

URINE AS A SOURCE OF CONSPECIFIC DISTURBANCE SIGNALS IN THE CRAYFISH *PROCAMBARUS CLARKII*

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Summary

Chemical signals are an important aspect of ecological interactions in crustacean systems. Repellent chemical signals can be classified into three context-specific categories: chemicals released directly from a repellent stimulus (avoidance chemicals), chemicals released from damaged conspecifics (alarm chemicals) and chemicals released from stressed but undamaged conspecifics (stress chemicals). Our study examines the existence and putative source of the stress signals in crayfish. We hypothesize that *Procambarus clarkii* can recognize stressed individuals through chemical signals and also that the source of the signal that provides *P. clarkii* with information on the behavioral state of the sender is the urine. We collected urine and gill water from stressed and non-stressed

animals, and chemicals from damaged conspecifics. Chemical cues were introduced into a test arena while several behavior patterns of *P. clarkii* were recorded. Stressed crayfish produce significantly more urine than non-stressed crayfish, and this urine caused crayfish to walk significantly faster and farther and away from the source of the signal. These results demonstrate that predator-stressed crayfish release urine that causes other crayfish to move away from the source of the signal. Responses to stress chemical signals may allow receiving organisms to avoid the fate of the signal sender.

Key words: chemical signal, crustacean, behaviour, stress signal, crayfish, *Procambarus clarkii*, urine cue, urine collection.

Introduction

An important facet of species interactions is communication through sensory signals, which can be mechanical, electrical, visual or chemical. For example, wood termites, *Zooternopsis nevadensis*, drum their heads against the substratum when disturbed, which causes termites that sense the vibrations to flee (Kirchner et al., 1994). Juvenile rainbow trout, *Oncorhynchus mykiss*, can detect the presence of a damaged conspecific through chemicals released from the damaged conspecific's skin (Brown and Smith, 1997). Male fiddler crabs, *Uca rosea*, use visual cues in the form of lateral claw-waving to attract females to their burrows to mate (Murai et al., 1987). For aquatic organisms, these communication signals often take the form of chemical stimuli because of limitations in visibility and ambient mechanical noise in flowing systems.

More specifically, the use of chemical signals has been well studied in the context of attractive and repellent stimuli. Many crustaceans use chemical signals to locate food resources in the environment (Moore et al., 1991; Weissburg and Zimmer-Faust, 1993). Similarly, moths are attracted to potential mates by pheromone signals (Vickers and Baker, 1994). In contrast, when stressed by humans (electrical shocking and handling), both rats and earthworms, *Lumbricus terrestris*, release chemicals that can be detected by and serve as a warning to

conspecifics (Valenta and Rigby, 1968; Ressler et al., 1968). Chemical cues present in the damaged tissue of conspecifics cause salamanders, *Ambystoma macrodactylum*, to avoid areas where conspecifics have been injured (Chivers et al., 1996). Chemicals released from a predatory fish (northern pike, *Esox lucius*) or an injured conspecific cause odonate larvae, *Enallagma boreale*, to change their foraging behavior (Wisenden et al., 1997). These studies all examined changes in the behavior of the receiver induced by a chemical signal.

Repellent chemical cues can be categorized into three general categories; avoidance, alarm and stress chemicals. Avoidance chemicals are well studied in the context of predation. Chemicals released from a predator cause potential prey actively to hide or move away from the predator, resulting in a lower risk of predation. A proposed functional benefit to predator detection through chemicals is the initiation of behavior patterns that decrease the predator's ability to detect the prey. Both crayfish, *Pacifastacus leniusculus*, and larval amphibians, *Eurycea bislineata* and *Hyla chrysoscelis*, exhibit avoidance behavior in response to chemical cues from fish predators (Petranka et al., 1987; Blake and Hart, 1993). These chemicals function to decrease the probability of an encounter with a predator.

Alarm signals have been examined in a number of systems and may play an important role in the awareness of conspecific injuries. The ability of an organism to detect and respond behaviorally to a damaged conspecific may allow it to avoid a similar fate. Hazlett (1994) and Sih (1986) examined the behavioral responses induced by alarm chemicals from damaged conspecifics. Hazlett (1994) found that crayfish, *Orconectes virilis*, respond to these chemicals with little or no movement. Sih (1986) observed a similar phenomenon in larvae of the mosquito *Culex pipens*. These investigators hypothesized that detection of damaged conspecifics allows organisms to avoid a similar conspecific predation event.

