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THE METABOLISM OF ECDYSONE AND ITS PUTATIVE ROLE AS THE FEMALE SEX - PHEROMONE IN THE GREEN SHORE CRAB CARCINUS MAENAS L.

by

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A B S T R A C T

— Both male and female crabs excrete large amounts of ecdysteroids and related metabolites during all major stages of the molting cycle.

A sex - specific pattern in the metabolic pathways could not be established.

These findings contradict the hypothesis that ecdysone functions as the female sex pheromone. —

R E S U M E

— Les Crabes des deux sexes excrètent de grandes quantités d'ecdystéroides et de leurs métabolites au cours des principales phases de la mue.

Les voies du métabolisme ne présentent aucune différence liée au sexe.

Ces résultats sont en désaccord avec l'hypothèse selon laquelle l'ecdysone jouerait le rôle de phéromone sexuelle femelle. —

K E Y W O R D S : Carcinus maenas, ecdysteroids, metabolism, sex - pheromone

M O T S - C L E S : Carcinus maenas, ecdysteroides, métabolisme, phéromone sexuelle

INTRODUCTION

Chemical communication plays an important role in marine communities, however, only a few compounds showing either attractive or repellent activity are chemically characterized so far. The steroid ecdysone, the common molting hormone of the crustaceans, seems to be one of these exceptions. KITTREDGE et al. (1971) concluded from their behavioral experiments that ecdysone additionally acts as the sexual pheromone in brachyuran crabs. The hormone is released by females to attract the males (discussed in DUNHAM, 1978).

Ecdysone and its related compounds are the only steroids identified so far in the phylum of the invertebrates. Ecdysone was first found in insects and characterized by BUTENANDT and KARLSON (1954). Currently it is believed to be the precursor of the active molting hormone, which bears an additional hydroxyl group, situated on the sidechain : 20-OH-ecdysone. A number of studies on the biosynthesis, metabolism and mode of action of the ecdysteroids are already published (for ref. see HOFFMANN 1981).

KITTREDGE et al. (1971) were able to induce mating behavior in male crabs of different species by adding ecdysone to the medium. Only ready-to-molt females are attractive and copulation can only take place if the females are freshly molted (RYAN, 1966). Accordingly, two functions of the steroid seem obvious : it is active internally, eliciting molting, and at the same time serves as an external attractant signalling the preparedness to molt and thus to copulate. But this assumption implies one prerequisite. To avoid intraspecific confusion only ready-to-molt, adult females should release ecdysone. Consequently a marked difference in the mode of the metabolism and the excretion of the steroid must exist between the two sexes. We can try this argument with the help of biochemical methods such as Thin-Layer-Chromatography (TLC), Radioimmunoassay (RIA) and radiotracer experiments. The excretion of the free hormone was mainly studied by SEIFERT (1982) of our working group, whereas its metabolism is the subject of the present publication.

MATERIAL AND METHODS

Mature C. maenas of both sexes of a narrow size range, 27 ± 3 mm, were used. They were collected on the island of Nordstrand. In the experiment depicted in Fig. 2 the size class was 22 ± 1 mm. All animals were kept under controlled aquarium conditions (ADELUNG & PONAT, 1980) and underwent one molt before being taken into the experiment. Molt staging was done according to ADELUNG (1971). The resulting stages can also be expressed in terms of the classical Drach-scheme, see SPINDLER, et al. (1974).

Injections were performed by inserting the needle of a 10 µl Hamilton Syringe (701) through the membrane separating Coxa and Basiischium of a walking leg. Ecdysone was purchased from Simes,

Milano. The generally labelled ^{3}H-ecdysone, specific activity 63 Ci/mMol, was produced by the Zoecon Corporation, USA. It was purified from autolysis products by TLC before use. Generally 1 µg ecdysone was injected. In the case of the tracer experiments this amount contained between 78 and 84 µCi ^{3}H-label. Animals were extracted by a n-butanol/water partition as described by ADELUNG (1971). Extracts were streaked in narrow bands onto Silicagel-TLC plates (Merck 11798, 0.25 mm, Kieselgel 60 F 254) and were developed in an equilibrated chamber in chloroform/methanol, 4 : 1. The resolution was greatly enhanced by using precoated high performance plates with a "concentration zone". All of the described metabolites appeared well separated and highly reproducible as could be verified by statistical treatment (BUCHHOLZ, 1980). In the first experiment (see Fig. 1) 22 fractions were scraped off the plate and eluted three times with the solvent, which then was evaporated and the residue subjected to Radioimmunoassay using the antibody H#3 (specification and technique see GOODWIN, 1977 and BUCHHOLZ, 1980).

