

## DISCRIMINATION OF CONSPECIFIC MALE MOLT ODOR SIGNALS BY MALE CRAYFISH, *ORCONECTES RUSTICUS*

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### A B S T R A C T

For many organisms, chemoreception plays a key role in numerous aspects of daily life. Crayfish use chemical signals to find mates, warn conspecifics of predators, and relay social status. While many of these situations have been studied in detail, behavior of conspecifics toward chemical signals from molted individuals has not been thoroughly examined. The physiology of crayfish changes dramatically during molting (ecdysis), which in turn may change both the chemical content and concentrations of the chemical cues released into the water. We hypothesized that conspecifics are sensitive to chemicals released during molting. A Y-maze experimental design was used to test for differential responses to various molt-related chemical stimuli presented to intermolt male crayfish (*Orconectes rusticus*). The sources of chemical stimuli were recently molted male crayfish, intermolt male crayfish, control (aged tank water), and food (fish). Behavioral indices of response included initial arm choice, time spent in each arm, time spent at each nozzle, number of arm changes, and meral spread at each nozzle. Experiments were also conducted where crayfish were presented the same chemical stimuli in each arm to obtain measures of locomotor activity in the different stimuli. In addition, orientation parameters such as walking speed, walking speed to source, and distance to source were analyzed. Intermolt individuals spent more time in the presence of molt signals, although the food stimulus was more attractive than any other stimuli tested. Crayfish showed a significant initial arm choice when molt stimulus was paired with control. During the identical presentation of chemical stimuli, crayfish showed an increase in locomotor activity in the molt and food chemical stimuli than in the intermolt and control chemical stimuli. There were no significant differences in orientation parameters between chemical stimuli. These results show that crayfish can discriminate molted male conspecifics from the other chemical stimuli tested.

Chemical signals mediate many behaviors in Crustacea. Crustaceans can use chemoreception to identify and localize food (Derby and Atema, 1982; Moore *et al.*, 1991; Weissburg and Zimmer-Faust, 1994), predators (Willman and Hill, 1994; Keller and Moore, 1999), and conspecifics (Copp, 1986; Zulantz Schneider *et al.*, 2001). Crustaceans appear to release alarm signals through urine secretion (Zulantz Schneider and Moore, 2000). In addition, crustaceans are sensitive to chemicals such as crushed conspecific cues (Hazlett, 1990; Rittschof, 1992; Hazlett, 1994; Pijanowska, 1997). Mate or sex recognition (Ameyaw-Akumfi and Hazlett, 1975; Hazlett, 1985; Atema, 1986; Dunham and Oh, 1992; Corotto *et al.*, 1999) and social interactions are influenced by chemical signals (Zulantz Schneider *et al.*, 2001). Chemical signals are important for

crustaceans because aquatic animals are “leaky bags” (Atema, 1996), where information about the internal state of an animal is transmitted to the external environment via chemical cues, either actively or passively released. Any change in the physiology of a crustacean will cause a subsequent change in the chemicals “leaking” out into the environment.

Physiological and physical changes occur during ecdysis, or molting. During ecdysis, crustaceans have increased concentrations of hormones, including ecdysone and 20-hydroxyecdysone, in the hemolymph (Chang, 1995). These hormones initiate many physiological responses such as the reuptake and sequestering of inorganic chemicals and ions, loosening of the old exoskeleton, and the generation of chemicals that form the new exoskeleton underneath the old one (Waddy *et al.*, 1995).

During and after ecdysis, chemical compounds lost to the aquatic environment may be at altered concentration and composition. The soft exoskeleton following ecdysis is more permeable than a hard exoskeleton to water and perhaps other compounds (Aiken, 1980; Chang *et al.*, 1993; Waddy *et al.*, 1995). Compounds that would not diffuse across an intermolt exoskeleton may be able to pass after ecdysis before the new exoskeleton has completely hardened. This would alter the composition of the chemicals entering the water. Also, because the soft exoskeleton is not an efficient barrier, higher concentrations of chemicals could pass into the water. Because many ecological interactions are influenced by chemical signals, the chemical changes associated with molting may bring about differences in those interactions.

The purposes of this study were to determine whether male crayfish, *Orconectes rusticus* (Girard, 1852), could distinguish between the chemical signals from recently molted *versus* intermolt male crayfish. To answer this question, crayfish were presented chemical food signals paired with control, intermolt or food signals. This study will elucidate whether crayfish respond to the chemical changes that accompany molting in conspecifics.

