SEX PHEROMONE ACTIVITY OF THE MOLTING HORMONE, CRUSTECDYSONE, ON MALE CRABS

(*Pachygrapsus crassipes*, *Cancer antennarius*, AND *C. anthonyi*)

JAMES S. KITTREDGE,[1] MICHELLE TERRY,[1] AND FRANCIS T. TAKAHASHI[2]

ABSTRACT

The pheromone released by premolt female *Pachygrapsus crassipes* is a heat stable non-ionic polar lipid. The coincidence of the release of the pheromone and the nubial molt suggested that the molting hormone, crustecdysone, may also function as a sex pheromone. Adult male crabs were observed to display typical precopulatory behavior when exposed to dilute solutions of crustecdysone. The threshold concentration for behavioral response was found to be 10^{-13} M for *P. crassipes*, 10^{-10} M for *Cancer antennarius* and 10^{-8} M for *C. anthonyi*. These findings provide the basis for a theory of the evolution of pheromone communication in the Arthropoda.

The dominant position of chemoreception in the behavior of marine invertebrates and the implication of sex pheromones in the reproductive activities of many species is supported by many behavioral observations, but no pheromone has yet been characterized from the marine environment. In many marine decapod Crustacea copulation takes place immediately after the female molts. The male of the species recognizes the premolt condition of the female, is attracted to her, and usually seizes and carries her until she molts. This recognition at a distance has been reported for many genera of Crustacea (Hay, 1905; L. Agassiz in Verrill, 1908; Needler, 1931; Burkenroad, 1947; Williamson, 1953; Hughes and Matthiessen, 1962; Knudsen, 1964; Snow and Neilsen, 1966). Ryan (1966) described the search and display behavior exhibited by male *Portunus anguinotentus* when a premolt female crab was placed in the holding tank with them. Each male became active, walked about on the tips of its dactyls, elevated its body, and extended its chelae. When thus stimulated they often attempted to pull any crab they met into a precopulatory carrying position. Ryan demonstrated that this behavior is released by a sex pheromone in the urine of the premolt female crab.

METHODS

BIOASSAY

Observation vessels for determining the response of male *Pachygrapsus crassipes* to dilute solutions of molting hormone were constructed from 4-liter beakers. With a glass blowing torch and the edge of a carbon flat we formed an indent in the side of each beaker approximately 4 cm deep, parallel to and 3 cm above the bottom of the beaker. The outside of the beakers was masked with black paint with the exception of an 8 cm window opposite the indent. When a crab was placed in seawater in the observation chamber, they always scurried into the niche between the bottom of the beaker and the indent. If the seawater contained molting hormone, the crabs were stimulated to come out of the niche and assume a premating stance. The time elapsing after adding a solution of crustecdysone in seawater to an empty vessel containing a male crab until the crab elevated its cephalothorax in a typical stance was noted. Six crabs were timed at each concentration of

[1] Division of Neurosciences, City of Hope National Medical Center, Duarte, Calif. 91010.
[2] Zoology Department, Oregon State University, Corvallis, Oreg. 97331. The data in this paper are taken in part from a dissertation by F.T.T. to be submitted to Oregon State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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crustecdysone, and fresh male crabs were used for each different concentration. The male crabs were held for several days in isolation from female crabs before testing.

ISOLATION COLUMNS

Columns (5 × 50 cm) of Amberlite XAD-2, a divinylbenzene polymer, were found to be effective in the recovery of polar steroids from seawater. The columns were washed with three void volumes of water to remove the salts, and the polar steroids were eluted with three volumes of 60% ethanol. The more nonpolar lipids were removed with 95% ethanol. After repeated use the columns were reconditioned by cycling through 95% ethanol, diethyl ether, hexane, diethyl ether, ethanol, and water.