In the case of the radiotracer experiments 100 µg of ecdysone and 20-OH-ecdysone were added to the homogenate as carrier and TLC-Standard. The developed plates were read with a Berthold radiothinlayer scanner II. In a second run the radioactivity could be quantified by the built in integration system. Ecdysone and 20-OH-ecdysone were identified on the plates by UV-scanning at 254 nm, and additionally by a derivatisation procedure : the respective bands were eluted and acetylated in acetic anhydride/pyridine, 1 : 1, for 75 min at 22°C. The products were separated by TLC in chloroform/ethanol 4 : 1, and the bands identified by radioscanner and UV-light at 254 nm. The bands were compared with those of correspondingly derivatized, radiolabelled standard hormones. ^{3}H-20-OH-ecdysone, specific activity 3.8 Ci/mMol, was purchased from NEN for this purpose. All other metabolites were identified by their R_f-values.

The seawater in which the crabs were kept was extracted three times with water saturated n-butanol which was washed twice with butanol saturated water. The yield of the procedure was 75 ± 8%, determined in triplicate. The extracts were subjected to TLC and evaluated as described.

RESULTS AND DISCUSSION

In order to test whether unaltered ecdysone was excreted directly, four male crabs were kept in a container with seawater for 36 hours. The water was extracted and the condensed extracts subjected to TLC. The 22 fractions were tested for ecdysone activity by RIA. The chromatographic separation step was introduced to exclude misinterpretation of the results caused by unspecific crossreaction of the antiserum with unknown substances. The main RIA activity found, corresponded exactly to the cochromatographing 20-OH-ecdysone standard. The titer was so unexpectedly high that the upper limit of detection was surpassed by far. Therefore the absolute values could not be determined with sufficient precision (Fig. 1). Calculated from data of SEIFERT (1982) animals of the same molting stage (D_2/D_3) excrete about 80 ng during the 36 hour period. No important crossreaction was found besides some minor peaks in the vicinity of the main peak indicating the presence of closely related degradation products of ecdysone.

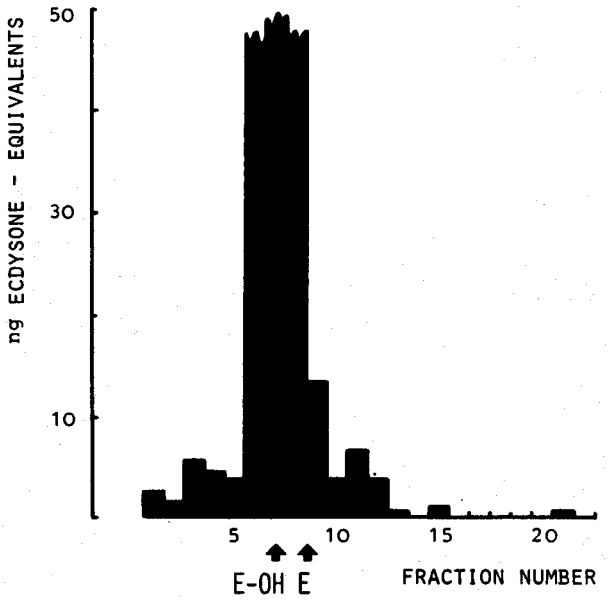

Fig.1 TLC-separation of an extract of aquarium-water in which 4 male crabs in late molting stages, D_2/D_3, were kept for 36h. Determination by Radioimmunoassay.

