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Austin W. Pritchard

Great importance has been attributed to the visual aspects of sexual communication in semi-terrestrial crabs and although P. crassipes may utilize visual signals, pheromones and chemotactile signals are important in the release of mating behavior. In the subtidal C. magister pheromones seem to mediate both courtship and mating behavior. Visual input, however, is also necessary for the release of courtship related behavior and tactile or chemotactile signals may be necessary for the consumation of mating.

Crustecdysone and its more "polar" metabolite function as mating pheromones in P. crassipes. Crustecdysone may also serve as a chemotactile releaser of mating when the crabs are out of the water. Foam bathing may be the mechanism by which this steroid is spread over the exoskeleton of the crabs. Crustecdysone has low

pheromonal activity in C. magister but its more polar metabolites may function as the pheromone. Precopulatory behavior may be released by a neutral or negatively charged metabolite and mating may be released by the positively charged metabolite. The functional and evolutionary significance of crustecdysone as the sex pheromone is discussed.

The sex pheromones and crustecdysone inhibit the feeding responses in both species of crabs. This appears to be a centrally mediated phenomenon occurring perhaps in the medulla terminalis ganglia and represents a simple form of integration of two stimuli. The effects of inhibition persist after removal of the pheromone of crustecdysone stimuli. Feeding responses in crabs are also inhibited on exposure to dopaquinones and naphthaquinones. The transitory effects, however, indicate that the inhibition is peripheral.

Chemical Communication and Chemoreception in the Dungeness Crab, *Cancer magister*, and the Lined Shore Crab, *Pachygrapsus crassipes*

by

Francis T. Takahashi, Jr.

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Professor of Zoology
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Redacted for Privacy

Head of Department of Zoology

Redacted for Privacy

Dean of Graduate School

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CHEMICAL COMMUNICATION AND CHEMORECEPTION IN THE DUNGENESS CRAB, CANCER MAGISTER, AND THE LINED SHORE CRAB, PACHYGRAPSUS CRASSIPES

INTRODUCTION

The aquatic medium abounds with a great variety of chemical substances from biotic and non-biotic sources making the ability to "read" the pertinent chemical substances and to respond in an adaptive manner a requisite for the well-being and survival of an organism. Chemical communication, utilizing this chemosensory ability, first evolved with the primordial protozoans and constituted the earliest form of communication (Haldane, 1954).

Since this research involving the chemosensory modality deals with both the communicative and non-communicative functions of chemicals, it is best at this point to make the distinctions clear. Chemical communication, the purposeful transfer of information between organisms wherein the response elicited in the receiver is such that it is advantageous for the signaler or its group to release such a signal, must be separated from chemoreception, the perceptive ability which confers advantage to the receiver but does not involve mutual organismic interactions (Burghardt, 1970). The chemical communicants have been further defined either as pheromones, intraspecific message converying chemicals secreted by an

individual and resulting in the elicitation of a specific response by another individual or individuals of the same species (Karlson and Butenandt, 1959), or as allomones, interspecific message conveying chemicals which, when secreted by an individual, elicit a response from a recipient individual or individuals of another species that is advantageous to that signaling individual (Brown, 1968). This investigation into chemical communication will concentrate principally on the role of the sex pheromones of Cancer magister and Pachygrapsus crassipes in mating and feeding inhibition while also testing the feeding inhibition caused by: 1) dopa; 2) dopaquinone, a crustacean epicuticle tanning agent (Dennell, 1947), which also is a precursor to melanin in octopus ink; and 3) naphthaquinones and spinochromes (substituted naphthaquinones) from the sea urchin. Both the sea urchin and the octopus are co-inhabitants of the rocky intertidal where P. crassipes lives, and these quinones may serve as defensive allomones inhibiting feeding. The investigation will also deal with the chemoreception of food stimuli by the crabs.

Communication by means of chemical substances occurs in a multitude of plants and animals. The focus, however, has largely been centered on the social insects where a complex social interaction necessitates a highly evolved message transferring capability. Reviews by Butler (1967), Schneider (1966), and Shorey (1973) show the extensive use and significance of the aggregation, sex, alarm, and trail pheromones and the defensive allomones in insect interactions.

Among the crustaceans, close relatives to the insects, behavioral evidence strongly suggests the presence of sex pheromones in reproductive interactions. C. magister, C. oregonensis, and C. productus seem to chemically signal the approaching molt perhaps by the release of molting hormone (Knudsen, 1964; Snow and Nielsen, 1966) which elicits the precopulatory embrace of the female by the male. This is sequentially followed by the molting of the female and then the act of copulation between the male and female. This pattern is also seen in C. pagurus (Edwards, 1966). Like the cancrid crabs reviewed above, the portunids follow the same sequence of behavior during reproduction. However, with the exception of Portunus sanguinolentus (Ryan, 1966), other portunids so far investigated seem to lack a diffusible female sex pheromone and instead rely upon tactile and chemotactile information. A more detailed survey of the literature on sex recognition in the portunids has been written by Teytaud (1971) who makes a case for a male pheromone and visual cues as communicants in Callinectes sapidus allowing the female to select a suitable mate. The grapsid crabs, Hemigrapsus oregonensis and P. crassipes, rarely interact sexually prior to mating and copulate shortly before egg deposition. In H. oregonensis mating is probably mediated by a female sex pheromone (Knudsen, 1964) but in P. crassipes visual signals are said to be the channel of communication (Bovbjerg, 1960).

The first direct evidence that pheromones release precopulatory behavior was shown by Ryan (1966) who sealed the excretory pores of premolt P. sanguinolentus females and thereby eliminated the precopulatory responses of the males to these females. He subsequently showed that the pheromone was in the urine. Since then a pheromone secreted by a newly molted female lobster (Hommarus americanus) which attracted males (Atema and Engstrom, 1971; McLeese, 1970), and a post paturial molt secretion from the sternal gland of the fresh water prawn, Paleomon paucidens, which initiates the search for copulation-ready females by the males (Kamiguchi, 1972) have been reported.

Evidence for the presence of the sex pheromone in crustaceans is mostly behavioral, with only one attempt (Christofferson, 1970) at isolation and characterization. An attempt was made here to describe and separate sexual behavior from non-sexual behavior, show the presence of a diffusible sex pheromone, create quantifiable bioassays for the sex pheromone and food stimuli, correlate the sexual behavior with the female's reproductive cycle and its release of the sex pheromone, and isolate, fractionate, and characterize the pheromones of the Dungeness crab, Cancer magister and the lined shore crab, Pachygrapsus crassipes. The effects of the individual pheromones of the two crabs on their respective feeding responses and the effects of dopa, dopaquinone, naphthaquinone, and spinochromes on the feeding

response of P. crassipes were studied. Since molting and mating are intimately associated, it has been hypothesized that the molting hormone, crustecdysone, also has a role as the sex attractant either directly or in a conjugated or catabolized form. In order to test this hypothesis, tritiated crustecdysone was injected into pre- and post-molt females of both crabs and the isolated pheromone-sea water fractions monitored for pheromone activity and radioactivity. Crustecdysone was bioassayed for its inhibitory effects on feeding. Eyestalks of C. magister were also ablated in order to determine the effect of an induced molt on the pheromone production.

Sexual communication among aquatic forms seems to be tactile and chemical while among semi-terrestrial forms it appears to be predominantly visual (Schone, 1968). Although Bovbjerg (1960) concluded that sexual communication was visual in P. crassipes, it seems improbable that its ability to communicate chemically would have been completely lost in transition to the semi-terrestrial life. The crab still spends much of its time in the waters of the tide pools and habituates crevices and rocky terrain which hampers vision. C. magister is aquatic in its habit and taxonomically is a close relative of P. crassipes. Both belong to the Superfamily, Brachyrhyncha. The comparative approach utilized in this study will hopefully shed further light on the question of whether sexual communication differs significantly between aquatic and semi-terrestrial crabs.

GENERAL METHODS

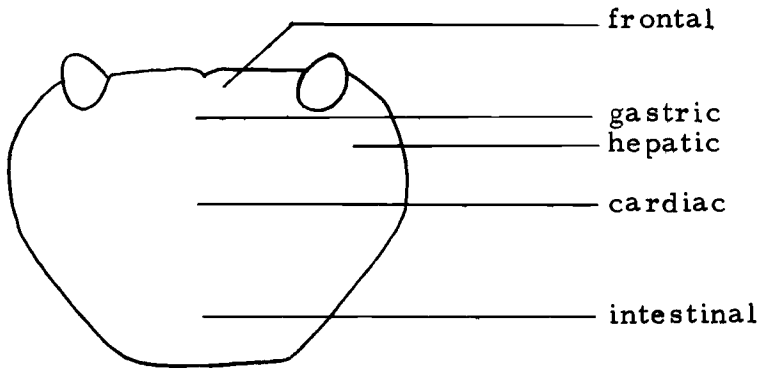
Collection of Crabs

The Dungeness crab, Cancer magister, was collected in Yaquina Bay, Oregon by three methods: 1) using a crab pot off the Marine Science Center small boat dock; 2) trawling the bay's main channel and various sloughs with an otter trawl; and 3) from commercial sources. Trawling provided the bulk of the crabs and had the advantage of providing crabs in every stage of the molt cycle. The lined shore crab, Pachygrapsus crassipes, was collected at low tides from the rocky intertidal areas of Corona del Mar and Point Vicente, California and at Newport, Oregon, its northern limit, by hand capture after turning over the rocks. After collecting, the two species of crabs were staged and maintained either in running sea water tanks at the Marine Science Center or in the thermoregulated, recirculating Ocean Systems aquaria and spare bath tubs at the City of Hope Medical Center in Duarte, California and fed three times a week on fish, bivalves, or shellfish. The crabs were separated according to species, sex, and stage in the intermolt cycle. Several males were always kept with the intermolt females for the purpose of selecting the females as they entered premolt and became sexually attractive.

Staging of Crabs

The establishment of the criteria for the determination of the stage in the intermolt cycle of the Dungeness and lined shore crabs was modification of the methods developed by Drach (1939). The method utilizes changes in the external morphology. Compressibility and rigidity of the dorsal carapace, sub-branchial region, and appendages roughly determines the stage of the crabs (Fig. 1, Table 1). A thinning exoskeleton makes visible the newly forming setae on the abdomen of a premolt animal (Fig. 1B). In early premolt only a faint pink line can be seen. During the intermediate stages of premolt the line widens and darkens with the progressive growth of the new setae and the progressive thinning of the exoskeleton. Finally in the latter stages of premolt a darker red line is visible. The abdomen also swells somewhat in female crabs, a condition especially pronounced in P. crassipes. This may be due to the formation of more abdominal appendages. Lethargy and refusal to feed are behavioral manifestations of late premolt.

The distinction between degrees of compressibility and rigidity is a qualitative one and although more precise methods of staging, changes within the layers of the exoskeleton and changes in the calcium level of the blood and urine were explored, the changes in the external morphology were used due to its simplicity and minimal



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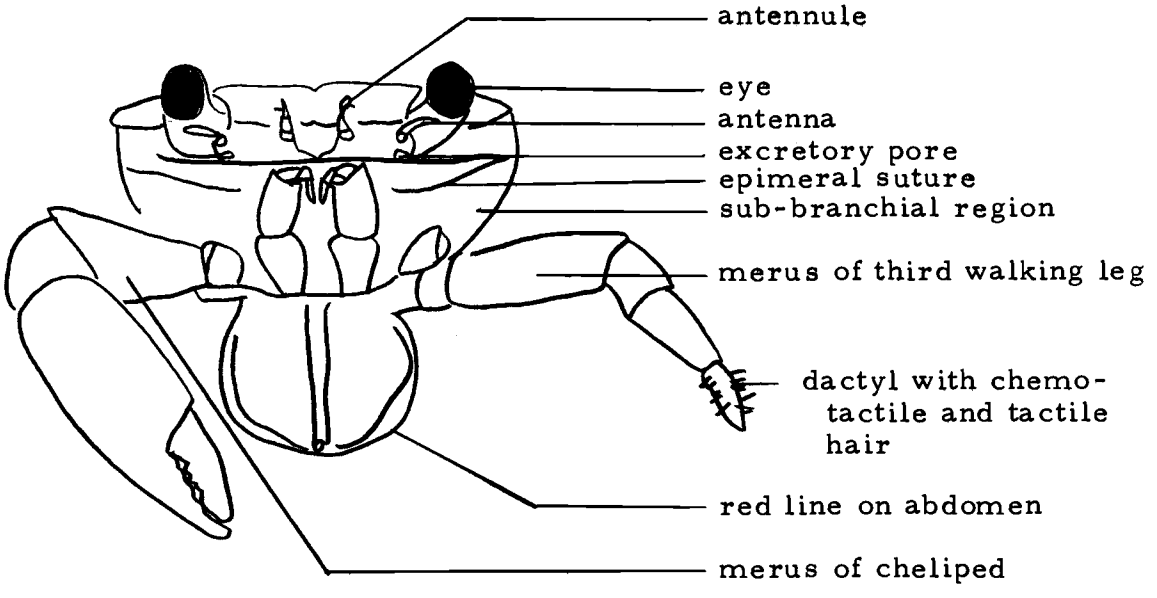

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Figure 1. Morphological features important in the staging of P. crassipes and C. magister. Regions of the dorsal carapace (A). Frontal aspect of a crab showing the unflexed abdomen (B).

Table 1. Changes in morphological and behavioral characteristics during the course of the intermolt cycle. Modified from Drach (1939).

Stage	Index	Dorsal carapace	Other morphological features	Behavior
Premolt	D_{1-4}	Thinning but rigid in all areas.	Sub-branchial area thinning and progressively more compressible but full of muscle. Abdomen soft and swollen with new bristle formation along the edges.	No feeding and lethargy in latter stages.
Molt	E	As above	Split along epidermal suture.	No feeding and very limited movement; exuvation.
Early postmolt	A_{1-2}	All areas soft and compressible.	All areas compressible.	Limited movement. No feeding.
Late postmolt	B_{1-2}	Gradual hardening beginning with the frontal and gastric areas.	Sub-branchial and merus compressible. Merus not filled with muscle.	Normal
Intermolt	C_{1-3}	All areas rigid except intestinal area.	Sub-branchial somewhat flexible; merus less compressible but not quite filled with muscle.	Normal
	C_4	All areas rigid.	Sub-branchial area rigid; merus rigid.	Normal

trauma to the crabs used for the isolation of the pheromone and for bioassays. The amount of pressure applied to P. crassipes had to be limited due to its fragility.

The determination of the premolt stage (stage D) was of greatest interest since precopulatory behavior began when the female reached premolt. Subsequently exuvation (stage E) occurred followed by copulation (stages A & B). In order to reduce experimental variability, the males used in bioassays were also staged and only intermolt crabs were used.

Observations of Crab Behavior

The behavioral responses of individuals, pairs, groups, and combination of sexes of C. magister and P. crassipes in a variety of situations preceding or associated with feeding and reproductive behavior were observed under laboratory conditions. The behavioral expressions of the two species of crabs in situations of resting, sitting, standing, ambulation, excitation, aggression, defense, feeding, precopulation and copulation were documented to form a partial behavioral ethogram. Behavioral responses to chemosensory, chemotactile, and tactile stimulation were also recorded. Using this repertoire of behavior, behavioral bioassays for the sex pheromone and feeding stimuli were established.

Behavioral Bioassay for the Sex Pheromone

In order to test for "activity" (presence of the sex pheromone), several types of bioassay chambers were utilized. Bioassays for "active" P. crassipes females were conducted by placing a male and female crab in a 16 x 25 x 20 cm rectangular aquarium filled with 5 liters of sea water and by observing the response of the male to the presence of the female. Non-quantitative bioassays of pheromone-sea water, artificial sea water in which an "active" female was immersed for 6 hours, could also be conducted in this manner utilizing only the male crabs. C. magister females were bioassayed in 22 x 45 x 15 cm rectangular aquaria each filled with 12 liters of sea water. A flowing water system (Fig. 2) was also used for C. magister to correlate the behavioral patterns with the release of the sex pheromone.

In an effort to quantify the mating response of male shore crabs to the sex pheromone a 4-liter glass beaker was indented and blackened on one side to provide a "secure" hiding place for the male P. crassipes (Fig. 3A). Pheromone-sea water which had been isolated and fractionated on various columns (see section on collection and isolation of sex pheromone and fractionation columns) and also crustecdysone, the hypothesized sex pheromone could then be bioassayed and the time dependency of the response could be recorded.

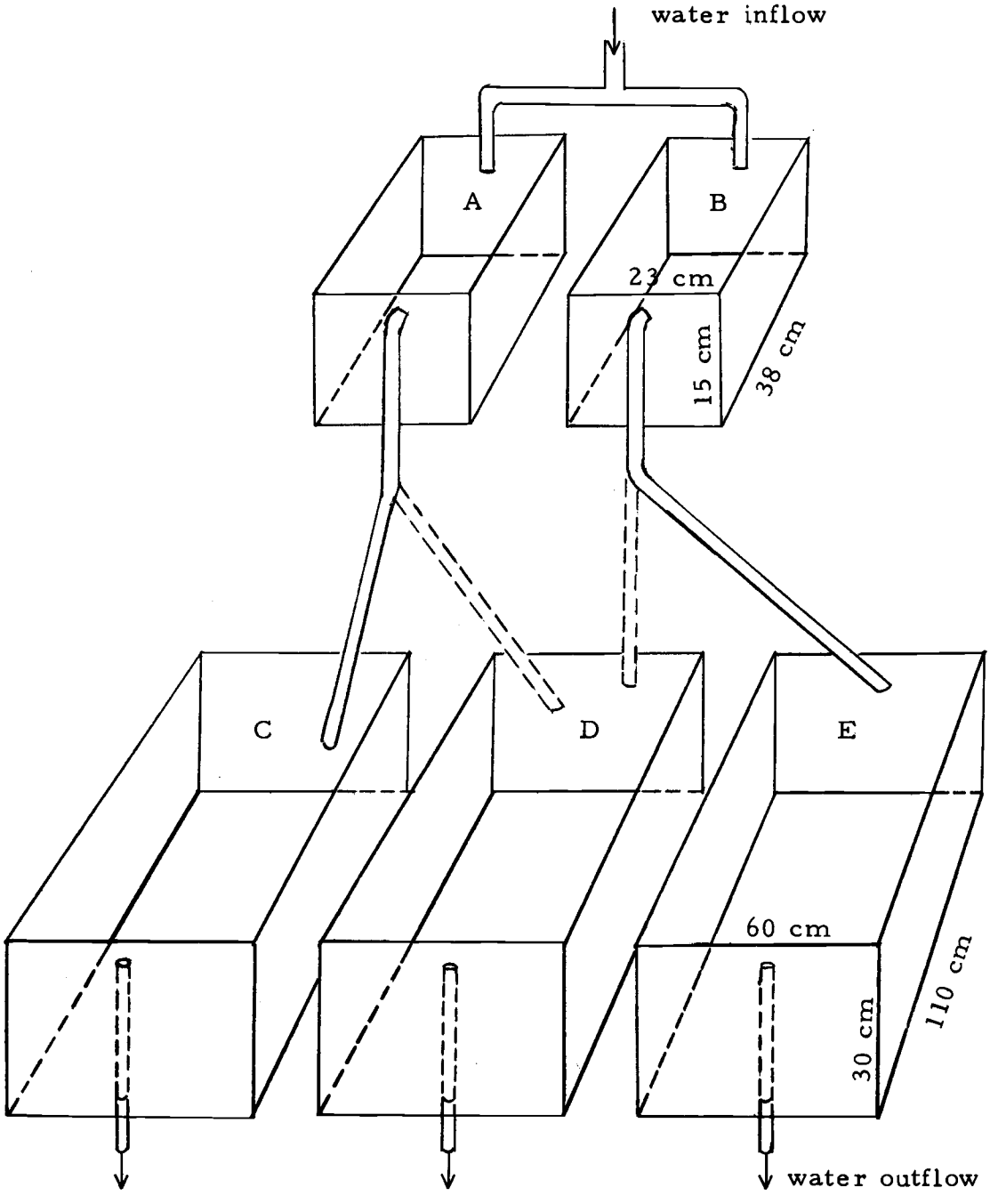
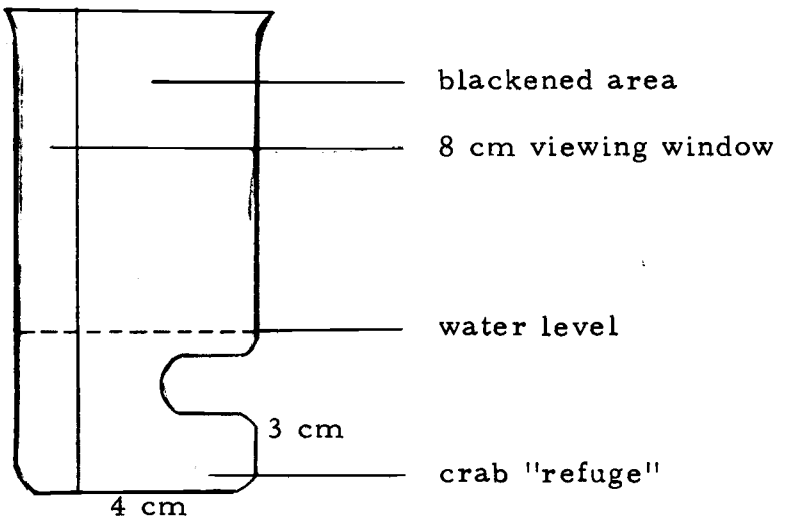


Figure 2. Flowing water bioassay system used to test the responses of C. magister crabs in the "reaction" tanks (C, D, E) to chemical signals released by a conspecific mating pair or "active" female in the "action" tank (A) during each reproductive event. Intermolt male and female conspecifics held in the control "action" tank (B).



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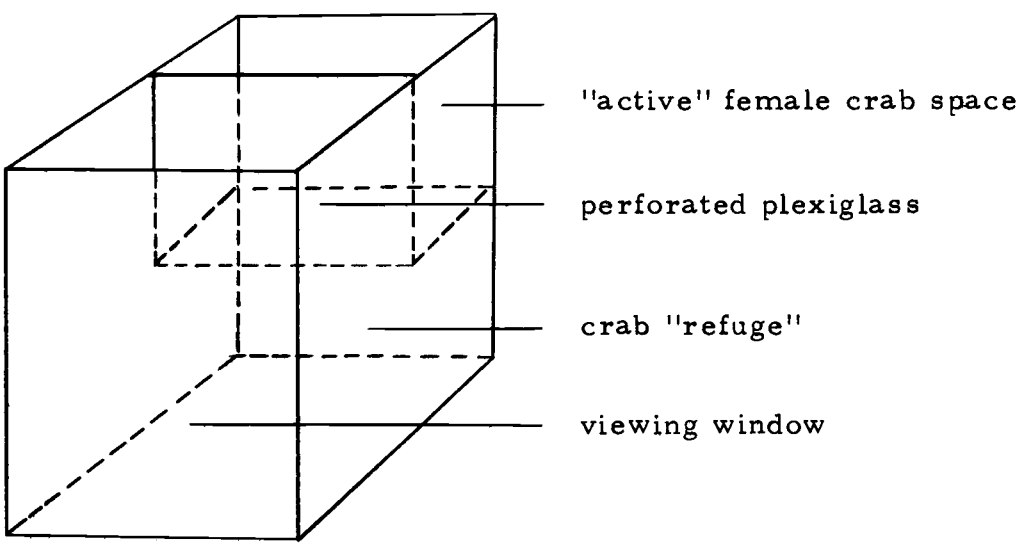

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Figure 3. Sex pheromone bioassay chambers for P. crassipes (A) and for C. magister (B).

C. magister fractions were quantitatively bioassayed in a 30 x 50 x 25 cm rectangular plexiglass aquarium which was blackened on three sides and equipped with a ledge under which the male crabs could retreat (Fig. 3B). All bioassays were conducted in a semi-dark room where only diffused indirect illumination fell on the bioassay chambers. This was necessary in order to minimize the "mirror-effect" caused by the blackened walls of the chambers and also to eliminate the shadows cast by the movement of the observer. All test animals were fed to satiation 1 day prior to the bioassays.