Finally, stress chemicals may also play an important role in the ecology and behavior of organisms and are the least studied of the three types of repellent cues. The ability of a receiver to recognize a conspecific under stress may allow it to avoid the circumstance that has caused the stress, whether it is predatory or social in nature. Hazlett (1985, 1990a) found that crayfish, *O. virilis*, are able to detect the presence of disturbed but undamaged conspecifics and disturbed members of other taxa through chemical cues. This behavioral response was not seen in the closely related species *O. rusticus* and *O. propinquus* (Hazlett, 1990a). *O. virilis* spent more time in a neutral posture in the presence of odor from a stressed conspecific and showed similar responses to disturbed leech (*Macrobdella decora*), darter (*Etheostoma exile*) and bass (*Ambloplites rupestris*). The response of *O. virilis* to these stress odors presumably allows them to avert a fate similar to that of the signal sender.

For crustaceans, these stress cues may be located in the urine. Breithaupt et al. (1999) found that lobsters could control the amount of urine they released depending upon the behavioral context and that the release could be pulsatile in nature. They suggested that the urine is a possible source of information in social encounters. Since crayfish are known to excrete urine, we speculate that urine is the source of a stress chemical that provides information on the behavioral state of the sender.

Our study was designed to determine whether recognition of stressed conspecifics through chemical signals occurs in the crayfish *Procambarus clarkii*, which have been shown to utilize chemical signals in basic ecological decisions. They can distinguish females from males and recognize dominance status solely on the basis of chemical information (Ameyaw-Akumfi and Hazlett, 1975; Zulandt Schneider et al., 1999). Because chemical communication has been demonstrated to be an important aspect of the behavior of *P. clarkii*, we hypothesize that *P. clarkii* can recognize stressed individuals through chemical cues located in the urine.

Materials and methods

Crayfish

Crayfish, *Procambarus clarkii* (Girard), were acquired from commercial suppliers (Carolina Biological Supply Company; Atchafalaya Biological Supply Company Inc.). They were separated by sex, their carapace length was measured, and

they were housed individually in 10 gallon aquaria (51 cm×25 cm×32 cm). Crayfish were maintained at a constant temperature of 23 °C, on a reversed 14h:10h L:D photoperiod and fed 0.5 cm³ pieces of frozen fish fillets (pollock, cod, herring, smelt) three times a week throughout the experimental period. Thirty-two crayfish were used in urine and gill signal collection averaging 5.04±0.15 cm (mean ± S.E.M.) in carapace length, and 48 crayfish were used in the bioassay of the putative chemical signal (4.06±0.18 cm carapace length).

Catheterization technique

Male crayfish were removed from their tanks during the middle of their light cycle and immobilized with rubber bands at the beginning of the catheterization process. Catheters were constructed from 10001 Pipetteman tips. The first 8 mm at the narrow end of the tips was cut off and attached over the nephropores of the crayfish using 5 min epoxy resin (Devcon) (Fig. 1A). The crayfish was restrained for approximately 5 min or the time it took for the resin to harden. The crayfish was then placed in a small holding tank containing water to a depth of 5 mm for 1 h while the resin dried completely. During this time, a small square of Velcro was glued to the carapace for the attachment of the collection bag. Catheters were checked for leaks by injecting a small amount of food coloring into them. Crayfish possessing catheters without leaks were returned to their tanks; the process was repeated if leaks were found.

The collection bags were constructed from the last 4 cm of latex non-lubricated condoms. The collection bag was rinsed