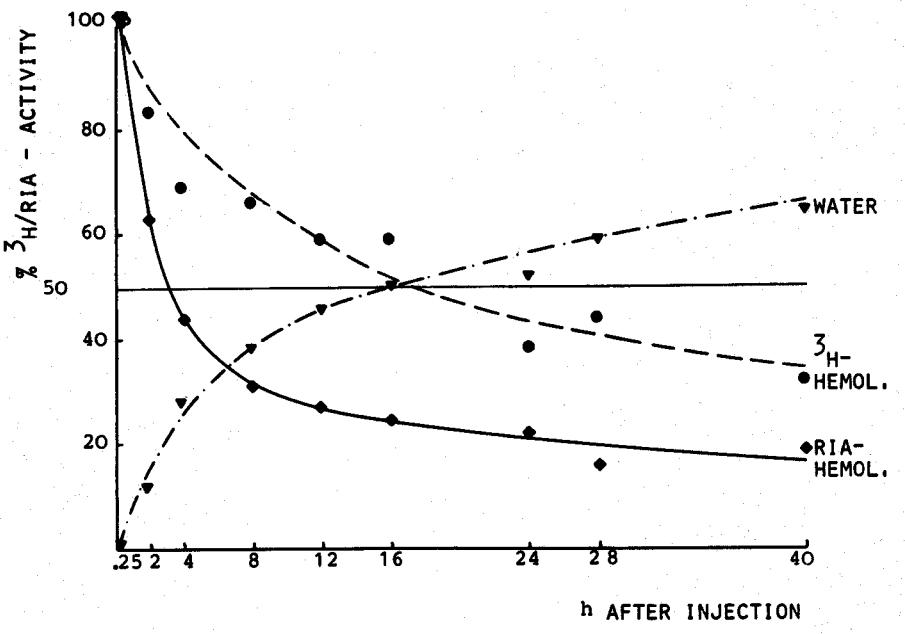

Fig.2 The relative hormone-contents (value after 15 min ≙ 100%) in the hemolymph, and excreted hormone-activity in the aquarium water after injection of 1 µg ecdysone. Determination by RIA and Tritium-labelled hormone.

In a further set of experiments the retention time of ecdysone in the organism was determined by injection of 1 ug/animal. In Fig. 2 the upper curve shows the diminishing values of Tritium activity in hemolymph samples taken from 12 injected crabs of both sexes. The excreted Tritium activity, found in the aquarium water the animals were kept in, is represented by the curve which runs mirror-inverted to the latter. The rate of elimination is rather slow, as 50% of the activity is still present after 16h. The solid curve shows the rate of elimination tested by injection of unlabelled ecdysone determined by RIA. A steep drop is evident. This can be explained by the fact that the injected ecdysone is converted rapidly to 20-OH-Ecdysone (see below). The used antibody is only half as sensitive to 20-OH-ecdysone as to ecdysone. Therefore the conversion adds to the elimination and this causes the steeper slope of the graph. The latter test was necessary, because it was assumed earlier (ADELUNG, 1967) that the injection of exogenous ecdysone stimulates the natural synthesizing structures to produce endogenous hormone. As the curve does not rise again after the initial drop, such a positive feedback mechanism can now be excluded.

In the following the results of the main set of experiments are described. The inactivation and excretion of the molting hormone was investigated in detail. To this end ecdysone, the metabolic precursor of 20-OH-ecdysone, was injected and by employing radioactive label the metabolisation of the injected hormone was examined. According to the slow elimination rate the crabs in this experiment were maintained for 36 h after injection and then extracted. The water the animals were kept in was also examined. In Fig. 3 the metabolites demonstrated on a radiochromatogram are depicted. In both diagrams shown, a substantial peak corresponding to 20-OH-ecdysone is seen besides unchanged ecdysone. In addition, three minor peaks P_1-P_3 are apparent in both extracts. In the water extracts an additional unpolar peak $P_{3.1}$ is evident. Apart from these other minor metabolites appear, but only the described ones are chosen for further study as these are encountered in substantial amounts in all extracts.