Behavioral Bioassay for the Feeding Response

Various concentrations of fish juice, glutamic acid, and taurine were applied onto the antennules of crabs in volumes of 20 µl using a Hamilton repeating syringe fitted with a 25 cm needle in order to determine what behavioral responses were to be used in setting up a scale of feeding intensity. The concentration of feeding stimulant to be used in the bioassays was then adjusted to the lowest level at which a strong feeding response was shown by a crab starved for 2 days. A standardized bioassay technique was then set up.

Eyestalk Ablation Experiments

To determine whether pheromone production commenced after the advent of a premolt induced by eyestalk ablation, both eyes of

intermolt females were removed with a curved scissors and the ocular sockets plugged with cotton balls. These females were then monitored for the onset of premolt and pheromone release.

Thermostability and Dialyzability of the Sex Pheromones

After the initial bioassays had established the presence of a diffusible sex pheromone in both C. magister and P. crassipes, tests for thermostability were conducted by boiling the "active" pheromone-sea water from pre- and post-molt crabs for 15 minutes. The pheromone-sea water was then cooled and aerated before being re-bioassayed. "Active" pheromone-sea waters of pre- and post-molt crabs were dialyzed for 48 hours in a 10°C cold room.

Collection and Isolation of the Sex Pheromone

"Active" female crabs were held for 6 hours in 1 liter of Instant Ocean artificial sea water treated with 0.1 ml of the antibiotic, Garamycin sulfate, which was shown to be effective against the two Pseudomonas species which were not destroyed by 10^{-3} M sodium fluoride treatment. The water was then passed through a 0.65 μ millipore filter in preparation for either liquid-liquid extraction or adsorption onto Amberlite XAD-2 resin.

For isolation by liquid-liquid partition, the pheromone-sea water was extracted three times with an equal volume of n-butanol or diethyl ether:isopropanol (1:1). Each of the organic solvent phases was then twice back washed with equal volumes of distilled water, combined, evaporated, and then either put back into sea water in order to bioassay for activity along with the water phase, or used for further analysis.

The isolation column was set at a slow flow rate allowing the filtered, degassed pheromone-sea water sample to equilibrate with the Amberlite XAD-2 as it passed through the 5 x 50 cm glass column. Amberlite XAD-2, a cross-linked polystyrene polymer with beads of 20-50 mesh and an affinity for the hydrophobic portions of water soluble organic molecules, had the dual function of adsorbing the organic compounds and desalting the sample. This resin was shown to be especially effective in removing polar steroids from salt solutions (Shackleton, Sjövall, and Wisén, 1970). The column was then washed with three void volumes of degassed distilled water to remove the salts. Initially the compounds adsorbed to the column were stripped by elution with three void volumes of 60% ethanol but later it was developed by a stepwise elution using three void volumes each of degassed 5%, 10%, 20%, 40% and 95% ethanol. Each fraction was then evaporated on a rotary evaporator to a small volume, and then either used in bioassay to determine the active fraction or used for further analysis.

Fractionation Columns

Based on an automatic column chromatographic technique for insect steroids developed by Hori (1969), a 0.9 x 100 cm column was packed with Chromosorb 102 (Amberlite XAD-2 ground to 80-100 mesh size) for the purpose of further purifying and fractionating the fractions eluted from the isolation column. An ethanol gradient was pumped at 40 ml per hour for 16 hours by a dual pistoned Isco Dialagrade programmed pump. The eluant was monitored at 254 mμ by an Isco Model UA-2 ultraviolet (UV) monitor and collected on a fraction collector.

The range, steepness, and shape of the gradient varied according to the polarity of the sample being fractionated and was programmed into the pump by adjusting dials controlling the percentage of eluant pumped by piston "B" which was fed by the reservoir containing the initial concentration. The remaining percentage was pumped by the second piston "A" which was fed by the reservoir containing the final eluant concentration. A 20%-80% ethanol gradient was formed by setting piston "B" to the percentage settings of 100, 100, 90, 80, 70, 60, 50, 30, 15, 0, and 0. The adsorbents eluted from the isolation column with 60% ethanol were run with this gradient and the adsorbents eluted stepwise were run with linear gradients of various ranges. Crustecdysone was used as a standard. The fractions were determined by their UV absorbance.

Analysis and Bioassays of the Fractions

After evaporation the fractions from the Chromosorb-102 columns were either tested for "activity" in the pheromone bioassay chambers (Fig. 3) or analyzed by UV spectroscopy, scanning from 340-220 mμ on a Beckman DK-2, and by chromatography on silica gel thin layer sheets. The sheets were developed with n-butanol:acetic acid:water (24:3:10) in a thermostatically controlled, 37°C, thin layer chromatography (TLC) chamber.

Experiments with Tritiated Crustecdysone

Tritiated crustecdysone (crustecdysone-H^3) with a specific activity of 3 curies per mM was purchased from New England Nuclear. The quantity to be injected was drawn off with a Hamilton microliter syringe and mixed with millipore-filtered artificial sea water. The ethanol: benzene (1:9) packaging solution was then evaporated with a fine nitrogen gas stream. P. crassipes intermolt, premolt, and postmolt females were given a dose of 0.8 μg crustecdysone-H^3 containing 1 microcurie (μCi) of tritium while C. magister females in the three stages mentioned and an intermolt C. magister male were given a dose of 2.5 μCi (2.0 μg) crustecdysone-H^3. The dosages were injected into the thoracic cavity through the membrane between the coxa and the merus of the fifth walking leg. The injected crabs were

rinsed twice to remove traces of radioactive material which may have leaked out before the needle hole clotted, and then immersed in sea water for collection of the pheromone. The pheromone-sea water was collected at intervals of 12 hours initially, and subsequently at intervals of 24 hours as the levels of radioactivity decreased.

The pheromone-sea water was next isolated on Amberlite XAD-2 with a stepwise elution of 5%, 10%, 20%, 40% and 95% ethanol. After measuring its conductivity the 5% fraction was fractionated on two 12-ml Dowex-50 x 8 (cation exchanger) column and a 12-ml Dowex-1 (anion exchanger). The fractionation scheme for intermolt-water and pheromone-sea water from crustecdysone-H^3 injected crabs is shown in Figure 4. The various fractions from the ion exchange columns and from the XAD-2 column were halved; each half fraction was then evaporated to 1 ml, added to 10 ml of Bray's solution and counted on a Packard Tricarb 3375 Scintillation Spectrometer. Since the fractions varied widely in polarity, the automatic external standard and a series of tritium standards were used to correct for quenching by the samples.

The other half fractions were either bioassayed, or chromatographed on silica gel (Brinkman F254 or Quantum LQD) using a chloroform:90% ethanol (1:1) solvent system. The thin layer strips were then scanned on a Packard Radiochromatogram Scanner.

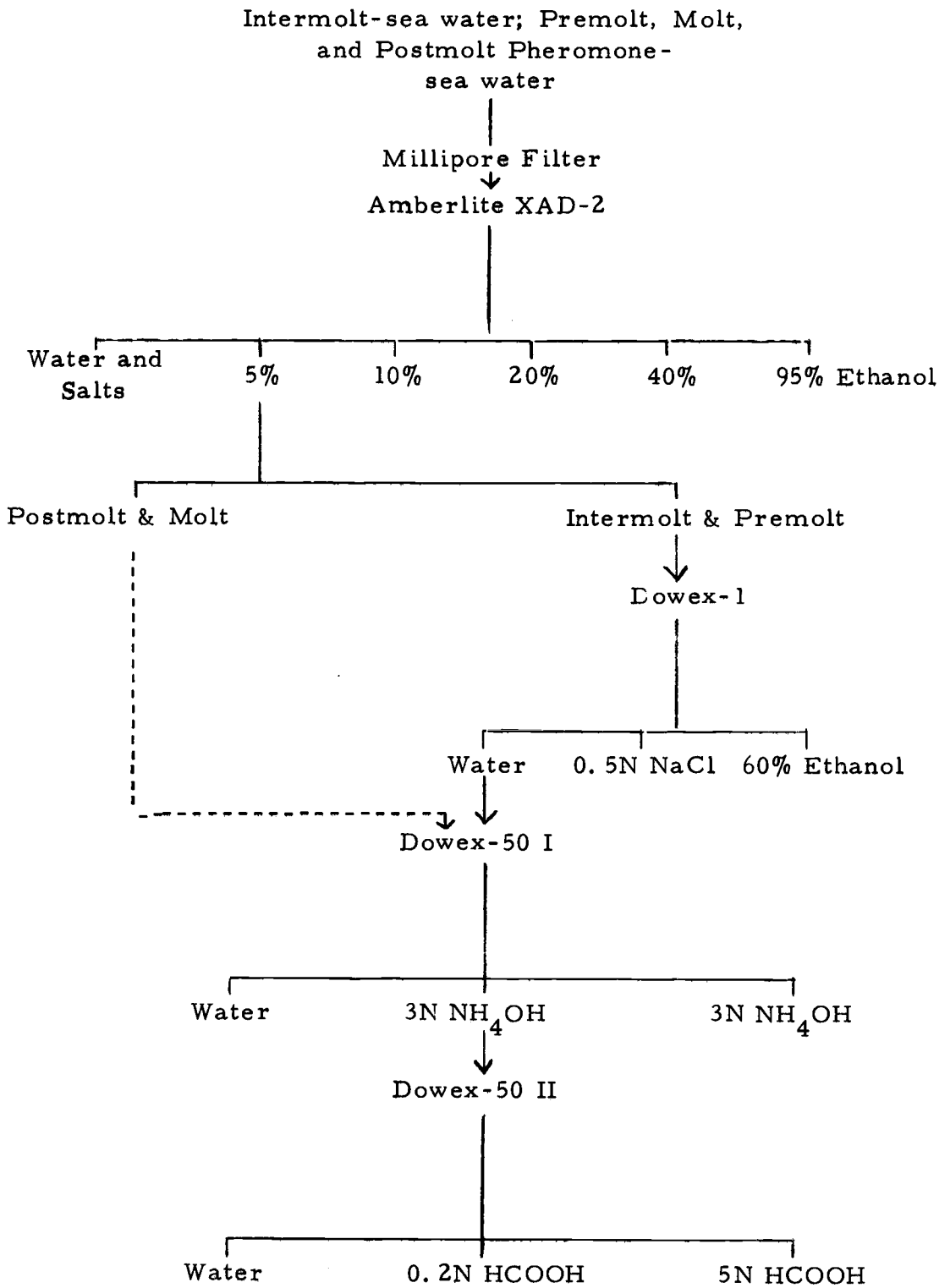


Figure 4. Isolation and fractionation procedure for crustecdysone-H^3 injected C. magister and P. crassipes crabs.

Feeding Inhibition by the Sex Pheromone and Quinones

In the initial observations, it was noted that the sex pheromone, besides releasing mating activity in the male, also seemed to suppress the feeding response. To test this phenomena, C. magister males were immersed in either sea water, pheromone-sea water, or crustecdysone for 20 minutes and then stimulated chemotactilely on the antennules, third maxillipeds, and chelae with a cotton swab dipped in 10% fish juice. The time elapsed from the start of stimulation to the elicitation of the feeding response was recorded. This stimulation was terminated if no response was seen within 30 seconds. A cotton swab with no food stimulus was also used to test the effect of tactile stimulation.

Since the initial experiments with C. magister showed a definite suppression of the feeding response by the sex pheromone and crustecdysone, more extensive inhibition studies were conducted using the standardized feeding bioassay technique (p. 27). P. crassipes males were immersed in 300 ml of test solution in 1-liter beakers layered with 1 inch of glass beads. Since the pheromone-sea water from P. crassipes "active" females inhibited the feeding responses of male conspecifics and intermolt female water did not, crustecdysone concentrations ranging from 10^{-9} - 10^{-6} M were used to determine how concentrations affected the rate at which inhibition of feeding occurred.

Crustecdysone, the postulated sex pheromone of P. crassipes, was shown to give a time-dependent release of mating stance (Fig. 15, p. 52). The effects of short term exposure (1 hour) and long term immersion in dopa, dopaquinone and spinochromes, and substituted naphthaquinones were also investigated. The spinochromes were provided by Dr. Paul Scheuer of the Chemistry Department at the University of Hawaii and the dopaquinone was synthesized by the silver oxide oxidation of 3, 4-dihydroxyphenyl-D, L-alanine (dopa) according to methods devised by Mason (1948). Again, as with the pheromone bioassays, these feeding bioassays were conducted in a semi-dark room. Crabs utilized in these experiments were starved for 2 days, isolated, and tested for positive feeding response prior to use.

RESULTS

Behavioral Observations and Bioassays

Although the expressions released during courtship and feeding and their sequence of release were of primary interest, it was necessary to document all other behavioral expressions or action patterns which preceded or were associated with courtship and feeding in C. magister and P. crassipes, before sex pheromone and feeding bioassays could be established. What is presented here and summarized in Figure 5 is not a complete ethogram. In the case of P. crassipes, the laboratory observations were augmented by field observations. Since the behavioral expressions and sequences of both species, with the exception of the courtship and reproductive behavior sequences, are quite similar, they will be discussed together and illustrated with generalized crab drawings. The sexual behavior sequence of both species will be compared and the courtship behaviors discussed separately.

The release of many of these action patterns is probably triggered by external stimuli. The sensory receptors that are utilized by the crabs in courtship (chemosensory hair on antennules) and feeding (antennules and chemotactile hairs on the mouth parts, dactyls, and chelae) are illustrated in Figure 1B. Also illustrated are the anatomical features which are important in the description of several of the action patterns.

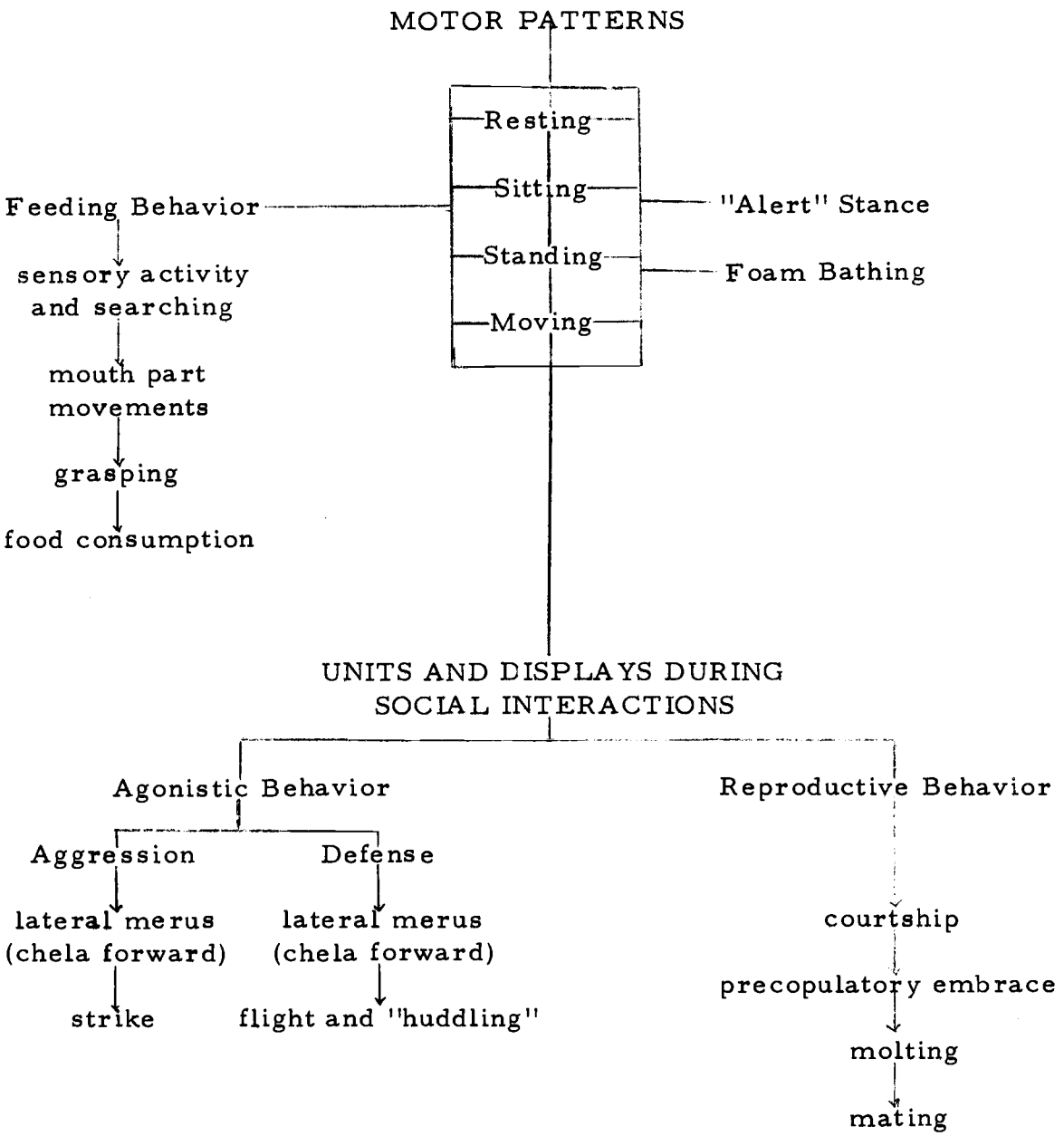


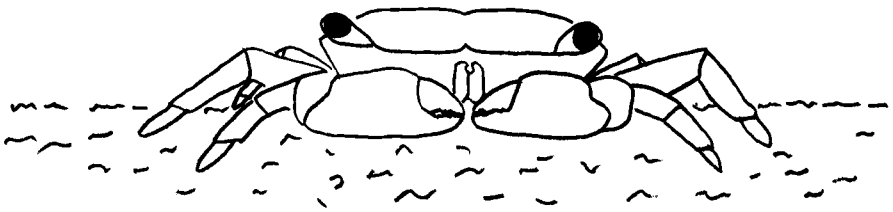
Figure 5. Units and sequences of motor patterns and social displays which were observed in C. magister and P. crassipes. "Alert" stance not seen in C. magister.

Motor Patterns

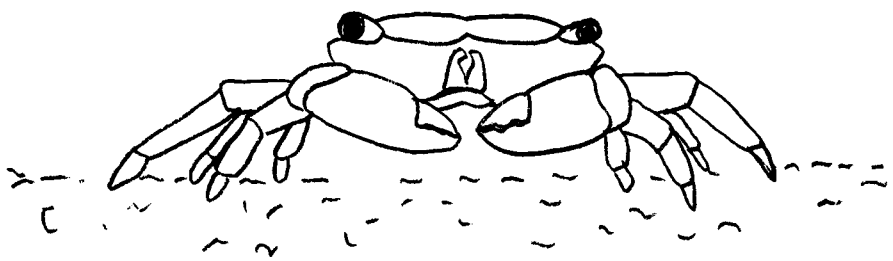
Crabs left undisturbed under laboratory conditions are generally seen resting, sitting, standing, or moving about. In the resting state the crab sits on the substrate with its eye tucked into its ocular pits, antennae motionless, and antennules withdrawn seemingly oblivious to its environment. When sitting, standing, or moving about (Fig. 6A & B) the crab monitors environmental stimuli through its raised eyes and low frequency flicking of its antennules. Antennae movements are minimal.

A motor pattern seen quite often and mainly in P. crassipes is an "alert" posture (Fig. 6C) which varies somewhat from the normal standing posture. Seemingly, in the absence of any releaser stimuli, the crab elevates itself high above the substrate by standing on the tips of its dactyls. The chela may be flexed against the cephalothorax or, as observed more frequently, extended forward with the chelaped tips on the substrate. In this posture the eyes, antennae, and antennules are raised to their maximum elevations. This does not necessarily lead to any other form of activity.

Foam bathing, also observed in other semi-terrestrial crabs (Brownscombe, 1965; Schone and Schone, 1963; Wright, 1966), occurs when P. crassipes is out of water. The foam originates in the region of the mouth parts and is spread over the body of the crab.



A



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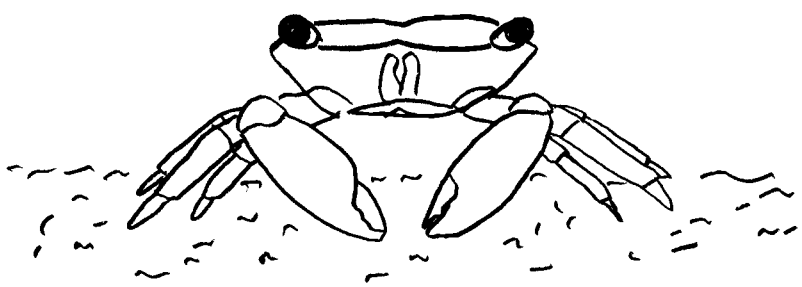


C

Figure 6. Motor patterns observed in C. magister and P. crassipes. Sitting (A), standing or ambulation (B). "Alert" posture (C) observed in P. crassipes only.

The feeding sequence, similar in both species, was most closely observed in P. crassipes males. Introduction of fish juice into the tank led initially to an increased frequency of antennular flicking, followed by mouth part movements and search behavior. With a higher stimulus intensity, grasping motions and motions of chelae toward the mouth parts were observed. If a piece of fish was offered and subsequently grabbed, the sequence ended in the consumption of the fish.

The Feeding Bioassay

On the basis of Case's (1964) and Levendowsky and Hodgson's (1965) results with the dactyl chemoreceptors of C. magister, initial tests were performed using L-glutamic acid as a stimulant. These tests proved inconclusive when applied to the antennules at 10^{-3} and 10^{-4} M. After testing various concentrations of taurine and using the observations previously recorded, a standardized bioassay technique was established. The criteria for this standardized quantified bioassay was the use of: 1) 1-liter beakers lined with 1/2 inch of glass beads; 2) crabs which were starved for 2 days and which gave positive feeding responses prior to use in an experiment; 3) a semi-dark, indirectly illuminated room; and 4) 3×10^{-3} M taurine applied in 20 µl quantities on the antennules of crabs starved for 2 days, and grading the responses which occurred within 5 seconds on a feeding intensity

scale of 0-5 based on movements of the mouth parts and chelae (Table 2). Although higher concentrations of taurine up to 10^{-2} M released a more intense and persistent feeding response, it was felt that a minimum concentration should be used in order to avoid the possible stimulation of chemotactile receptors elsewhere on the crab.

Agonistic Displays

Unlike the motor patterns hitherto presented, agonistic and courtship displays serve a communicative function and are closely related in appearance to each other. This is especially the case in C. magister where the "aufbaum reflex" (Bethe, 1897), renamed and redescribed as the lateral merus displays by Wright (1968), are used with minor differences for both courtship and agonistic displays. In accordance with other examples of lateral merus displays described in the survey of the Brachyrhyncha (Wright, 1968), the lateral merus displays in C. magister begin with the meri extended laterally, the chelipeds extended anteriolaterally and in a horizontal plane from the anterior portion of the cephalothorax against which it is normally held. The intensities of the displays (low, mid, and high) are determined by the extent of the extension and the high intensity displays are depicted in Figure 7A & B. A strike against the target of this display is carried out by the rapid flexion of both chelipeds. Occurring at times with the lateral merus display is a quick jerk of the

Table 2. Quantification of the feeding response of P. crassipes.

Feeding response	Feeding intensity	Description
Negative	0	No movement associated with feeding except antennular motion.
Negative	1	Minimal movement of maxillipeds and maxillae.
Positive	2	Movement of maxillae; maxillipeds sweep over antennules.
Positive	3	Movement of maxillae; maxillipeds sweep from oculars inward.
Positive	4	Movement of maxillae and maxillipeds and grasping and movement of chelae to the mouth parts.
Positive	5	Movement of the maxillae and maxillipeds; active grasping and movement of the chelae to the mouth parts which persists for a time. *

*Rarely seen at the low concentration of 3×10^{-3} M of taurine used.