FRACTIONATION COLUMNS

Chromosorb 102, which is an 80/100 mesh fraction of Amberlite XAD-2, in a 0.9 × 100 cm column was used to fractionate the polar lipids. This column was eluted with a gradient of ethanol (20% to 80%) (Hori, 1969). The gradient was formed by an Isco Dialagrad dual pump[3] with the following settings: 40 ml/hr; reservoir for "A" pump, 80% ethanol; reservoir for "B" pump, 20% ethanol; percentage settings for "B" pump, 100, 100, 90, 80, 70, 60, 50, 30, 15, 0, 0 (this pump makes five intermediate linear steps between each setting); total time 16 hr. The fractionation was monitored at 254 μ with an Isco Model UA-2 UV monitor and fractions were collected in an Isco Model 327 fraction collector.

A silicic acid column (0.6 × 30 cm) eluted with chloroform-ethanol (5:1, v/v) and monitored in the UV was employed for further fractionation (Horn et al., 1968).

OBSERVATIONS ON THE PREMATING BEHAVIOR AND THE SEX PHEROMONE OF *Pachygrapsus crassipes*

Although the premating behavior of the lined shore crab, *Pachygrapsus crassipes*, as described by others (Hiatt, 1948; Bovbjerg, 1960), did not include the typical stance of other Brachyura, the abundance and ease of collection of this species prompted us to re-examine their behavior. We found that male *P. crassipes*, in the presence of a premolt female, exhibit an easily recognizable stance. The males elevate their cephalothoraxes and tilt the anterior margin up. They walk on the tips of the dactyls of their first three pairs of walking legs and extend their fourth pair horizontally backwards. The chelipeds are partially extended but lowered, as opposed to the elevated defensive position. When thus stimulated they will often attempt to seize any other *P. crassipes* they encounter, male or female, and turn them over into the holding position with which they maintain control of a premolt female. This behavior compares with that described by Ryan (1966) for male *P. sanguinolentus*. There were two additional characteristics of the male *P. crassipes* behavior that paralleled the premating behavior of *Cancer magister* as described by Snow and Neilsen (1966). They observed that the male *C. magister*, while carrying the female, frequently extended his fourth pair of walking legs straight back. We have observed that *C. magister* will thus extend his legs while holding his body elevated when stimulated by the sex pheromone before he seizes the female, as does *P. crassipes*. Snow and Neilsen (1966) also noted that "on occasion the male would rise up on the tips of his walking legs and raise the female up into an elevated position nearly 6 inches off the bottom of the tank. This movement would be accompanied by a continuous flexing of the male's abdominal flap." A frequent observation with pairs of *P. crassipes* was that they would stand facing each other, both with their legs extended and body elevated, but with the male higher. In this position the female would lower her abdominal flap slowly and then flex it rapidly but not into a completely retracted position. She would repeat this movement several times. The male would then repeat an identical movement of his abdominal flap.

[3] Reference to commercial products does not imply endorsement.

This aspect of their behavior could be interpreted as a courting gesture, but in the context of chemical communication we prefer to interpret this as a fanning motion facilitating the distribution of pheromones that may be aphrodisiac in nature. Commercial fishermen for both the American lobster, *Homarus americanus*, and the California spiny lobster, *Panulirus interruptus*, have suspected that the males of each species could be used to attract the females.

A single active premolt female *Pachygrapsus crassipes* released sufficient pheromone to stimulate all of the males in a 25-gal recirculating aquarium. A single female *P. crassipes* in an aerated 2-gal container released a pheromone which stimulated a male *Cancer antennarius* to exhibit a premating stance.

With these observations providing the bioassay, we examined the nature of the sex pheromone. Water which had contained a premolt female crab was boiled for 10 min, cooled, and aerated. The active principle was still present. The active principle was not retained by cation nor anion exchange resins nor by charcoal. The active substance could, however, be extracted from "active seawater" with isopropanol/diethyl ether. These observations, together with the premolt condition of the active females, led us to suspect that the females might be releasing molting hormone into the water, and that this steroid might be functioning as a sex pheromone.