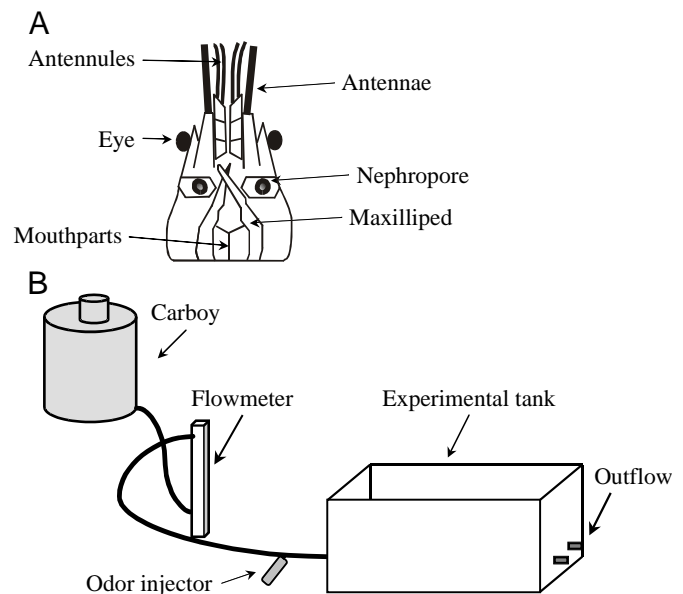


Fig. 1. (A) Frontal view of a crayfish showing the nephropore. Catheters were placed over the nephropores to isolate urine from the gill current. (B) Diagram of the experimental tank used in the behavioral portion of the study showing a side view. Water flowed from the carboy through the flow meter into the experimental tank. Odor was added to the water flow through the odor injector.

with distilled water and attached using a rubber band to a small plastic tube (approximately 8.5 mm diameter, 1.5 cm length). A 1 cm×1 cm square of Velcro was attached underneath the rubber band, and 10 cm of Nalgene tubing (3 mm inside diameter) was affixed to the plastic tube with hot-melt glue. Copper wire was placed inside the Nalgene tubing to support the collection bag and take the stress off the catheters upon attachment. Each Nalgene tube configuration was equipped with a collection bag filled with food coloring and attached to plugged pipette tips to check the structure for leaks. Each collection bag was used only once.

Urine and gill signal collection

The collection bags were attached to the crayfish 24 h after catheterization. Crayfish were then placed in 1 l of water in a Ziploc plastic bag (17.78 cm×20.32 cm). To collect stressed crayfish body fluids, crayfish were placed into the tank (102.9 cm×43.2 cm×41.9 cm) of a largemouth bass [*Micropterus salmoides* (Lacépède), approximately 30 cm standard length, animal protocol number 98016] for 10 min. During this 10 min trial, the crayfish and bass were able to interact. They could see one another and attempt contact through the plastic bag, which resulted in several predatory attempts by the bass during each trial. The presence of the bass induced the crayfish to exhibit threat displays with large meral spreads for long periods and to tail-flip, a well-documented escape behavior.

Collection of non-stress body fluids was similar to the procedure described above. Crayfish were equipped with collection bags and placed into a Ziploc plastic bag (17.78 cm×20.32 cm) which was put into an empty tank (102.9 cm×43.2 cm×41.9 cm) for 10 min. During these trials, the crayfish could move freely about the bag, as in the stressed trial, but there was no predation pressure present. Escape behavior was not seen in the non-stressed collection trials.

The plastic bag was removed from the tank after 10 min and the fluids were collected. The fluids collected from the Ziploc bag were termed gill water and those collected from the condom were termed urine. Gill water (125 ml) was funneled from the Ziploc bag into a plastic Nalgene container (125 ml) and frozen. The urine was collected using a 0.40 mm×12 mm needle attached to a 3 cm³ syringe. The needle was inserted through the latex bag, and urine was collected into the syringe. The amount of urine collected varied from 1.0 to 1.5 ml with the size of the organism. The urine was placed in a 1.5 ml microcentrifuge tube and frozen for later use. Crayfish were used only once in stress or non-stress trials, and catheters were removed after each trial. Twenty males were used in the stressed signal collection and 12 in non-stressed signal collection. Crayfish used for urine collection were not used in the behavioral study described below.

Collection of odor from damaged conspecific

The chelae of two crayfish were excised and placed in a mortar along with 100 ml of distilled water. The chelae were crushed using a pestle and strained through mesh screening to

remove large pieces of carapace and tissue. The remaining solution was placed into storage vials and frozen for later use. This protocol is similar to that used in other crayfish studies (Hazlett, 1994).

Bioassay of putative chemical signals

A flow-through tank (91 cm×26.5 cm×25 cm) with an experimental section (50 cm×26.5 cm×25 cm; Fig. 1B) was used for bioassays. A 10 l carboy provided the water for the tank and was connected to the experimental tank through an in-line flow meter (Manostat Riteflow no. 4). Water flowed into the experimental tank at a rate of 169 ml min⁻¹. The odors were added to the tank through a 3 cm³ syringe connected to the tubing 20 cm from the experimental tank inflow (Fig. 1B). The control treatment was 2 ml of dechlorinated water without crayfish odor added. Odor treatments consisted of introducing 2 ml of one of the following odors to the test tank; stressed gill water (Stress GW), non-stressed gill water (No stress GW), stressed urine (Stress U), non-stressed urine (No stress U), damaged conspecific water (Damaged Conspecific), a mixture of stressed gill water and urine (Stress U-GW) and a mixture of both non-stressed gill water and urine (No stress U-GW). The No stress U-GW treatment was collected by the methods described above, but the collection bag was not attached to the catheters so that any urine released would be mixed with the gill water. The syringes, tank and flow meter were rinsed with distilled water after each trial and the carboy and test tank were refilled with fresh water.