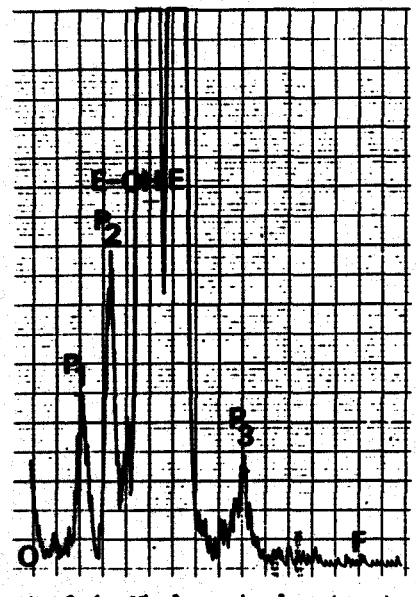

Fig.3.1. Whole-animal extract

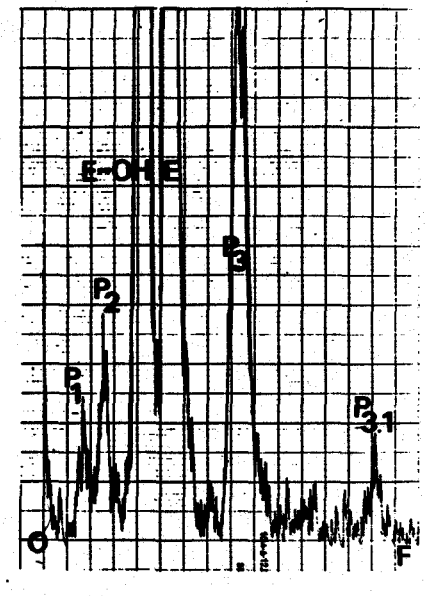

Fig.3.2. Extract of aquarium water

Radio-TLC chromatograms of metabolites in ^{3}H-ecdysone injected crabs and their aquarium water.

If the rate of metabolisation is observed with increasing time of incubation (see Fig. 4), the activity bound to 20-OH-ecdysone after six hours amounts to 50%. The other metabolites show little activity. After 36 h the animals contain only 6% unmetabolized ecdysone. The overall percentage of the minor derivatives increases to 18% of the 3H-activity. If the composition of these metabolites other than ecdysone are observed in more detail, their relative proportion appears to be changing with the duration of the incubation (Fig. 5). At first the unpolar derivatives emerge, but after 36 h the proportion is shifted towards the polar and for this reason slow moving substances.

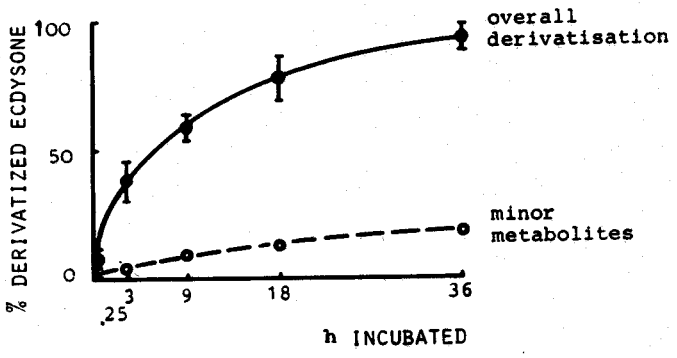

Fig.4 Percentage of metabolized ^{3}H-ecdysone in whole-animal extracts with increasing incubation-times. n = 3, mean and standard deviation indicated.

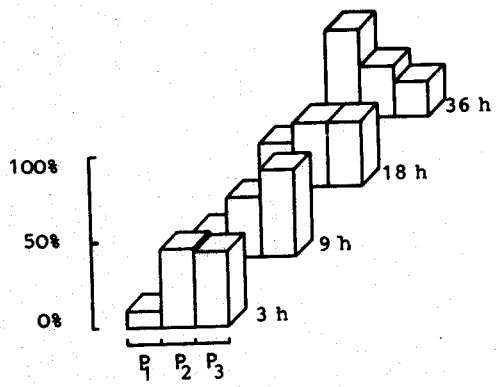

Fig.5 Proportion of metabolites other than 20-OH-ecdysone with increasing incubation-times

In the course of our studies of the molt physiology in C. maenas the changing titer of the ecdysones was determined in relation to the molting cycle (ADELUNG, 1971). By RIA I found a steady increase of the hormone concentration in the hemolymph towards a sharp rise just prior to molting (BUCHHOLZ, 1980; see also ANDRIEUX, 1976). This peak was followed by a steep drop, occuring about one day before the molt. The question was then, whether the rate of conversion and excretion as well as the

relative proportion of the metabolites would also change during the molting cycle. In respect to elucidate the function of the pheromone it is of particular interest, whether a sex-specific difference in the metabolism related to the cycle can be demonstrated.

Accordingly, in the following injection experiments three groups of crabs of different molting phases were used. The groups consisted of three male and three female crabs showing the stages of intermolt, early, and late premolt respectively. In considering the results, the extracts of whole animals contain 76 ± 6% 20-OH-ecdysone and 16 ± 4% of the other metabolites. No significant sex-specific as well as stage dependent differences appear between the investigated groups. The crabs of each group were kept together for the standard period of 36 h in seawater, which was extracted and analysed as described. The results are shown in Fig. 6. The white bars represent the portion of 20-OH-ecdysone and the black areas the overall percentage of the other described metabolites. The difference of the bars from 100% accounts for unmetabolized excreted ecdysone.

