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Identification of a disturbance signal in larval red-legged frogs, *Rana aurora*

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Animals that are warned about the presence of a predator are more likely to avoid and/or survive an encounter with a predator. Chemical signals released by disturbed or injured conspecifics may provide prey animals with an early warning. In this study we conducted experiments to determine whether larval red-legged frogs respond to chemical stimuli produced by disturbed conspecifics and to examine the chemical compounds that may act as the alarm signal. In laboratory tests, groups of tadpoles responded with antipredator behaviours when exposed to chemical cues of disturbed conspecifics but not when exposed to chemical cues of control (undisturbed) conspecifics. In subsequent tests, disturbed animals increased ammonium (the main metabolic waste of tadpoles) excretion relative to undisturbed individuals. When tadpoles were exposed to low-level ammonium concentrations (1 mg NH_4^+/litre), they responded by increasing antipredator behaviours. Our results suggest that red-legged frog tadpoles release a chemical that provides conspecifics with an early warning of predator presence, and that ammonium (NH_4^+) may be a component of the disturbance signal.

The likelihood of an animal avoiding a predator's attack should increase if the predator's presence is detected early in the predation sequence (Lima & Dill 1990). Many aquatic prey animals use chemical signals to assess predation risk (e.g. Petranka et al. 1987; Chivers & Smith 1994; Kiesecker et al. 1996; Kiesecker & Blaustein 1997; see for review Chivers & Smith 1998; Kats & Dill 1998). Cues may arise from predators or they may be released by other prey animals when they detect or are captured by a predator.

Chemical cues of either source can act as signals to warn prey of nearby predators. Warned prey may be better able to survive an encounter with a predator. For example, fathead minnows, *Pimephales promelas*, exposed to chemical alarm cues have higher survival rates in the presence of a predatory northern pike, *Esox lucius*, than those not exposed to such cues (Mathis & Smith 1993). Similarly, western toad, *Bufo boreas*, larvae exposed to chemical alarm signals are better able to survive in the presence of an invertebrate predator (Hews 1988). The earlier prey are aware of a predator's presence, the greater the potential advantage to the prey.

In a recent review, Chivers & Smith (1998) divided chemical alarm signalling systems into two general categories based on the point in the predation sequence when signals are emitted. Damage-released alarm signals are those chemicals released by prey animals only upon being captured by a predator. In contrast, disturbance signals are chemicals that are released by senders that have been disturbed but not captured by a predator. Damage-released alarm signals are widespread in aquatic taxa (e.g. fish: Smith 1992; Chivers & Smith 1994; amphibians: Pfeiffer 1966; Hews & Blaustein 1985; Wilson & Lefcort 1993; Chivers et al. 1996, 1997; insects: Sih 1986; crustacea: Hazlett 1994; Williams & Moore 1985; sea anemones: Howe & Sheikh 1975; echinoderms: Lawrence 1991; gastropods: Appleton & Palmer 1988). Chemical disturbance signals have received little attention. However, recent experiments suggest that they may be widespread (Hazlett 1985, 1989, 1990a, b; Wisenden et al. 1995).

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Hazlett (1985, 1990b) demonstrated that both crayfish, *Orconectes virilis*, and hermit crabs, *Calcinus lavimanus*, increase antipredator behaviour when exposed to chemical cues released by disturbed conspecifics. In addition, *O. virilis* responds to disturbance signals produced by numerous heterospecifics, including other crayfish species, leeches, newts and fish (Hazlett 1985, 1989, 1990a). Moreover, Iowa darters, *Etheostoma exile*, increase antipredator behaviour when exposed to chemical cues of disturbed conspecifics (Smith 1979; Wisenden et al. 1995).

The disturbance signal in crayfish and Iowa darters may be ammonium, excreted from the gills or urine during periods of increased metabolic activity (Hazlett 1989, 1990a; Wisenden et al. 1995). Smith (1979) documented that Iowa darters lose their ability to produce disturbance signals, indicating that the supply can be depleted. This implies that the disturbance signal is not excreted continuously, but rather may be stored and released as a pulse. A likely candidate for the source of a disturbance chemical signal is urine.

