

LABORATORY EXPERIMENTS TO DETERMINE IF CRAYFISH CAN COMMUNICATE CHEMICALLY IN A FLOW-THROUGH SYSTEM

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(Received January 7, 1980; revised March 14, 1980)

Abstract—The importance of chemical cues for transmitting information concerning sexual identity, agonistic state, and stress-related condition in the crayfish *Procambarus clarkii* (Girard) was examined in a flow-through system. Experiments tested the effects of “conditioned water” from stimulus tanks on the behavior of solitary male or female crayfish. Twenty males and 20 females were subjected to a random sequence of five treatments: unconditioned water (control), conditioned water flowing through tanks containing a solitary male or female, and conditioned water from tanks holding either two males or two females. Durations of the following behaviors were recorded: chelae up, chela(e) in baffle hole, chela waving, climbing, digging, grooming, gross body movement, and meral spread. Results indicated that crayfish chemically detected another animal within 0.25 m without additional visual or tactile stimuli; however, crayfish apparently did not “communicate” information on sexual identity, agonistic state, or stress condition, nor does this detection necessarily imply discrimination between stimuli from crayfish and other taxa (e.g., fish). Our conclusions are contrasted with the two previous reports on chemical communication in crayfish in which experimental animals were tested in static systems. We suggest that a temporal separation of molting and copulation and a long reproductive receptivity period for females (which would allow abundant intersexual encounters) could account for a lack of selective pressure to evolve long-distance sex pheromones.

Key Words—Agonistic behavior, chemical communication, crayfish, pheromone, *Procambarus clarkii*.

INTRODUCTION

Biological communication as defined by Wilson (1970) is “. . . action on the part of one organism (or cell) that alters the probability pattern of behavior in

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another organism (or cell) in an adaptive fashion" (see also Burghardt, 1970). The critical portion of this definition is the requirement for a real or perceived advantage to the signaler (or its kin) to encode the message. In other words, information transfer (e.g., mere chemical detection of another organism) does not necessarily imply communication.

Pheromones are a form of chemical communication functioning within species or, in certain instances, between closely related species (Karlson and Lüscher, 1959). In Crustacea, experimental evidence for a sex pheromone is limited to only two of the eight major subclasses, the Malacostraca and the Copepoda (for a review, see Dunham, 1978). The majority of such studies have dealt with marine species with much less attention directed toward freshwater crustaceans (e.g., Decapoda: Kamiguchi, 1972; Ameyaw-Akumfi and Hazlett, 1975; Little, 1975; Thorp and Ammerman, 1978; Amphipoda: Dahl, et al. 1970a,b). Little (1975) showed that third-stage larvae of the freshwater crayfishes *Cambarus virilis* (now *Orconectes*), *Orconectes sanborni*, and *Procambarus clarkii* discriminate chemically between brooding and nonbrooding females. Ameyaw-Akumfi and Hazlett (1975) and Ameyaw-Akumfi (1976) suggested that sex recognition by males of the crayfish *Procambarus clarkii* was mediated by a sex pheromone. Males responded agonistically to male chemical stimuli and submissively to female stimuli. Thorp and Ammerman (1978), however, found no chemical sex recognition in *Procambarus a. acutus* and suggested that the ecological importance of pheromones for adult crayfish was species specific and perhaps niche specific.

Dunham (1978) reviewed experiments on sex pheromones in Crustacea and concluded that "The lack of appropriate control procedures casts doubt upon most of the available evidence for a sex pheromone in Crustacea." He cited a lack of (1) "blind" observation techniques, (2) novel stimuli, and (3) randomized sequences of treatments. Some previous studies, in our opinion, also failed to eliminate vibrations as a means of communication nor did the design of the apparatus take into account a pheromone's dispersal through its active space (area of threshold concentration for behavioral response; Bossert and Wilson, 1963; Wilson, 1970).