BIOASSAY OF CRUSTECDYSONE

We soon confirmed that dilute solutions of crustecdysone (β-ecdysone, 20R-hydroxyecdysone, ecdysterone, isoinokosterone), which is one of the molting hormones of Crustacea (Horn et al., 1968), elicited a typical response from male *P. crassipes*. In order to establish the threshold concentration that would release response we standardized the conditions for the bioassay. As described under "Methods," the test adopted allowed the male crab to be flooded with a known concentration of the steroid in seawater, in contrast to the diffusion techniques often employed. *P. crassipes* proves to be ideal for this mode of testing because they are an intertidal species and in nature normally leave the tide pools at low tide to feed on the rocks. They were not disturbed on being placed in a wet empty observation vessel and sought out the artificial niche provided. On flooding with seawater they would remain in the niche for long periods or occasionally come out to explore briefly and then return to the niche. When a male *P. crassipes* was flooded with a solution of crustecdysone in seawater, he was stimulated to come out of the niche and explore the vessel and then to assume the premating stance. The time elapsing until the crab raised its body to assume the stance was found to be a function of the concentration of crustecdysone. Although the male crabs would often exhibit a full stance in a brightly illuminated laboratory at the higher concentrations of crustecdysone, the response was often erratic. All of the bioassays were conducted in an isolated room illuminated with an Eastman darkroom lamp with a 15-w bulb and an Eastman No. 00 yellow filter. The observer was stationed quietly before the "window" of the test vessel. On removal from the test vessel the male crabs were transferred to a 2-liter beaker of seawater to rinse off the extraneous crustecdysone and then were transferred to a small aquarium for further observation. In spite of the handling during the two transfers, male crabs that had been stimulated to display a premating stance in the observation vessel usually resumed this posture shortly after being transferred to the aquarium. When thus stimulated they often attempted to seize any other male crab in the aquarium.

All of the male *P. crassipes* utilized in establishing the response curve were collected at the same time and in the same area where we had just succeeded in collecting a number of premolt females. They were all held for three or more days in isolation from any female crabs. The curve was started with a concentration of 10^{-5} M crustecdysone in filtered seawater. At this concentration the response was rapid, averaging 7 sec. Succeeding tests were performed with ten-fold dilutions of the crustecdysone. Fresh solutions of crustecdysone were prepared after three steps of dilution or at the start of each day's testing. Previous experience had

demonstrated that dilute solutions in seawater lost some or all of their activity on storage even at 0° C, presumably through bacterial degradation or adsorption. It was planned that six male crabs would be tested at each concentration and that the five most consistent times would be averaged; however, the response was found to be remarkably uniform and in all but three cases all six crabs responded within a narrow time range. There was no sharp threshold of concentration. The average response times plotted as a smooth curve extending to 10^{-3} M crustecdysone concentration where the average response time was 22 min (Figure 1). No response was observed at 10^{-14} M. The scatter of response times was greatest at 10^{-5} M, probably because of the short-term disturbance of the male crab during flooding. The standard deviation of the normalized response times for 45 crabs, from 10^{-6} M to 10^{-13} M crustecdysone, was 8.6.

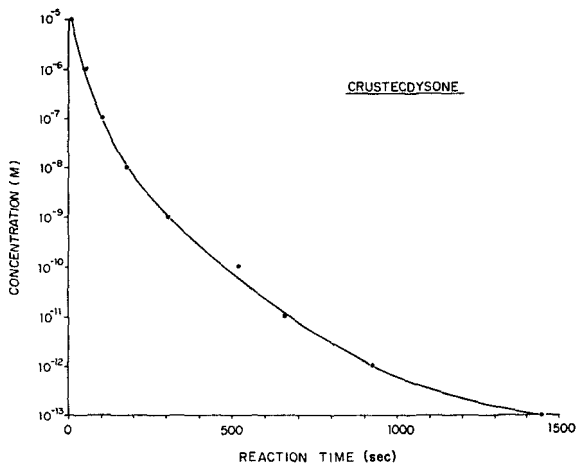

FIGURE 1.—Time elapsed following immersion of male *Pachygrapsus crassipes* in seawater solutions of crustecdysone before the body elevation phase of the precopulatory behavior.

Since the precopulatory behavior of males in the presence of premolt females appears to be general among the Brachyura, we examined the response of two species of *Cancer* to crustecdysone. Both *C. antennarius* and *C. anthonyi* displayed typical premating behavior when exposed to dilute solutions of crustecdysone. When the response time vs. concentration study was carried out with these two species each yielded a response curve similar to that developed by *P. crassipes*. There was, however, a marked difference. There was an abrupt break in the response yielding a distinct threshold at 10^{-10} M for *C. antennarius* and 10^{-8} M for *C. anthonyi*.

