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Behavioral Response of the Crayfish Procambarus clarkii to the Crustacean Molting Hormone 20-Hydroxyecdysone

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Honors Senior Project

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By: William D. Hornsby

Directed by: Dr. Richard Modlin

THE UNIVERSITY OF ALABAMA IN HUNTSVILLE

Honors Program

HONORS SENIOR PROJECT APPROVAL FORM

(To be submitted by the student to the Honors Program with a copy of the Honors Project suitable for binding. All signatures must be obtained.)

Name of Candidate: William D. Hornsby

Department: Biology

Degree: Bachelor of Science

Full Title of Project: Behavioral Response of the Crayfish Procambarus clarkii to the Crustacean Molting Hormone 20-Hydroxyecdysone

Approved by:

Project Advisor — Date: 5/13/97

Department Chair — Date

Honors Program Director for Honors Council — Date: 5/13/97

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Abstract

The importance of 20-hydroxyecdysone, a crustacean molting hormone, in the chemotaxic stimulation of mating behavior has been a matter of controversy. In order to yield more conclusive data on this matter, this project made a careful assay of the compound, along with other compounds with possible stimulatory effects. Sample groups of the crayfish *Procambarus clarkii* were held in acclimatization aquariums for a week before testing was conducted. The testing involved injection of test compounds into an aquarium that contained a single specimen and was equipped with a shielded observation port. Specimens were monitored for a predetermined set of behavioral criteria. These criteria were compared to normal behavior at resting states so that changes in behavior could be observed.

The results revealed that 20-hydroxyecdysone did not elicit a high level behavioral response in the crayfish, but only a low level feeding-type response. This finding did not support the theory that 20-hydroxyecdysone functions as a universal crustacean sex pheromone. Testing with other compounds did produce an initial hypothesis that a primary hydroxyl group may trigger a chemotaxic response in crayfish. This theory was tested using the compound glucuronamide, but conclusive results were not obtained. The author recommends further study of this postulation, as it may yield valuable information concerning the communication systems of crustaceans.

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Introduction

Background

The role of ecdysone derivatives as sex stimulants in crustaceans has long been controversial. The controversy began in the early 1970s when experiments showed that female post-molt urine elicited mating behavior in many marine crustaceans (1). Prior to this, the steroid hormone 20-hydroxyecdysone, or crustecdysone, had been identified as the molting hormone for many crustaceans. As this hormone was present in the post-molt urine, there was reason to believe that it could play a role in the mating process. Subsequent studies suggested that this compound functioned as the trigger for mating behavior in several species of marine crustaceans (2). Because of these experiments, it was widely theorized that 20-hydroxyecdysone could function in a universal role as the sex pheromone of crustaceans.

However, a careful bioassay of 20-hydroxyecdysone and its closely related derivatives conclusively proved that it did not stimulate a sexual response in male lobsters (3). A further study of the broken down metabolites of 20-hydroxyecdysone also failed to cause significant sexual responses in male lobsters (4). These findings discredited the theory that 20-hydroxyecdysone functioned as the universal sex pheromone for crustaceans. Since these studies, no definitive experiment has yielded a plausible theory for the role of 20-hydroxyecdysone in the mating process.

Purpose

New methods for isolating the ecdysones from plant sources have recently been discovered (5). These methods yield more abundant and purer samples than had previously been available. This new source of 20-hydroxyecdysone was chosen to assay a previously untested crustacean, the crayfish *Procambarus clarkii*, in order to discover if this compound may function as a sex stimulant in freshwater crustaceans. It was postulated that data for a freshwater species, which were closely related to the lobsters utilized in earlier tests, may yield valuable clues to the solution of the controversy concerning the role of 20-hydroxyecdysone as a crustacean sex pheromone.