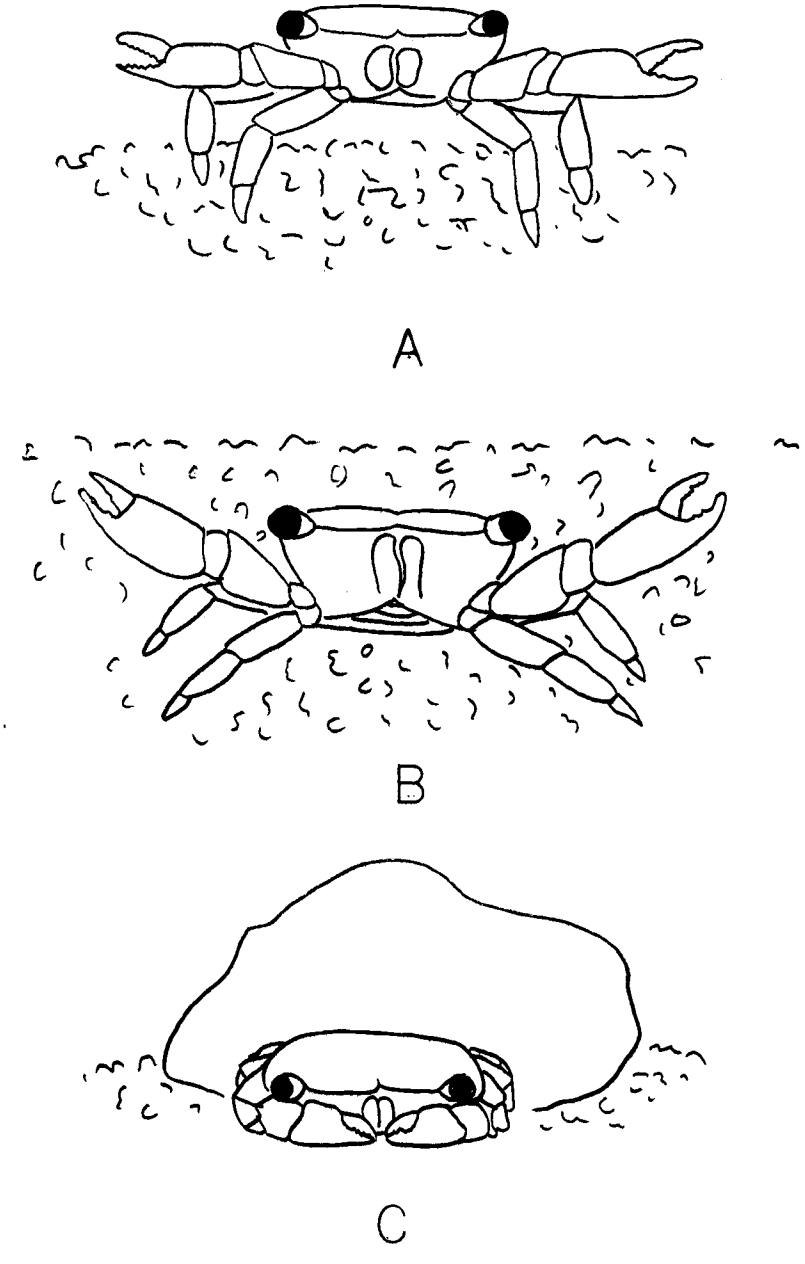


Figure 7. Agonistic displays observed in C. magister and P. crassipes. Agressive posture (A); posture containing both aggressive and defensive elements (B); defensive posture (C).

anterior portion of the cephalothorax. This jerking is effective as an agonistic signal even when both chelipeds are missing. In contrast to the lateral merus displays which contain both aggressive and defensive elements, is the seemingly purely defensive or submissive posture termed "huddle posture" by Wright (1968). In this behavior pattern the crab assumes a low profile, generally with its posterior to a rock or other protective object, drawing its appendages under and its chela close against the anterior of its cephalothorax, exposing only its hard dorsal carapace and chelae to attack (Fig. 7C). These agnostic displays, which are significant inter- and intraspecifically among many decapods, can also be elicited from P. crassipes. Another form of agonism, pushing with flexed chelae, has been observed in P. crassipes. The chelae are much larger in proportion to the body in P. crassipes than in C. magister. Shone (1968) describes this as a more formalized agonistic display.

Sequence of Events during Reproduction

Whenever reproductive activities of the decapods are discussed, the intermolt cycle must be considered since the two events although agonistic are closely coordinated. The scheme presented in Figure 8, which focuses on the female ecdysis as a zero point, compares in C. magister and P. crassipes the onset and duration of various events associated with reproduction--the span of "attractiveness" of

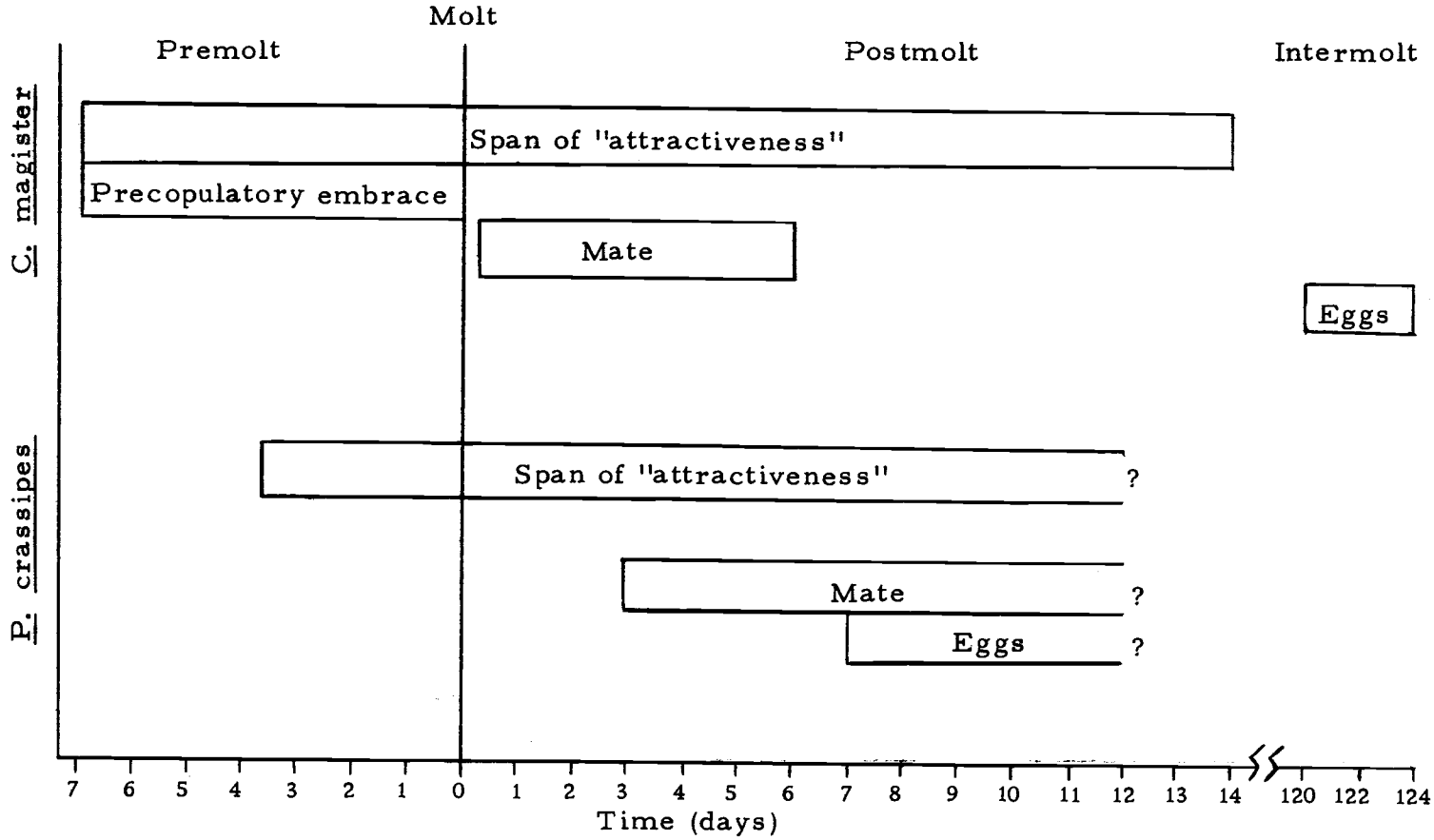


Figure 8. Comparison of the onset and duration of reproductive events in relation to molt (time 0) in C. magister and P. crassipes females. Data represent earliest observation and longest duration of crabs' individual behavior occurring under laboratory conditions.

the female to the male and the onset and duration of time when the male is induced to release precopulatory and mating behavior.

In C. magister, the sequence of events observed began with the precopulatory embrace (Fig. 9A) of premolt females by the conspecific males. After being held in this embrace for 5-7 days, the females molted within the protection of the males (Fig. 9B). The molting times ranged from 2-6 hours. After a short delay (up to 3 hours in some cases) copulation (Fig. 10) commenced and lasted from 1/2 - 1 hour after which the males either held the females in the precopulatory position or released them. One male may mate several times with a female, resting between each copulatory act. The females remain "attractive" to males for approximately 1-2 weeks after molting, but tolerate grasping by the male and copulation for only 5-6 days postmolt. The "attractiveness" of the females seems to wane gradually and they are better able to escape the grasp of the males. Approximately 4 months later, during the intermolt period, the eggs are deposited.

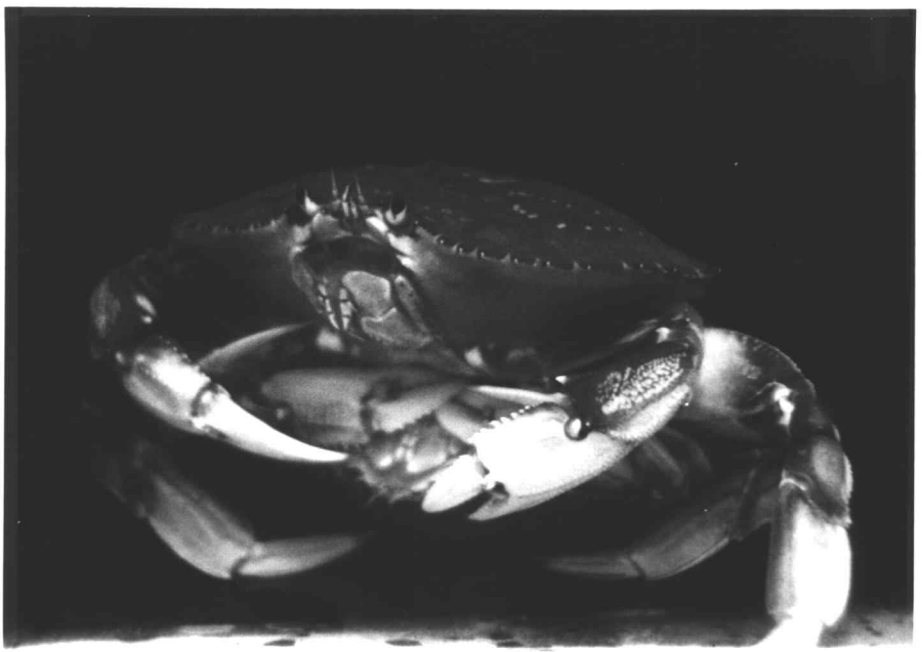
In P. crassipes, precopulatory embraces were observed only infrequently in the field and never in the laboratory although the premolt females' presence released courtship displays by the males. Females found in the premolt state molted within 3-4 days and did so within 15 to 30 minutes. Although the presence of the postmolt females continued to release courtship displays by the males, the

A

B

Figure 9. Reproductive behavior. Premolt C. magister female held in a precopulatory embrace (A) and female molting within the protective grasp of a male (B).

A



B

Figure 10. Reproductive behavior. Copulatory act in C. magister. Anterior (A) and posterior (B) aspects.

females avoided the males and mating did not occur until approximately 3-5 days had elapsed. This delay allowed time for the soft postmolt female's exoskeleton to harden somewhat. At this time a qualitative change in the female's "attractiveness" was observed making the males more persistent and the females more apt to participate in courtship activities which led to mutual grasping. Copulation which lasted from 15-45 minutes occurred 2-3 times with 15-20 minute rest intervals between each act of copulation (Fig. 11). The females remained "attractive" and mated with various males in the interval from 3 days postmolt to 10-12 days postmolt after which time the female deposited her eggs. Instances of copulation between gravid females and males were also observed.

Courtship Behavior and the Sex Pheromone Bioassay in *C. magister*

Courtship displays are closely related or may have been derived from agonistic displays according to Schone (1968) but, in contrast to agonistic displays, courtship displays are performed at a slow pace devoid of rapid and jerky motion except occasionally at the instant of capture of the female. Using a series of males in the 20-liter bioassay chamber (Fig. 3B), the "active" pheromone-sea water collected over a period of 6 hours from "active" pre- and postmolt females, the urine of these females, and these females themselves were bioassayed.

A

B

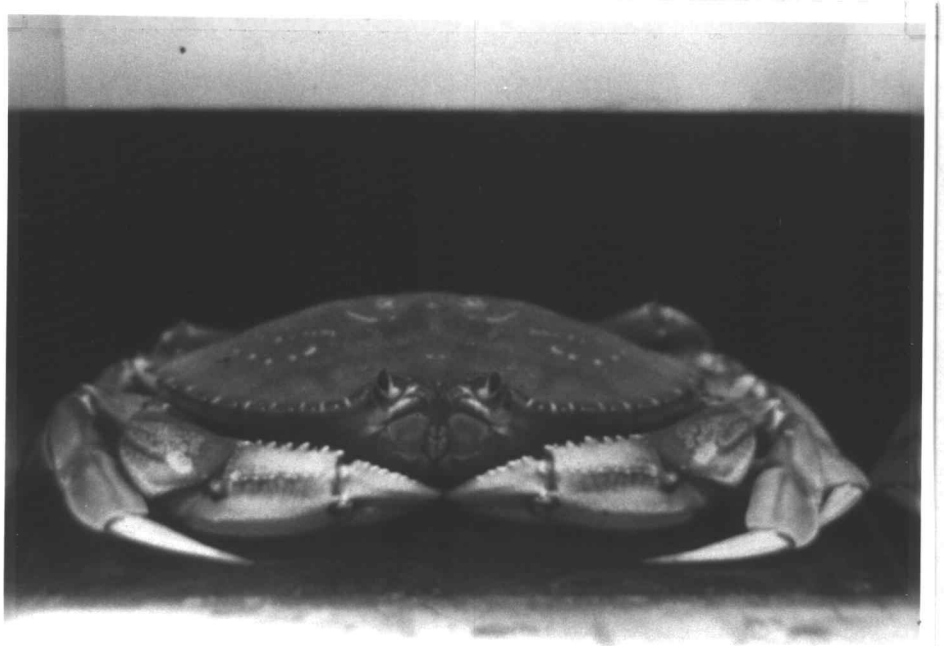
Figure 11. Reproductive behavior in P. crassipes. Copulatory act with female above the male. Posterio-lateral (A) and anterior (B) aspects.

The release of courtship behavior by the males as a result of exposure to the "active" pheromone water established the existence of the sex pheromone in C. magister females.

When stimulated by the pheromone, the males displayed searching, arm waving, and mating stances which included either low, mid or high intensity lateral merus displays (Fig. 12 & Fig. 13). The opening of the third maxilliped, best illustrated in Fig. 13B, vigorous pumping of water through the branchial chamber, and high frequency of antennular flicking accompanied the display. Waving of the abdomen was seen infrequently.

Introduction of an "active" premolt female elicited the courtship response described above. Although not always the case, the high intensity lateral merus was generally released on approach to the female and was followed by the grasping of the female. If the female attempted to escape the male would roughly slap the female's dorsal carapace with both chelae, an act which seemed to subdue the female. Aside from the rough slap, the male never pinched the female hard enough to cause injury when positioning or holding a resisting female. At times, the female initiates activity by approaching the male. The same sequence was followed when a postmolt female was introduced, but in this situation mating follows. If no "active" female was available and the male was highly stimulated by the pheromone, the

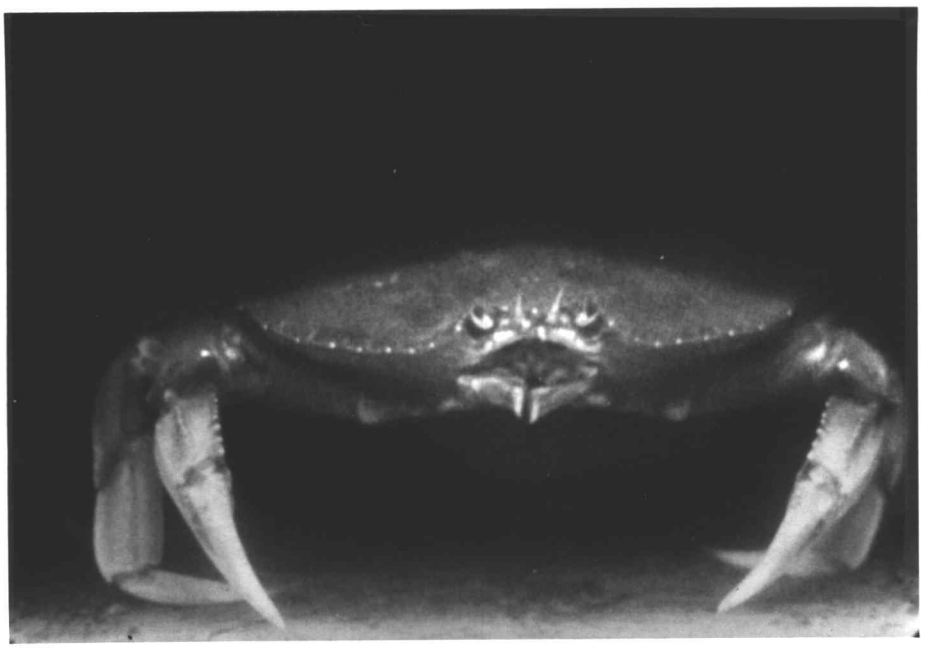
A



B

Figure 12. Courtship behavior in C. magister. Male sitting (A) and displaying a low intensity mating stance (B) in the 20-liter bioassay chamber.

A



B

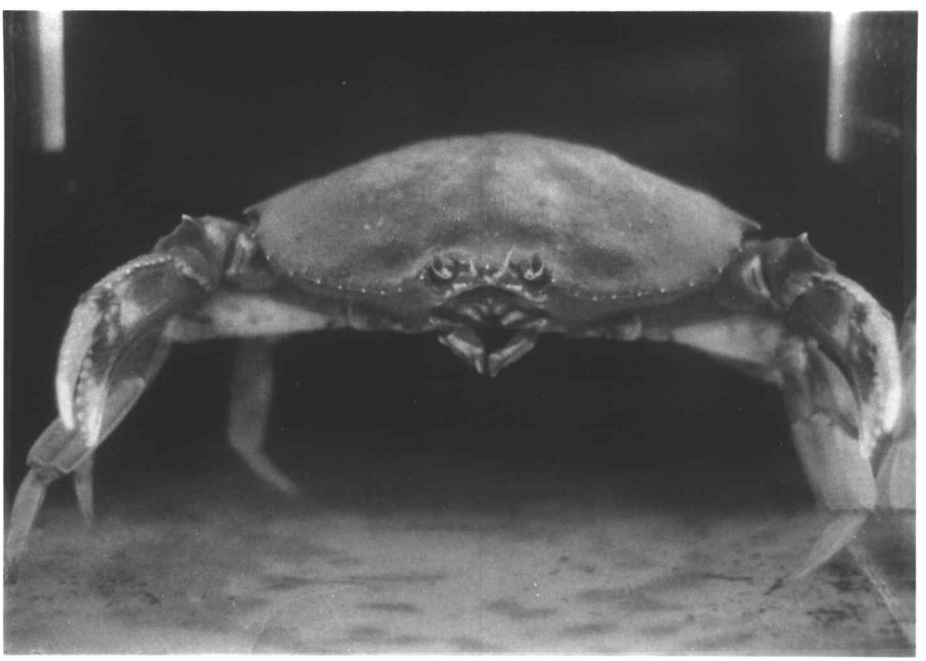


Figure 13. Courtship behavior in C. magister. Males displaying a mid intensity mating stance (A) and a high intensity mating stance (B) in the 20-liter bioassay chamber.

male grasped rocks, other inanimate objects, and other males. This behavior, however, does not persist.

Using these observations, an attempt was made to quantify the bioassay by timing the release of the mating stance. This attempt, for reasons which will be discussed later, gave inconsistent results so the bioassay for the pheromone of C. magister was conducted utilizing the release and intensity of the mating stance to indicate the presence or absence of the pheromone.

Courtship Behavior and the Sex Pheromone Bioassay in P. crassipes

In the presence of an "active" female or "active" pheromone-sea water collected over a period of 6 hours, the conspecific male stood on the tips of its dactyls, raised its body and the fifth pair of walking legs high above the substrate and thrusted its chelipeds forward at an angle of 45-90^{o} to the substrate (Fig. 14A). The tips of the chelipeds rested on the substrate. A high frequency of antennular flicking, rapid pumping of water through the branchial cavity, and the waving of the abdomen occurred.

Although the mating stance was released by the presence of premolt and early postmolt females, precopulatory embraces and copulation was not seen due to the reluctance of the female to be grasped and held. If a female in the later stage of postmolt was used, a

A

B

Figure 14. Courtship behavior in P. crassipes. Male in mating stance waving abdomen (A). Precopulatory interaction of a male and postmolt female (B).

courtship dance first described by Bovbjerg (1960) was observed. The male displayed the mating stance and waved his abdomen while very slowly and deliberately approaching the female, which was in a lower stance with her chelipeds held forward and vertical to the substrate at the same time waving her abdomen (Fig. 14B). A back and forth movement with the chelae of male and female occasionally touching occurred for periods up to 30 minutes or longer. The male also used the dactyls to "feel" the female's legs and carapace. During this behavior sequence, the male placed the tips of his chelae under his body obscuring all aggressive signals. The dance was concluded either by a mutual agreement to mate, in which case the male rolled over on its back as the female clambered over, or by the male grasping the female and then rolling on its back (Fig. 11). Often the male took a more direct approach by quickly grasping the female and copulating. Males released resisting females rather than pinching them. Any semblance of aggression was gone during copulation. This occasional absence of courtship activity prior to mating was also reported by Hiatt (1948).

The attempt to quantify the bioassay proved more successful for P. crassipes than for C. magister due to the fact that for a given concentration of "active" pheromone-sea water the series of males used released their mating stance at approximately the same time. The quantitative bioassay therefore recorded the length of time

necessary for the release and maintenance of the mating stance after exposure to the pheromone. The procedure established for the quantitative bioassay involved filling the 4-liter bioassay chamber (Fig. 3A) to a point above the indented niche with the solution to be tested, gently immersing a male crab which immediately scrambled under the niche, and observing and timing the response. When sufficiently stimulated, the male crab could be observed venturing forth and searching and eventually assuming the stationary mating stance.

Pheromone Activity in *C. magister* Assayed in a Flowing-water System

A flowing water bioassay system (Fig. 2) was utilized to make absolutely certain that the mating stance displays and subsequent sexual activities were mediated by diffusible chemical substances rather than by chemotactile or visual cues from the female, and to determine the times and nature of the release of these diffusible chemicals. The system allowed the observation of the activity of a mating pair or an "active" female in the "action" tank and the responses of the males in the "reaction" tanks to the effluent water from the "action" tank.

Low intensity mating stances, arm waving, and search behavior were observed in males exposed to the effluent from the "action" tank containing a premolt female. After an overnight cessation of

the flow with the premolt female remaining in the "action" tank, the flow was again resumed resulting in high intensity mating stances, search, and grasping of other males. After allowing 2 hours for the complete turnover of the water in the 12 liter "action" tank, the flow was redirected to another "reaction" tank containing unstimulated males which responded by releasing low intensity mating stances and search patterns. When a male, introduced into the "action" tank with the premolt female, grasped and slapped the female into a precopulatory embrace, the males in the "reaction" tank released high intensity mating stances and grasping behavior. Grasping and agitation by the observers did not elicit the same response from the males in the "reaction" tank. If a mating pair in a precopulatory embrace was left undisturbed in the flowing water system, the males in the "reaction" tank irregularly released low intensity mating stances and search behavior. These observations seem to indicate that the premolt female intermittently leaked a small quantity of pheromone when undisturbed or within the embrace of a male but released a larger quantity when grasped and slapped by the conspecific male.

