9. The social behavior of hand-reared canids does not differ significantly, if at all, from that of mother-reared animals during early development, and hand-rearing provides all animals with similar environments [M. Bekoff, *BioScience* **24**, 225 (1974)].
10. M. Bekoff, *Am. Zool.* **14**, 323 (1974).
11. _____, thesis, Washington University, St. Louis (1972).
12. In three of the other five linear pairwise analyses, the coyotes and New England canids showed the smallest D^2, indicating the closest relationship among the animals that were compared.
13. We thank H. Hilton for providing the litter of New England canids and D. Armstrong, R. E. Jones, and H. M. Smith for comments on an earlier draft of this report. Supported by a Biomedical Research Grant and a Faculty Research Initiation Fellowship from the University of Colorado to M.B.

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Sex Recognition in the Crayfish *Procambarus clarkii*

Abstract. *Male crayfish,* Procambarus clarkii, *show different behaviors toward males (aggression) and females (submission, courtship). Behavioral and neurophysiological tests with water in which the crayfish had been held demonstrated the existence of sex pheromones. The inner rami of the antennules are the site of reception, and the chemicals are probably carbohydrates.*

In the crayfish *Procambarus clarkii*, copulation is preceded by courtship behavior (*1*). Courtship consists initially of aggressive behavior by both male and female but is succeeded by a series of nonaggressive interactions. Male-male interactions are persistently aggressive. A similar system has been described in the banded shrimp *Stenopus hispidus* (*2*) and in snapping shrimp (*3*). The difference in male-male and male-female interactions in the crayfish indicates that sex recognition occurs. We now report the existence and nature of sex pheromones in *P. clarkii*.

After being shipped from Louisiana, specimens (40 to 61 mm, cephalothorax length) were kept in separate, aerated aquaria.

To show that a male can discriminate sex without physical contact, the following experiment was performed. For each trial, a male was held in a 10-gallon (38-liter) aquarium for 2 to 5 days. An opaque plastic container (15 cm in diameter, 19 cm tall, and perforated with 1-mm holes), was placed in the aquarium near the source of aeration. A stimulus animal was placed in the perforated container, and the behavior of the test animal in the aquarium was observed. Control tests using opaque but nonperforated containers were negative.

All tests were run between 1100 and 1500 hours, when this species is normally less active. After a stimulus animal was placed in the plastic container, the response latency was recorded, and the amounts of time spent by the test animal in the following activities were recorded for 60 minutes: (i) searching, either 1 cm or less from the test container or elsewhere; (ii) resting, either 1 cm or less from the container or elsewhere; (iii) chelae raised posture; (iv) feeding movements; and (v) curled telson and uropod. There were 15 stimulus replications, 10 male and 5 female (Table 1).

In neither searching time nor resting time did the test animals respond differentially to male and to female stimulus animals. However, males showed submissive behaviors (feeding movements, chelae down, curled telson) when females were in the test container (90.6 percent of observation period) while the raised chelae posture (an index of agonistic behavior) was prevalent when the stimulus animal was a male (84.0 percent of period; $\chi^2 = 77.9$, $P < .0001$).

A second series of experiments was made with the same test animals after they had been blinded (*4*). The behaviors were timed as in the first series (Table 1). Normal and blinded animals did not differ in the amount of time they spent searching. The blinded test males searched longer when females rather than males were in the perforated container ($t = 3.44$, $P < .005$). Again, males spent most of the observation period (82 percent) in aggressive postures when the stimulus animal was a male, but showed submissive behaviors (89 percent of observation period) when females were in the test container ($\chi^2 = 78.0$, $P < .0001$).

The first and second experiments showed that neither vision nor physical contact was necessary for sex recognition. In the remaining tests, chemical means of communication were studied. Aged, aerated water from a container in which a male or a female crayfish had been held for 24 to 48 hours (called male or female water) was used to test the responses of isolated males. Glass tubes were connected to two funnels, one used for the male or female water and the other for control water (aged, aerated, tap water). The flow of water from the funnels into the tank of an isolated male was at a rate of about one drop every 3 seconds. Durations of behavior patterns were recorded as above for 30-minute periods. We made 12 replications of this experiment (Table 1). There was a significant difference in the time spent within a 2-cm radius of the test source (male or female water) and the control source ($t = 28.6$, $P < .001$). Males showed aggressive behavior toward male water but were submissive when female water was introduced ($\chi^2 = 51.8$, $P < .0001$).

Ablation experiments were done to establish sites of chemosensory organs. Eight males whose antennae were removed responded appropriately to male or female water. When the antennules of these individuals were removed, they no longer responded to test water. In addition, ten males with intact antennae but antennules removed showed no response to test water (each male tested on three separate days). Furthermore, when just the inner flagella of the antennules were removed, test animals were no longer responsive.