Materials and Methods

Specimen Preparation

The preparation process for the test specimens was designed to cause the least amount of stress possible at the time of the test, so that unclouded data could be obtained. Group I specimens were obtained from a local commercial source. Subsequent groups were ordered from Carolina Biological Supply. All specimens were held in eight-gallon aquariums for at least one week prior to testing. Holding aquariums were filled to a depth of 7 centimeters. Watercress from a local stream was placed in each aquarium to provide both cover and food. To minimize fighting among the specimens, no more than six crayfish were held in any aquarium at once. After 5 days of holding, all specimens were fed green oak leaves and Purina Cat-Chow. Water in the aquariums was changed on average every three days. All water used in this experiment was obtained from a local upwelling site of a natural spring.

Experimental Design

This experiment was designed to carefully observe each specimen's response to a stimulus without presenting other influences on behavior. To this end, a small Plexiglas aquarium was constructed. The aquarium was designed so that 1 liter of water would create a depth of 7.5 cm, sufficient to completely cover the crayfish. This design allowed the specimens freedom of movement, but was small enough to channel them toward the source of the test compounds. Initial tests with the aquarium revealed that the crayfish responded to movement outside the aquarium, so an opaque shield from fiberboard was fashioned that covered all of the aquarium except for an observation port. With the shield in place, the specimens did not respond to any outside motion. The applicator system allowed introduction of a test compound into the aquarium without causing alarm to the specimens. Figure 1 provides an overview of the entire testing apparatus.

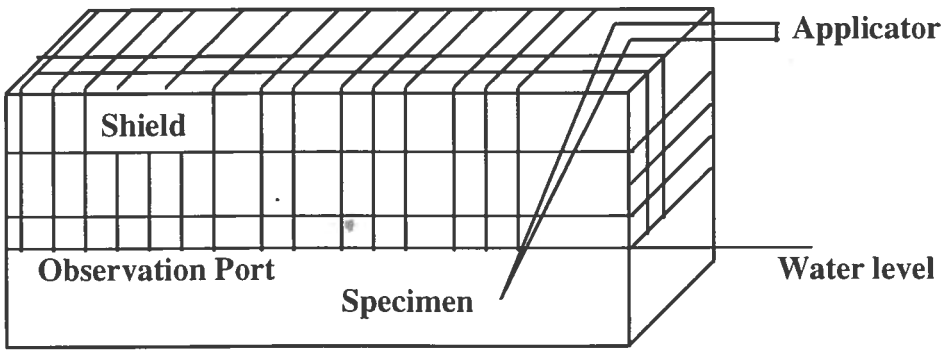


Figure 1- Test Apparatus

Testing Procedure

Prior to testing, all of the testing apparatus was thoroughly washed with hot tap water and then rinsed with deionized water. The apparatus was rinsed again with stream water immediately before a test. The test solutions were mixed daily to the concentrations listed in Table 1. The temperature of the test environment has significance in the behavior of the specimens, so all stream water used in testing was allowed to reach within one degree of the equilibrium room temperature of 20.5 degrees Celsius.

Once the apparatus was prepared, a test specimen was removed from its holding aquarium and placed in the test aquarium. The applicator and shield were then put in place. Following complete assembly of the testing apparatus, the test specimen was allowed to acclimate to the test environment for a minimum period of 45 minutes. Once this minimum time had passed, testing could not begin until the specimen had been observed to be completely stationary for at least 3 minutes. Furthermore, the specimen must be positioned facing towards and within 4 cm of the applicator tip. If all of these stipulations were not met at the end of the minimum acclimation period, the aquarium was tapped lightly until the specimen was in the proper position, then another 20 minute period was allowed in order to reduce stress and external influences on the test crayfish.

Table 1. Working Concentrations of Test Compounds

Compound	Test Concentration
Stream Water (Control)	Pure
20-Hydroxyecdysone	0.000104 Molar
α -Hydroxyecdysone	0.0001076 Molar
Sucrose	5 % Mass Solution
Ethyl Alcohol	5 % Volume Solution
Glucuronamide	5 % Mass Solution

Once the test specimen was acclimatized, the test compound was injected into the aquarium environment. A timer was started at the moment of injection. The average time for complete coverage of the aquarium by an injected compound was 98 seconds, as checked by dye diffusion tests. Beginning at the time of compound application, the behavior of the test specimen was carefully observed and noted for a period of at least 5 minutes. Observation time varied depending on the level of behavioral response the test crayfish exhibited; observation continued until significant response ceased.