As the female molted within the protection of the male in the "action" tank, males in the "reaction" tank displayed arm waving, low and mid intensity stances and grasping attempts. It cannot be said with certainty that there was, at this point, a release of another pheromone which could cause the male to loosen precopulatory

embrace and allow the female to molt sitting on the substrate within the loose protection of the male's chelae. From the observations of three molting females, it did appear that the males in the reaction tank displayed more frequently the mid intensity stance which closely resembled the loose hold that the male in the "action" tank had on the molting female.

While the male introduced into the "action" tank quickly grasped and copulated with the newly molted female, other males in the "reaction" tanks responded very strongly to the effluent, displaying arm waving, extensive searching, high intensity stances, and grasping of other males. Although the responses of the males in the "reaction" tank closely resembled the responses of the males to the effluent from the premolt females, it seemed plausible that another pheromone which elicited copulation was released by the female after molting. This was partially substantiated by the observation of a male grasping a 3-inch diameter clay plug and pushing it into the copulatory position. This response was never observed in males stimulated by the premolt pheromone.

In order to test the assumption that a copulation releasing pheromone different from that which released precopulatory behavior, a postmolt female was placed in one-half of a 12-liter aquarium separated by a clear perforated plexiglass divider from a premolt female and a male in a precopulatory embrace. The postmolt female

attempted to get into the part where the male was holding the premolt female. After an unspecified period (time necessary for the aerator to circulate water through both halves), the male pushed the premolt female into the copulatory position. No mating attempts followed, however. When the barrier was removed, the postmolt female approached the male and after contact with the male's chela, the male dropped the premolt female and grasped and mated with the postmolt female. There is, seemingly, a need for the input of either tactile or chemotactile information before the act is consumated.

Controls of intermolt males and females elicited absolutely no response from either the males or females in the "reaction" tank. Monitoring the females' response to the effluents indicated no response to the effluent from the premolt female but search and arm waving displays to the effluent from molting females and postmolt females.

A single male in a "reaction" tank often would show no response to pheromone effluents even though high intensity responses were observed to the effluent in another "reaction" tank containing several males. To test the effect of a visual input, an old washed oyster shell tied to a nylon line was dangled near the non-responding male. No motion or a rapid spin of the asymmetrical shell elicited no response from the male but a slow motion not unlike the motion of courting crabs quickly elicited a high intensity stance directed at the shell.

The flowing water experiments indicate the presence of two or possibly three pheromones released by the female over the course of its reproductive period encompassing premolt, molt, and postmolt and show the importance of the visual input in the elicitation of courtship behavior by the males.

Eyestalk Ablation Experiments

There is a close inter-relationship between molting and mating in C. magister females. In premolt a pheromone is produced which elicits from the male the precopulatory embrace of the female and subsequent to molting a pheromone is released which elicits the copulatory behavior of the male. An attempt was made, therefore, to induce the premolt condition in the females by eyestalk ablation in order to determine whether the premolt sex pheromone could be produced in this way.

Only three C. magister intermolt females out of a total of ten survived the eyestalk ablation. Mortalities were not directly attributable to the operation itself since most crabs survived a week or more and a few died due to failure of the flowing water and aeration systems. An average time of 21 days (ranging from 18-26 days) elapsed before pheromone production began as shown by the release of precopulatory behavior by the male. All these showed signs of premolt. Since mortalities were very high these experiments were not

pursued further. Autopsies indicated that ovarian development was stimulated. These limited results seemed to strengthen the hypothesis that either the molting hormone, crustecdysone, or a conjugated or metabolite of it was probably also the sex pheromone. Certainly during the period of premolt and molting it would be more "economical" to use one compound for both functions.

Bioassay of Crustecdysone

Since reproductive activity and molting are so closely interwoven, crustecdysone, the molting hormone of crustaceans, was bioassayed in order to determine whether or not it also functioned as the sex pheromone during the reproductive process. Using the standardized sex pheromone bioassay techniques, both C. magister and P. crassipes males were subjected to various concentrations of crustecdysone.

C. magister males released low intensity mating stances in four of the nine exposures to crustecdysone (Table 3). After 20 minutes a small intermolt male, which was forced to autotomize its chelae and two pairs of walking legs, was introduced to the non-responding males and this released grasping responses from three of the five hitherto non-responding males. This modification of the standardized bioassay procedure will be discussed later. Due to the high cost of crustecdysone, these bioassays were not pursued any further. The results,

Table 3. Bioassay of 10^{-6}M crustecdysone for sex pheromone activity using C. magister males. Time elapsed prior to the release of the mating stance and time required for non responding males to grasp "model female."

Crab no.	Time for release of the mating stance (min)	Time for grasping of "model female" (min)	Positive responses
1	8.5	n. r.*	+
2	n. r.	7.0	+
3	2.5	n. r.	+
4	n. r.	3.5	+
5	n. r.	n. r.	-
6	2.5	2.3	+
7	2.3	n. r.	+
8	n. r.	n. r.	-
9	n. r.	3.0	+
	4/9	4/9	7/9

*n. r. = no response

however, seemed to support the hypothesis that crustecdysone was or had properties similar to the sex pheromone. Alternate hypotheses, that the sex pheromone molecule was a catabolite of crustecdysone or that crustecdysone was conjugated to another compound, were proposed at this point.

P. crassipes, on the other hand, displayed a remarkably consistent release of the mating stance. The time dependent release of the mating stance as a function of concentration of crustecdysone is shown in Figure 15. Unlike C. magister, P. crassipes was highly stimulated by crustecdysone and continued the search, stance, and grasping of other crabs, stones, and other inanimate objects even when removed to the holding tanks, lending strong support to the hypothesis that it was indeed the sex pheromone.

Isolation, Fractionation, and Characterization of the Sex Pheromone

Due to their volatility, the airborne pheromones of insects readily lend themselves to cold trap isolation and gas chromatographic analysis. The sex pheromones of both C. magister and P. crassipes, on the other hand, must be freed from the salt in the urine or sea water fluids into which they are released, since salts interfere with the analysis of organic compounds. Salt removal and concentration of the sex pheromone were the initial problems encountered.

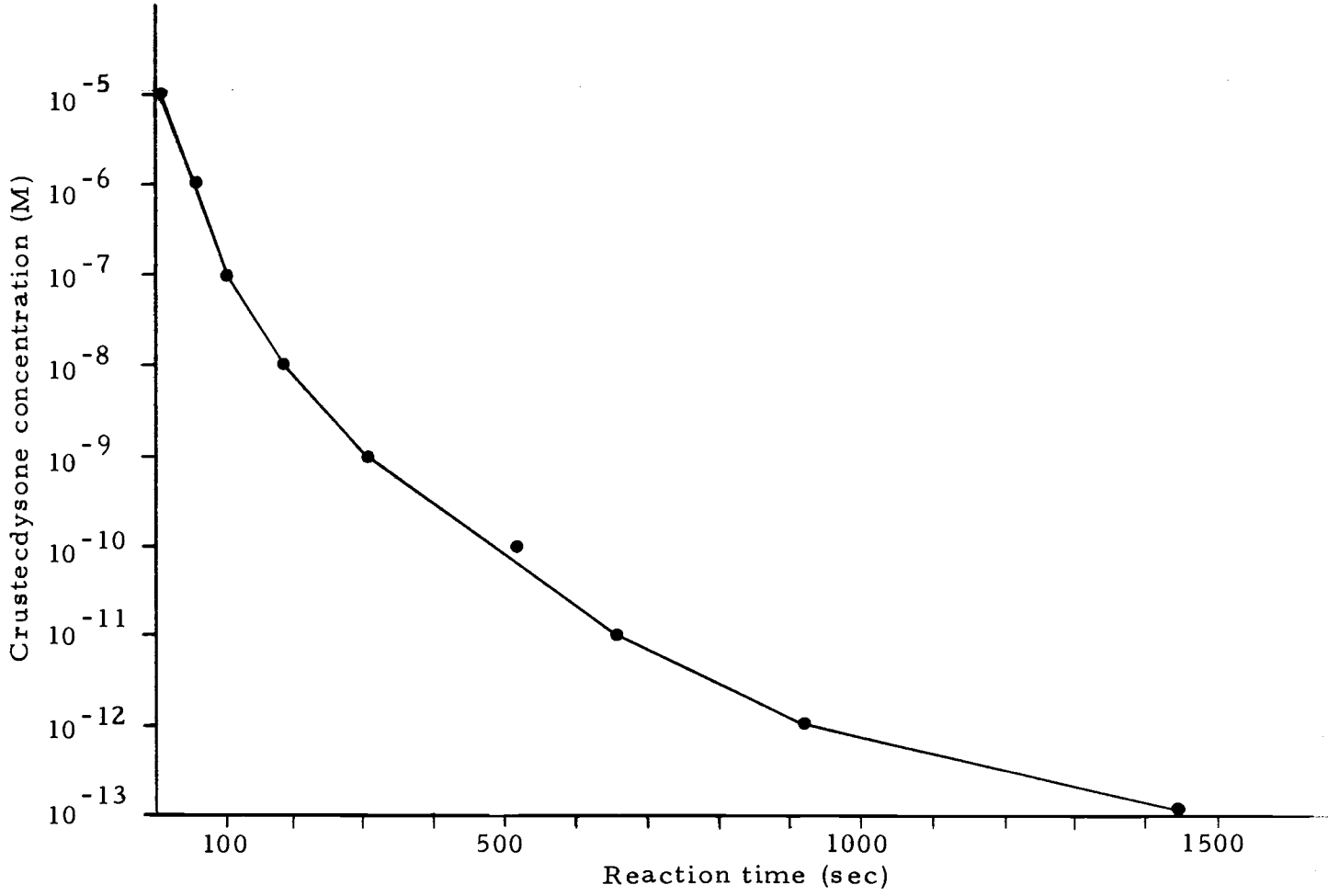


Figure 15. Bioassay of crustecdysone using P. crassipes males. Time elapsed prior to the release of the mating stance. (Kittredge, Terry, and Takahashi, 1971)

Thermostability, Dialyzability, and Solubility

The "active" pheromone-sea water, collected from pre- and postmolt females of both crab species, dialyzed and proved to be stable after 15 minutes of boiling at 95-100°C. C. magister males released search, stance, and grasping behavior and P. crassipes males released mating stances in response to both unheated and heated pheromone-sea water from the "active" pre- and postmolt females.

The partitioning of the sex pheromone-sea water collected from C. magister with either isopropanol-ether or n-butanol showed that the sex pheromone was soluble in both organic solvents. Bioassays of the organic phase elicited moderate and high intensity courtship displays while the aqueous phase elicited only low intensity displays. The partitioning of the pheromone-sea water collected from P. crassipes yielded similar results.

Initial Isolation, Fractionation, Analysis, and Bioassays

Sufficiently encouraged by the thermal stability and the somewhat hydrophobic nature of the pheromone, isolation of the pheromone-sea water proceeded using the Amberlite XAD-2 resin. Sea water in which intermolt, premolt, molting, or postmolt females had been

immersed for 6 hours was allowed to equilibrate with the resin and after washing off the salts, the pheromone and other compounds adsorbed to the resin were eluted with 60% ethanol, concentrated to 2 ml in 20% ethanol, and fractionated on Chromosorb 102 using a programmed ethanol gradient ranging from 20%-80%. One hundred micrograms of crustecdysone were also developed on the Chromosorb 102 column. The volume of eluate per fraction was the function of the U. V. adsorption displayed by the UA2 U. V. monitor and therefore the eluate, collected within the limits of the ethanol gradient program, was arbitrarily divided into five parts--fractions A, B, C, D, and E. Each fraction was then bioassayed for "activity" with the greatest interest focused on the "C" fraction since it was in this fraction that the crustecdysone standard also eluted.

For C. magister, the pheromone-sea water from premolt, molting, and postmolt females developed on Chromosorb-102, all showed strong U. V. absorbances in the "A" (20%-25% ethanol) and "E" (56%-73% ethanol) fractions (Figs. 16, 17, and 18). The premolt females (Fig. 16) showed a high U. V. absorbance (0. 17) in the "C" fraction (33%-40% ethanol), into which crustecdysone also eluted; the molting females (Fig. 17) showed essentially no absorbance (0. 01) in the "C" fraction, and the postmolt females (Fig. 18) showed a low absorbance (0. 04) which did not have the same peak as crustecdysone. Intermolt females showed low absorbances in fractions "A" (0. 05) and "C" (0. 02)

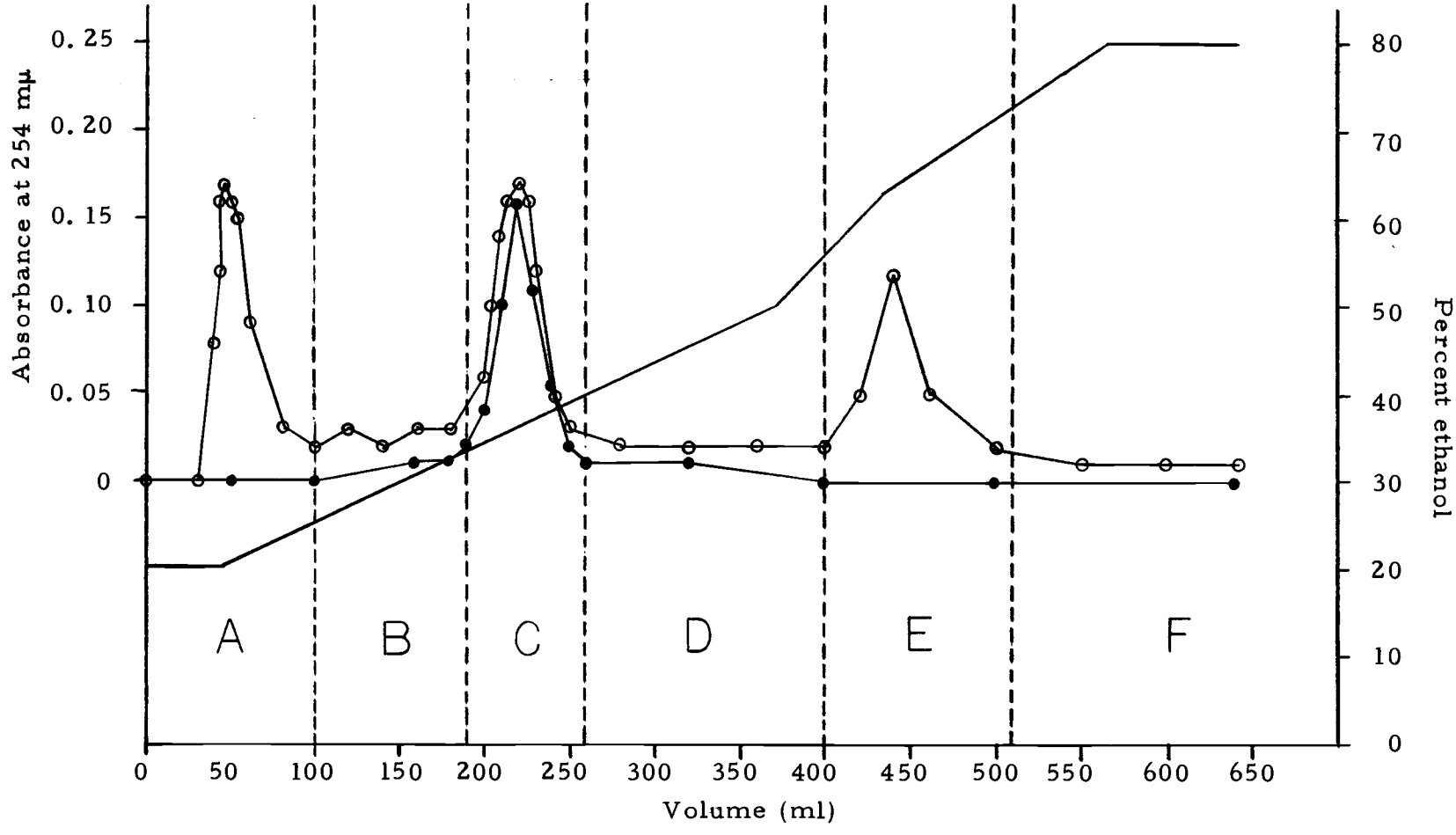


Figure 16. Premolt C. magister female pheromone-sea water isolate fractionated on Chromosorb-102. Capitalized letters denote fractions. Pheromone-sea water o—o; crustecdysone ●—●; alcohol gradient —— .

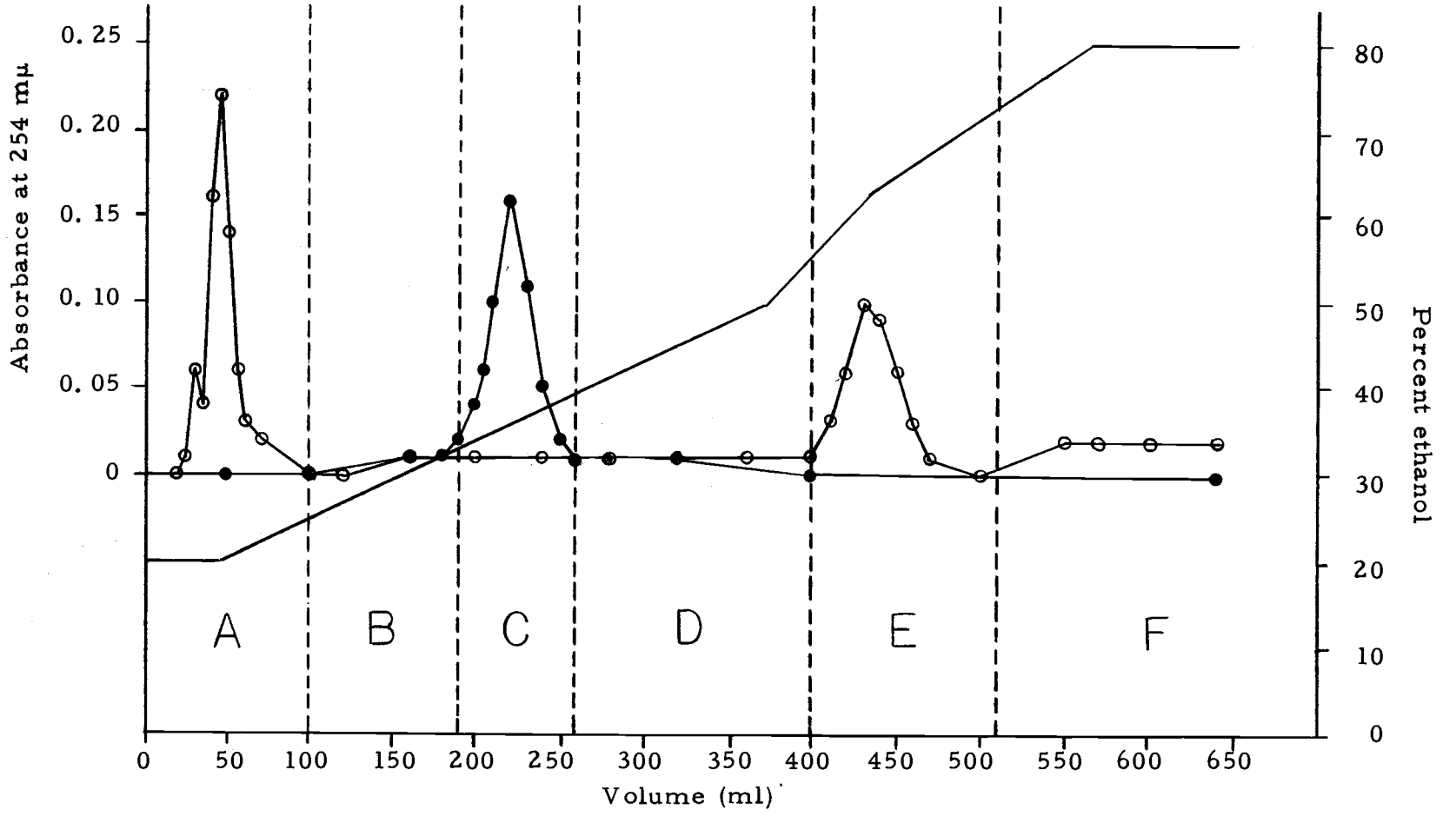


Figure 17. Molting C. magister female pheromone-sea water isolate fractionated on Chromosorb-102. Capitalized letters denote fractions. Pheromone-sea water o — o; crustecdysone ● — ● ; alcohol gradient — .

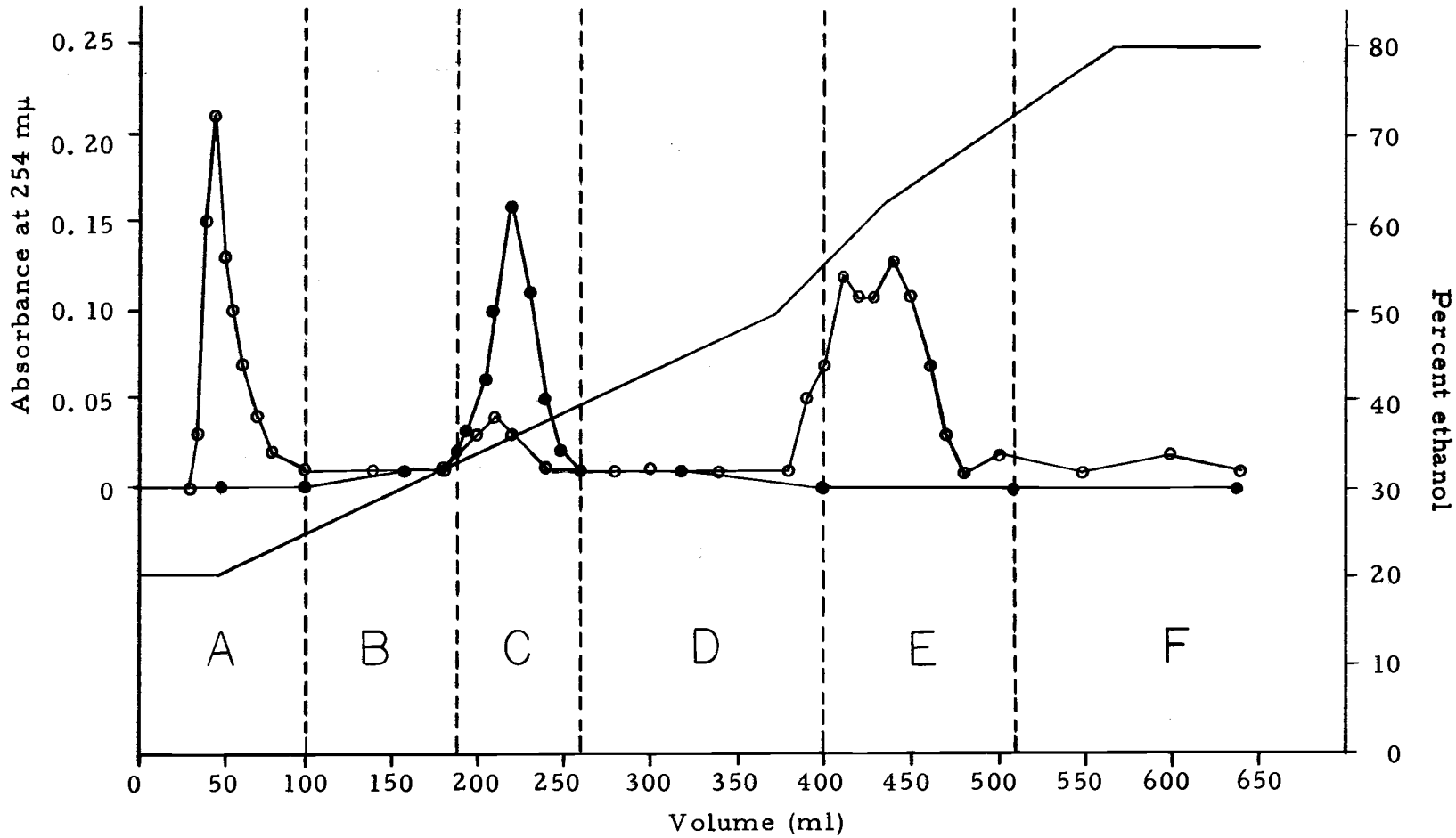


Figure 18. Postmolt C. magister female pheromone-sea water isolate fractionated on Chromosorb-102. Capitalized letters denote fractions. Pheromone-sea water o — o; crustecdysone ● — ● ; alcohol gradient — .

while showing a high absorbance (0.25+) in fraction "E." "E" was quickly identified on TLC and U. V. spectral analysis as a component of Garamycin, the antiobiotic which was used because of its wide ranging action against Pseudomonas spp. and other bacterial species.