Trials were begun 3–5 h after lights out. A male crayfish was measured to the nearest millimeter and marked with White Out to allow identification when digitizing the trials. Crayfish were allowed to acclimate to the experimental tank for 1 h before the trial began. The trial began when the water flow was turned on. Water was allowed to flow through the tank for 1 min prior to odor introduction for the pre-odor (control) portion of the trial. Odor was then introduced to the tank for 45 s at a rate of 2.6 ml min⁻¹. All trials were recorded from above using a Sony Hi-8 camcorder at 30 frames s⁻¹ for both the pre-odor and odor portions. Crayfish were used only once over the course of the experiments. Six trials were run per treatment.

Dye trials

Dye trials were run to examine the distribution of odor treatments in the experimental tank. Food coloring was used as the tracer and prepared by diluting 0.5 ml of food coloring in 100 ml of water; 2 ml was injected into the experimental tank in the same manner as the odor treatments. Ten dye trials were run to determine how long odor remained in the tank and whether odor distribution was uniform throughout the tank. The visible center, left and right front of the dye-plume were digitized, as were the left and right trailing edges. The dye was found to leave the tank 45 s after being introduced and visually to spread throughout the experimental tank.

Data analyses

The behavior of the crayfish during the pre-odor and odor

portions of the trials was assessed by digitizing (PEAK Motus motion-analysis system) four points on the body of the crayfish: the tip of the rostrum, the telson, and the tips of the right and left chelae. Both visible dye trials and behavioral trials were digitized at a rate of 1 frame s^{-1} for 45 s before and after odor introduction. The raw data coordinates were used to obtain walking speeds, meral spread ratio (meral spread/carapace length), total distance traveled and movement in relation to the odor source by measuring the distances between the 45 frames digitized before and after odor introduction.

To control for individual behavioral differences prior to odor stimulation, all values were normalized by subtracting the pre-stimulus behavioral values from the post-stimulus behavioral measures. All subsequent statistics were run on normalized data. Using this technique, a lack of behavioral response to the stimulus would result in a zero value for that variable. A two-tailed *t*-test was used to examine whether the odor treatments changed crayfish behavior (Zar, 1996). A multivariate analysis of variance (MANOVA) was used to compare across treatments, and a *post-hoc* test (least significant difference test) was performed using a commercial statistics software package (Statistica).

Results

Qualitative measures of stress

Behavioral differences were observed between the two urine collection treatments. Crayfish tended to freeze when first placed into the tank with the bass. This was followed by long periods of threat displays (large meral spreads, body high off the bottom) and fast flicking of antennules, and frequent tail-flips occurred. These animals were designated stressed. The control treatment organisms usually sat quietly, near the substratum, and some walked slowly around. Tail-flips and threat displays were not observed in these organisms. These animals were defined as non-stressed.

Urine collection

The amount of urine collected over a 10 min period from stressed animals was significantly greater than from non-stressed organisms (two-tailed *t*-test for unequal means, $t_{0.5(2)22}=2.0739$, $P<0.0001$, $N=18$ stressed, $N=12$ non-stressed). Stressed organisms produced 1.04 ± 0.12 ml of urine in the 10 min period. Non-stressed animals did not produce a measurable amount of urine either during the trial period or over longer periods of up to 1 h. Thus, non-stress urine animals were excluded from further analysis (note: urine could not be collected over periods longer than 1 h because of problems with collection bag attachment over extended periods).