Characterizations of Behavioral Response

The criteria that constituted a behavioral response were based on findings in a paper by Dr. D. W. Dunham on the chemosensory roles of crayfish antennules (6). These monitored responses consisted of the following:

- Antennule twitches- quick movements of the short antenna over short distances
- Large Amplitude Movements- slower sweeping of inner antennules over a wide angle
- Main Antenna Sweeping- large scale movements of the main outer antennae
- Large Claw Presentation- raising or sweeping of large claws
- Feeding Motions- rapid movements of maxillipeds and mandibles
- Searching Motions- searching with dactylate periopods (walking appendages)

The behavior of the test specimens was carefully observed for the above responses. A careful count of each type was recorded with a laboratory counter.

Results and Discussion

The behavior of each specimen was carefully observed during the three minute period at the end of the acclimatization period prior to testing. The resting behavior was observed in order to give a standard by which increases in behavioral criteria could be measured. Table 2 lists the increases in each behavioral type in response to each test compound. The sample size (N) of each test group is given with the test compound. Stream water functioned as the neutral control in this experiment. Therefore the levels of increase shown for stream water may be considered as standard response to any movement of current in the vicinity of the test specimens. The sucrose test was conducted in order to familiarize the observer with characteristic behavioral criteria of the crayfish, and may be considered a control.

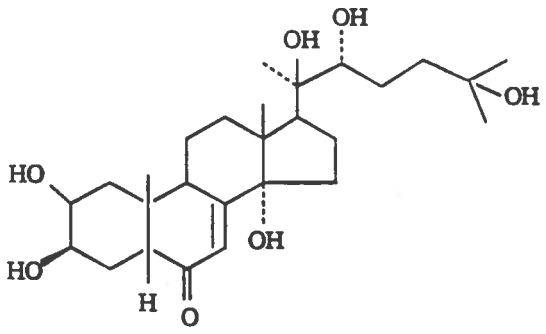
Several points of interest were found from the data. Primarily, the compound 20-Hydroxyecdysone did not stimulate a high level of behavioral response in the crayfish *Procambarus Clarkii*. In the categories most associated with mating behavior (Searching Motion and Large Claw Presentation), 20-Hydroxyecdysone elicited about the same response as the water control. This compound did however stimulate some low level feeding type responses. This finding is similar to the results of the studies with lobsters by Atema and Gagosian (3). Therefore, this experiment does not support the theory that 20-Hydroxyecdysone functions as a sex-pheromone for crustaceans.

The α–Hydroxyecdysone compound initially seemed to cause a high level of response that may have been mating oriented. However, the standard α–Hydroxyecdysone from which the working solution was made was dissolved in ethyl-alcohol. When this was discovered, the tests with the 5% ethyl alcohol solution were

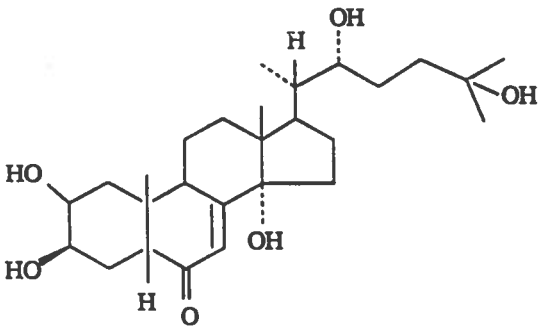
instituted as a control. The findings from this control test suggest that it is the ethyl-alcohol that causes the chemotaxic response in the crayfish. This finding may be significant- the common molecular structure between sucrose and ethyl alcohol is a primary hydroxyl group. **Figure 2** shows the structure of several of the test compounds used in this experiment, and illustrates the primary hydroxyl groups.
20-Hydroxyecdysone lacks this group, which may explain why it fails to trigger significant behavioral responses. This theory is supported by the findings of Atema and Gagosion in their tests with ecdysone metabolites. Their results stated that Compound 9 caused the greatest level of behavioral response in the test lobsters (4:119). This compound was the only tested substance with a primary hydroxyl group. The compound glucuronamide, which is glucose with an amide group substituted for the primary hydroxyl group, was assayed to test this theory . However, the control test with glucuronamide yielded somewhat inconclusive results: high level responses were not elicited in the test subjects, but the responses were greater than those caused by the water control. The results were very similar to the findings for 20-Hydroxyecdysone. These findings support the hypothesis that the primary hydroxyl group is important in the stimulation of high-level chemosensory response.