Pre- and postmolt P. crassipes samples were treated in a like manner but the results were not as clear cut. These crabs are not usually held in a precopulatory embrace and therefore remain active and feed until 1-2 days prior to molting. By the time the signs of the premolt state are evident and the "active" premolt females isolated for pheromone collection, their stomachs still contained food and they continually released feces into the water. Compounds from the feces, a large proportion of which contained algal matter, probably contributed heavily to the U. V. absorbing moieties and caused the many U. V. peaks seen in Figure 19. Nonetheless, strong "A" (0.25+) and "C" (0.25+) fraction peaks were observed. The "C" peak did not precisely correspond to the crustecdysone peak. Since postmolt animals did not feed for approximately 5-7 days, the postmolt pheromone water was much "cleaner" showing a strong "A" peak (0.24) but essentially no "C" (0.02) peak (Fig. 20).

The results at this point seemed to confirm the hypothesis of similarity in molting hormone and sex pheromone; i. e., the premolt C. magister females had a strong U. V. peak corresponding to that of crustecdysone and the premolt P. crassipes females showed U. V.

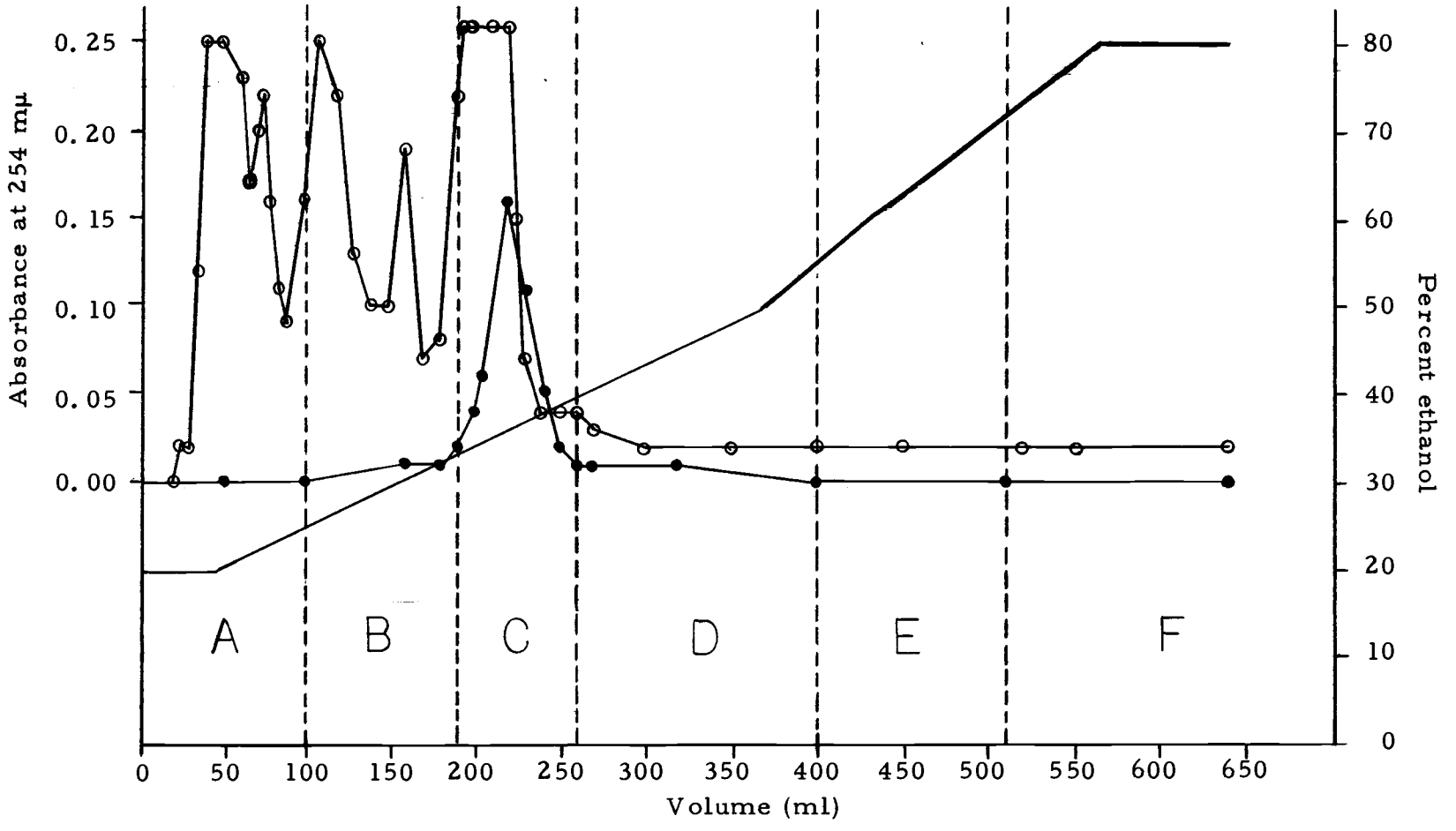


Figure 19. Premolt P. crassipes female pheromone-sea water isolate fractionated on Chromosorb-102. Capitalized letters denote fractions. Pheromone-sea water o–o; crustecdysone ●—●; and alcohol gradient —.

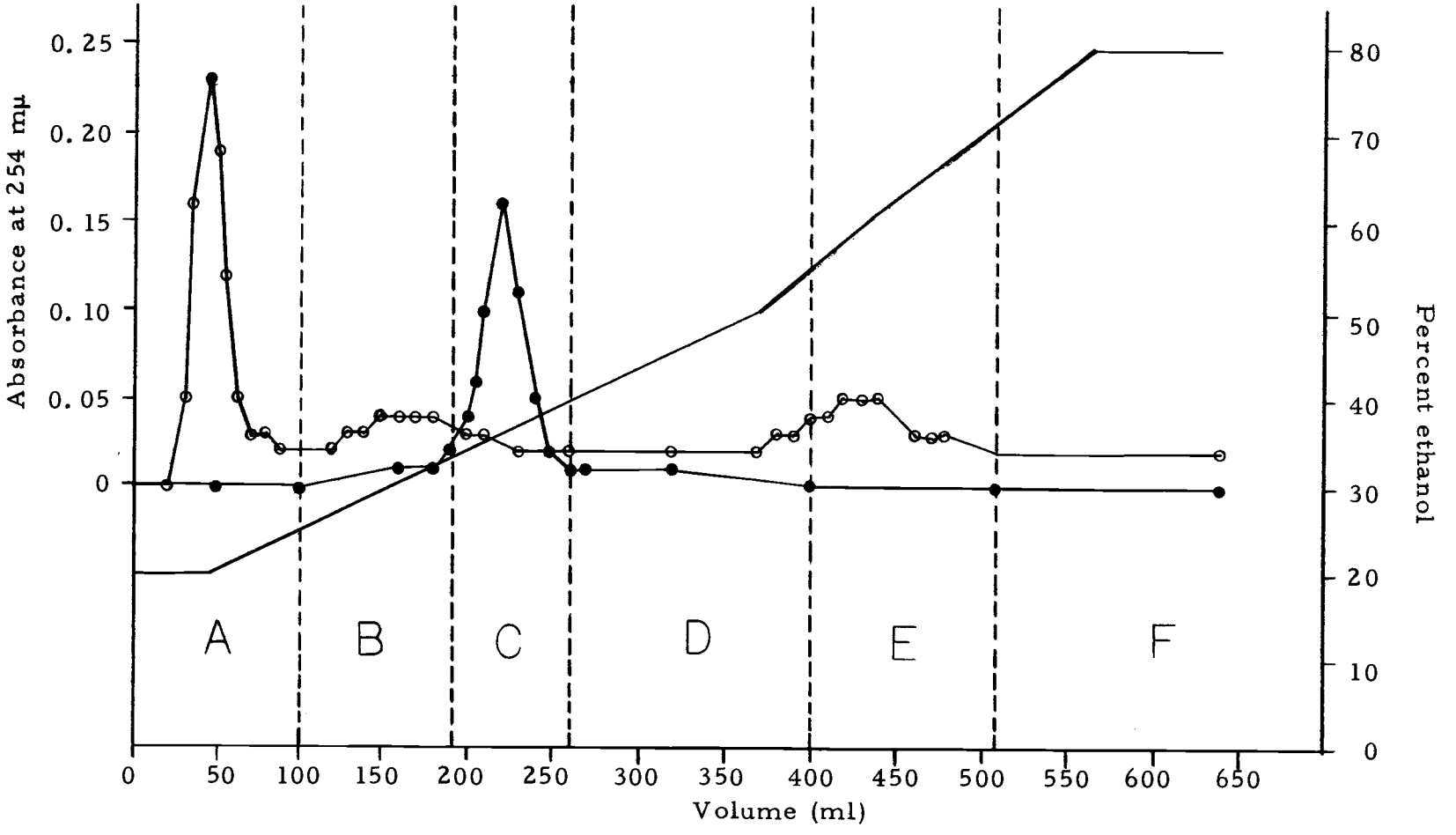


Figure 20. Postmolt P. crassipes female pheromone-sea water isolate fractionated on Chromosorb-102. Capitalized letters denote fractions. Pheromone-sea water o–o; crustecdysone ●–●; and alcohol gradient ___.

absorbance in the "C" fraction. The bioassays of these fractions from both species, however, indicated that although the "C" fraction showed low "activity," the strongest "activity" was in response to the "A" fraction (Table 4). Although the "A" fraction was shown to elute between 20%-25% ethyl alcohol according to the gradient programmed, it actually contained all the compounds eluting between 0-25% ethyl alcohol. Further fractionation and bioassays indicated that the "activity" was concentrated between 0-5% ethyl alcohol and therefore from this point on the XAD-2 isolation column was fractionated stepwise with 5%, 10%, 20%, 40%, and 95% ethyl alcohol.

The presence of crustecdysone in the "C" fraction from the crabs could not be confirmed by TLC on silica gel or by U. V. spectral analysis due probably to its low concentration and interference from other compounds in that fraction. Using an Absorbasil Prekote silicic acid TLC plate and developing for approximately 4 hours at 37°C with an n-butanol:acetic acid:water (24:3:10) solution system, the crustecdysone standard was visualized as a pink spot at rf .65 under U. V. light after being sprayed with a 100% (wt/vol) distilled water solution of p-toluene sulfonic acid and heated for 10 minutes at 100°C. This spray reagent which visualized different steroids by colors does not visualize lipids (Boisio and Ambrosetti, 1968). TLC of the concentrated "C" fraction and also the other fractions appeared as faint streaks on the plate.

Table 4. Bioassay of the eluants from the Chromosorb-102 column fraction of "active" pheromone-sea water of C. magister and P. crassipes females.

U. V. analyzer response (A) of fractions		Ethanol range over U. V. response (%)	Intensity of male's response	Average stance time (min)	Positive responses per no. of trials
		C. magister premolt pheromone-sea water			
A	(0.25+)	0-25	High	4.8	4/4
B1	(0.07)	26-28	-	-	0/0
B2	(0.10)	29-32	Moderate-high	11.7	3/4
C	(0.25+)	33-38	Moderate-low	9.2	5/6
D	(0.04)	39-54	None	-	0/3
E	(0.13)	55-66	None	-	0/3
		C. magister postmolt urine			
A	(0.02)	0-25	High	2.3	3/3
B	(0.01)	26-32	Low	20.0	1/2
C	(0.01)	33-40	Low	12.5	2/2
D	(0.00)	41-61	None	None	0/2
E	(0.03)	62-73	None	None	0/1
		10^{-7}M Crustecdysone			
C	(0.16)	33.5-37.5	Low	2.8	1/1
		Postmolt P. crassipes			
A	(0.38)	0-24	-	7.5	2/2
B	(0.18)	25-28	-	6.6	3/3
C	(0.02)	29-39	-	13.0	2/3
D	(0.10)	40-49	-	None	0/2
E	(0.04)	50-80	-	None	0/2

When crustecdysone in 60% ethyl alcohol was scanned (340-220 mμ) on a Beckman DK-2 spectrometer, it showed an absorption maximum of 247 mμ. Scans of the "C" fraction and other fractions from various crabs at each stage of the molt cycle did not show any consistent absorption pattern except for the "E" fraction which had an absorption maximum (255 mμ) and pattern identical to that of Garamycin. None of the scans matched that of crustecdysone. What was observed generally was a curve rising irregularly as the wave length decreased.

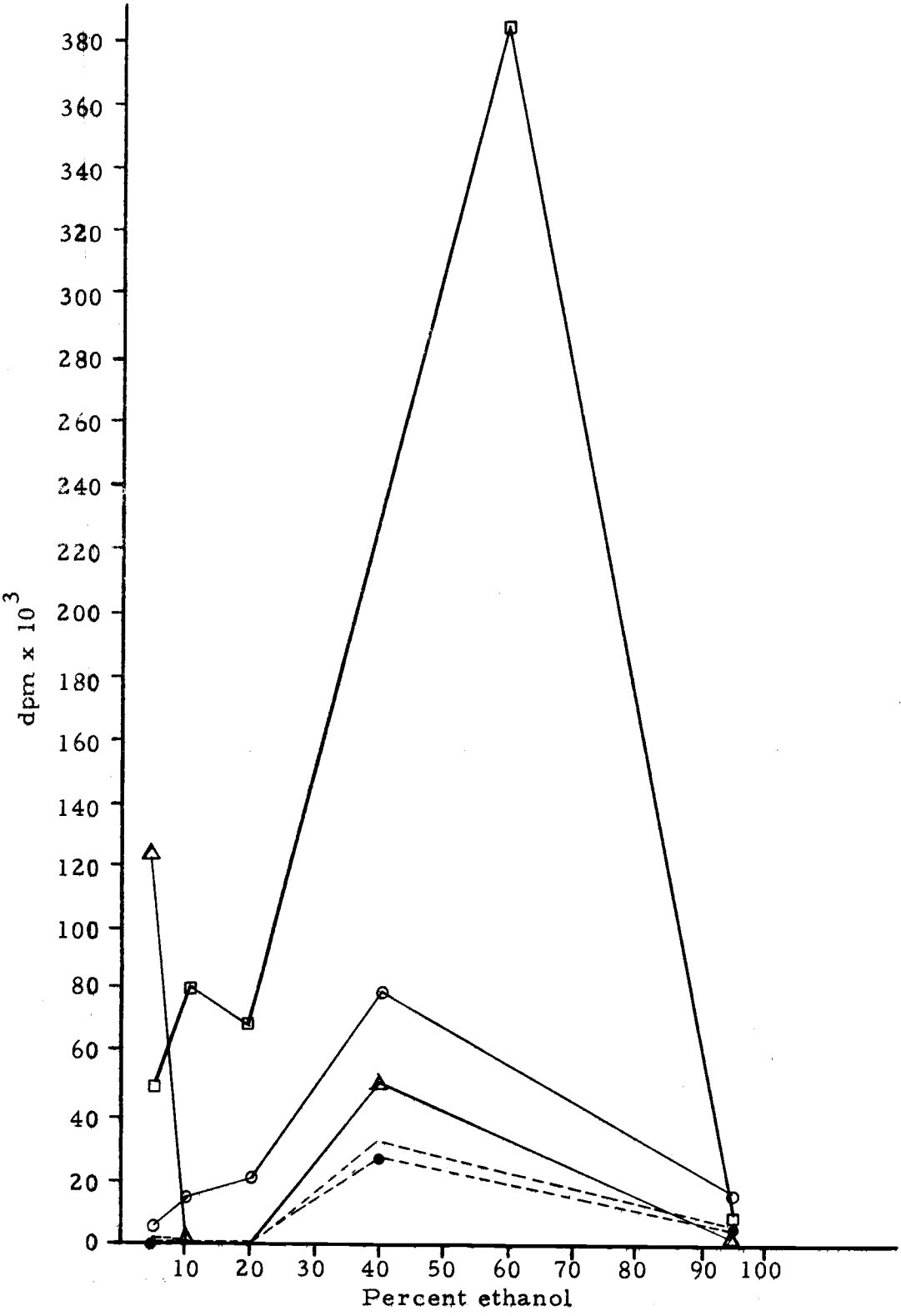
Since the presence of crustecdysone could not be confirmed on two different assay systems, it was decided that the injection of tritiated crustecdysone into the crabs would serve as a valuable tool for showing the possible release of crustecdysone by the crabs and its eventual fractionation into the "C" fraction. Also, since the highest "activity" was found to be in the much more polar fraction "A," rather than the low "activity" fraction "C," the tritiated crystecdysone would be useful in determining if the pheromonal compounds in the fraction "A" were in fact catabolites or conjugates of crustecdysone, in keeping with the original hypotheses. Even if neither were the case, it was felt that a valuable insight into the metabolism of crustecdysone could be gained. With this in mind "active" females were injected with the tritiated crustecdysone and their pheromone-sea water isolated and fractionated on Amberlite XAD-2. Ion exchange and electrophoretic techniques were also developed for the further fractionation of fraction "A."

Experiments using Tritiated Crustecdysone

Twelve hours after injection with 2.5 μCi of crustecdysone-H^3 pheromone-sea water collections from C. magister females in various stages of the molt cycle and from an intermolt C. magister male were fractionated on Amberlite XAD-2 with stepwise 5%, 10%, 20%, 40%, and 95% ethanol elutions. The tritium levels in each fraction of the pheromone-sea water collections are shown in Figure 21. The results indicate that during intermolt, both the male and female retained much of the injected crustecdysone-H^3, which elutes between 33% and 40% ethanol, "leaking" only a relatively small amount to the environment (29,017 dpm for the female and 33,422 dpm for the male). During premolt, on the other hand, crustecdysone-H^3 was released in quantities an order of magnitude larger than during intermolt. The results for the different molt stages are not directly comparable due to the fact that this crustecdysone fraction was eluted between 30% and 60% ethanol instead of 20%-40%. The major portion of the fraction, however, was shown by TLC to be crustedcysone. Along with the increased release of crustecdysone during premolt there was an increase of radioactivity in the 5% ethanol fraction where most of the biological activity of the pheromone is concentrated. Large increases were also recorded in the 10% and 20% ethanol fractions. Tritium levels, during molt, although still higher than intermolt levels, decreased

Figure 21. Tritium levels of fractionated pheromone-sea water collected from inter-, pre-, postmolt and molting C. magister crabs 12 hours after injection with 2.5 μCi crustecdysone-H^3.

- ● Intermolt females
- ▫ Premolt females
- △ Postmolt females
- o Molting females
- --- Intermolt male


dpm x 10^3
380
360
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
10
20
30
40
50
60
70
80
90
100
Percent ethanol

markedly in all the fractions from the premolt levels. Although very low levels of crustecdysone were recorded during the post-molt stage, a dramatic rise in radioactivity was seen in the 5% fraction where, as in premolt, the major portion of biological activity is concentrated. There appears to be a differential release of crustecdysone with different stages of the molt cycle. During premolt and postmolt some of the crustecdysone was converted to a more polar molecule.

In P. crassipes (Fig. 22) premolt and early postmolt females release approximately the same amount of crustecdysone. During late postmolt, when copulation occurs, the level of crustecdysone increases markedly. Again there was a differential release of crustecdysone with the different stages of the molt cycle. Only a small amount of crustecdysone seemed to be converted to more polar forms.

Although the high tritium levels in the 20%-40% ethanol fractions had been assumed to be from the crustecdysone-H^3 released by the crabs, it was necessary to make a more positive identification and show that a major portion of the radioactivity was indeed from the crustecdysone-H^3. To accomplish this, the 20%-40% ethanol fractions were mixed with "cold" crustecdysone, developed on Quantum LQD TLC plates in a chloroform: 90% ethanol (1:1) solvent system, and scanned on a Packard Radiochromatogram scanner (Fig. 23). By

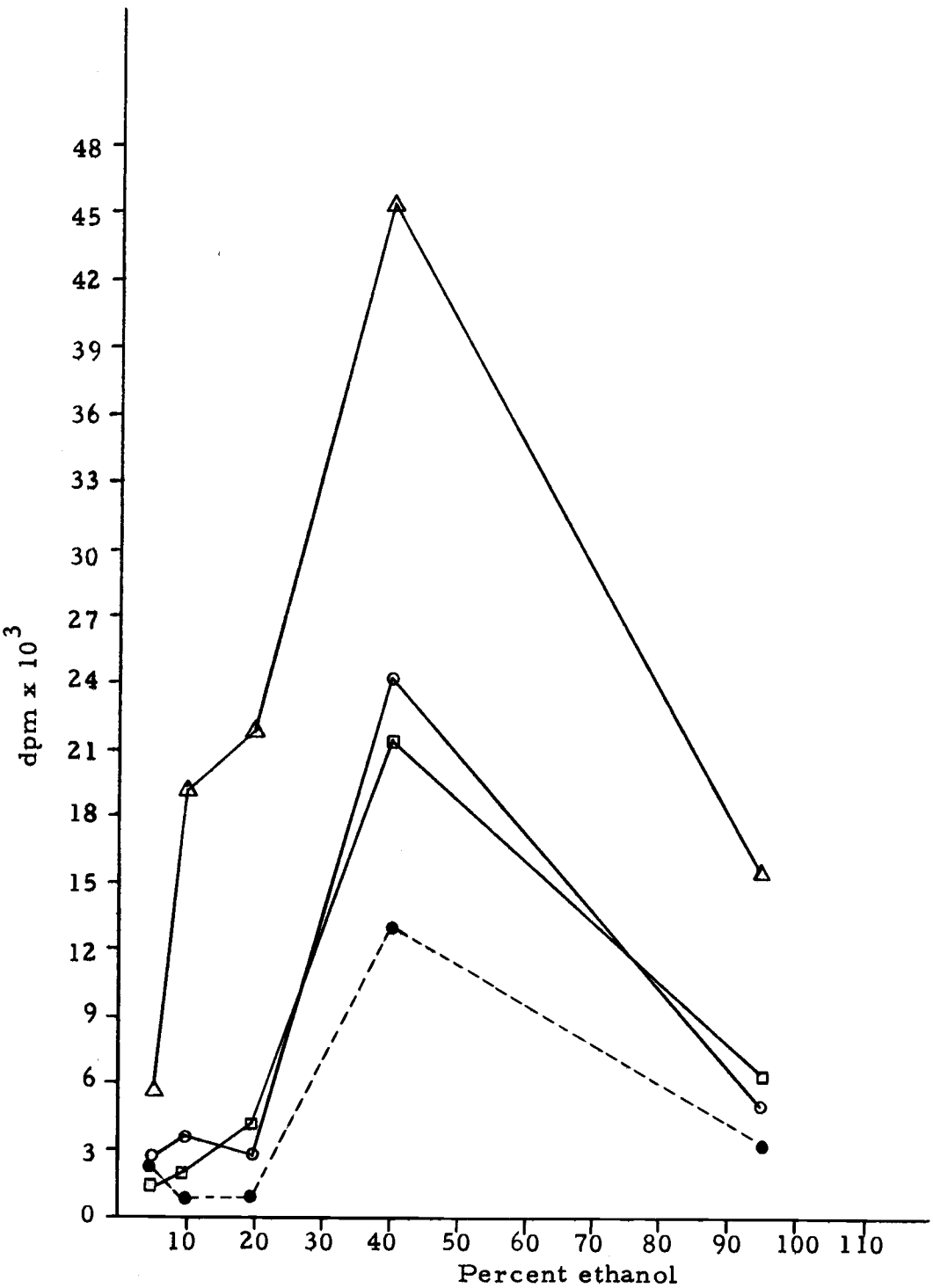


Figure 22. Tritium levels of fractionated pheromone-sea water from inter-, pre-, and postmolt P. crassipes females 12 hours after injection with 1.0 μCi crustecdysone-H^3. Intermolt ● ; premolt o ; 6 hours postmolt ▫ ; and 12 days postmolt △ females.