## MATERIALS AND METHODS

### Animals and Housing

Male crayfish (*Orconectes rusticus*) were collected from the Portage River in Wood County, Ohio, U.S.A. All crayfish were fed approximately 0.1 g frozen haddock every other day. Crayfish used in experiments were housed in ventilated pots (17.8 cm ID) stored in a flow-through holding tank (48 × 154 × 31 cm) where water in the pots was exchanged with water in the tank. Crayfish were 3.48 (± 0.45) cm carapace length and were mechanically and visually isolated for at least one week before experiments. Crayfish were starved for 48 h prior to the experiments.

Male crayfish chosen for stimulus collection were mechanically, visually, and chemically isolated in pots (17.4 cm ID) that were not exposed to external flow. Molting was induced in an environmental chamber at 22°C with a 14:10 L:D light cycle. Water in the stimulus pots was changed every other day (approximately 3 h after feeding).

### Y-maze Design

A flow-through Y-maze was used to test crayfish response to different chemical stimuli (tank = 61 × 29 × 31 cm, arm = 43 × 14.5 × 31 cm). Two reservoir tanks (plastic gallon jugs; 3.79 liters) supplied water and stimuli to the arms of the maze (see Fig. 1). Chemical stimuli flowed from reservoir tanks through 1.0 cm (ID) Nalgene® tubing. Two in-line flowmeters (Monostat Riteflow #4) controlled flow (20 ± 0.5 ml/min). Dye trials, using commercial food coloring, were run at 20 ml/min to ensure that the flow from

each holding tank was separate and equal when traveling through the arms of the maze. Water exited the tank at the opposite end through four outflow pipes that were controlled by valves. The temperature of both tank and stimulus water were the same to ensure that the vertical position of the odor plume was conserved along the entire arm of the tank. The outflow pipes were 5 cm above the bottom of the maze. Dye trials were also used to determine the time for the odor plume to travel to the end of the arm.

### Stimuli and Collection Methods

Water containing molt cue was collected from pots in which crayfish had undergone ecdysis within the previous 24 h and was frozen at -20°C for later use. Water with intermolt cues and control stimuli (aged tank water) were collected and frozen 24 h after the water had been changed. Preliminary behavioral experiments confirmed that there is no difference in crayfish response to frozen *versus* fresh chemical stimuli used in this study. Stimulus was collected from a single crayfish only once. Three stock solutions were prepared for each chemical stimulus (molt: 31, 33, and 35 individuals; intermolt: 40, 48, and 50 individuals; control: 31, 31, and 40 individuals). These stocks were used to eliminate the possibility that attraction was attributable to the chemical stimulus of particular individuals as opposed to a general population-wide molt status.

Food stimulus was prepared from frozen haddock. Haddock (4.7 ± 0.4 g) was added to 1 liter of aged tank water and liquefied using a blender. The solution was then strained to remove any solid matter. Food stimulus was chosen to elicit a baseline chemosensory response.

### Experimental Protocol

Experimental conditions consisted of either two different stimuli or two identical stimuli introduced into the arms of the Y-maze. Within the different-stimuli treatment, each trial was comprised of pair-wise combinations: molt vs. control, molt vs. intermolt, molt vs. food, intermolt vs. control, intermolt vs. food, and food vs. control. In the identical-stimuli treatment, the same chemical stimulus was presented in each arm of the Y-maze. These latter experiments help to explain differences in activity patterns within a stimulus without the confounding factor of other stimuli present in the tank.

Chemical stimuli were assigned randomly to each reservoir chamber by flipping a coin. The maze was filled with aged tank water, and the bottom was lined with gravel that had been rinsed for 10 min with hot, then distilled water. Crayfish were acclimated in a gated shelter for 10 min before flow was initiated. The shelter consisted of a bisected 2-inch PVC pipe with a gate of egg-crate and a mesh rear to ensure that the crayfish were exposed to the flow from the arms. Flow was then initiated and crayfish were further acclimated for 8 min (dye trials showed that the odor plume arrived at the end of the arm 7 min after flow initiation). After acclimation, the gate was lifted and crayfish were allowed to explore the tank for 15 min with a constant flow rate of 20 ml/min. A video camera (Panasonic WV-CL350) was set up above the maze so that the entire maze was in view. Trials were recorded on a VCR (Panasonic AG-1980) and displayed on a monitor (Sony PVM-1351G). The Y-maze and gravel were rinsed for 10 min with hot, then distilled water between trials. Trials in which the experimental animal did not move, escaped from the maze, or appeared visibly disturbed by the researchers were removed from analysis (5 of the total 65 trials, < 8%, were removed