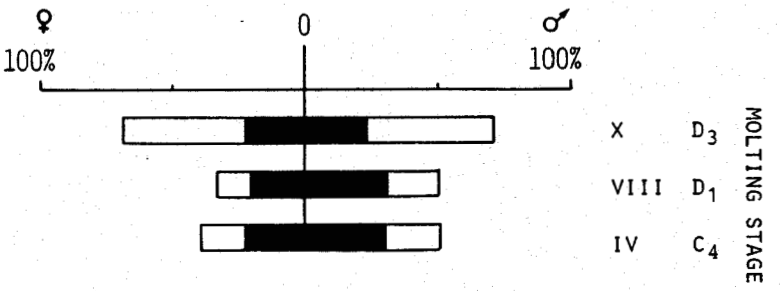

Fig.6 Percentage of metabolized ^{3}H-ecdysone in extracts of aquarium-water. Comparison of the two sexes of crabs in postmolt, early and late premolt stages. White bars: 20-OH-ecdysone; black bars: minor metabolites.

It can be noted that the metabolites other than 20-OH-ecdysone constituate a substantial part of the excreted hormone derivatives. In the earlier stages more than half of the derivatised ecdysone falls to the share of the unknown metabolites. Their relative amount does not vary significantly throughout the molting cycle. If 20-OH-ecdysone is considered, a stage dependent difference is obvious. Shortly before molting at least double as much 20-OH-ecdysone is generated than in the earlier stages. The high internal hormone titer at this stage is a sign for a high demand for 20-OH-ecdysone. Therefore the conversion rate increases, and consequently more metabolised ecdysone is found in the excreta. This derivatisation pattern is common to both sexes. An increase in the conversion rate was also noted by LA CHAISE et al. (1976) in C. maenas and McCARTHY and SKINNER (1977) in Gecarcinus lateralis.

The last diagram, Fig. 7, represents data from the same experiment. Here the percentages of the minor metabolites are considered, whereas 20-OH-ecdysone is omitted. The derivatives found

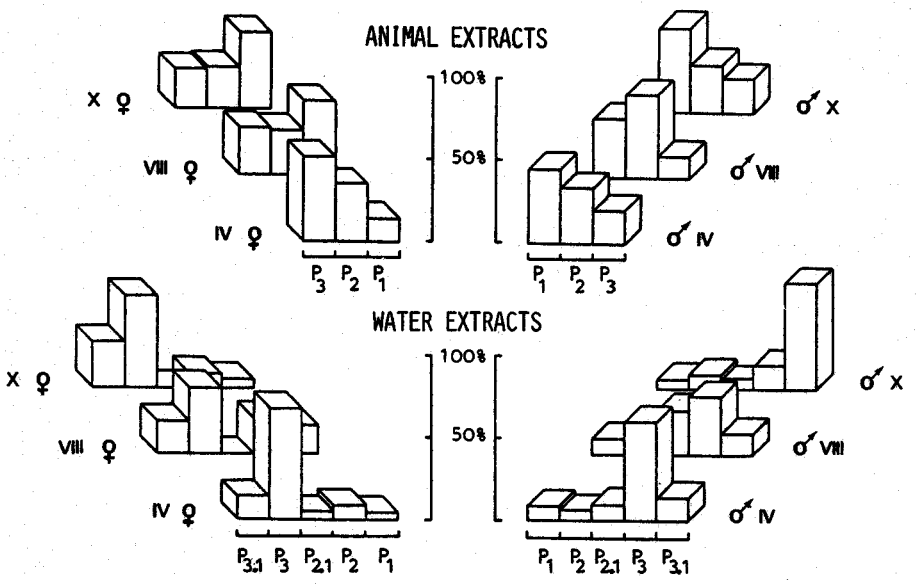

Fig.7 Proportion of metabolites other than 20-OH-ecdysone in whole-animal extracts and extracts of the aquarium water, 36h after injection of ^{3}H-ecdysone. Comparison of the two sexes of crabs in postmolt, early and late premolt stages.

in animals and their excreta are again compared. No major differences between the female and the male groups are apparent in the patterns of metabolites. Besides this result some interesting details can to be pointed out : In the water extract the unpolar derivative P_3 dominates, accounting for up to two thirds of the overall activity. It is the one metabolite which disappears quickly from the animal extracts with increasing incubation time as already shown in diagram 5. In the male animals with the highest molting stage X, the proportion is shifted towards derivative $P_{3.1}$. The same tendency appears on the side of the female crabs, yet the male animals are probably slightly more advanced in their molting stage.