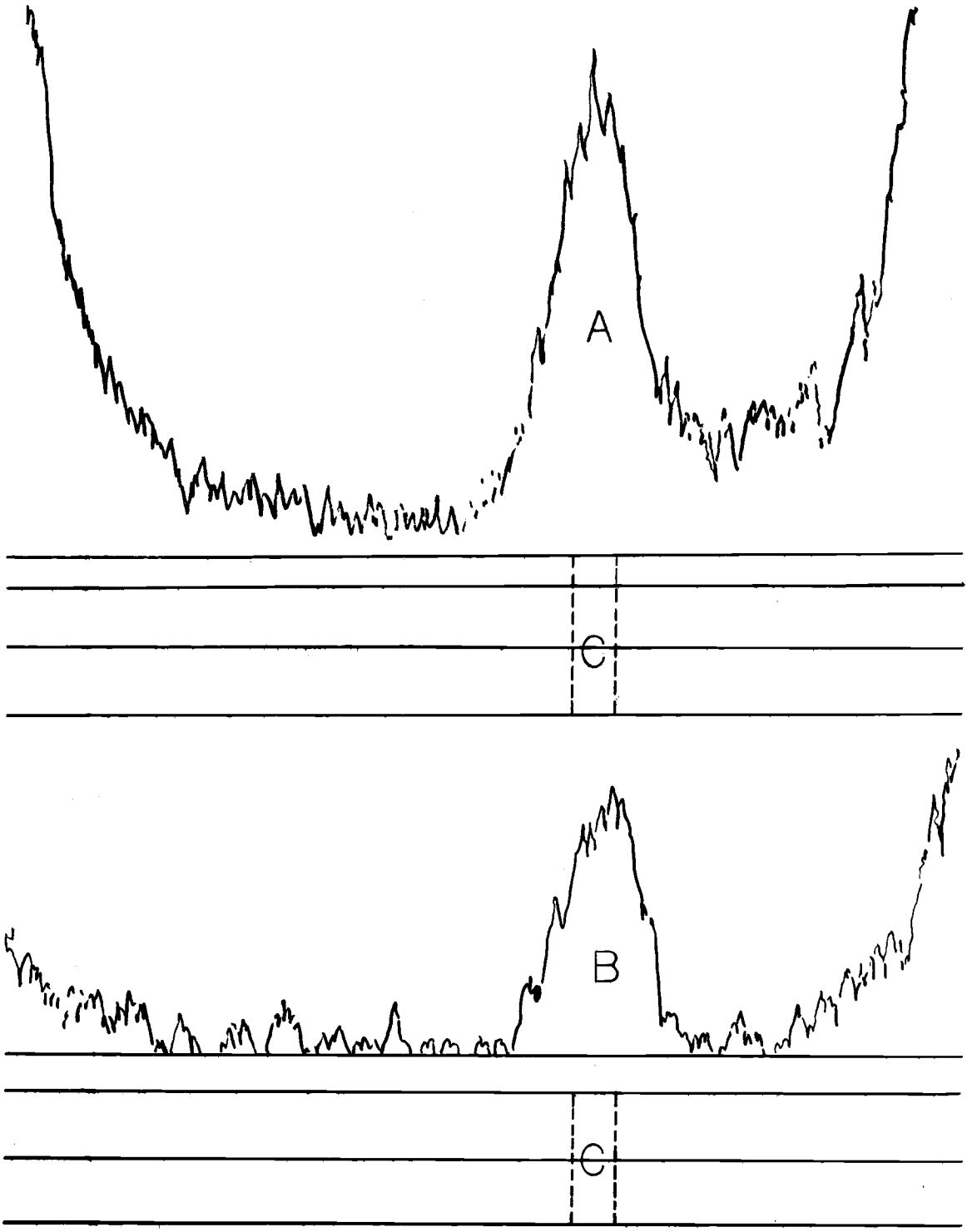


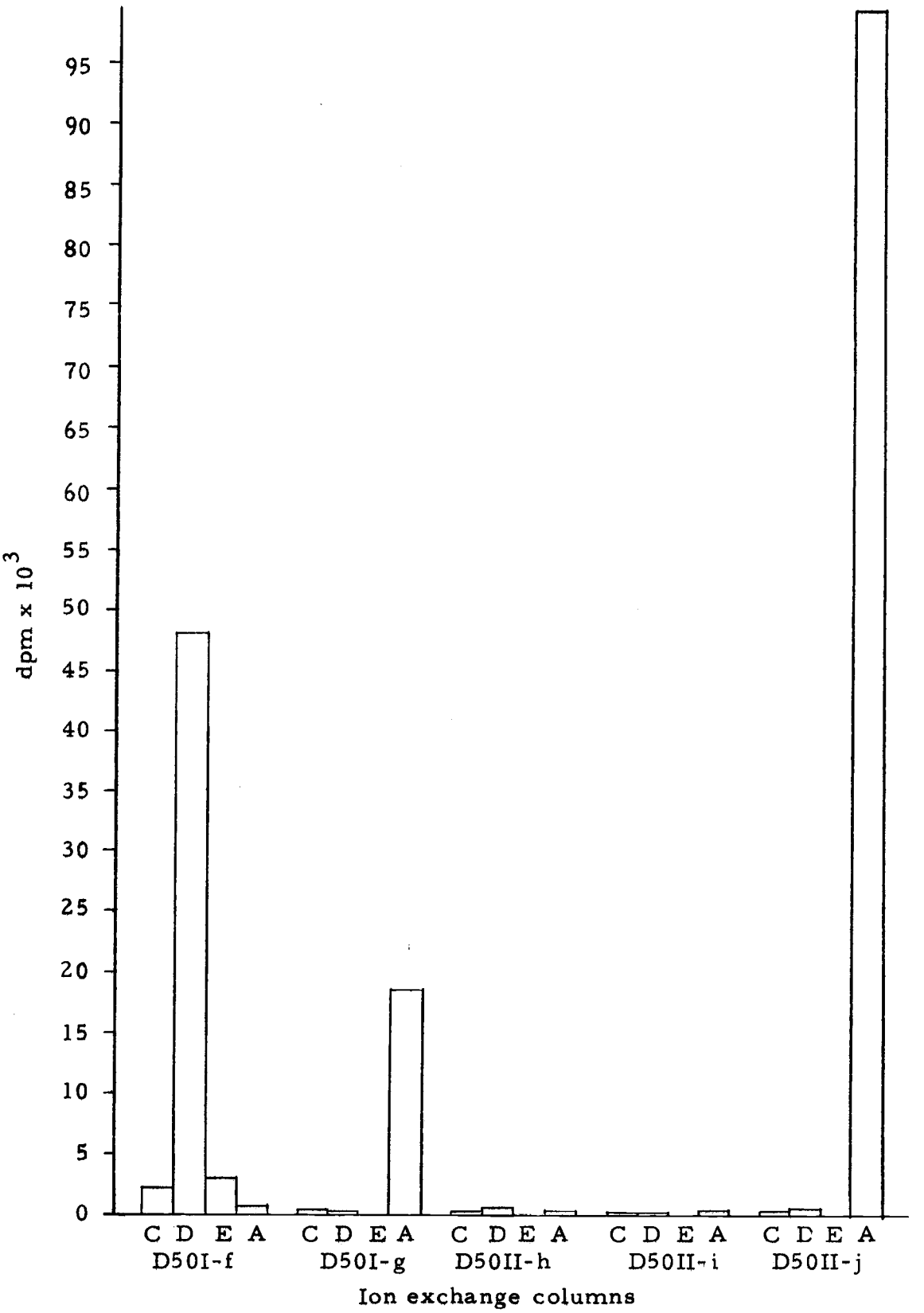
Figure 23. Radiochromatogram scans of 20%-40% fractions from crustecdysone-H^3 injected C. magister (A) and P. crassipes (B) mixed with "cold" crustecdysone and developed on Quantum LQD plates. Crustecdysone bands (C) visualized with U. V. light.

aligning the TLC plate under the scanner's recording by means of the tritium dye markers at the top and bottom of the plate, it could be clearly seen that the major portion of the tritium was directly attributable to the crustecdysone-H^3. This confirmed the release of crustecdysone by the crabs according to three different systems of analysis: 1) U. V. absorbance (254 mμ) of the eluate from the gradient programmed Chromosorb 102 columns; 2) scintillation of the eluate from the stepwise elution of Amberlite XAD-2; and 3) TLC showing the correspondence of "cold" and tritiated crustecdysone.

Ion Exchange Analysis of the
5% Ethanol Fraction

Although the 5% fraction had a fairly low conductivity, it was decided to use ion exchange analysis for further resolution, due to the relative simplicity of this method. An "active" 5% fraction, which was run on high voltage thin-layer electrophoresis, indicated that the "active" molecule had a positive charge. Therefore, 5% fractions from crustecdysone-H^3 injected crabs were fractionated on two Dowex-50 x 8 cation exchange columns according to the scheme presented in Figure 4. The results are presented in Figures 24 and 25. After sample application, the first column (D50I), which was used to somewhat purify the sample, was washed with three void volumes of water (D50I-f) and then stripped with two void volumes

Figure 24. C. magister female's 5% fraction run on Dowex-50. Letters C, D, E, and A refer to molt cycle stages; D50I column eluted with water (f) and 3N NH_4OH (g), and D50II column eluted with water (h), 0.2N HCOOH (i), and 5N HCOOH (j).


95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
dpm x 10^3
C D E A C D E A C D E A C D E A C D E A
D50I-f
D50I-g
D50II-h
D50II-i
D50II-j
Ion exchange columns

of 3N NH_4OH. This fraction was applied to the second column (D50II) after the NH_4OH was evaporated off. Three more void volumes of NH_4OH (D50I-g) were used to strip any remaining material from the D50I column. The D50II column was then fractionated using three void volumes each of H_2O (h), 0.2N HCOOH (i), and 5N HCOOH (j).

Both intermolt and molting C. magister females (Fig. 24) had very low and similar levels of radioactivity in each of their fractions. However, both the premolt female's D50I-f fraction and the postmolt female's D50II-j fraction contained very high levels of radioactivity which gave positive bioassays, releasing courtship activity from males. The existence of a negatively charged or neutral premolt pheromone and a positively charged postmolt pheromone correlates well with the behavioral observations which indicate the presense of precopulatory embrace as well as copulation releasing pheromones (see flowing water experiments, p. 44-48).

Since the C. magister 5% fraction contained "active" molecules not retained by D50, it was decided to develop the P. crassipes 5% fraction first on Dowex-1 anion exchanger (D1, Fig. 25). After the H_2O ($D1A_2$) wash was applied to D50I, 0.5N NaCl was used to strip the D1 of any anions present. This was followed by 60% ethanol ($D1A_3$, Fig. 25) to remove any adsorbed particles. The cation exchangers were developed as they were with the C. magister fractions.

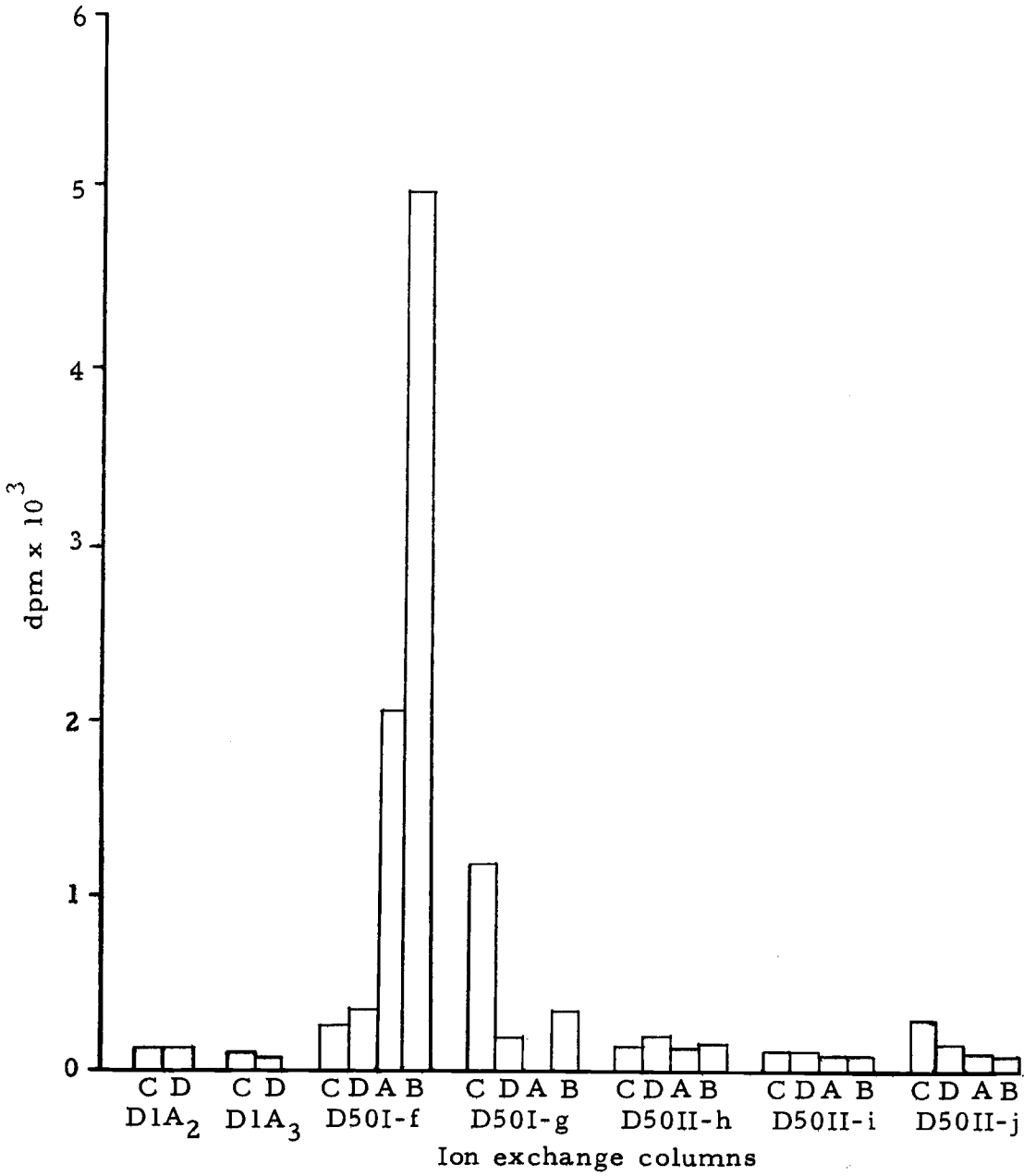


Figure 25. P. crassipes female's 5% ethanol fractions run on Dowex-1 and Dowex-50 ion exchangers. Letters C, D, A, and B refer to molt cycle stages; D1 column eluted with 0.5N NaCl (A_2) and 60% ethanol (A_3); D50I eluted with water (f) and 3N NH_4OH; D50II column eluted with water (h), 0.2N HCOOH (i) and 5N HCOOH (j).

The P. crassipes 5% fraction did contain anionic (or neutral) and adsorbed compounds but only in very low concentrations. The fraction with the major portion of the tritium label (D50I-f) was also biologically "active" in stages D, A, and B.

The use of crustecdysone-H^3 has shown that the molting hormone is released in different quantities by both crabs during pre- and postmolt. Mating activity, however, could only be released in conspecific males when the "crustecdysone fraction" of a late, postmolt (P. crassipes) female, which contained relatively more crustecdysone, was used. No precopulatory or copulatory activity was released by like fractions from C. magister.

Both crab species release "active" ionic (or neutral) compounds (D50I-f)--relatively large quantities in premolt C. magister and postmolt P. crassipes. A positive ionic compound (D50II-j) recovered in relatively large quantities from postmolt C. magister fractions and to a lesser extent from P. crassipes fractions show biological "activity" in C. magister but no "activity" in P. crassipes. Whether the compounds are catabolites or conjugates of crustecdysone has not been proven but since α and β glucosidase digestions of the 5% fractions have failed to free crustecdysone, the feeling is that these compounds may be catabolites of the molting hormone, crustecdysone.

Feeding Inhibition by the Sex Pheromones and Quinones

The initial perception of food or prospective mating partners is mediated through the chemosensory modality. Under normal (control) conditions, stimulation of the antennules and third maxillipeds of C. magister with fish juice elicits motion of maxillipeds and chelae. The average response time (Table 5) upon maxilliped stimulation was much faster (0.4 seconds) than on antennular stimulation (3.2 seconds). Under the influence of the sex pheromone, however, average response time on maxilliped stimulation was slowed (1.3 seconds) but on antennular stimulation no response was seen. Results using 10^{-6} crustecdysone were essentially the same except that complete suppression was not achieved on antennular stimulation. When 2×10^{-6} M crustecdysone was used, however, there was a complete suppression of the feeding response.

P. crassipes males respond in the same manner to their own sex pheromone and crustecdysone. When increasing concentrations of crustecdysone were used the suppression rate increased in direct proportion to the concentration (Fig. 26) perhaps indicating that some threshold had to be reached before inhibition occurred.

Although crustecdysine, the postulated sex pheromone, was shown to inhibit feeding responses, it had not been positively identified in pheromone-sea water while this aspect of the research was being

Table 5. Inhibition of the feeding response of C. magister males after a 20-minute exposure to the sex pheromone, 10^{-6}M, and 2 x 10^{-6}M crustecdysone. Times represent rapidity of feeding response. Test was terminated if no response was seen within 30 seconds.

Sample	Crab no.	Controls				Pheromone or crustecdysone			
		Antennular stimulation (sec)		Maxilliped stimulation (sec)		Antennular stimulation (sec)		Maxilliped stimulation (sec)	
		Tactile	Chemo-tactile	Tactile	Chemo-tactile	Tactile	Chemo-tactile	Tactile	Chemo-tactile
Pheromone	1	30.0+	2.7	30.0+	0.6	30.0+	30.0+	30.0+	1.6
	2	"	1.4	"	0.5	"	"	"	0.6
	3	"	6.0	"	0.4	"	"	"	1.6
	4	"	3.2	"	0.4	"	"	"	0.6
	5	"	2.5	"	0.3	"	"	"	2.0
$\bar{X}$		30.0+	3.2	30.0+	0.4*	30.0+	30.0+	30.0+	1.3
10^{-6}M crustec-dysone	1	30.0+	3.2	30.0+	0.6	30.0+	20.0+	30.0+	3.3
	2	"	1.5	"	1.1	"	30.0+	"	5.8
	3	"	3.4	"	0.5	"	18.0+	"	0.5
	4	"	1.6	"	0.9	"	28.0+	"	6.2
	5	"	1.6	"	0.5	"	30.0+	"	5.0
$\bar{X}$		30.0+	2.6	30.0+	0.7	30.0+	25.0+	30.0+	4.2
2 x 10^{-6}M crustecdysone	1	30.0+	3.2	30.0+	1.0	30.0+	30.0+	30.0+	2.5
	2	"	1.2	"	0.3	"	"	"	18.0**
	3	"	3.0	"	0.5	"	"	"	2.3
	4	"	1.3	"	0.7	"	"	"	0.5
	5	"	1.9	"	0.6	"	"	"	0.5
$\bar{X}$		30.0+	2.1	30.0+	0.6	30.0+	30.0+	30.0+	4.7

*Accuracy limited by speed at which stopwatch could be turned on and off.

**Not included in mean--larger than 2 S. D.

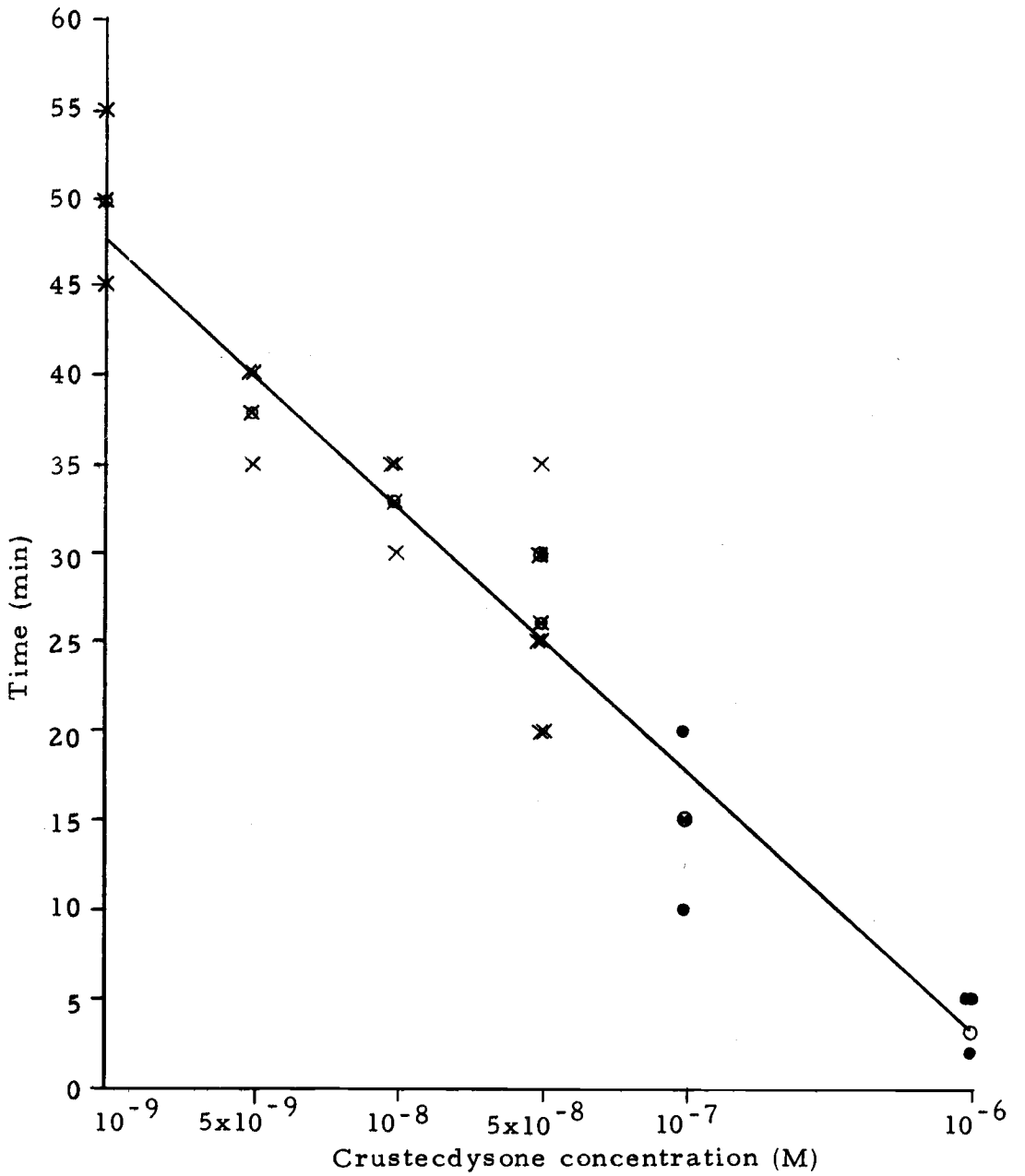


Figure 26. Time elapsed prior to the inhibition of the feeding response in P. crassipes exposed to sea water solutions of crustecdysone. Each series of bioassays is represented by a distinct symbol. Averages are indicated by circle symbols (Takahashi and Kittredge, 1973).

conducted and it was quite possible that other compounds released with the sex pheromone could have been the inhibitors. Therefore the inhibitory effects of other compounds, especially the dopa quinone which have been shown to function in the tanning of crustacean epicuticles (Dennell, 1947) and spinochromes were investigated.

When five P. crassipes males, each in 1-liter beakers, were immersed in 300 ml of 10^{-4} dopa-sea water solution, spontaneous positive feeding responses were observed for about 30 minutes (Fig. 27). Positive feeding responses were also observed when the crabs were stimulated with 3×10^{-3} M taurine. As time progressed, however, and the solution turned pink (3-6 hours) the crabs were less inclined to respond to the feeding stimulus. After 15 hours had elapsed, the crabs were completely inhibited and showed no response to the feeding stimulus. By this time the sea water solution had turned purple and melanin polymers had begun to settle. No responses were observed during intermittent tests and at 48 hours the water was changed and recovery from the treatment began. Although a marginal, positive (plus 2) mean feeding intensity was seen at 111.5 hours, full recovery did not occur until 303.5 hours had elapsed. Throughout the test the control's responses fluctuated between high (plus 4) and low (plus 2) positive mean feeding intensity responses and only once showed feeding inhibition.