Effect of odor on walking speed

Crayfish increased their walking speeds significantly over pre-odor values in only the Stress U treatment (two-tailed *t*-test, $t_{0.5(2)6}=2.571$, $P<0.05$, $N=6$) (Fig. 2). Organisms walked

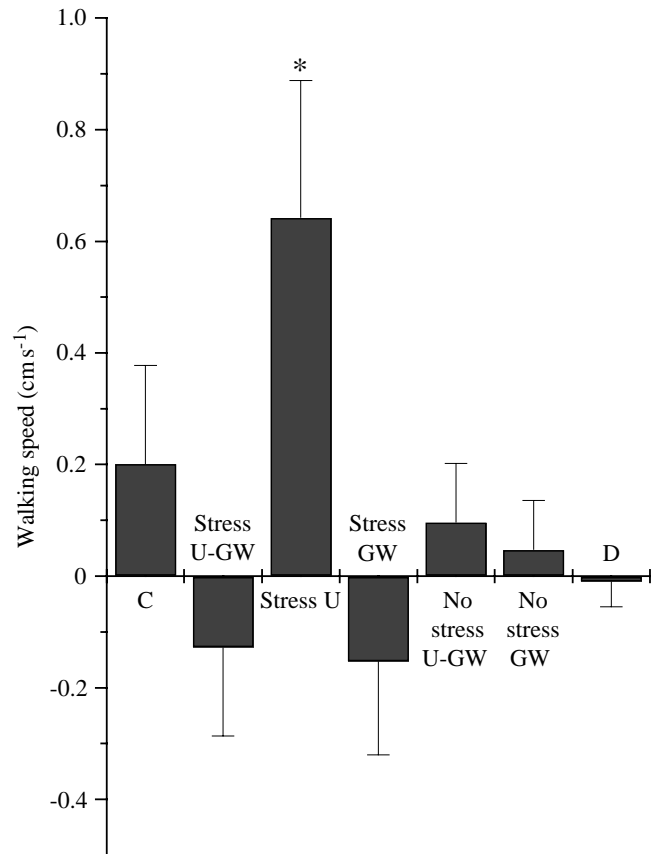


Fig. 2. Normalized walking speed for crayfish presented with different chemical stimuli. Columns are normalized mean values + S.E.M. for $N=6$ crayfish. Values are normalized to pre-stimulus walking speed for each crayfish. An asterisk indicates a value that is significantly different from zero ($P<0.001$ two-tailed *t*-test) and a significant difference between odor treatments ($P<0.05$, MANOVA). The chemical stimuli were gill water (GW) or urine (U) collected from either stressed or non-stressed crayfish or derived from crushed chelae (damaged conspecific). C, control; D, damaged conspecific.

significantly faster, at a normalized speed of 0.64 ± 0.2 $cm\ s^{-1}$, during the Stress U treatment than in all other odor treatments (multivariate analysis of variance, MANOVA, $P<0.001$, $N=6$) (Fig. 2).

Effect of odor on total distance traveled

The Stress U treatment was the only odor treatment to show a significant change from pre-odor values in total distance traveled (two-tailed *t*-test, $t_{0.5(2)6}=2.571$, $P<0.05$, $N=6$) (Fig. 3). Organisms walked significantly farther, a normalized distance of 31.4 ± 10 cm, during the Stress U treatment than in all other odor treatments (MANOVA, $P<0.01$, $N=6$) (Fig. 3). Organisms in the Stress GW treatment walked a significantly shorter distance, 8.08 ± 6.1 cm, than the control and Stress U treatments (MANOVA, $P<0.01$, $N=6$) (Fig. 3).