The following tests were performed (by the procedure of the third experiment) to establish some characteristics of the chemical factors involved.

1) Water from five males and five females was filtered separately through Sargent filter paper No. 500, and the filtrates were tested on ten males. All males responded differentially toward the filtrates.

2) The water from the males (six samples) and females (four samples) was boiled, cooled, and tested. Three male and two female samples were boiled for 5 minutes, the others for 30 minutes. The results were positive and appropriately different for the two sexes.

3) The water from males (four samples)

Table 1. Average time (in minutes) spent by a male crayfish in different behaviors. Numbers in parentheses represent number of trials with male and with female stimuli. The timing of the observation period began with the initial response. In the third experiment, times in parentheses are response times to aged, aerated, tap water.

Stimulus	Latency	Searching		Resting		Chelae up	Feeding	Curled telson
		Near source	Elsewhere	Near source	Elsewhere			
Normal males, stimulus animals in container, 60-minute observation periods								
Male (10)	9.8	49.9	3.8	2.9	3.4	50.4	0.5	2.8
Female (5)	10.2	43.0	3.2	5.2	8.6	1.8	52.2	54.4
Blinded males, stimulus animals in container, 60-minute observation periods								
Male (10)	6.6	45.6	6.8	3.4	4.2	49.3	0	0.7
Female (5)	11.8	50.6	4.0	3.6	1.8	0.6	53.4	52.2
Normal males, test water added, 30-minute observation periods								
Male (7)	4.0	24.9 (1.1)	3.3	0.4 (0.4)	0.6	27.6	0	1.4
Female (5)	7.8	22.6 (1.0)	2.8	2.4 (0.4)	1.0	0.8	27.6	26.8

and females (two samples) was digested with Pronase for 6 to 7 hours at 37°C. The resulting solution elicited the appropriate responses.

4) Polysaccharides in four male and two female water samples were hydrolyzed in 1*M* HCl at 100°C for 5 hours in sealed vessels. The hydrolyzate was neutralized with KOH to a *p*H 7 to 7.2. The products of the hydrolysis when tested on six crayfish gave negative results—there was neither aggressive behavior elicited by the hydrolyzate from male water, nor was there courtship (or any overt change in behavior) elicited by the hydrolyzate from female water. The experimental animals responded to untreated male and female water appropriately.

From the above tests, we can infer that the chemical factor is a carbohydrate, although we have not yet identified it.

The response of male antennules to male and female water were studied electrophysiologically. Antennulogram recordings (*5*) were made from ablated antennular flagella (Fig. 1). The eight male subjects responded similarly. The characteristic shape of the wave (Fig. 1c) resulting from stimulation with both male and female water was also seen as a response to male or female water treated with Pronase (Fig. 1d). The responses to the acid hydrolyzates from male and female water (Fig. 1e) and to distilled water treated with acid (Fig. 1f) are probably responses to the chemical treatment of the water. The outer flagella of the antennules did not respond to test water, although the basis for the differential responses of the inner and outer flagella is not yet known.

Among the ways the sexes recognize and respond to one another are through differences in morphology (as in sexually dimorphic species), behavior (posture, sound production), or chemicals (pheromones). A combination of these may be found in the same species, as in the genus *Uca*, in which morphology and behavior (sound production and posture) are used (*6*).

In crayfish it has been assumed that the male recognizes the female only through the submissive behavior of the latter (*7*). In species without clear sexual dimorphism, it is extremely difficult to investigate the features peculiar to the male that cause the female to adopt a submissive posture during an encounter. There must be other cues involved, especially since these animals are nocturnal. The presence of a sex pheromone is an obvious sexual identification mechanism.

The possible presence of a sex pheromone has been reported for several crustaceans (*3, 8, 9*), although experimental verification has been found for only a few (*10*). Prior to copulation, the male of a number

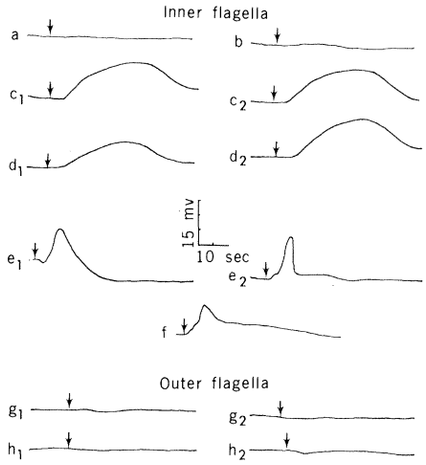

Fig. 1. Representative electroantennulogram records from the inner and outer antennular flagella. Arrows indicate the introduction of stimulus water. The solutions used were: a, distilled water; b, aged tap water; c_1, normal water from females; c_2, normal water from males; d_1, Pronase-treated female water; d_2, Pronase-treated male water; e_1, acid-hydrolyzed female water; e_2, acid-hydrolyzed male water; f, acid-treated distilled water; g_1, distilled water; g_2, aged tap water; h_1, normal female water; h_2, normal male water.

of species can detect the female before she molts or just afterward at a distance (*3, 9*). In crayfish, the female does not molt just before copulation.