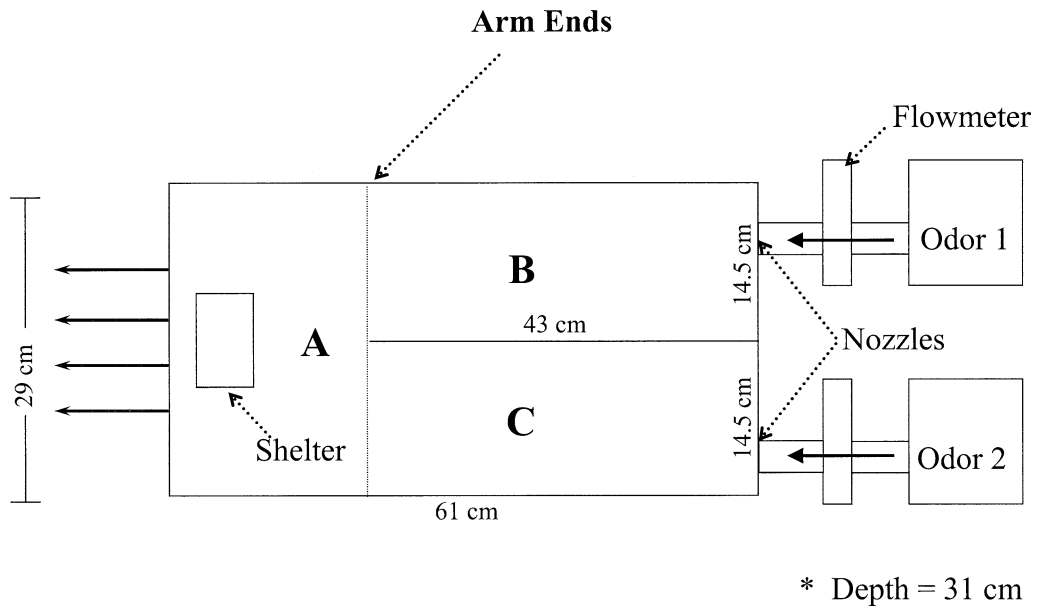


Fig. 1. Y-maze setup for attraction experiments. Tank is divided into three sections (A, B, C). Two odor sources are introduced through Nalgene<sup>®</sup> tubing (flow is right to left). Flow is regulated by an in-line flow meter. The odors then enter either arm B or C of the tank. Outvalves on the opposite side of the tank draw the odors through the arms in a straight path.

from analysis). A sample size of  $n = 10$  was used for all pair-wise combinations and  $n = 6$  for the double presentation of chemical stimuli.

#### Data and Statistical Analysis

Videotapes were analyzed using Peak Motus Motion Analysis software (Peak) to digitize the (X, Y) coordinates of the crayfish. The rostrum position was digitized once every second for the total length of the trial. A crayfish was considered in or out of an arm when its rostrum passed a line separating the two arms from the back of the tank (see Fig. 1, line labeled "Arm Ends").

From X, Y coordinates, behavioral parameters, including initial arm choice, proportion of time spent in each arm, proportion of time spent at each nozzle (within 10 cm of the inflow nozzle), and number of arm changes were calculated. Initial arm choice was defined as the first arm the crayfish entered. A binomial test ( $n = 10$  for all treatments) was used to determine if initial arm choices in each treatment were different from random (Zar, 1999). The number of arm changes (after initial arm choice) were counted for each trial and analyzed for differences between treatments with a one-way ANOVA. This parameter will give a measure of the amount of exploration of the entire tank during trials. The proportion of time spent in each arm is the total time spent in one arm divided by the total time spent in either arm. The proportion of time at the nozzle was defined as the total amount of time a crayfish spent within 10 cm of the nozzle, provided that it was directly touching (with at least one chela) or facing the nozzle, divided by the total amount of time spent in either arm. The proportions calculated were arcsine-square root transformed to normalize the data, then tested for significance with two-tailed paired  $t$ -tests. Meral spread is the distance between the tip of the right and left

chela and has been used as a measure of aggressiveness (Thorpe and Ammerman, 1978; Bruski and Dunham, 1987; Zulandt Schneider *et al.*, 1999). Meral spread ratio (distance between chelae/carapace length) was used to account for differences in body size between experimental animals. Two-tailed paired  $t$  tests or Mann-Whitney  $U$  tests were used to detect significant differences in average meral spread at the nozzle for each treatment, depending on the normality of the data ( $n = 10$  for each treatment within each measure).