To maximize active space and minimize "background noise" from chemical signals overlapping spatially and temporally, highly motile crayfish may have evolved pheromones with short fade-out times (both lotic and lentic species) and/or they could release them into currents (lotic species) (see related discussion in Wilson, 1970). Unfortunately, few pheromone experiments have either examined behavioral responses of aquatic organisms when the temporal separation between stimulus release and reception are reduced (thus obviating response to frequently unrealistic accumulations of wastes in laboratory experiments) or have employed flow-through conditions (fewer wastes and also rapid dispersal of pheromones).

In this study we evaluated whether information concerning sexual identity, agonistic state, or stress-related condition is transmitted chemically in the crayfish *Procambarus (Scapulicambarus) clarkii* (Girard, 1852). Crayfish were tested in flow-through systems in which conditioned water passed immediately from stimulus tanks (or head tanks, after Thorp and Ammerman, 1978) to observation tanks. Behaviors which were used as experimental indicators of chemical detection and/or communication were applicable to laboratory situations and were not necessarily completely representative of normal crayfish behavior in natural environments, although we have observed similar behaviors in the field and many have frequently been described in laboratory experiments on crayfish behavior (e.g., Bovbjerg, 1970).

METHODS AND MATERIALS

Reproductively active male (form I; wet weight 13–44 g) and female (wet weight 12–30 g) *P. clarkii* were purchased from a commercial supplier in Louisiana and tested in the summer of 1978. Crayfish were acclimated for several weeks under a 12-hr L:D photoperiod at $22 \pm 2^\circ\text{C}$ in individual aquaria and were fed commercially pelleted fish food 3 times a week.

Five experiments were designed to test the effects of "conditioned water" (possibly containing pheromones) from head tanks (stimulus tanks) on the behavior of solitary male or female crayfish in observation tanks. A random group of 20 males and 20 females were subjected individually to a random sequence of five treatments: unconditioned water (control), water flowing through tanks containing a solitary male or female, and water from tanks holding either two males or two females. The series of experiments was formulated to reveal (1) any sexual differences in production and reception of chemical cues, and (2) any differential production of chemical cues from solitary (relatively undisturbed) and communally housed (stressed) crayfish.

Each experiment consisted of a 2- to 3-day isolation period, a 90-min period for acclimation to observation and head tanks, and a 20-min observation period during the light phase of the photoperiod. The acclimation period allowed crayfish to adjust to their new surroundings and, in most cases, to complete "exploration" of the tanks (thus increasing the likelihood of detecting treatment-related behavior). For experiments with two head tank animals, the second crayfish was not introduced until the beginning of the observation period to promote maximum stress (from agonistic contact) at this point. The two head tank crayfish were separated by a transparent flow-through baffle (Figure 1) to prevent physical contact and the loss of body fluids from potential wounds. Head tank animals were observed to respond agonistically to the other stimulus crayfish during at least the initial portions

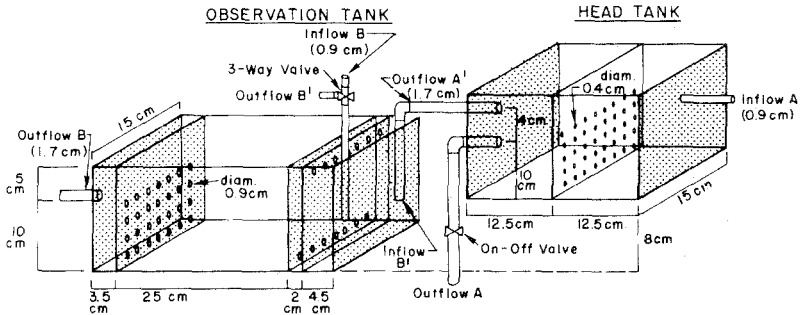


FIG. 1. Flow-through test chambers. During acclimation, water flowed through inflows A and B, and out through outflows A and B. During subsequent observation periods outflow A and inflow B were closed which caused water to pass out of the head tank through outflow A' and into the observation tank through inflow B'. When two male or two female stimulus crayfish were in the head tank, they were separated by a transparent partition. Tanks were constructed of $\frac{1}{4}$ -in. Plexiglas and contained 1 cm of fine gravel on the bottom.