While a chemoreceptive function of the antennules of crayfish during feeding has been demonstrated in some species (*11*), *P. clarkii* does not rely on antennules for distance chemoreception during feeding (*12*). Presumably the structures are used in contexts other than feeding, and one such context is intraspecific communication.

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References and Notes

1. C. Ameyaw-Akumfi, in preparation.
2. V. R. Johnson, Jr., *Pac. Sci.* **23**, 40 (1969).
3. B. A. Hazlett and H. E. Winn, *Crustaceana* **4**, 25 (1962).
4. Successive coats of fingernail polish were applied until the crayfish did not respond to visual stimuli. This method avoided the problems and trauma associated with eyestalk ablation in decapod crustacea [B. A. Hazlett, *Z. Vgl. Physiol.* **71**, 1 (1971)].
5. The antennulogram method used was a modification, for an aquatic organism, of the method of D. Schneider[*J. Insect Physiol.* **8**, 15 (1962)]
6. M. Salmon, *Zoologica (N.Y.)* **50**, 123 (19 → M. Salmon and S. Atsaides, *Am. Zool.* **8**, 623 (1968).
7. E. A. Andrews, *Am. Nat.* **29**, 867 (18 → *ibid.* **38**, 165 (19 → A. S. Pearse, *ibid.* **43**, 746 (19 → F. E. Chidester, *ibid.* **46**, 279 (1912); J. C. Mason, *Can. J. Zool.* **48**, 969 (1970).
8. G. H. Parker, *Bull. U.S. Fish Comm.* **21** (2), 103 (1901).
9. W. P. Hay, *U.S. Bureau of Fisheries Report for 1904* (Government Printing Office, Washington, D.C., 1905), p. 397; M. D. Burkenroad, *Am. Nat.* **81**, 392 (1947); D. I. Williamson, *Br. J. Anim. Behav.* **1**, 83 (1953); D. B. Carlisle, *J. Mar. Biol. Assoc. U.K.* **38**, 481 (1959); J. W. Knudsen, *Pac. Sci.* **18**, 3 (1964); R. G. Hartnoll, *Crustaceana* **15**, 165 (1968).
10. E. P. Ryan, *Science* **151**, 340 (19 → J. Atema and D. G. Engstrom, *Nature (Lond.)* **232**, 261 (1971); Y. Kamiguchi, *J. Fac. Sci. Hokkaido Univ. Ser. VI Zool.* **18**, 347 (1972).
11. E. S. Hodgson, *Biol. Bull. (Woods Hole, Mass.)* **115**, 114 (1958).
12. C. Ameyaw-Akumfi, *Crustaceana*, in press.

13. We thank Dr. G. Jones, who gave advice and materials used in chemical characterizations, and Dr. R. Shafer, who gave advice and equipment for the electrophysiological recordings.

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Molecular Vehicle Properties of the Broad Host Range Plasmid RK2

Abstract. *The plasmid RK2 is stably maintained in a broad range of gram-negative bacteria. The RK2 DNA has a single Eco RI restriction site. The insertion of a DNA fragment into this site does not interfere with either plasmid maintenance or self-transmissibility. Because RK2 has a broad host range, it should be useful for the construction in vitro of hybrid plasmid molecules capable of being established by conjugal transfer or transformation into many genera of gram-negative organisms.*

Bacterial plasmids of compatibility class P may conjugally transfer into many different host genera of gram-negative bacteria in which they are stably maintained (*1, 2*). These plasmids are therefore useful for introducing new genes into these organisms. For this reason, we examined the molecular vehicle properties of the P plasmid RK2, in the hope that it may be used to construct in vitro new plasmids that would retain the broad host range of the parental molecule.

The plasmid RK2 specifies resistance to the antibiotics ampicillin, kanamycin, and tetracycline (*2*). Supercoiled, tritium-labeled RK2 DNA was purified by preparative scale ethidium bromide–cesium chloride equilibrium centrifugation of "cleared" (*3*) lysates of JC411(RK2), a strain of *Escherichia coli* K-12 carrying the plasmid. The DNA was exhaustively digested by the restriction endonuclease Eco RI (*4*) and analyzed both by sedimentation through a neutral sucrose gradient and by agarose gel electrophoresis. Undigested RK2 DNA is homogeneous in size (Figs. 1A and 2A), with $s_{20,w}$ values of 60 and 43, corresponding to the covalently closed and nicked circular forms of the plasmid, and a calculated molecular weight of 40×10^6 (*5, 6*). Restricted RK2 DNA migrated at a single position on the