Walking speed, time spent in either arm, time spent at either nozzle, time spent moving, and walking speed while moving in the arms was measured for the identical chemical stimulus presentation. Time spent in either arm or at either nozzle was calculated for each treatment. Crayfish, while chemically stimulated, travel in a pattern consisting of periods of movement and periods of no movement. Therefore, the proportion of time spent moving and the walking speed while moving in the arms were measured. Proportions were normalized by arcsine-square root transformation and analyzed with a one-way ANOVA. Walking speed while moving in the arms was calculated by analyzing whether the crayfish was both in an arm and was moving. From these data points, average walking speeds were calculated and analyzed with a one-way ANOVA across chemical stimuli.

Odor sources often induce changes in locomotion patterns of chemosensory animals (Kleerekoper, 1967; Teyke *et al.*, 1992; Breithaupt *et al.*, 1995; Moore and Lepper, 1997). Behavioral analysis that looks at changes in those patterns provides insight into ecologically important responses to chemical stimuli. Orientation parameters calculated from the digitized videos were average walking speed, average distance to source, and average walking speed to the source. These parameters have been used in the

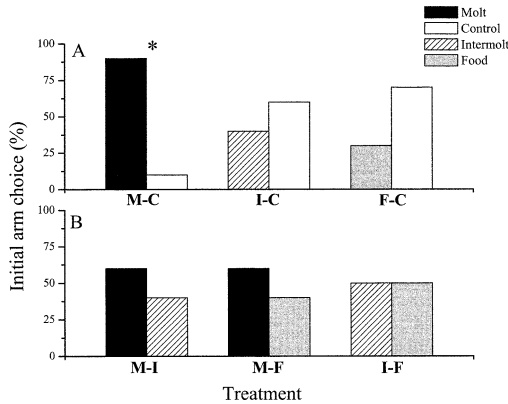


Fig. 2. Percentage of initial arm choice for control (A) and experimental (B) treatments. Stimuli are molt odor (black), control (white), intermolt (hatched), and food (gray).  $N = 10$  for all treatments. Asterisks show significance by binomial tests ( $P < 0.05$ ).

past to characterize orientation behavior to a food source in crayfish (Moore *et al.*, 1991; Keller and Moore, 1999; Moore and Grills 1999). Two-tailed paired  $t$ -tests were used to detect significant differences between spatial analysis parameters for each treatment ( $n = 10$  for each treatment within each measure).

Because the experimental conditions for each treatment were different (different paired stimuli present), parameters cannot be compared across treatments and only within a treatment. Therefore, paired tests were chosen instead of multivariate statistics.

## RESULTS

### Initial Arm Choice

In the molt vs. control treatment, the molt arm was selected first in 90% of trials and the control arm in 10% of trials (binomial test,  $P < 0.01$ ,  $n = 10$ ) (see Fig. 2). In all other treatments, the initial arm choice did not show a significant difference (binomial test,  $P > 0.05$ ).

### Time Spent in Each Arm

Crayfish spent significantly more time in the arms with the molt and food stimuli when paired with control chemical stimuli (see Fig. 3a). There was not a significant difference in the time spent in the intermolt arm versus the control arm in that treatment ( $t$  test,  $t = 0.76$ ,  $P > 0.05$ ,  $d.f. = 9$ ). Crayfish spent significantly more time in the molt arm over the intermolt arm in the molt vs. intermolt treatment ( $t$  test,  $t = 3.09$ ,  $P < 0.01$ ,  $d.f. = 9$ ) (see Fig. 3b). However, when offered the molt and food chemical stimuli, crayfish resided in the food arm rather than the molt arm for a significantly