Steroids like the ecdysteroids can be extracted selectively with n-butanol. Polar substances which show a higher solubility in water remain at least partly in the extracted water phases. For this reason samples were taken from the extracted water phases and their radioactivity determined. Apparently (see Tab. 1) the remaining activity in the animal extracts does not change significantly with the molting stage. About 28 $\pm$ 8% stays in the extracts. The picture is different concerning the remaining activity encountered in the extracts of the aquarium water, where a significant increase with the progressing molting stage can be noted. It was not possible to further analyse the nature of these excreted substances which carry up to half of the injected activity. Therefore the results of this part of the derivatisation experiments have to be considered preliminary.

		%	
		a	b
♂	IV	36	7
♂	VIII	27	50
♂	X	39	10
♀	IV	26	1
♀	VIII	25	35
♀	X	16	44
$\bar{x} \pm s$		28±8	

Tab. 1 Remaining activities (^3H) in the extracted waterphases
a) extracts of whole animals (n = 3)
b) extracts of the aquarium water

Taking findings of other authors into account my results can be interpreted as follows : McCARTHY and SKINNER (1979) were able to treat the nonbutanolsoluble excreta of crabs enzymatically. This was done in the land crab Gecarcinus lateralis. They could demonstrate that these substances mainly consist of conjugates of ecdysone, 20-OH-ecdysone, or 20,26-di-OH-ecdysone and a sulfate or glucoside residue. It is highly probable when comparing the R_f-values with results of other authors (KOOLMAN et al., 1979), that the derivative P_1 in my TLC separations corresponds to 20,26-di-OH-ecdysone. This last ecdysteroid is known to be an inactivation product of 20-OH-ecdysone.

In my experiment the polar substances contain high amounts of the injected activity and their importance increases with the molting phase. The male animals of the highest stage again are an exception from the pattern. But I doubt that this is an expression of a systematic sex-dependent difference in the catabolism. It is more likely that these animals, as already mentioned above, are more advanced in their molting stage. I suppose that the male crabs are beyond the highest peak of the hemolymph titer, when large amounts of inactivation products are excreted. Interpreting the findings of McCARTHY and SKINNER (1979) the polar substances probably consist of conjugates of only three ecdysteroids. These ecdysteroids appear as free substances in the radiochromatograms and, as I could demonstrate, their proportion does not show any sex-specific differences. Therefore I consider it unlikely that their respective conjugates behave otherwise.

CONCLUSION

Not only female crabs but also males excrete large amounts of 20-OH-ecdysone directly and unchanged. SEIFERT (1982) confirmed by RIA that prior to molting the excretion increases greatly in adult females and juveniles of both sexes. The rate of excretion is reflected in the changing pattern of the internal hormone titer.

In conjunction with the high concentration in the late molting stages the rate of conversion from ecdysone to 20-OH-ecdysone is increased and the excretion of the metabolite enhanced. The direct excretion of unaltered ecdysteroids seems to be an important way to inactivate the hormone. Moreover, large amounts of further metabolized ecdysteroids as well as polar substances, presumably conjugates, were found in the excreta. All those substances appear in concentrations which would allow them to function as attractants, bearing in mind the impressive chemosensitivity of C. maenas : ABEL (1980) proved that mixtures of certain amino acids elicit a reaction in the animals down to a concentration of 10^{-12} mol.

However, only female crabs are attractive for males and only for a short time just prior to molting. Consequently the sex-pheromone should only be released by premolt females. As 20-OH-ecdysone and other metabolites are produced and excreted during all major phases of the molting cycle by crabs of both sexes, the prerequisite for the function of the pheromone is not provided in the case of these substances. Behavioral experiments (SEIFERT, 1982) equally contradict the hypothesis that an ecdysteroid acts as the female sex-pheromone but unequivocally point to the existence of such an attractant. Experiments to identify this unknown compound are under way in our laboratory.

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