We conducted a series of experiments to determine whether larval *R. aurora* respond to chemical stimuli released by disturbed conspecifics and to ascertain what chemical compounds may act as the alarm signal. We quantified changes in antipredator behaviour of *R. aurora* larvae exposed to chemical cues from disturbed conspecifics. We then assessed whether disturbed tadpoles increase ammonium output, the principal metabolic waste of tadpoles (Duellman & Treub 1986). Finally, we examined whether changes in ammonium concentration induced antipredator behaviour of larval *R. aurora*.

METHODS

Experiment 1: Nondamage Alarm Signalling

We collected *R. aurora* embryos on 20 December 1995 from a pond situated 18 km south of Waldport, Oregon. Embryos were collected and transported to a laboratory at Oregon State University where they were reared in 38-litre aquaria. After hatching, tadpoles were raised at approximately 16°C on a 12:12 h light:dark cycle and fed alfalfa pellets and fish food ad libitum. We began tests when all tadpoles reached Gosner (1960) stage 25.

Testing took place in 38-litre aquaria that were divided with a fibreglass screen partition and a black plastic blind that prevented movement of tadpoles and transmission of visual cues, but allowed the transmission of chemical cues. One end of the aquaria held stimulus animals (hereafter senders), while the other end held focal animals (hereafter receivers) whose behaviour was observed. Senders were placed into cages (15.5 × 10.0 cm) that restricted their movement and prevented them from being seen by focal animals. The cages were composed of three sides of opaque Plexiglas and a fourth side of fibreglass screen. The screen end was placed away from the focal animals. We forced air through an air stone near the cage to facilitate the movement of chemical cues from sender to receiver animals. Focal animals were allowed to move freely within their end of the tank. Two lines divided the floor of the receiver compartment into four equal sections. A shelter made of opaque Plexiglas (12 × 12 × 5 cm high) was placed in the compartment with the focal animals. The water in the tank was 10 cm deep, thus, tadpoles had a choice of swimming above or below the shelter.

A test began when we placed 15 senders into the cages and allowed them to acclimate for 12 h. After 12 h, we placed the five focal tadpoles (receivers) into the tank and allowed them a 10-min acclimation period. Each trial included two 15-min periods (a pre- and poststimulus response). During each 15-min period, we observed focal animal activity, position (relative to senders) and shelter use. As a measure of activity, we counted the number of times any of the five tadpoles crossed any of the lines during both pre- and poststimulus periods. We also counted the number of focal animals under shelter and the number of animals away from the senders' half of the aquaria at 30-s intervals. Focal animals were considered to be away from the senders if they were in either of the two sections of the receivers' compartment that were furthest from the senders' compartment. These 30-s counts were then averaged for pre- and poststimulus trials.

The experiment consisted of two treatments. Focal animals were either exposed to senders that were frightened (experimentals) between the pre- and poststimulus period, or to senders that were left alone (controls). To frighten tadpoles, a wooden heron model was placed into the senders' cage by an experimenter. The experimenter stood behind the blind that separated the stimulus and focal ends of the tanks and thus could not have been seen by the focal animals. The wooden model was moved around the cage for 30 s to simulate a predator attack. Care was taken not to touch or damage any of the tadpoles. Care was also taken to ensure that the addition of the predator model did not add noise and/or mechanical stimuli that exceeded that of the active air stones. Thus, both control and experimental receivers were exposed to similar levels of noise and mechanical stimuli from the senders. An opaque barrier prevented the receiver tadpoles from seeing the movement of the wooden model.

We conducted a total of 30 trials (15 experimental, 15 control). The treatments were presented in random order. Testing took place during daylight hours on 3 consecutive days (19–21 February 1996). All animals were naive to the experiment and were used only once as either a sender or receiver animal. Tanks were drained and washed between trials. For all trials, stimulus and focal animals were arbitrarily drawn from stock tanks. All animals were size-matched between trials and treatments ($\bar{X} \pm$ SE mass in grams: control focal=0.447 ± 0.037; control stimulus= 0.487 ± 0.047; experimental focal=0.414 ± 0.021; experimental stimulus=0.414 ± 0.072; F=0.04, P=0.874).