of the "observation period" despite their inability to achieve direct physical contact. Observation and head tanks were designed to maintain constant flow rates (approximately 1.5 cm/sec) near the bottom of the tanks (as indicated by dye tests of dispersion rates). Stable flow was achieved with valves which kept pressure constant in the entire system during both the acclimation period, when tanks were independently supplied with well water (20°C), and the observation period, when tanks were connected in series. Observation and stimulus tanks were placed close enough together so that maximum separation of animals was less than 0.5 m but were not touching in order to prevent intertank vibrations. The 0.5-m distance was selected somewhat arbitrarily on the basis of behavioral observations of an approximate, maximum detection (chemical or tactile) distance for some blind cave crayfish (James H. Thorp and H. H. Hobbs III, personal observation) which were used in other pilot experiments. After each replicate the tanks were flushed for 5–10 min, and any remaining detritus or fecal matter was removed with a small mesh dipnet. All animals were tested between 1000 and 1800 hr and only intermolt crayfish were used. Because of a shortage of suitable crayfish, some observation animals also served as stimulus crayfish. Crayfish were returned to their aquaria for at least 2 days prior to a new experiment. Behavioral data for crayfish which died within 3 days of their trial were discarded.

Behaviors of crayfish in observation tanks were recorded with a Sanyo videotape system and were analyzed for duration and/or frequency of individual behaviors. The 20-min observation periods were subdivided for analysis into 80 intervals of 15 sec each. During each 15-sec interval the

occurrence of any of eight defined crayfish behaviors were noted (Table 1). In addition, observation tanks were subdivided for analysis (no physical barriers) into three equal-sized zones (zone 1 was upstream); and, the position of the crayfish was noted at the end of each 15-sec interval.

Data were analyzed for statistical significance ($P < 0.05$) with the general linear model procedure (GLM; analysis of variance portion) of the statistical analysis system (SAS). GLM procedures were run comparing treatment effects on duration of the eight behaviors listed in Table 1 as well as on the probability of a crayfish being in zone 1 (upstream third) and on the mean of the quantity "average duration per trial" for the following behaviors: chela(e) up, chela wave, and meral spread. Tendencies toward "handedness" (i.e., right or left chela waving) were also analyzed. Treatments were designated with the letters A through J as described in Figure 2 and Table 2. Grouped letters (e.g., AD or ACDF) indicate that these treatments were statistically compared with the GLM procedure.

TABLE 1. BEHAVIORAL RESPONSES RECORDED IN EACH EXPERIMENT

Chelae up	Both chelae raised off the substratum.
Chela(e) in baffle hole	Insertion of one or both chelae in hole of the inflow or outflow baffles.
Chela waving	Moving the right or left chela back and forth horizontally in front of the body with propodus of chela held approximately perpendicular to substratum; crayfish not simultaneously grooming that appendage.
Climbing	Cephalothorax pointed at angle above the horizontal with at least 4 walking legs off the substratum.
Digging	Digging in the gravel on the bottom of the tank using walking legs and chelae.
Grooming	Rubbing, picking, or scratching at parts of the body with one or more walking legs.
Gross body movement	Movement of the entire body around the tank (climbing is a subset of this behavior).
Meral spread	Both chelae raised above level of coxae of walking legs and spread laterally from normal walking position. Cephalothorax may be raised but crayfish not climbing (meral spread is a subset of chelae up).