We then attempted to determine if *C. antennarius* males could detect a gradient in the concentration of crustecdysone. For this purpose we employed a simple "T" maze with a baffle at the head of each arm separating the seawater sources and forming mixing chambers. Five male *C. antennarius* were placed at the head of the maze and the water flows from each arm balanced. While seawater alone was flowing through the maze the *C. antennarius* remained quiet in the two corners at the origin of the maze. When a flow of crustecdysone solution was added to the mixing chamber at the head of one arm all five of the male *C. antennarius* soon became active. They explored up and down each arm of the maze but demonstrated no tendency to select the arm containing the crustecdysone. While these tests did not indicate any ability to detect or follow up a gradient, there was a positive response to the crustecdysone. All five of the male crabs were stimulated simultaneously to undertake an active exploratory behavior when the crustecdysone was introduced.

DEVELOPMENT OF THE TECHNIQUES FOR THE ISOLATION AND FRACTIONATION OF THE SEX PHEROMONE(S)

Liquid-liquid extraction procedures are inefficient for the recovery of trace quantities of polar lipids. Columns of Amberlite XAD-2 have been employed for the recovery of steroids from urine (Bradlow, 1968; Shackleton, Sjövall, and Wisén, 1970). Recently, Hori (1969) has employed a column of this resin eluted with a li-

near gradient of ethanol in water for the fractionation of the phytoecdysones. We have found that a column of XAD-2 could be used to recover traces of crustecdysone from seawater and from crab urine. Using the above two columns we have examined the seawater in which female *P. crassipes*, *C. magister*, and *C. productus* had been maintained for 3 to 6 hr. The product of individual *Cancer* were assayed, while the seawater from two or more *P. crassipes* was combined before extraction. We have also examined the urine of female *C. magister*. The *Cancer* were staged according to Drach (1939), and the female *P. crassipes* were selected for activity by observing the behavior of males in their presence. During our observations we found the female *C. magister* continued to release a pheromone for up to 2 weeks post molt.

The material recovered from the isolation column in 60% ethanol was reduced in volume to a few microliters and transferred in 20% ethanol to the Chromosorb 102 column. Elution of this column yielded an ultraviolet-absorbing peak near the front and two or more succeeding peaks. Each of three stage "D" *C. magister* and one stage "D" *C. productus* studied were found to have released an ultraviolet-absorbing compound that eluted from the column at the same ethanol concentration that a crustecdysone standard did. Both extracts of *P. crassipes* seawater yielded a peak in the position of crustecdysone. One stage "A" *C. magister* also yielded a peak in this position. A stage "C-4" *C. magister* did not yield any peak in the position of crustecdysone nor did urine from a stage "C-4" *C. magister* yield a peak in this position. Also one of the *C. magister* females that had yielded material eluting as crustecdysone while in stage "D" did not yield this substance during molting (stage "E"). The ultraviolet-absorbing fractions corresponding to crustecdysone from the above columns did not have an ultraviolet-absorption spectrum corresponding to that of crustecdysone. The absorption peak included the spectrum of crustecdysone, but had a double peak at a lower wave length. These fractions were concentrated and applied to a silicic acid column. The elution of this column with chloroform-ethanol yielded an ultraviolet-absorbing peak near the front and a peak eluting in the same volume as a crustecdysone standard. The material from this column has an ultraviolet-absorption spectrum that corresponds closely to that of crustecdysone.

We are now developing a derivitization technique that will permit us to subject our final samples to gas chromatography-mass spectrophotometry for structural conformation. Katz and Lensky (1970) have published a technique for the silylation of α-ecdysone for GLC analysis. We have employed silylation techniques with crustecdysone and observed decomposition during GLC.