Experiment 2: Identification of Proposed Alarm Signal

Several studies have suggested that disturbance signals of aquatic organisms may include nitrogenous wastes that are excreted during duress (Hazlett 1989, 1990a;

Wisenden et al. 1995). We conducted a laboratory experiment to examine whether tadpoles disturbed by a simulated predator attack increase ammonium output, relative to undisturbed controls. Embryos were collected on 27 January 1997 from the same pond described in experiment 1. All animals were handled and reared in a manner identical to that described in experiment 1. We began tests when all tadpoles reached Gosner (1960) stage 25.

Testing took place in 40 6-litre plastic containers ($27 \times 16 \times 11.5$ cm) that were filled with 3 litres of dechlorinated tap water. Two 25-cm pieces of tygon tubing were secured to each container. This allowed experimenters to remove water samples from each container without disturbing the tadpoles. Three tadpoles were added into each of the 40 containers. Before being placed into containers, tadpoles were held in a net for 15 s and rinsed with dechlorinated tap water. Tadpoles were left to acclimate for 6 h, after which time, we removed a 25-ml sample of water. We then disturbed the tadpoles in 20 of the containers. To frighten tadpoles, a clean glass rod was placed into the container by an experimenter. The rod was moved around for 1 min to simulate a predator attack. Care was taken not to touch or damage any of the tadpoles. After an additional minute, we removed a second water sample from all control and experimental containers.

All water samples were frozen until analysis. Ammonium concentrations (mg/litre) were determined colourimetrically by the salicylate-hypochlorite method (Wall et al. 1975) on a Lachat flow-injection autoanalyser (Lachat Instruments, Milwaukee, Wisconsin).

Testing took place during daylight hours on 7 May 1997. All animals were used only once in this experiment and treatments were assigned randomly. Animals were size-matched between treatments ($\bar{X} \pm$ SE mass in grams: control=1.08 ± 0.057; experimental=1.07 ± 0.068; t=1.088, P=0.283).

Experiment 3: Reaction of Tadpoles to Proposed Alarm Signal

If ammonium acts as a chemical alarm signal, we would expect *R. aurora* larvae to display antipredator behaviour when exposed to a pulse of ammonium. We conducted an experiment to test the ability of ammonium to induce antipredator behaviour in larval *R. aurora*. Animals used in this experiment were from the same group of animals collected for use in experiment 2 and were handled in an identical manner.

Testing took place in 29 rectangular plastic containers ($32 \times 18 \times 8$ cm) that were filled with 3 liters of dechlorinated tap water. We added three tadpoles to each of the 29 containers (15 experimentals and 14 controls). Tadpoles were given a 1-min acclimation period. At the start of each trial, we observed tadpoles for 3 min. We measured activity by recording the total time in seconds that any of the three tadpoles were moving. We then added a 15-ml solution of either 200 mg NH_4^+/litre (experimental) or distilled water (control). The addition of ammonium solution raised the final concentration to 1 mg NH_4^+/litre. After 1 min, we observed tadpoles for 3 min and recorded the total time spent moving in seconds by each tadpole.

Treatments were presented in random order. Testing took place during daylight hours on 3 July 1997. All animals were used only once in this experiment. Animals were size-matched between treatments ($\bar{X} \pm$ SE mass in grams: control=1.14 ± 0.098; experimental=1.12 ± 0.13; t=1.047, P=0.317).

Statistical Analyses

For statistical comparisons, we tested for differences between experimental and control treatments in the change (between pre- and poststimulus) of either activity, shelter use, position (experiment 1), ammonium concentration (experiment 2) or time spent moving (experiment 3). We used the change in behaviour (experiment 1 and 3) or ammonium concentration (experiment 2) between pre- and poststimulus periods to produce a single, independent datum for each response variable per trial. We then used Mann–Whitney U tests (Siegel & Castellan 1988) to compare differences in these changes between experimental and control treatments. We also used Wilcoxon signed-ranks tests (Siegel & Castellan 1988) to look for differences between pre- and poststimulus measures for both experimental and control treatments. Significance level for all tests is 0.025, adjusted for use of between-subject (Mann–Whitney U test) and within-subject (Wilcoxon signed-ranks test) comparisons.