D, L dopa (10^{-4} M in 10 ml pH 5.6 phosphate buffer) was oxidized

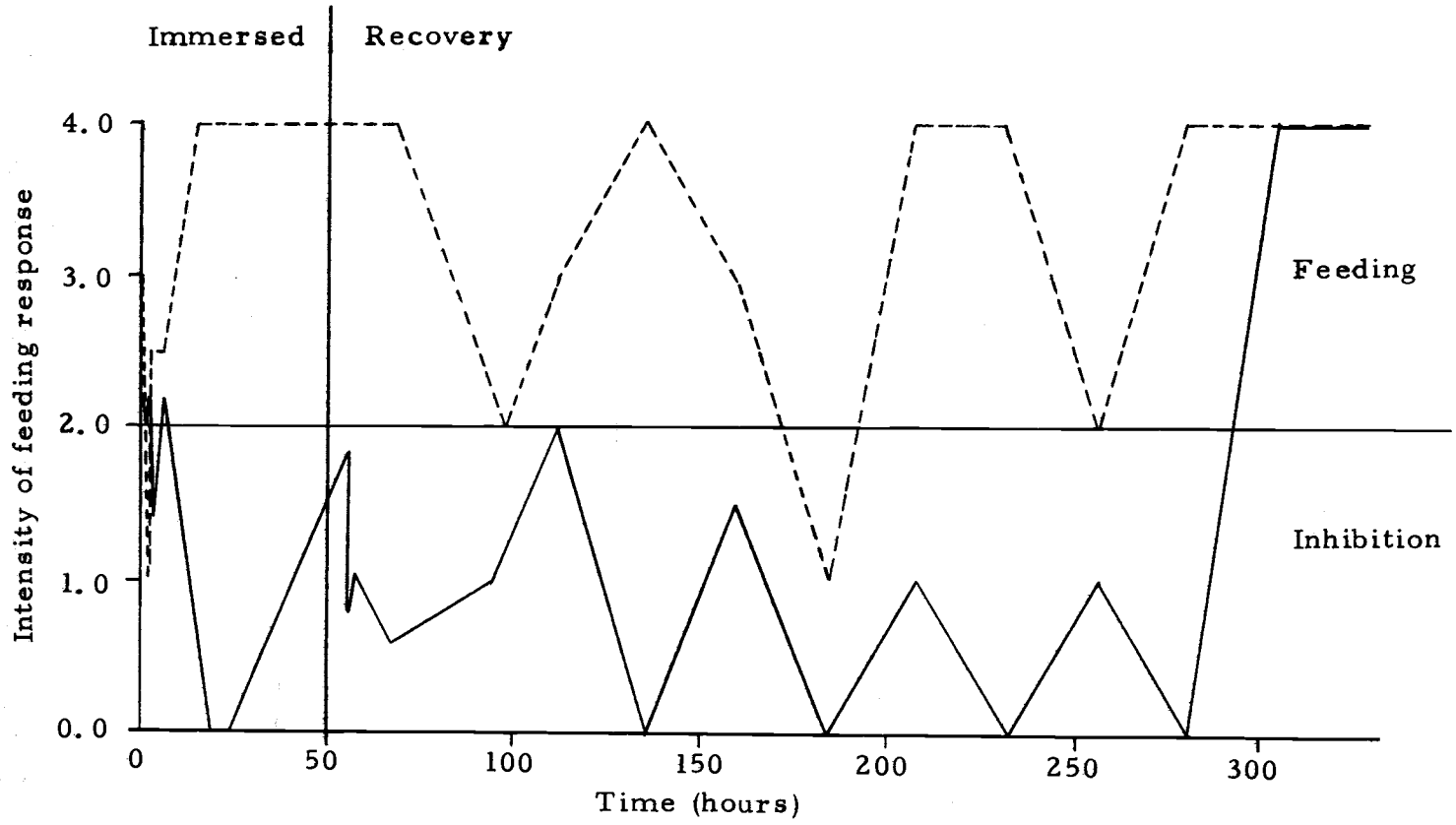


Figure 27. The inhibition of the feeding response in P. crassipes males by 10^{-4}M 3, 4-dihydroxy phenyl-D, L-alanine. Control ----; immersion and recovery —— .

with 50 mg Ag_2O. The mixture was millipore-filtered onto Dowex-50 to remove the traces of silver and diluted 100 times and bioassayed. Inhibition occurred within 30 minutes in all cases and all recovered within 24 hours although still immersed in the test solution.

In further search of clues as to the identity of the compounds which would act as feeding inhibitors, 1, 4-naphthaquinones and 5-OH-1, 4-naphthaquinone (juglone) were bioassayed. Since these compounds were effective in inhibiting feeding, several spinochromes (substituted 1, 4-naphthaquinones) isolated from the sea urchin were bioassayed.

In general, the crab's feeding responses were inhibited when immersed in these spinochrome solutions (Figs. 28, 39, 30), but recoveries (mean feeding response intensities of plus 2 or higher) were rapid when the crabs were put into fresh sea water. Full recoveries did not occur immediately, however, and several days elapsed before the mean feeding response intensity matched that of the control crabs. Crabs kept immersed in the test solutions remained inhibited but showed a gradually increasing feeding response intensity. The dopaquinones or naphthaquinones probably polymerized.

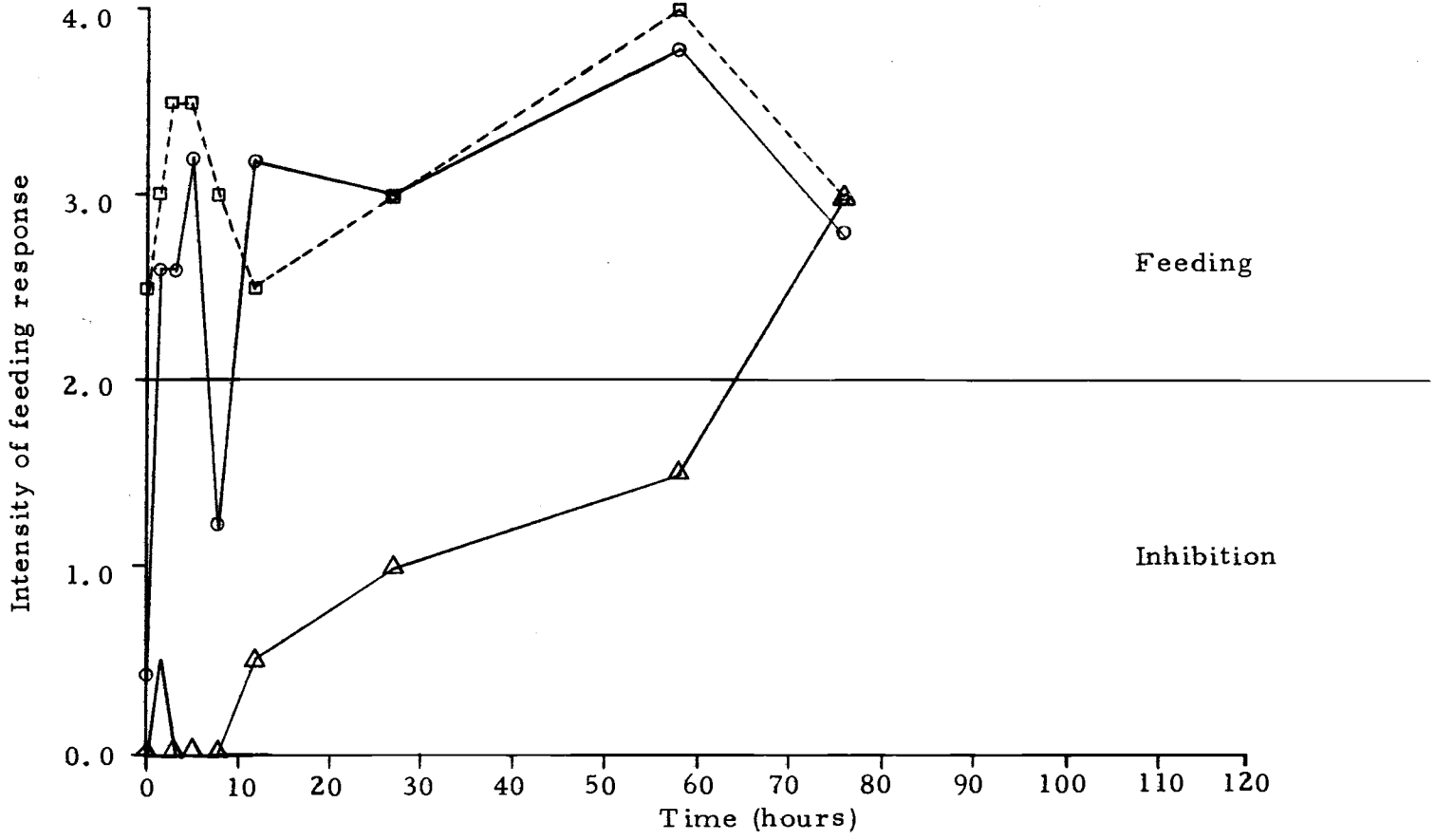


Figure 28. The inhibition of the feeding response in P. crassipes males by 10^{-6}M 5-MeO-1, 4-naphthaquinone. Control □ ; recovery after an hour's immersion o ; and continuous immersion △ .

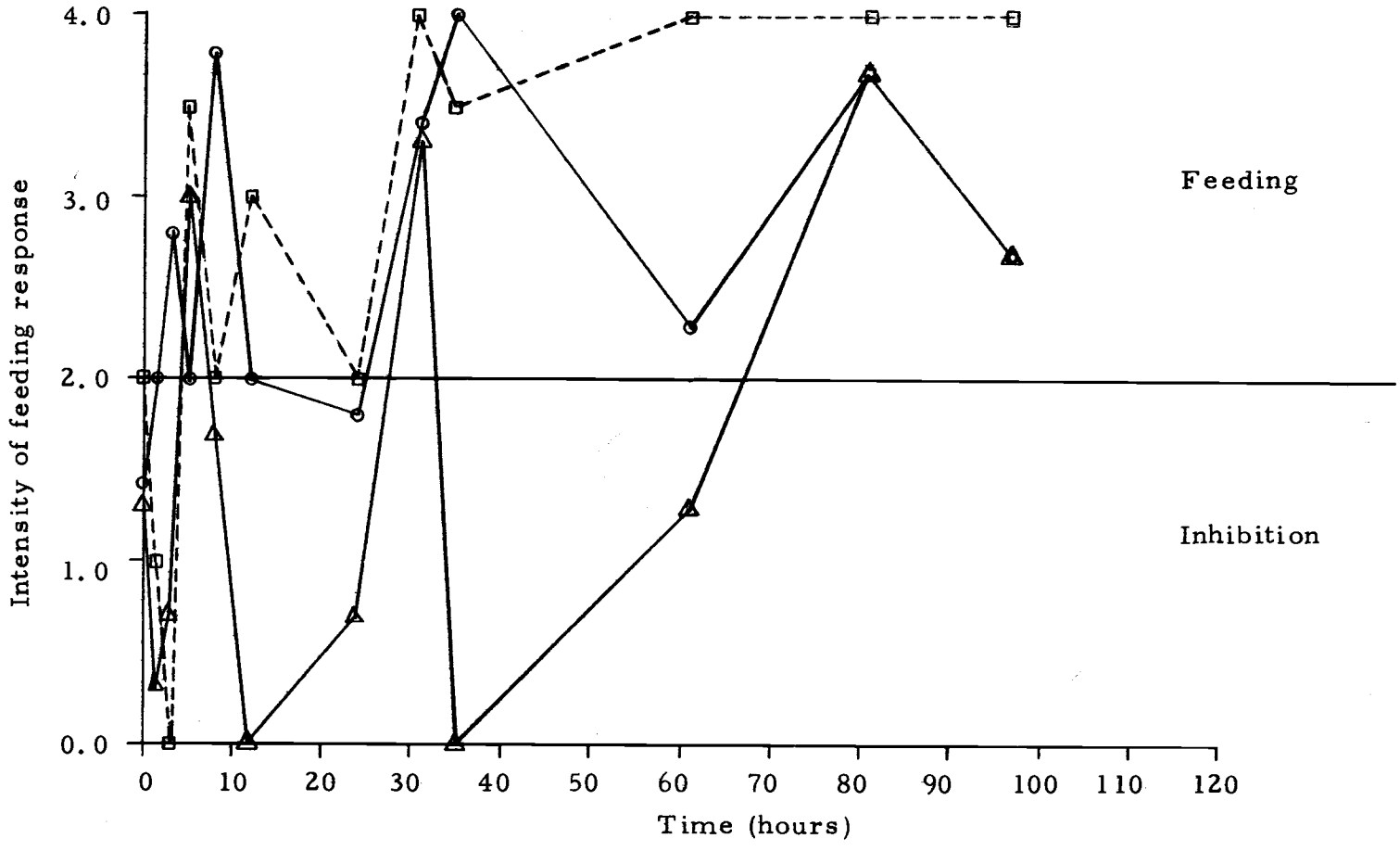


Figure 29. The inhibition of the feeding response in P. crassipes males by 10^{-6}M 2, 5-OH-1, 4-naphthaquinone. Control □ ; recovery after an hour's immersion o ; and continuous immersion Δ .

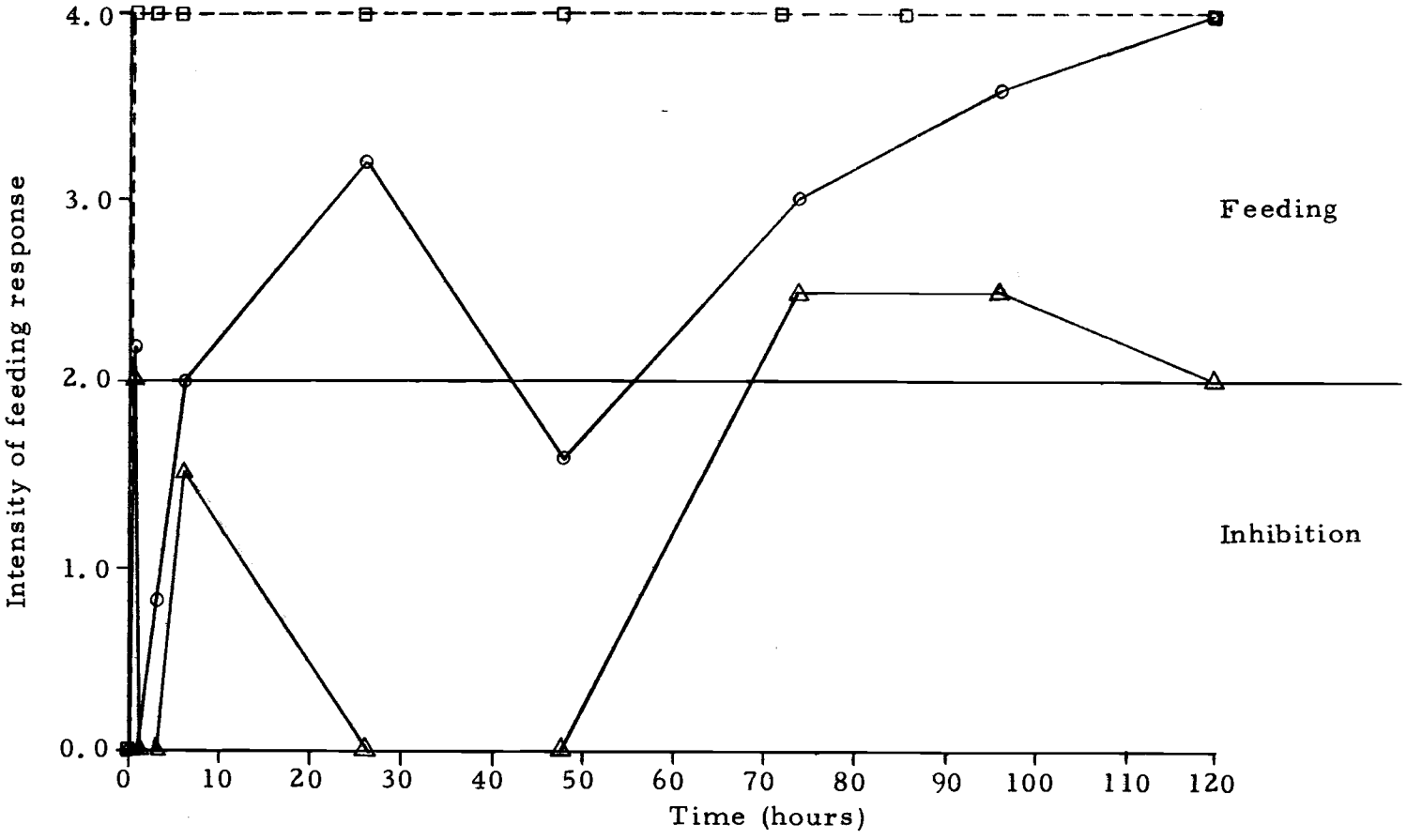


Figure 30. The inhibition of the feeding response in P. crassipes males by 10^{-6}M 2-Et-1,4-naphthaquinone. Control □ ; recovery after an hour's immersion o ; and continuous immersion Δ .

DISCUSSION

Initially, it seemed that learning the "language" of the crabs would be a fairly straightforward problem. As it turns out, the "language" is obscured and hidden and perhaps it should well be, for in order that the message conveying capability be advantageous to that species it must be correctly perceived by only those for whom it was specifically targeted. Man's rapidly expanding understanding of the sexual "language" of insects is causing the demise of an increasing number of insects. It is ironic that the "advantageous" chemoreceptive ability of the Dungeness crabs led to their capture and use in this attempt at understanding how they perceive part of their world and communicate.

The Breeding Seasons

Although a majority of the subtidal C. magister crabs molt during the summer and fall months, females kept in the laboratory molted and mated all year round. Due to the fact that premolt females rarely entered the crab pot in response to the bait and that trawling was not conducted on a systematic monthly basis, no conclusion could be drawn as to when the majority of females became "active" and bred. Since eggs are deposited between October and March (Waldron, 1958), breeding season probably extends from June to December.

With P. crassipes, on the other hand, records kept of monthly collecting trips indicated that females began entering the premolt stage during March and by May great numbers of premolt females could be found. This breeding season may be triggered by the low-low tides shifting from late afternoon and night to a period between mid-morning and mid-afternoon during February and early April, exposing these crabs, which are vertically distributed from the -7 foot tide level and to the high intertidal (Hiatt, 1948), to higher air temperatures for longer periods. During June and July many gravid (eggs on the abdominal pleopods) females were found. There also seemed to be a shorter second period of molting of females during September when the time of the low-low tides begin to fluctuate from very early morning to very late at night on its way to becoming late afternoon and night tides. Hiatt (1948) indicated that the molting season extended from March to November. These observations for P. crassipes are based on a limited sample and are biased by the collecting method which selected the slowest and most exposed crabs or those under rocks that could be overturned. These observations on P. crassipes and C. magister were important, however, since they set the timing for the performance of the mating sequence.

Analysis of Crab Behavior and the Bioassay Systems

Given the multisensory environment in which they lived, the two

species of male crabs studied are motivated to release many different behavioral patterns and displays, the manifestations of which are integral of endogenous and exogenous stimuli inputs (Marler and Hamilton, 1966). This research concentrated mainly on two exogenous inputs--the female sex pheromone which communicated the sexual receptivity of the female to a conspecific male and the food stimuli which had a chemoreceptive value to a hungry crab. The effect of the sex pheromone on the mating behavior and on the reception of the food stimuli and the release of behavior associated with feeding was also studied. Since there were many things to keep in mind it was useful to have a simple model of behavior and necessary to organize the units and sequences of behavior displayed by the crabs.

The model assumes the existence of an internal homeostatic mechanism which, upon displacement from equilibrium by endogenous and/or exogenous factors, functions to correct itself by selecting from a hierarchy of response options (Roberts and Matthysse, 1970). Thus, given a food stimulus, a hungry crab searches and releases feeding behavior; if stimulated by the sex pheromone, a male crab searches and displays courtship behavior; and given both stimuli, a male crab selects courtship over feeding.

Food stimuli coming from a distant source and sex pheromones are transduced by the antennules which serve a chemosensory or olfactory function (Lockwood, 1967; Dahl et al., 1970). On contact

with the food, the stimuli are transduced by the chemotactile hair (taste) on the dactyls, chelae, and mouth parts (Lockwood, 1967). These dactyl chemotactile hairs may also help P. crassipes males select a copulation ready female.

The ethogram, the repertoire of crab behavior, from which the feeding and sex pheromone bioassays were created, was divided into two parts--the motor patterns and social displays (Fig. 5). The motor patterns, which included resting, sitting, standing, the feeding sequence, foam bathing, and the "alert" posture, described the behavior which lacked communicative function. It was difficult to decide whether the so-called "alert" posture was really a unique display of P. crassipes or just a variation on standing caused by the disproportionately larger chelae. It did seem that stimulation by glutamate and aspartate as well as other substances brought about this posture. It also closely resembled the mating stance except no abdominal waving was observed.

The sequence of events which make up the feeding response (Fig. 5) is in response to food giving off a high chemosensory signal, i. e., flesh and bleeding animals. This sequence, for which detailed mouth part movements are described (Table 2), reflexively signals the reception of the food stimuli. The plus 2 feeding response is considered as a marginal positive response since it is occasionally seen when the animal is stimulated with non-food chemical stimuli. If

capture of a prey is required, then grasping precedes the mouth part movements which act in the process of aiding consumption rather than manifestation motions which indicate the desire to feed.

The social displays include agonistic and courtship behavior, both of which have communicative functions and utilize postures that seem closely related. Aggression and defense are operational terms describing agonistic behavior; all variants intermediate to aggression and defense are also agonistic displays (Schone, 1968). The similarities of agonistic and courtship displays are not surprising since the same postures and movements must be used whether the object of capture is an "active" female or some prey or aggression animal. The notable difference that characterizes the courtship behavior is the absence of violence except when there is a need to make the female submissive. Movements during courtship are generally slow and even and the use of the chela to pinch is kept at a minimum; in contrast, agonistic movements are rapid and uneven and injuries are common if the threat progresses to actual fighting. The observations that C. magister uses the lateral merus reflex (chelae lateral or unflexed) for both agonistic and courtship encounters, and that P. crassipes (although using the lateral merus in high threat situations) uses flexed chelae (chelae forward, Wright, 1968) to communicate both agonistic and courtship intentions, are in concurrence with the conclusions of Schone's (1968) survey which indicated that the subtidal

genera generally used the lateral merus for courtship and agonism while the semi-terrestrial forms used a flexed chelae. Flexed chelae movements are not exclusive to the semi-terrestrial crabs, however, since it has been observed in C. magister males stimulated by the sex pheromone.

The lateral merus displays, although considered primitive (Schone, 1968; Wright, 1968), seem to be widely recognized by many different species of clawed crustacea. Crayfish, occasionally used as food for C. magister, can stop a hungry crab by using the lateral merus display. P. crassipes and H. nudus, the two semi-terrestrial crabs observed, generally use the "more highly evolved and formalized" (Schone, 1968; Wright, 1968) chela forward displays but under high threat situations resort to the lateral merus displays.

The releasers for the social displays discussed above are visual, tactile, or chemical stimuli. Agonistic displays are generally released by visual stimuli although a noxious chemical stimulus can also be a releaser. Tactile and chemical stimuli are generally considered to be the releasers for courtship displays in subtidal forms (see Literature Review in Introduction). In semi-terrestrial forms, the releasers seem to be visual signals (Bliss, 1968; Crane, 1958; Schone, 1968; Wright, 1968) rather than chemical ones. These authors tend to preclude chemical releasers since mating of most semi-terrestrial crabs occurs out of water.