RESULTS AND CONCLUSIONS

The pheromone released by *P. crassipes* stimulates premating behavior in *C. antennarius*. Male *C. magister* are excited into seizing and clasping female *C. productus* by their pheromone. Crustecdysone mimics the pheromone in its effects on male *P. crassipes*, *C. antennarius*, and *C. anthonyi* in the release of the premating stance. After exposure to crustecdysone all these species of male crabs attempt to seize other crabs, male or female, and pull them into a precopulatory position. In addition crustecdysone triggers a search behavior in male *C. antennarius*. These observations demonstrate a lack of specificity in the sex pheromones of these species and either that crustecdysone is the sex pheromone or sufficiently similar in molecular structure to the natural pheromones to mimic them.

A possible explanation of the discrepency between our results and those of Ryan (1966) that indicated a species specificity for the sex pheromones in the three species of crabs that he studied may be that some species may respond to deoxycrustecdysone, callinecdysone A (inokosterone) or callinecdysone B (makisterone), other ecdysones that have been isolated from Crustacea, (Gailbraith et al., 1968; Faux et al., 1969), or they may respond to one of the metabolic products of crustecdysone detected in insects (Gailbraith et al., 1969; Moriyama et al.,

1970; Cherbas and Cherbas, 1970; Heinrich and Hoffmeister, 1970).

The isolation and analysis of the material released into seawater by active female *P. crassipes*, *C. magister*, and *C. productus* demonstrated that a compound could be detected that is eluted from two different columns in the same position as crustecdysone and has a UV absorption spectra that is similar to that of crustecdysone.

The semilog plot of the response times for male *P. crassipes* to varying concentrations of crustecdysone is approximately parabolic, and the scatter of response times at each concentration is remarkably narrow. This, the range of response times, and the continued response of the male crabs after removal from the stimulus, permit an interpretation of the chemoreception of pheromones from dilute solutions. The observations suggest that the pheromone has a high affinity for the receptor site resulting in a long half life for the receptor-pheromone complex. Indeed, one might have postulated that even a polar steroid might be strongly bound to a lipoid receptor in an aqueous medium. It is apparent that the crabs are capable of summating the chemical information for a considerable period of time before a threshold which releases response behavior is reached. Though summation may take place at any level in the nervous system, the simplest interpretation suggests that this takes place at the receptors. This summation of "information quanta" can function either in extremely dilute solutions or, in nature, it would permit the accumulation of subthreshold amounts presented in random turbulences of the current from the source.

This finding has significance in a consideration of the evolution of pheromone communication. It has been suggested that chemical signals between cells were evolved before the evolution of the metazoans and that these signals were later internalized as hormones and synaptic transmitters (Haldane, 1955; Wilson, 1968). In the present instance we have a reversal of this internalization. The Crustacea, having evolved polar steroid hormones to regulate molting, on externalization of the receptor site onto chemoreceptor organs and on alteration of the resorption process in the antennual gland during the premolt stage of the females were then capable of signaling the approach of the nubial molt. This interpretation obviates the concern over the improbability of the simultaneous *de novo* origin of both the genetic information directing the biosynthesis of the pheromone and that concerned with the architecture of the receptor site. We may assume that an unmasking of that portion of the chromosome that specifies the receptor site for the hormone on the membranes of the target organs occurred in the chemosensory neurons. A masking of the active transport system for the hormone from the fluid of the antennule gland of the female is also assumed. These two innovations are reasonably small evolutionary steps and conceptually preferable to the two *de nova* origins that must be assumed otherwise. This evolutionary step, the pheromone function of a hormone, may have been the origin of pheromone communication in the Arthropoda, for once fixed because of its reproductive value, it was then susceptible to a gradual evolutionary drift toward a variety of more specific pheromones.

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LITERATURE CITED

BOVBJERG, R. V.
1960. Courtship behavior of the lined shore crab, *Pachygrapsus crassipes* (Randall). Pac. Sci. 14: 421-422.

BRADLOW, H. L.
1968. Extraction of steroid conjugates with a neutral resin. Steroids 11: 265-272.

BURKENROAD, M. D.
1947. Reproductive activities of decapod crustacea. Amer. Natur. 81: 392-398.

CHERBAS, L., AND P. CHERBAS.
1970. Distribution and metabolism of α-ecdysone in pupae of the silkworm *Antheraea polyphemus*. Biol. Bull. (Woods Hole) 138: 115-128.