Effect of odor on movement relative to the odor source

The Stress U and Stress GW treatments were the only

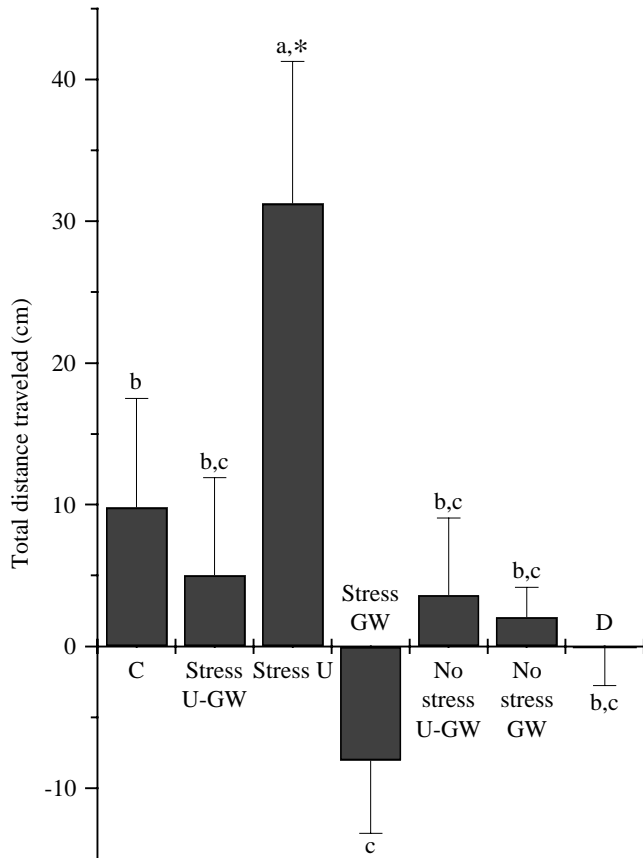


Fig. 3. Normalized total distance traveled for crayfish presented with different chemical stimuli. Columns are normalized mean values + S.E.M for $N=6$ crayfish. Values are normalized to pre-stimulus distance traveled for each crayfish. An asterisk indicates a value that is significantly different from zero ($P<0.01$ two-tailed t -test). Values with the same letter are not significantly different; values with different letters are significantly different ($P<0.01$, MANOVA). The chemical stimuli were gill water (GW) or urine (U) collected from either stressed or non-stressed crayfish or derived from crushed chelae (damaged conspecific). C, control; D, damaged conspecific.

treatments to show a significant change from pre-odor values (two-tailed t -test, $t_{0.5(2)6}=2.571$, $P<0.05$, $N=6$) (Fig. 4). Crayfish in the Stress U and Stressed GW treatments moved significantly farther away from the source than in all other treatments at an average speed of $0.62\pm 0.21\text{ cm s}^{-1}$ and $0.87\pm 0.12\text{ cm s}^{-1}$, respectively (MANOVA, $P<0.001$, $N=6$) (Fig. 4).

Discussion

Crayfish respond to urine collected from stressed animals in a manner different from other odor treatments. Crayfish increased their walking speed and total distance traveled significantly from the pre-odor treatment when stress urine was introduced into the test tank (Figs 2, 3). These responses are significantly greater than those for all other odor treatments (Figs 2, 3). Stress urine causes the crayfish to move away from

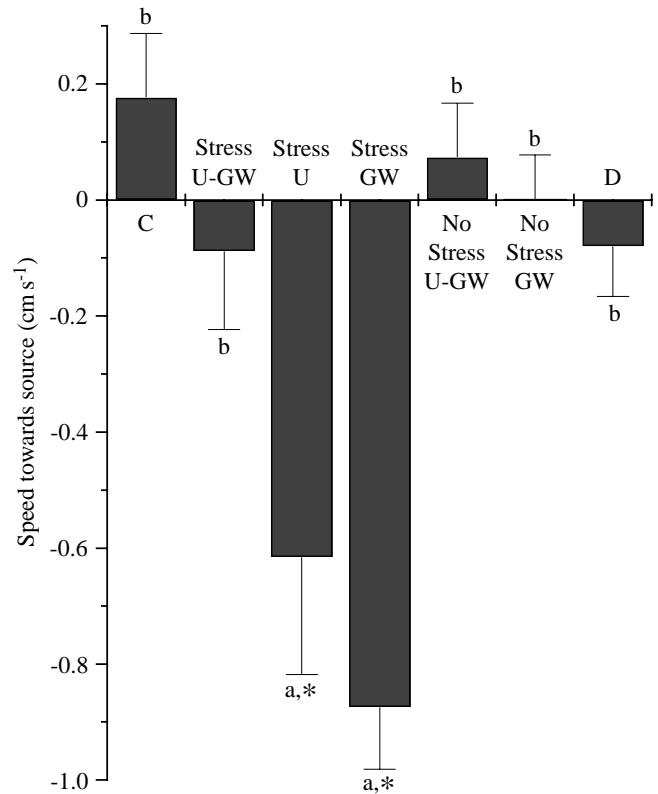


Fig. 4. Normalized speed of movement relative to the odor source for crayfish presented with different chemical stimuli. Columns are normalized mean values + S.E.M for $N=6$ crayfish. Values are normalized to pre-stimulus walking speed for each crayfish. Positive values indicate net movement towards the source, while negative values indicate net movement away from the source. Asterisks indicate values that are significantly different from zero ($P<0.05$ two-tailed t -test). Values with the same letter are not significantly different; values with different letters are significantly different ($P<0.001$, MANOVA). The chemical stimuli were gill water (GW) or urine (U) collected from either stressed or non-stressed crayfish or derived from crushed chelae (damaged conspecific). C, control; D, damaged conspecific.

the source significantly faster than for all treatments except the stress gill water treatment (Fig. 4). These data therefore support our hypothesis that *P. clarkii* respond to a stressed conspecific chemical signal and that urine is the source of the signal.