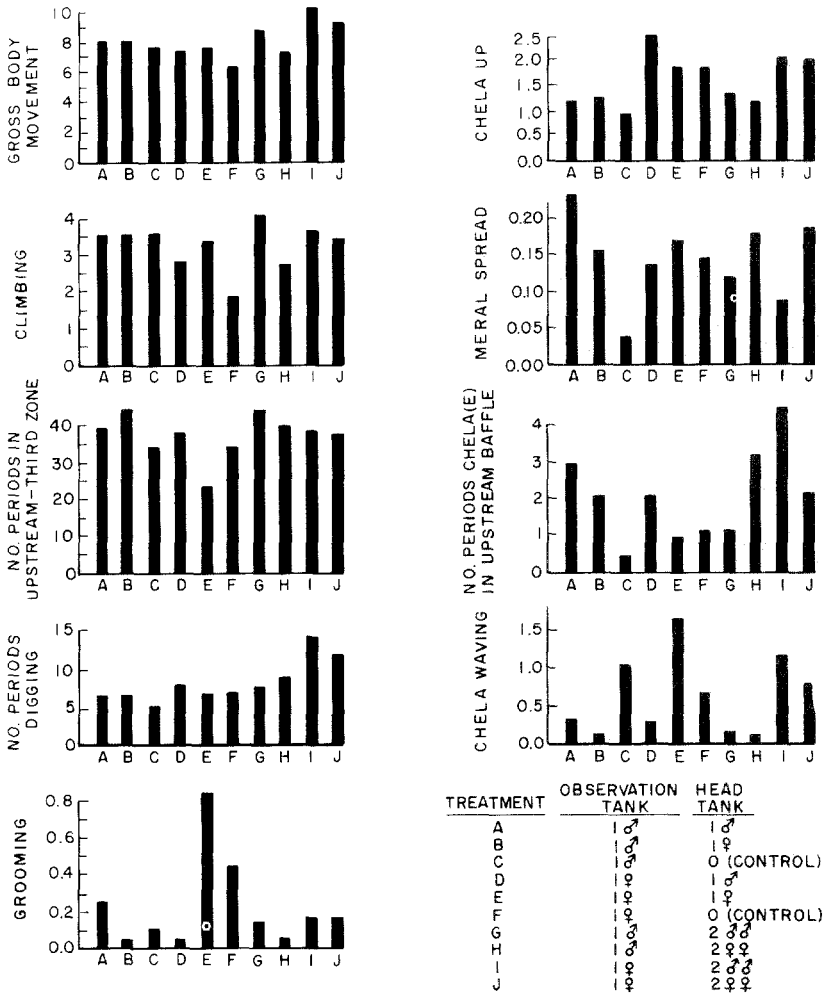


FIG. 2. Effect of conditioned water from stimulus animals in head tanks on specified behaviors of male or female crayfish in observation tanks. Letters on abscissa indicate treatment groups as described below. Behaviors are described in Table 1. Values on ordinates expressed as mean duration in minutes for each behavior per 20-min experiment or as mean number of 15-sec periods out of 80 that behavior occurred. Results of statistical comparisons of various treatments are in Table 2.

RESULTS

Chemical Detection of Conspecifics. Observation tank crayfish appeared able to detect chemically animals in head tanks. Despite the large probability of type I errors (from over 300 GLM procedures), the frequency of significant

TABLE 2. RESULTS OF GENERAL LINEAR MODEL PROCEDURES FOR COMPARING VARIOUS TREATMENT EFFECTS ON DURATION OF SPECIFIED CRAYFISH BEHAVIORS^a

TRT	Observation tank	Head tank	TRT	Observation tank	Head tank
A	1 ♂	1 ♂	F	1 ♀	0 (control)
B	1 ♂	1 ♀	G	1 ♂	2 ♂♂
C	1 ♂	0 (control)	H	1 ♂	2 ♀♀
D	1 ♀	1 ♂	I	1 ♀	2 ♂♂
E	1 ♀	1 ♀	J	1 ♀	2 ♀♀