RESULTS

Experiment 1

There were significant differences in how experimental and control animals changed their behaviour between pre- and poststimulus periods. There were differences in the change of activity (Z=3.59, N=30, P<0.001; Fig. 1a), shelter use (Z=2.70, P=0.007; Fig. 1b) and avoidance of senders (Z=3.69, P<0.001; Fig. 1c) between experimentals and controls. Focal animals exposed to chemical cues of disturbed senders decreased their activity (Z= − 2.616, N=15, P=0.009; Fig. 1a), increased shelter use (Z=2.556, P=0.011; Fig. 1b) and increased time spent away from senders (Z=3.351, P=0.001; Fig. 1c). Whereas, focal animals exposed to chemical cues of nondisturbed senders increased activity (Z=2.617, N=15, P=0.009; Fig. 1a) and experienced no changes in shelter use (Z= − 1.478, P=0.139; Fig. 1b) or proximity to senders (Z= − 0.502, P=0.615; Fig. 1c).

Experiment 2

There were significant differences in the change of ammonium concentration for tadpoles exposed to a simulated predator attack compared with that of nondisturbed tadpoles (Z=2.124, N=40, P=0.017; Fig. 2). Poststimulus ammonium concentrations were higher for disturbed tadpoles compared with prestimulus levels (Z=2.053, N=20, P=0.04; Fig. 2). In contrast, there were

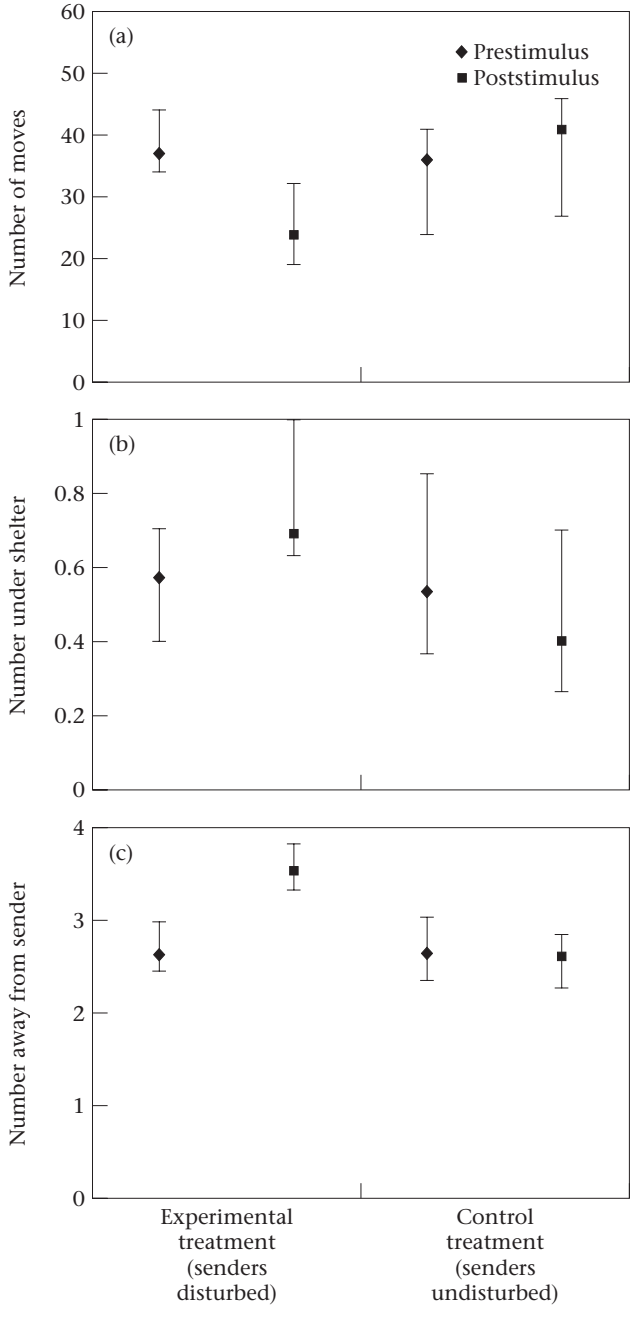

Figure 1. Median (and upper and lower quartiles) of (a) number of moves, (b) number of animals under shelter and (c) number of individuals away from sender compartment for tadpoles during pre- and poststimulus periods exposed to either cues of disturbed or control stimulus animals.