Although it is probable that the semi-terrestrial forms could have changed from the chemical to the optical channel of communications, it seems improbable that the capacity to communicate chemically would be lost completely. Many of the semi-terrestrial crab interactions described (Bovbjerg, 1960; Krammer, 1967) indicate that touching is involved at various times prior to the actual copulatory embrace. This fact seems to lend credulence to the existence of chemotactile releasers in these crabs. During the courtship dance, P. crassipes males generally touch the female's legs with the dactyl tips in what could be described as a "feeling" motion. The fact that a water soluble pheromone which releases mating behavior subtidally has been isolated and identified from P. crassipes females and the fact that mating can also be initiated and consumated on land is consistent with the author's contention that chemical signals are the main releasers of mating behavior in the semi-terrestrial P. crassipes, since that same water soluble pheromone could be spread over the chelae, legs and sub-branchial region by the process of foam bathing occasionally observed in the crabs that are out of water. The visual signals may initiate courtship and allow for the close contact of the crabs.

During foam bathing, a frothy material perhaps released from the antennal gland covers the region of the mouth parts. This is spread by the legs and chelae and allowed to dry (Wright, 1966). A cleaning

function was ascribed to this process by Schone (1963) and Brownscombe (1965) but as Wright (1966) postulated this may be the means by which the pheromone released in the urine is spread over the body so as to serve as a chemotactile mating signal.

C. magister females can control the release of their sex pheromones. As described in the flowing water experiments (p. 44-48), active premolt or postmolt females not in the grasp of a male release very low concentrations of pheromone. When being grasped by a male they release a high concentration of pheromone which signals their readiness to either molt or mate and indicates to the male that he had made the right choice. Once held by the male, the female stops releasing pheromone or releases only a small amount. A premolt Portunus sanguinolentus female held by a male or by a clamp does not release the sex pheromone (Christofferson, 1970).

The advantages fo the females are immediately apparent since by "leaking" a low concentration of pheromone when not being held, large quantities of the pheromone need not be produced and quantities can be stored and released when high concentrations are needed to reinforce the courtship behavior of a searching or grasping male. Stopping the flow of pheromone on being held by a male stops the courting activities of other males and therefore prevents potentially disruptive or destructive acts which might have occurred if other males had tried to take the female away from the first male. Releasing

low concentrations or no pheromone at all when alone or when being held during her period of greatest vulnerability, the female prevents predators from exploiting the pheromone as a feeding attractant.

The probability that other aquatic forms also control the release of their pheromones makes it necessary to use a flowing water system (Fig. 2) where action and reactions can be observed. Teytaud (1971), who kept single premolt Callinectes sapidus females in plastic buckets for 2-1/2 hours prior to siphoning the water into the male's tank, concluded that no premolt sex pheromone was produced. It is possible that these females (portunids like P. sanguinolentus, which control their pheromone release) either did not release their pheromone due to the fact that they were placed in an environment which they were not adapted to and which had no visual images of crabs to which they could signal, or the single subject male on reception of the pheromone did not have to require visual input which may have been necessary for the release of precopulatory activity. Mating stimuli which appear to be tactile or chemotactile must also be reevaluated due to the fact that the touch may be the signal which causes the release of a diffusible pheromone.

Besides differing in the time of the breeding seasons and the forms of agonistic and courtship displays, C. magister and P. crassipes also differed in premolt, molt, postmolt copulatory behavior as well as to the timing of copulation during the reproductive sequence.

The precopulatory embrace of a premolt female, released by a premolt sex pheromone in C. magister, is rarely observed in P. crassipes although a premolt attractant seems to be present. With semiterrestrial forms such as P. crassipes, this behavior would be nonadaptive, since on leaving the buoyant water, the male must bear the full weight of the female making locomotion over the rocks and escape from predators difficult. P. crassipes females do not molt within the protection of a male and do not mate in the soft shell condition (stage A) as do many subtidal forms which include C. magister. Instead, late premolt and early postmolt females stay hidden under rocks in well protected and well watered tide pools mating in a female superior position during a period from the end of postmolt (stage B) through early intermolt (stage C_3). All the copulating females which Hiatt (1948) examined were in the C_3 stage of the molt cycle.

Since the exoskeletons of the copulating females were not fully hardened, the female superior position protects the female from injury by objects in the rocky intertidal. Bliss (1968) noted that many other intertidal crabs have assumed this position even though the females mate when the exoskeleton is fully hardened. This position has the added advantage in that it facilites copulation between crabs of disparate sizes.

Soon after mating the P. crassipes females deposit their eggs onto their abdominal pleopods. Egg deposition in C. magister occurs

after a 4-month delay. The timing of copulation in relation to egg deposition seems to indicate that the mating pheromone in C. magister is produced in association with factors controlling molt while the mating pheromone in P. crassipes is perhaps produced in association with ovary maturation factors.

The many hours of behavioral observations resulted in the feeding and sex pheromone bioassays which were to be used in the study of feeding inhibitions and for the determination of "activity" in fractions of isolated sex pheromones. The feeding bioassay (Table 2) worked well since the feeding response was easily released under normal conditions by "food" stimuli. Feeding is probably a high priority response option in crabs which have been starved for 2 days. The smallness of P. crassipes crabs, however, made it difficult to apply the 20 µl dose of taurine onto the tiny antennules.

Unfortunately, the sex pheromone bioassays did not work as well as expected. A quantified bioassay, although not absolutely essential, is useful in the analysis of the various fractions since it gives indications of the concentration, purity, and biological activity. Of the many action patterns released by the crab stimulated by the sex pheromone, the release of the mating stance seemed best suited to quantification (timing its release). This worked well in the initial bioassays of crustecdysone (Fig. 15) which showed that the release of the mating stance of P. crassipes males was a time-dependent function

of the crustecdysone concentration indicating, perhaps, the effects of summation at the lower concentrations of crustecdysone. As a result, this bioassay was adapted to C. magister.

Problems arose when it was discovered that other fractions more polar than crustecdysone also elicited this stance behavior and seem to have sex pheromone activity. The stance bioassay did not indicate which or if all of these fractions would actually release the complete mating sequence from courtship to copulation. Prior to thie point in the research, it was assumed that only one pheromone was necessary for the release of precopulatory and mating behavior. While this standardized bioassay did not supply the observer with enough information, it also denied the crab important input--visual image of another crab to which courtship activity could be directed. This failing was especially obvious with C. magister where some males would show no response at all or just show searching behavior until another male or moving object (p. 47) was presented. This importance of visual input to C. magister was not suspected since all accounts in the literature had minimized the use of visual releasers in courtship activity of subtidal crabs while emphasizing the importance of a chemical releaser.

Crab-shaped stone "models" were used in an attempt to give the crab an image to display at but generally no response was given and the models were not grasped unless the male was highly stimulated.

Air stones with the stream of bubbles providing motion were displayed to more often than were the models. Small males with the chelae and two pairs of walking legs autotomized were not entirely successful as models since they were still able to create rapid and uneven motions and jerk the anterior portion of the dorsal carapace upward as in an agonistic signal.

Mating stances are postures released prior to grasping in order to facilitate grasping in the event that a graspable object is encountered. The mating stance bioassay failed to indicate if grasping would take place or in the event that it did whether the grasp was for the purpose of holding, as in the precopulatory embrace, or for the purpose of mating. The flowing water experiments (p. 44-48) using C. magister indicated the presence of a premolt pheromone which releases the precopulatory embrace and a postmolt pheromone which releases copulatory behavior. This also seems to be the case with P. crassipes since premolt females are "attractive" to males releasing stances and abdominal movements. The significance or importance of this signal as a releaser of precopulatory behavior seem to be lost or minimized and only the postmolt mating hormone is functional in P. crassipes.

Since there was evidence for more than one sex pheromone and since visual information was also shown to be important in the release of courtship activity, the mating stance bioassay was modified in order that more information could be provided to the observer and crabs.

Fractions to be bioassayed were poured into 4 liters of sea water in which two food sated P. crassipes males were sitting. Their responses were rated a positive or negative on the basis of whether they engaged in the courtship dance or whether one attempted to grasp the other male and roll over on his back in attempted to copulate. With C. magister, again using food sated crabs, another much smaller male or a small intermolt female was added to the males' bioassay chamber. If the fraction being bioassayed was "active," attempts to grasp the smaller crabs followed and although resistance was put up by these smaller crabs they usually could be slapped into submission and held. Of course, quantification was not possible since some males were better at grasping the "models" than others. The grasping behavior gave no indication whether the fraction being bioassayed was one releasing precopulatory or mating behavior due to the fact that besides having an unwilling partner, chemotactile or tactile inputs may be necessary before mating behavior is released.

The behavioral "black box" was more difficult to open than anticipated. The chemical analysis which will be discussed next aided greatly in unlocking many of the secrets of crab behavior. P. crassipes, which was reported to use visual signals during courtship, was shown here to communicate by chemosensory and chemotactile means. C. magister was shown to produce a premolt pheromone and also a postmolt pheromone and could control their release.

Analysis of the Chemical Data

The chemical analysis of the sex pheromone-sea water began while the crabs' behavior was still being investigated, making it possible to view each of these phases of the research within the context of the other. The initial results indicated that the pheromone was heat stable, dialyzable, and soluble in organic solvents, strengthening the hypothesis that the crustacean molting hormone crustecdysone was also functioning as the sex pheromone. Crustecdysone has been isolated and identified (Faux et al., 1969; Hampshire and Horn, 1966; Horn et al, 1966, 1968) and shown to induce molting of various crustaceans (Blanchet, 1972; Graf, 1972; Krishnakumaran and Schneiderman, 1969; Lowe et al., 1968; Rao et al., 1972).

Column chromatography of the pheromone-sea water from premolt and postmolt C. magister females produced fractions with U. V. adsorbing compounds with the same retention time as crustecdysone (Figs. 16 & 18). Premolt P. crassipes females fractions (Fig. 19) showed absorbance that overlapped crustecdysone's but did not have identical retention time while postmolt females (Fig. 20) showed low absorbance in that area due to the fact that they were only a few days postmolt.

The injection of crustecdysone-H^3 into C. magister and P. crassipes females during different stages of the molt cycle confirmed

the release of crustecdysone into the pheromone-sea water, and also showed that the quantities released differed with the different stages. The premolt C. magister female released a large quantity of crustecdysone and also lesser amounts of more polar metabolites (the metabolites in the 5% fraction showing the highest pheromonal activity). The postmolt female showed a decreased output of crustecdysone but a very much elevated level of polar metabolites in the 5% fraction which, like the premolt 5% fraction, contained most of the pheromonal activity (Fig. 21). Premolt and early postmolt P. crassipes females released crustecdysone at approximately twice the intermolt level and also released low levels of polar metabolites. The females during these periods are "attractive" but do not mate. The late postmolt (stage B) female which did copulate released approximately four times the crustecdysone of intermolt and higher amounts of polar metabolites (Fig. 22). The crustecdysone fraction and 5% fraction were successful in releasing mating behavior.

Adelung (1969) has shown that the levels of crustecdysone in the carb Carcinus maenas vary over the duration of the intermolt cycle. Crustecdysone (μg/g wet weight) is secreted at four different times in the molt cycle: 1) 110 μg 6-8 days premolt to induce apolysis; 2) 80 μg 1-2 days premolt to induce molting; 3) 47 μg following molt to harden the cuticle; and 4) 31 μg during intermolt for larval development and the induction of the next molt. The correlations are indirect at best

since these values do not represent levels in the blood. However, if there is validity to these correlations, it could explain the high crustecdysone level in the premolt, and the relatively lower amount in postmolt, females of C. magister. The molting frequency of P. crassipes depends on the crab's relative size but it does occur frequently over the long molting season extending from February to November (Hiatt, 1948), and therefore the intermolt period is called diecdysis where intermolt is hardly distinguishable and postmolt gradually merges into premolt (Highnam and Hill, 1969). The increased release of crustecdysone by P. crassipes late in postmolt, as in this case, or into intermolt when many other females mate (Hiatt, 1948), could be a reflection of the crustecdysone levels necessary for larvae maturation or induction of the next molt.

Fractionation of the C. magister and P. crassipes female's 5% ethanol fraction on ion exchange showed that the radioactivity was concentrated in those fractions showing the highest pheromonal activity. During premolt the major portion of the C. magister activity was found to pass through the Dowex-50 resin, indicating a negatively charged or neutral molecule, while during postmolt the activity seemed to be in the form of a positively charged molecule, which eluted between 0.2N and 5N HCCOH (Fig. 21). Both compounds gave a positive bioassay but the bioassays did not distinguish between a precopulatory and mating pheromone.

P. crassipes females' 5% ethanol samples were chromatographed (Fig. 22) in the same manner as were the C. magister samples except that the intermolt and premolt samples were first run through Dowex-1. In the early (stage A) and late (stage B) postmolt crabs, the activity seemed to be the result of either a negatively charged or neutral molecule, which showed the same elution pattern as the C. magister premolt female pheromone molecule. Since copulation occurred only in the late premolt stages, it was probably the result of the higher concentration of this compound at this time. The positively charged compound which showed the same elution pattern as the "active" C. magister positively charged compound (Figs. 24 & 25) had very low radioactivity and no pheromonal activity.

Interestingly, however, the positively charged compound and the neutral or negatively charged compound from P. crassipes females showed positive bioassays when bioassayed on C. magister males. Although the C. magister ion exchange fractions were not conversely bioassayed using P. crassipes males, bioassays of ethanol fractions from "active" C. magister females using P. crassipes males showed that the 5% and 40% ethanol fractions released mating stances in P. crassipes males. The existence of several "active" pheromonal compounds partially explains the lack of specificity of the individual pheromones-sea water of C. magister and P. crassipes. Crustecdysone and perhaps its negative (or neutral) metabolite could be functional

as P. crassipes mating pheromones, while a negative (or neutral) compound, perhaps the releaser of precopulatory behavior, and a positive compound, perhaps the releaser for mating behavior, seem to be functional as C. magister pheromones. Crustecdysone, which may be the precursor of the C. magister pheromones, also has some pheromonal activity. Whether any or a combination of these compounds act synergistically in releasing reproductive activity in these crabs is not known.

Although research in the field of crustacean pheromones continues, no other crustacean sex pheromone has been positively identified. Christofferson (1970) has characterized the premolt pheromone of the crab Portunus sanguinolentus as a molecule with ionic characteristics and a molecular weight of approximately 1000 while Rajulu et al. (1973) have suggested from their work that 5-hydroxytryptamine or a compound similar to it may function as the sex pheromone in the crab Paratelphusa hydrodromus. The bioassay, in this case, was based on searching behavior rather than any behavioral display specific to reproduction.

It is perhaps significant from the functional as well as evolutionary viewpoint that crustecdysone was shown to be one of the mating pheromones in P. crassipes. Its multifunctional role as a molt inducer, larvae maturation factor (Adelung, 1969), and a sex pheromone makes it metabolically economical. Its structural complexity

gives it a high specificity and although it is a relatively "polar" steroid, its low solubility in aqueous mediums gives it a high binding constant for receptor membranes and facilitates detection at low concentrations (Kittredge and Takahashi, 1972). Its low solubility also facilitates its binding to the epicuticular lipids (Dennell, 1960) during foam bathing, in order that it may serve as a chemotactile mating signal when the crab is out of the water.

In the evolution of pheromone communication, the use of the molting hormone, crustecdysone, as the sex pheromone may have been the result of minor genetic changes, i.e., the "leakage" of the molt hormone into the external medium and the externalization of the receptor sites that were already present on the molt hormone's target cells. This avoids the highly improbable "de novo" evolution of a pheromone and its receptor site (Kittredge and Takahashi, 1972).

Inhibition of the Feeding Response by the Sex Pheromone, Crustecdysone, and Quinones

The simple model of behavior presented earlier in the discussion indicated that when a crab was presented with both the sex pheromone and feeding stimuli, it would release behavior patterns associated with reproductive behavior. The preliminary observations of pre-mating pairs of C. magister and males placed in "active" pheromone-sea water indicated that the presentation of natural food stimuli did

not release the feeding response. Although the males had been starved for a week, no feeding response was released when the antennules were touched with a piece of fish or a cotton swab dipped in 10% fish juice. Part of the feeding response, the manipulation of the piece of fish or the swab with the maxillipeds and maxillae, could be released if the chemosensory hairs on the third maxillipeds were touched by the fish or swab dipped in fish juice, but this response was quickly terminated without the consumption of the food. This phenomena, which is an advantageous adaptation during reproductive interactions, provides an example of a very simple form of central integration of two stimuli.

Since the individual pheromones-sea water from "active" C. magister and P. crassipes females inhibited the feeding responses of their conspecific males while the individual sea waters in which intermolt females were immersed did not, it was decided to bioassay crustecdysone in regards to feeding response.

As mentioned previously, crustecdysone, the copulation releasing pheromone in P. crassipes, also had some pheromonal significance to C. magister. The results (Table 6 and Fig. 26) indicate inhibition of the feeding responses in males of both species.

P. crassipes, exposed to crystecdysone concentrations ranging from 10^{-9} to 10^{-6}M, showed an increasing rate of feeding inhibition with a logarithmic increase in the crustecdysone concentration. The

longer time required for feeding inhibition at lower concentrations probably indicates a summation to some threshold value before inhibition is effected.

The considerably faster feeding response on stimulation of the maxillipeds (0.4 seconds) of C. magister males with a food stimulus points to the reflexive nature of the feeding response at the gustatory level. On the olfactory level the longer feeding response time (3.2 seconds) may be indicative of several interneuronal synapses. The two systems are coordinated, however, since the inhibition of the olfactory system by the sex pheromone delays the feeding response time (1.3 seconds) observed at the gustatory level. This "series of synapses" may very well be the center controlling the integration of the two stimuli.

Although there is little information concerning the morphology and neurophysiology of the antennular chemoreceptors, the assumption has been made, in accordance with existing chemoreceptor studies (Dethier, 1968; Ghiradella, Case and Cronshaw, 1968; Mellon, 1968), that there are a multiplicity of sex pheromone receptor sites independent of the taurine and amino acid receptor sites. The calceoli (sexual dimorphic characteristics), which are located on the second antennae of the male amphipod, Gammarus duebeni, have been shown autoradiographically to accumulate the tritium label from sexually "active" female conspecifics which had been fed the livers of trout

injected with acetylglucosamine-H^3 (Dahl, Emanuelsson and von Mecklenburg, 1970). Although decapods lack calceoli, specialized hairs, the aesthetascs on the outer flagellum of the antennule, have been shown to have chemosensory function in the lobster, Panulirus argus (Laverack, 1964). The aesthetascs on the antennules of the crab, Portunus sanguinolentus, seem to be sensitive to the sex pheromone and other chemical stimuli (Christofferson, 1970). The time-dependency of the inhibition of P. crassipes suggested a high binding affinity of the crustecdysone molecules for the receptor sites. More label incorporated into the antennules of the amphipod G. duebeni after longer exposure to the pheromone tends to support this assumption (Dahl et al., 1970).

No clear understanding has been established for the mechanism by which the sex pheromone and feeding activator inputs are integrated releasing mating behavior and blocking antennular response. In crabs the entire sequence from the activation of feeding by stimulation of the chela with an object, through the feeding process of manipulation with the chela, maxillipeds, and maxillae, and finally to ingestion, can occur after removal of the brain (Bethe, 1897). Copulation can also occur after similar extirpation (Wiersma, 1960). The specific control center regulating the release of the appropriate behavioral response when more than one stimulus is presented, may well be located within the medulla terminalis, a segment of the brain located

within the eyestalks (Bullock and Horridge, 1965). Thus, the lobster, P. argus, loses its antennular chemosensitivity to food stimuli after eyestalk ablation and subsequently shows discoordination in feeding and tendencies to ingest non-food items (Maynard and Sallee, 1970). This anosmic antennular response to food stimuli after eyestalk ablation also occurred in C. magister and P. crassipes. In the absence of appropriate stimuli, eyestalk ablated P. crassipes displayed random feeding movements, mating stances, and aggressive postures. It is quite possible that the pheromone inhibition and the regulation of other behavioral patterns such as social interaction and migration occurs in the medulla terminalis as suggested by Maynard and Yaeger (1968).

Since dopa has a role as a tanning agent in crustacean cuticles (Dennell, 1947), there was a possibility that it might be released by the crab either prior to or after molting. Dopa is also a precursor to melanin in octopus ink and since MacGintie and MacGintie (1968) had reported that the olfactory abilities of the moray eel were inhibited by exposure to the ink (an allomone in this case), a decision was made to test the inhibitory effects of dopa on P. crassipes.

After initial positive feeding responses to the 10^{-4}M dopa solution (Fig. 27), there was a complete inhibition as dopa catalyzed by the trace heavy metals of sea water oxidized to dopaquinone and then polymerized to melanin. Recovery of the feeding response started after 48 hours immersion in the dopa solution but full recovery did not

occur until 300 hours had elapsed. By subsequent silver oxide oxidations of dopa it was determined that the dopaquinone (2-carboxy-2, 3 dihydroindole-5, 6-quinone) was probably the compound responsible for the inhibition. Its inhibitory effects at the low concentrations (10^{-6}M, if one could assume full oxidation) were transitory and recovery occurred within 24 hours.

The inhibition caused by the dopaquinones, naphthaquinones and spinochromes persisted only when the crab was continually immersed in them. Recoveries of the feeding response were rapid once removed from the test solution. Full recoveries of the feeding response to the levels of the controls, however, did not occur for several days. Crabs immersed in the test solutions recovered also after several days since the naphthaquinones probably gradually polymerized.

The use of chemicals as feeding deterrents and in defensive mechanisms is widespread and may influence the evolution of species and the organization of communities (Kittredge et al., 1974; Whittaker and Feeny, 1971). The study of these systems has been termed allelochemics. Juglone (5-OH-1, 4-naphthaquinone), a feeding inhibitor of a bark beetle (Gilbert et al., 1967), and 1, 4-naphthaquinone, a feeding inhibitor of the cockroach (Norris et al., 1970), has a transitory inhibitory effect on P. crassipes feeding, similar to that of the dopaquinone and spinochromes. In contrast the sex pheromone

has a persistent effect after the removal of the stimulus. Whether the feeding inhibitions by the dopaquinone and spinochrome are centrally mediated inhibitions or examples of Haldane's (1955) "cryptic" odors which act peripherally in "blinding" the chemoreceptors, remains an unanswered question. The transitory nature of the inhibition suggests the latter explanation. Whatever the mechanism, this inhibition of the feeding response in P. crassipes provides a useful tool for the study of integrative systems at a simplified level and for the study of natural compounds and compounds such as the water soluble components of oil (Takahashi and Kittredge, 1973) which may have a profound influence on aspects of the animals' feeding and reproduction.

SUMMARY

1) The action patterns observed in C. magister and P. crassipes were recorded and categorized as either motor patterns or social displays.

2) A feeding response bioassay guaging the intensity of feeding and quantified mating stance bioassays for both crab species was created.

3) Behavioral evidence indicated that C. magister females released a premolt pheromone, which elicited precopulatory behavior, and a postmolt pheromone, which elicited matinb behavior in males. The premolt pheromone of P. crassipes, although still present, had lost its significance since mating, elicited by the mating pheromone, did not occur immediately after molting as it did in C. magister.

4) C. magister females controlled the release of their pheromone. Visual as well as chemical signals were important for the release of courtship behavior by the male.

5) Although visual signals were important for the initiation of courtship behavior, chemosensory and chemotactile information were more important in the release of mating activity in P. crassipes.

6) The rate of release of the mating stance in *P. crassipes* was directly proportional to the concentration of crustecdysone. *C. magister* releases a low intensity response to crustecdysone.

7) Crustecdysone was identified as a component of the pheromones-sea water of both *C. magister* and *P. crassipes*. It appeared to act as the mating pheromone for *P. crassipes*.

8) Compounds more polar than crustecdysone were found to have pheromonal activity. A negatively charged (or neutral) compound seems functional as the precopulatory pheromone and a positively charged compound releases copulation in *C. magister*. A negatively charged (or neutral) compound also releases copulation in *P. crassipes*.

9) *C. magister* releases positive courtship responses when stimulated by the negatively charged (or neutral) compound and also the positive compound isolated from *P. crassipes*. *P. crassipes* responds to the 5% and 40% fractions isolated from *C. magister*. This explains the lack of specificity of these pheromones.

10) The sex pheromone and crustecdysone inhibit the feeding responses of both species of crabs. Inhibition was postulated to occur in the medulla terminalis ganglia present in the crabs' eyestalks.

11) Quinones and naphthaquinones also inhibit feeding but the short termed effect was not believed to be centrally mediated but instead an example of Haldane's (1955) "cryptic" odors.

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