Comparisons ^b	Significant behaviors ^c	Rank order	Total <i>DF</i>	<i>F</i> ratio	Prob. > <i>F</i>
ABC, ACG, EFJ, AG, BH, DI, HJ, AB, GH, and IJ	All nonsignificant (<i>P</i> > 0.05)				
DEF	Chela wave	(E>F>D)	62	3.90	0.026
CGH	Chela(e) in upstream baffle	(H>G>C)	60	3.73	0.030
FIJ	Gross body movement	(I>J>F)	62	4.72	0.013
	Climbing	(I>J>F)	62	3.51	0.036
	Chela(e) in upstream baffle	(I>J>F)	62	3.22	0.047
BCH	Chela(e) in upstream baffle	(H>B>C)	59	3.42	0.040
DFI	Gross body movement	(I>D>F)	61	4.01	0.023
	Grooming	(F>I>D)	61	3.34	0.042
	Chela(e) in upstream baffle	(I>D>F)	61	3.38	0.041
ACDF	Grooming	(F>A>C>D)	81	2.78	0.046
	Chelae up	(D>F>A>C)	81	3.08	0.032
BCEF	Chela(e) in upstream baffle	(B>F>E>C)	83	3.10	0.031
CFGJ	Digging	(I>G>F>C)	83	3.20	0.270
	Chelae in upstream baffle	(I>G>F>C)	83	5.78	0.001
CFHJ	Grooming	(F>J>C>H)	82	3.27	0.025
	Chelae up	(J>F>H>C)	82	2.69	0.051
	Chela(e) in upstream baffle	(H>J>F>C)	82	2.58	0.059
EJ	Time in upstream zone	(J>E)	40	5.77	0.021
AD	Chelae up	(D>A)	38	3.79	0.059
BE	Chela wave	(E>B)	40	8.13	0.007
	Time in upstream zone	(B>E)	40	9.77	0.003
GI	Chela(e) in upstream baffle	(I>G)	40	5.03	0.031
CF	Chelae up	(F>C)	42	4.35	0.043
	Chela(e) in upstream baffle	(F>C)	42	4.23	0.046
	Average chelae up per trial	(F>C)	39	4.94	0.032
DE	Chela wave	(E>D)	39	6.07	0.018
	Time in upstream zone	(D>E)	39	4.87	0.034
A-J	Grooming		203	1.97	0.044
	Chelae up		203	2.04	0.036
	Chela(e) in upstream baffle		203	1.97	0.044

^aThose tested were chelae up, chela(e) in upstream baffle, chela waving, climbing, digging, grooming, gross body movement, meral spread, presence in upstream zone, and mean of the quantity "average duration per trial" for chelae up, chela wave and meral spread.

^bGrouped letters indicate that means of these experiments were compared statistically.

^cBehaviors described in Table 1. Probability values listed only for significant behaviors.

comparisons (Table 2) for the behavior "chela(e) in upstream baffle" supports our conclusion about chemical detection. This behavior suggests that observation crayfish may have been orienting towards the chemical cues and were attempting to approach the source. Only one behavior (chela wave) out of 28 possible was significant for the statistical comparisons ABC and DEF (see treatment descriptions in Figure 2 and Table 2), which suggests that neither males nor females react outwardly to a single male or female in head tanks. When either two males or two females were in the head tank, however, observation males and females tried to reach through holes in the upstream baffle (comparisons CGH and FIJ), moved (FIJ), and climbed (FIJ) significantly more than in controls. In general, observation crayfish were more responsive when two head tank crayfish were present than when a single animal was in the head tank.

Although chemical detection of the head tank animals was shown, these experiments do not imply that crayfish can discriminate between conspecifics and either congeneric crayfish or, in fact, noncrustaceans. Likewise, significant demonstration of "detection" does not necessarily imply "communication" as defined in the introduction.

Communication: Sexual Recognition. Sexual recognition would be demonstrated by either quantitative or qualitative differences in behavioral response to the chemical presence of males or females. In most comparisons with significant differences, females were more active than males to water conditioned by either one or two males (comparisons AD, GI, ACDF, CFGI), or one or two females (BE, HJ, BCEF, CFHJ) (Figure 2, Table 2). This partially resulted because females in control treatments (C and F) showed significantly more "chela(e) up" and "chela(e) in upstream baffle" than did males. Despite this result, however, for almost all behaviors either no significant differences among treatments occurred or, when differences were significant (ACDF, CFGI, BCEF, CFHJ), treatment values were greater than controls.