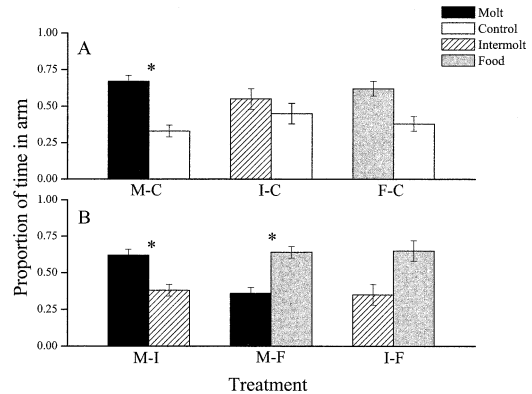


Fig. 3. Mean ( $\pm$  SEM) proportion of time spent in each arm(s) for control (A) and experimental (B) treatments. Stimuli are molt odor (black), control (white), intermolt (hatched), and food (gray).  $N = 10$  for all treatments. Asterisks show significance by two-tailed paired  $t$ -tests on transformed proportions ( $P < 0.05$ ).

longer period ( $t$  test,  $t = -3.17$ ,  $P < 0.01$ ,  $d.f. = 9$ ). There was no statistical difference between the times spent in the intermolt versus food arm ( $t$  test,  $t = -2.01$ ,  $P > 0.05$ ,  $d.f. = 9$ ).

### Time Spent at Nozzle

Crayfish spent significantly more time at the nozzle of the molt chemical stimulus when paired with either control ( $t$  test,  $t = 5.49$ ,  $P < 0.001$ ,  $d.f. = 9$ ; see Fig. 4a) or intermolt chemical stimuli ( $t$  test,  $t = 3.11$ ,  $P < 0.02$ ,  $d.f. = 9$ ; see Fig. 4b). Crayfish did, however, spend more time at the food nozzle rather than the molt nozzle ( $t$  test,  $t = -3.79$ ,  $P < 0.004$ ,  $d.f. = 9$ ). There were no significant differences in time at the nozzle for the intermolt vs. control ( $t$  test,  $t = 0.70$ ,  $P > 0.05$ ,  $d.f. = 9$ ), intermolt vs. food ( $t$  test,  $t = -2.01$ ,  $P > 0.05$ ,  $d.f. = 9$ ), and food vs. control treatments ( $t$  test,  $t = 1.06$ ,  $P > 0.05$ ,  $d.f. = 9$ ).

### Number of Arm Changes

All tests resulted in at least three arm changes between the paired stimuli. Animals did not choose one arm and stay within that arm. Overall, the one-way ANOVA did not show any difference between treatments ( $P > 0.11$ ).

### Identical Chemical Stimulus Presentation

There was a statistical difference between stimuli for the parameters of the proportion of time spent moving (see Fig. 5a) and walking speed while moving in the arms (see Fig. 5b) (MANOVA, Rao's  $R_{6,36,0.05} = 8.18$ ,  $P < 0.001$ ).

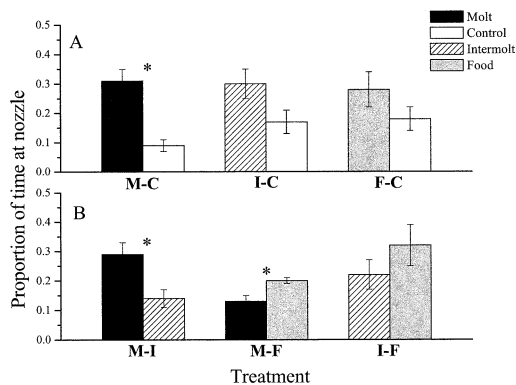


Fig. 4. Mean ( $\pm$  SEM) proportion of time spent at each nozzle(s) for control (A) and experimental (B) treatments. Stimuli are molt odor (black), control (white), intermolt (hatched), and food (gray).  $N = 10$  for all treatments. Asterisks show significance by two-tailed paired  $t$ -tests on transformed proportions ( $P < 0.05$ ).

The proportion of time moving was statistically significant between molt chemical stimulus and both intermolt (LSD,  $P < 0.02$ ) and control (LSD,  $P < 0.01$ ) chemical stimuli, but was not different between molt and food chemical stimuli (LSD,  $P > 0.05$ ). While moving in the arms, crayfish walked faster in the molt chemical stimulus than in the intermolt chemical stimulus (LSD,  $P < 0.05$ ). There were no differences in the overall walking speed, time spent in either arm, or time spent at either nozzle between chemical stimuli.