DRACH, P.
1939. Mue et cycle d'intermue chez les crustacés décapodes. Ann. Inst. Oceanogr., New Ser. 19: 103-391.

FAUX, A., D. H. S. HORN, E. J. MIDDLETON, H. M. FALES, AND M. E. LOWE.
1969. Moulting hormones of a crab during ecdysis. J. Chem. Soc. (London), Sect. D, Chem. Commun., p. 175-176.

GAILBRAITH, M. N., D. H. S. HORN, E. J. MIDDLETON, AND R. J. HACKNEY.
1968. Structure of deoxycrustecdysone, a second crustacean moulting hormone. J. Chem. Soc. (London), Sect. D, Chem. Commun., p. 83-85.

GAILBRAITH, M. N., D. H. S. HORN, E. J. MIDDLETON, J. A. THOMPSON, J. B. SIDDAL, AND W. HAFFERL.
1969. Catabolism of crustecdysone in the blowfly *Callifora stygia*. J. Chem. Soc. (London), Sect. D, Chem. Commun., p. 1134-1135.

HALDANE, J. B. S.
1955. Animal communication and the origin of human language. Sci. Progr. 43: 385-401.

HAY, W. P.
1905. The life history of the blue crab (*Callinectes sapidus*). Rep. [U.S.] Bur. Fish. 1904: 395-413.

HEINRICH, G., AND H. HOFFMEISTER.
1970. Bildung von Hormonglykosiden als Inaktivierungsmechanismus bei Calliphora erythrocephala. Z. Naturforsch. 25b: 358-361.

HIATT, R. W.
1948. The biology of the lined shore crab, *Pachygrapsus crassipes* Randall. Pac. Sci. 2: 135-213.

HORI, M.
1969. Automatic column chromatographic method for insect-molting steroids. Steroids 14: 33-46.

HORN, D. H. S., S. FABBRI, F. HAMPSHIRE, AND M. E. LOWE.
1968. Isolation of crustecdysone (20 R-hydroxyecdysone) from a crayfish (*Jasus lalandei* Milne-Edwards). Biochem. J. 109: 399-406.

HUGHES, J. T., AND G. C. MATTHIESSEN.
1962. Observations on the biology of the American lobster, *Homarus americanus*. Limnol. Oceanogr. 7: 414-421.

KATZ, M., AND Y. LENSKY.
1970. Gas chromatographic analysis of ecdysone. Experientia 26: 1043.

KNUDSEN, J. W.
1964. Observations of the reproductive cycles and ecology of the common Brachyura and crablike Anomura of Puget Sound, Washington. Pac. Sci. 18: 3-33.

MORIYAMA, H., K. NAKANISHI, D. S. KING, T. OKAUCHI, J. B. SIDDALL, AND W. HAFFERL.
1970. On the origin and metabolic fate of α-ecdysone in insects. Gen. Comp. Endocrinol. 15: 80-87.

NEEDLER, A. B.
1931. Mating and oviposition in *Pandalus danae*. Can. Field Natur. 45: 107-108.

RYAN, E. P.
1966. Pheromone: evidence in a decapod crustacean. Science (Washington) 151: 340-341.

SHACKLETON, C. H. L., J. SJÖVALL, AND O. WISÉN.
1970. A simple method for the extraction of steroids from urine. Clin. Chim. Acta 27: 354-356.

SNOW, C. D., AND J. R. NEILSEN.
1966. Premating and mating behavior of the Dungeness crab (*Cancer magister* Dana). J. Fish. Res. Bd. Can. 23: 1319-1323.

VERRILL, A. E.
1908. Decapod crustacea of Bermuda, I. Brachyura and Anomura. Their distribution, variations, and habits. Trans. Conn. Acad. Arts Sci. 13: 299-474.

WILLIAMSON, D. T.
1953. Mating behaviour in the Talitridae (Amphipoda). Brit. J. Anim. Behav. 1: 83.

WILSON, E. O.
1968. Chemical systems. *In* T. A. Sebeok (editor), Animal communication, p. 75-102. Indiana Univ. Press, Bloomington.