Most importantly, males did not recognize differences between water conditioned by head tank males or females (comparisons ABC, AB, GH) nor, in general, did females distinguish chemically between males or females (DEF, DE, IJ). Although females waved their chelae more often (DE) to solitary females than to solitary males, there were no differences in response to water conditioned by either two males or two females (IJ).

Communication: Agonistic Behavior. Although each of the behaviors graphed in Figure 2 could represent agonistic responses (fight, flight, or displacement activities), only meral spread is consistently associated with agonistic behavior (personal observation). Meral spread behavior, however, was infrequently shown (Figure 2) and never significantly affected by experimental treatment (Table 2).

Communication: Stress. Differential response to one versus two head

tank crayfish could indicate (1) a special "stress pheromone" was released as a result of agonistic encounters between head tank animals, (2) another pheromone, not specifically coding for stress, was released intermittently, e.g., when two crayfish interacted, or (3) the response threshold for a pheromone in the flowing system required output from at least two crayfish. Although responses were generally greater to two animals than one in head tanks, comparisons of single versus multiple crayfish treatments (AG, BH, DI, EJ) were rarely significant.

DISCUSSION

The crayfish *Procambarus clarkii* seems able to detect chemically the presence of another animal at a distance of at least 0.25 m without additional visual or tactile stimuli. The information received produced significant, but slight, quantitative differences from controls in releaser behavior (Wilson and Bossert, 1963) and in chemokinetic and chemotaxic reactions (Dunham, 1978) but no significant change in qualitative behavior. Increasing the number of head tank animals tended to enhance the response of the observation crayfish. Despite evidence for chemical detection, however, the results do not suggest that crayfish "communicated" information on sexual identity, agonistic state, or stress condition, nor does this detection necessarily imply discrimination between stimuli from crayfish and other taxa (e.g., fish).

On the basis of experiments with larval crayfish (Little, 1975), it appears that crayfish possess at least one pheromone; however, the existence of sex pheromones or other pheromones acting between adults remains controversial at best. Ameyaw-Akumfi and Hazlett (1975) and Ameyaw-Akumfi (1976) concluded that *Procambarus clarkii* produced sex pheromones, but Thorp and Ammerman (1978) reported no significant sex recognition in *P. a. acutus*, although they demonstrated significant chemical detection. An obvious question at this point is "Why are the findings of these studies on interadult sex pheromones in conflict?"

All studies were conducted under artificial conditions in the laboratory; however, in our opinion, the apparatus and methods employed in the present study produced physical and chemical conditions (and thus behaviors) more representative of those occurring in the natural environment. Flow-through systems (1) provide better directionality for chemical cues than do static systems, (2) allow dispersal of chemical cues at rates more comparable to natural lotic conditions (a factor particularly important for pheromones with short fade-out times), and (3) prevent possible pheromones and toxic waste products from accumulating at levels unnaturally high for either lotic or most lentic environments. In previous studies with static systems, however, metabolic waste products and possible pheromones were allowed to build up

for 1 day (Thorp and Ammerman, 1978) or 1-2 days (Ameyaw-Akumfi and Hazlett, 1975) before addition to the observation chamber. Pheromones with 1- to 2-day fade-out times could create confusing "signal noise" in aquatic systems, and therefore selective forces should prevent their evolution in motile species. Separating the responses caused by either pheromones or toxic wastes would be difficult in studies employing static systems and long acclimations.