#### Meral Spread Ratio at Nozzle

There were no significant differences in the average meral spread ratio at the nozzle in the presence of chemical stimuli for any treatment. Across all of the treatments, the average meral spread ratios calculated ranged from 0.5 to 1.5.

#### Spatial Analysis of Orientation Paths

Three spatial orientation parameters were analyzed: walking speed, walking speed to source, and distance to source. Walking speed in the molt vs. intermolt treatment showed a significant difference. Crayfish walking speed was higher in the intermolt arm than in the molt arm ( $t$  test,  $t = 2.85$ ,  $P < 0.01$ ,  $df = 9$ ). No other spatial analysis parameters were significantly different in any treatment.

#### DISCUSSION

The results from our study provide an example where environmental signals possibly

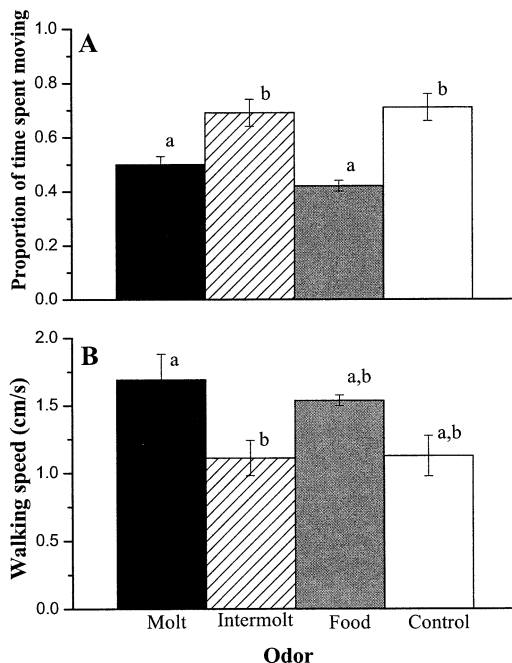


Fig. 5. Parameters measured during identical odor presentation of stimuli. Parameters included (A) mean ( $\pm$  SEM) proportion of time spent moving (walking speed  $< 0.5$  cm/s) and (B) walking speed while moving in the arms (cm/s). Stimuli are molt odor (black), intermolt (hatched), food (gray), and control (white). Bars with the same letters are not significantly different from each other (MANOVA,  $P < 0.05$ ).

provide information about the internal state of animals. Crayfish distinguished, solely through chemical cues, between conspecifics in different physiological stages of the growth cycle. It is clear that conspecifics discriminate between the chemical cues released into the environment from crayfish that are undergoing the internal changes accompanying ecdysis.

Discrimination of molt stimulus from other stimuli tested was demonstrated by intermolt crayfish during treatments where molt chemical stimulus was paired with the other chemical stimuli. Crayfish spent more time in the molt arm and at the molt nozzle when paired with control or intermolt stimuli. Conversely, when molt stimulus was paired with a food stimulus, crayfish spent less time in the molt arm and at the nozzle. There were no statistical differences in other treatments where intermolt, control, and food chemical stimuli were paired with each other, suggesting that crayfish do not discriminate between those chemical stimuli. These results demonstrate that crayfish can distinguish

chemical stimuli from recently molted crayfish when paired with the other chemical stimuli tested.

With this experimental design, it is possible that crayfish are not attracted to recently molted chemical stimulus but are simply avoiding the other stimulus with which it is paired. Analysis of the behaviors when test chemical stimuli were paired with control chemical stimuli allows us to differentiate between these two interpretations. In the intermolt *versus* control treatment, animals did not avoid the intermolt arm. According to the number of arm changes, crayfish also continued to explore the maze. Therefore, the results from the experiments where chemical stimuli are paired against control stimuli demonstrate that crayfish are attracted to the recently molted crayfish stimulus and are not avoiding the intermolt stimulus.

In the identical-stimuli treatments, crayfish behavior was significantly different in the presence of molt chemical stimulus as compared to intermolt and control stimuli. Animals spent less time moving while exposed to molt or food stimuli than with intermolt or control stimuli. Conversely, crayfish walked faster while moving in the arms during trials with molt chemical stimulus than with intermolt chemical stimulus. These measurements can gauge the locomotor activity level of the animals. Though they spend less time moving in the molt chemical stimulus, crayfish were more active overall, walking through the arms faster and perhaps exploring a greater portion of the test arena. The same response is seen when comparing the food chemical stimulus to intermolt or control chemical stimuli. The display of differential behaviors in the molt *versus* the intermolt chemical stimulus also supports the discrimination by crayfish between the two chemical stimuli.