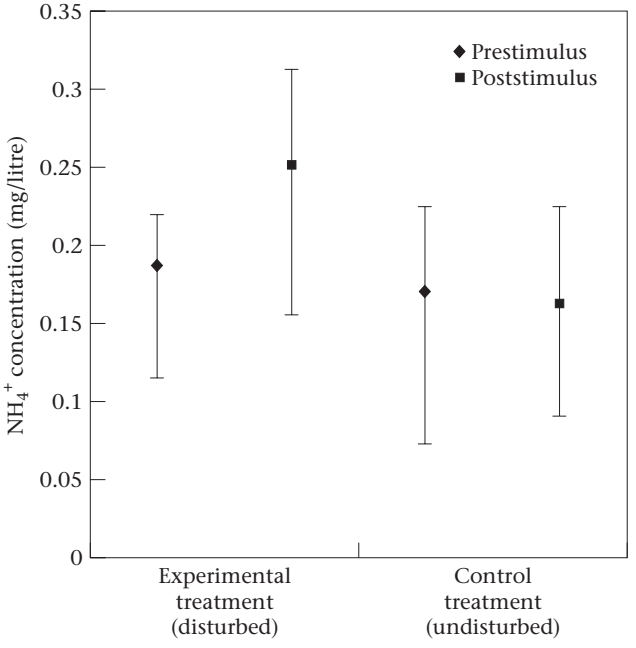

Figure 2. Median (and upper and lower quartiles) of ammonium concentration for control and disturbed tadpoles during pre- and poststimulus periods.

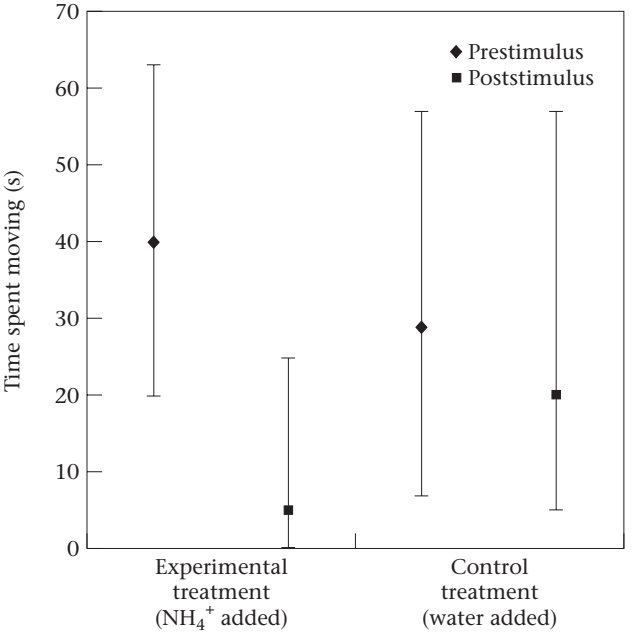

Figure 3. Median (and upper and lower quartiles) of total time that any of the three focal tadpoles were moving, for control and experimental tadpoles during pre- and poststimulus periods.

no differences in ammonium levels between pre- and poststimulus periods for control tadpoles ($Z=-0.168$, $N=20$, $P=0.867$; Fig. 2).

Experiment 3

Experimental tadpoles exposed to an addition of ammonium significantly changed time spent moving compared with control tadpoles receiving only water ($Z=2.008$, $N=29$, $P=0.022$; Fig. 3). Tadpoles exposed to added NH_4^+ decreased time spent moving during the poststimulus period compared with the prestimulus period ($Z=-3.266$, $N=15$, $P=0.001$; Fig. 3). In contrast, tadpoles exposed to a water control showed no significant change in time spent moving between pre- and poststimulus periods ($Z=1.712$, $N=14$, $P=0.087$; Fig. 3).