Methodological problems and differences in experimental design make it difficult to compare results of studies on interadult pheromones in crayfish. For example, acclimation and test temperatures affect agonistic behavior (Thorp, 1978), and acclimation time in test tanks can alter dramatically the expressions of many behaviors (personal observation). Unfortunately, information on acclimation temperature, acclimation period in test tanks, prior "housing" conditions, and sequences of experimental treatments (including randomization procedures) was not reported in Ameyaw-Akumfi and Hazlett (1975) nor in Ameyaw-Akumfi (1976). Additional problems may be differences in definition of behaviors (e.g., chelae up versus meral spread) and in interpretation of what constitutes agonistic and submissive behaviors. Methodological differences could perhaps explain why Ameyaw-Akumfi (1976) reported for *P. clarkii* high frequencies of agonistic behavior with low variability (e.g., average minutes chelae up per 30-min experiment = 27.6 ± 0.976 SD), whereas we found reduced agonistic response with high variability. However, Thorp and Ammerman (1978) and unpublished pilot studies by Thorp have shown similar low responses for other crayfish species in both static and flow-through conditions (e.g., with *Cambarus latimanus*, *Procambarus troglodytes*, and with the blind, troglotic crayfish, *Orconectes i. inermis*).

If we are correct in stating that adult crayfish do not communicate with each other over long distances with pheromones, then two questions arise. First, are there interadult sex pheromones with small active spaces? Second, what selective pressures were missing which, had they been present, would have promoted evolution of "long-distance" pheromones?

Behavioral displays by crayfish suggest that visual and tactile stimuli are important for intra- and interspecific communication, but it is not clear which means (i.e., chemical, tactile, visual, and/or behavioral) are utilized for sex recognition. At least as early as 1909, investigators were questioning the ability of crayfish to discriminate visually or chemically between sexes. Pearse (1909) reported amplexus between two males and between a live male of one species and a dead female of another (although the latter behavior was rare). Mason (1970) observed intrasexual copulation of crayfish in the laboratory and reported finding spermatophores on the exterior of a mature male in the field. Copulation between *Procambarus (Ormannicus) hirsutus* and *P. (O.) acutus* has been noted in our laboratory (personal observation). Occurrences of successful and even unsuccessful attempts at interspecific and intrasexual

amplexus argue against "facile" acceptance of sex pheromones in crayfish. Sex recognition and successful intersexual copulation appear to involve lowered levels of agonistic behavior by receptive females (Chidester, 1912; Mason, 1970) with the possible partial contribution of sex pheromones which are operative only over short distances.

Natural selection should promote development of sex pheromones for species in which mate location is difficult or haphazard, or in which females are receptive for only relatively short periods. For most epigeal crayfish species, however, locating suitable mates would probably never limit a species because females are reproductively active over a period sufficiently long (several months) to allow encounters with a large number of form I males (see Penn, 1943, for life history information on *Procambarus clarkii*). In contrast to mating in crayfish, copulation between lobsters is assumed usually to be a molt-related phenomenon restricted to a 24-hr period following the female's molt (Templeman, 1934, 1936, in Dunham, 1978) although copulation has been observed at other times (Dunham, 1978). Evidence for sex pheromones has been reported for lobsters and other crustaceans, although these results are not definitive (Dunham, 1978). If mating attempts are more likely to be successful during intermolt periods (e.g., because of reduced agonistic behavior and/or heightened receptivity in the female), then it would be an advantage for that species to have evolved a molt-related, sex pheromone.

In summary, the results of this study suggest that chemical communication between adult crayfish does not occur or is not efficient at distances greater than the effective range for visual communication. A temporal separation of molting and copulation and a female receptivity period which is sufficiently long to allow abundant intersexual encounters could account for a lack of selective pressure to evolve long-distance sex pheromones. Finally, there is no definitive evidence in crayfish for interadult pheromones which act over short distances or which are related to nonsexual responses (e.g., alarm, stress, and/or agonistic behaviors).

Acknowledgments—Critical reviews of an earlier draft of this manuscript by Alan Covich and Roy Stein were very beneficial. We are completely responsible, however, for the conclusions in this study. We are grateful to Horton H. Hobbs III, who donated his time in collecting blind cave crayfish which were used in an unpublished pilot study. Jean Coleman was indispensable in drawing figures for this paper. Research support came from contract EY-76-C-09-0819 between the University of Georgia and the U.S. Department of Energy. The first author was provided stipend support while at SREL by cooperative education grants to SREL from the U.S. Department of Energy and the National Science Foundation.

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