Results of the spatial analysis of orientation behaviors did not reveal any significant differences between orientation patterns in any treatment. It appears as if crayfish are not actively orienting toward the source (Moore *et al.*, 1991; Keller and Moore, 1999; Moore and Grills, 1999) in order to locate the source of the molt chemical stimulus. It may be that crayfish are not perceiving molt chemical stimulus as a food source, for there is an absence of the typical food orientation responses. It is also possible that the length of the Y-maze was too short of a distance to observe orientation patterns in the spatial parameters measured.

Also, the hydrodynamic conditions of the Y-maze may have been different than those of previous studies examining orientation behavior. Instead, crayfish may be attracted to the source of molt chemical stimulus in order to investigate the chemical stimulus source for possible social consequences as well as resource consequences.

Further experiments are needed to test whether the chemical signal from a molted individual is quantitatively or qualitatively different from an intermolt individual. The soft exoskeleton is an insufficient barrier to the passage of ions and other molecules out of the animal (Chang *et al.*, 1993; Waddy *et al.*, 1995). Many physiological processes accompany the molt cycle, so chemical components of the hemolymph and body fluids are altered (Chang, 1995). Possibly, traces of these additional components can diffuse across the exoskeleton and qualitatively alter the chemical signal.

Cannibalism of an individual during its molt cycle has been observed in the laboratory where crustaceans are in close proximity and at high population density (Atema and Cobb, 1980). Crayfish often reach high densities in natural populations (Mather and Stein, 1993; Lodge and Hill, 1994; Stewart *et al.*, 1998), and it is suspected that cannibalism may occur in the wild as well. The ability to distinguish a molted individual by chemoreception alone would be useful to a hungry crayfish with limited resources. A recently molted crustacean is likely to remain in its shelter if undisturbed until the hardening of its exoskeleton (Tamm and Cobb, 1978). However, molt signals in the environment may allow a conspecific to find vulnerable individuals and subsequently capture use of resources previously held by the molted individual.

The ability to discriminate a molted individual may influence agonistic interactions with conspecifics. After molting, American lobsters (*Homarus americanus* Milne Edwards, 1837) have a soft exoskeleton and reduced motor capability following ecdysis (Tamm and Cobb, 1978; Chang, 1995; Waddy *et al.*, 1995), suggesting that an intermolt opponent would have an advantage. In experiments with the stomatopod crustacean, *Neogonodactylus* (as *Gonodactylus*) *bredini* (Manning, 1969), molted individuals will participate in agonistic encounters but experience a much higher risk of fatal injury (Steger and Caldwell, 1983; Adams and Caldwell, 1990). In the current experiment, the

meral spread ratios calculated within treatments suggest no change in aggressive response to a molting *versus* intermolt conspecific. Additional experiments have shown that fight dynamics were altered when one or more of the individuals in an encounter have recently undergone ecdysis (Adams and Moore, in review). In these experiments, molted and intermolt crayfish interacted in a small arena, where molted crayfish could not escape and were forced to fight. Because chemical stimuli do play a role in agonistic interactions of Crustacea (Thorpe and Ammerman, 1978; Atema, 1986; Hughes, 1996; Zulantz Schneider *et al.*, 1999; Zulantz Schneider *et al.*, 2001), further studies are needed to elucidate whether chemical signals from a molted individual alter fight dynamics when crayfish interact in a larger fight arena where interactions are not forced.

The results from these experiments have demonstrated not only that conspecifics can distinguish between molted and intermolt crayfish by chemoreception alone, but also that the internal state of one individual impacts how it is perceived by another. By extending our results, it may be possible that chemicals passively transported to the environment have the potential to carry information about individuals to other social situations. Chemical signals may indicate aggressive state and social position (Karavanich and Atema, 1991; Karavanich and Atema, 1998; Breithaupt *et al.*, 1999; Zulantz Schneider *et al.*, 1999), sex and reproductive status (Ryan, 1966; Hardy and Shaw, 1983; Bouchard *et al.*, 1996; Bushmann and Atema, 2000), metabolism (Bryant and Atema, 1987), and from this study, molt stage of animals. Other animals can react to this information and alter behavior not only to environmental circumstances but to the state of a conspecific as well.

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