DISCUSSION

These results show that red-legged frog larvae display antipredator behaviours in response to chemical cues released from disturbed conspecifics. The antipredator responses observed included a reduction in movement, avoidance of the stimulus compartment, and an increase in shelter use. All of these responses are commonly reported responses of prey to the presence of predators (Sih 1987; Lima & Dill 1990). Increased shelter use and decreased movement may be especially important behaviours against predators that locate their prey by detecting movement. In contrast to the decrease in activity shown by experimental animals in experiment 1, control animals showed an increase in activity between the pre- and poststimulus periods which may represent exploratory behaviour. The decrease in activity by experimental animals emphasizes that the cost of conspicuous behaviour probably exceeds the benefit of information gathering when under the risk of predation.

The results of experiment 1 must be interpreted with some caution. The presentation of the predator model to experimental senders between the pre- and poststimulus periods may confound the effect of a disturbance signal with incidental mechanical stimuli associated with the movement of the predator model. Nevertheless, we feel that this is unlikely given the high background levels of noise and mechanical stimuli released from the active air stones placed in with both control and experimental senders. Moreover, the results of experiments 2 and 3 are in no way confounded. Experiment 2 clearly shows that tadpoles release ammonium when disturbed by a predator, and experiment 3 shows that receiver tadpoles respond to ammonium with antipredator behaviour. We suggest that further studies should be conducted to assess the role that predator movement may play in facilitating the diffusion of disturbance signals to focal animals.

Our results also suggest that ammonium may be a component of the disturbance alarm signal of *R. aurora*. Disturbed animals increased ammonium output relative to undisturbed individuals. Moreover, red-legged frog larvae display antipredator behaviour (reduced activity) in response to increases in ammonium concentration. Ammonium is the chief metabolic waste of anuran larvae (Duellman & Treub 1986), and thus may represent an effective means by which to communicate chemically. However, we are aware that the design of experiment 3 does not allow us to distinguish between behavioural changes related to changes in concentration of any chemical and those specifically related to ammonium. Further experiments are needed before alternative compounds can be excluded completely.

Several studies (Hazlett 1985, 1989, 1990a, 1994; Wisenden et al. 1995) have suggested that a nitrogenous waste product, possibly ammonium, may function as a general disturbance signal used by aquatic organisms. In fact, Hazlett (1990a) has demonstrated that disturbed crayfish, *O. virilis*, increase ammonium output compared with undisturbed conspecifics. Moreover, *O. virilis* displays antipredator behaviours in response to increased ammonium concentration (Hazlett 1990a). The observation that *O. virilis* responds to chemical cues of disturbed animals from a variety of taxa suggests that the chemical(s) involved are not species specific (Hazlett 1989, 1990a). We might expect cross-species alarm responses to occur between prey species that are syntopic and exposed to the same suite of predators. Ammonium is a waste product of many aquatic organisms, and thus may represent a general means by which they can detect disturbance. A range of background levels of ammonium can probably be found in most natural bodies of water. Consequently, the relevant disturbance cue to which tadpoles respond is probably not the overall concentration of ammonium, but instead a sudden pulse of nitrogenous metabolites, especially in gregarious species where urine pulse is summed over many individuals. Some level of experience may be required for animals to associate predation risk with such a pulse.

The release of disturbance signals may not be intentional but may represent a normal physiological process to which other individuals have become sensitive. Releasing ammonium during a predator encounter may be the direct result of an increase in metabolism that is required for effective escape. In fact, in our study disturbed animals appeared to have elevated respiration rates during poststimulus periods compared with prestimulus periods.

The release and detection of disturbance signals have important implications for predator–prey interactions. Prey animals that detect disturbance cues have an early warning of the presence of a predator and may be able to avoid an encounter by leaving the area or by reducing movement and becoming cryptic. Early detection of a predator's presence will allow prey to increase vigilance, which will probably result in an improved chance of survival should the encounter escalate to an attack (Hews 1988; Mathis & Smith 1993).

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