The utilization of a stress chemical has been examined in the hermit crab *Calcinus laevimanus* (Hazlett, 1990b) and the crayfish *Orconectes virilis* (Hazlett, 1985). These studies examined the behavioral responses of conspecifics in non-contextual situations, and observed an overall general avoidance of the chemical by the exposed organism.

Avoidance behavior may play an important functional role in the ecology of these organisms. It has been suggested that the ability of organisms to recognize stressed conspecifics can affect their ability to avoid predation (Hazlett, 1985). Organisms that detect and respond to a stressed conspecific by avoiding the area where the chemical is emitted can decrease

their probability of being detected by a predator or of encountering a similarly threatening scenario. The behavior patterns observed during the present study, an increase in the rate of movement, moving a greater distance and moving away from the urine source, suggest that the crayfish actively avoid the urine source. In an ecological context, all these behavior patterns would move the organism away from potential predators or other stressful situations.

Our results also support the conclusion that the urine is source of the stressed conspecific signal. Because we were unable to test behavioral responses to non-stress urine alone, we cannot conclude whether this is a context-specific response. We can conclude that urine is the source of the behavioral responses seen in this study. Given the evidence for other chemical signals being released through urine (Ryan, 1966; Gleeson, 1980; Bushmann and Atema, 1997), it is likely that urine is the source of the stress conspecific signal. By examining the characteristics of signals in general, and chemical signals more specifically, we will further support this conclusion.

The simplest definition of a signal is a code or stimulus pattern containing information that is transmitted through the environment (Dusenbury, 1992). There are several qualities of a signal that are important when attempting to classify a chemical as a signal: it must (1) be associated with a behavioral state in the sender; (2) be released at a context-specific time; (3) elicit a behavioral response in the receiver; and (4) have a meaning attached (Smith, 1977; Dusenbury, 1992). The urine from the stressed crayfish meets these criteria.

The chemical mixture released in stressed crayfish urine is associated with the behavioral state of the crayfish. Stressed crayfish were more active and exhibited both avoidance and escape behavior in the presence of the predator. This behavioral state was different from that seen in non-stressed crayfish, which did not exhibit avoidance or escape behavior, but instead sat motionless or slowly explored the collection bag. Therefore, a component in the urine released from a stressed organism could be associated with this change in behavior.

Second, stressed crayfish released a significantly greater amount of urine than non-stressed crayfish; non-stressed crayfish did not produce a measurable quantity. Since the urine released from stressed crayfish was associated with the presence of a predator, this suggests the release of a signal at a context-specific time.

Third, the stress urine produced a significant behavioral response in crayfish: they walk faster and farther and move away from the source of the stress urine. This behavioral response was not seen for any other odor treatment.

Finally, a chemical signal must have a meaning attached. The urine signal may provide information about an immediate or other danger. The behavior recorded indicates avoidance behavior. The meaning of the signal is clearly to move away from the source or face impending danger.

Our findings using an odor from a damaged conspecific appear to conflict with previous findings (Hazlett, 1985, 1990b) showing that crustaceans become stationary or increase

shelter use in the presence of this signal. Our experimental design differs from previous work in that our animals were not provided with a shelter and were already moving when the chemical was introduced, whereas the crayfish in other studies were habituated to tanks with adequate shelter (Hazlett, 1985, 1990b).

In addition, we found no behavioral response when stress urine was combined with stress gill water (Figs 2–4). This could be due to several factors. It could be explained by the resulting reduction in concentration of urine in the combined sample. Also, the gill water was thoroughly mixed with the urine before introduction into the tank, which is different from the natural situation where temporal effects may have a role. Also, the relative concentrations of gill water and urine may have differed from those a crayfish normally encounters in nature.

In summary, crayfish in a non-stressful situation do not exhibit threat displays and avoidance behavior and do not produce a noticeable amount of urine. Urine collected from stressed crayfish causes other crayfish to increase their rate of movement, move farther and move away from its source when it is introduced into the experimental tank. Because this urine has the qualities of a chemical signal, we suggest that it is the source of the stressed conspecific signal found in *P. clarkii* and that this signal may allow crayfish to avoid stressful situations.

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