fathead minnows (Brown and Smith 1996) that have been food-deprived for periods of 24–48 h fail to show an overt fright reaction when presented with conspecific alarm pheromones. In the case of fathead minnows, food-deprived individuals are still able to learn to recognize predator stimuli (Brown and Smith 1996). These data underscore the importance of understanding the role of the various costs and benefits associated with alarm signalling systems.

It is still not known if the chemical alarm signal demonstrated in the current study constitutes a true alarm pheromone (as in Williams 1992). Magurran et al. (1996) argue that the ostariophysian alarm pheromone system does not meet the "classic" definition of an alarm pheromone, since it may be context-dependent (though for contradictory arguments see Smith 1997). As a result, they call for further field testing to verify the phenomenon. Further work, especially field trials, is therefore required before we can consider the trout chemical alarm signal to be a true alarm pheromone.

In this study, hatchery-reared trout were purposely used as both a chemical signal source and as test fish. Hatchery-reared salmonids tend to show reduced levels of antipredator behaviour in both laboratory (Johnsson et al. 1996) and field (Berejikian 1995; Shively et al. 1996) studies. This may be a result of relaxed selection pressure within hatchery populations (Johnsson et al. 1996) or a lack of opportunity to learn predator recognition cues (Suboski and Templeton 1989; Berejikian 1995). Our current results can been seen as having potential applications to salmonid stocking programs. It may be possible to pair the chemical alarm signal with either chemical or visual cues from potential predators to condition predator-naive hatchery-reared trout to recognize predation risk (acquired predator recognition; Chivers and Smith 1994a, 1994b; Chivers et al. 1995).

These data clearly demonstrate that under laboratory conditions, juvenile rainbow trout increase antipredator behaviour when presented with conspecific skin extracts. Further tests are required to determine the role such a system might play in the natural behaviour of salmonids.

Acknowledgements

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