Table 2. Percentage of Specimens Exhibiting an Increase of Behavioral Criteria in Response to Test Compounds

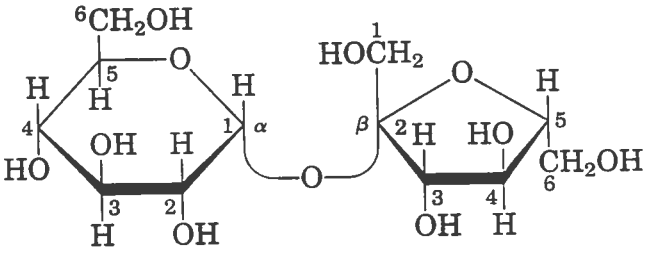
	Stream Water N= 8	20-Hydroxy Ecdysone N= 7	α -Hydroxy Ecdysone N= 8	Sucrose Solution N= 6	Glucuronamide N=7	Ethyl Alcohol N= 6
Antennule Twitches	100%	100%	100%	100%	100%	100%
Large Amplitude Movements	25%	42%	100%	100%	57%	100%
Main Antenna Sweeping	12%	14%	87%	83%	29%	83%
Large Claw Presentation	0%	0%	62%	50%	14%	66%
Feeding Motion	25%	42%	100%	100%	42%	100%
Searching Motion	12%	14%	75%	100%	29%	83%



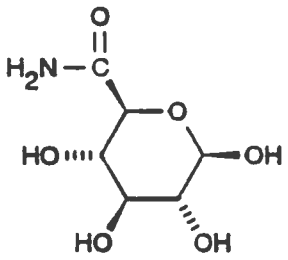
Structure of 20-hydroxyecdysone



Structure of α-ecdysone



Sucrose



Glucuronamide

Figure 2-Molecular Structure of Test Compounds

Conclusion

Although the main test compound, 20-Hydroxyecdysone, failed to trigger a significant response, this project yielded data and results which may provide significant advances in the understanding of chemotaxic induced behavior in crustaceans.

The postulation that it is a primary hydroxyl group which triggers the chemotaxic response in crustaceans is a theory that deserves more attention and study. Therefore, while this project failed to substantiate the role of 20-Hydroxyecdysone as a universal sex-pheromone for crustaceans, perhaps more valuable knowledge resulted.

The participants in this project recommend further research in order to explore the validity of the theory concerning the effect of primary hydroxyl groups. Specifically, other compounds which resemble structurally the response stimulating compounds ethyl alcohol and sucrose, but lacking a primary hydroxyl group, should be assayed. If these compounds fail to trigger behavioral responses, it will be almost certain that the primary hydroxyl group is the triggering agent.

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Appendix: Summary of Raw Data

Test Compound- Water

Specimen #	Antennule Twitches	Large Amplitude Movements	Main Antenna Sweeping	Large Claw Presentation	Feeding Motion	Searching Motion
1	Y	Y	N	N	N	N
2	Y	N	Y	N	N	N
3	Y	Y	N	N	Y	N
4	Y	N	N	N	Y	Y
5	Y	N	N	N	N	N
6	Y	N	N	N	N	N
7	Y	N	N	N	N	N
8	Y	N	N	N	N	N

Y indicates a recorded positive increase in behavioral type.

Note: Specimens 1-4 tested on first day of tests. Thereafter specimens were allowed to acclimate longer and test spring water was held for a longer period prior to testing. These circumstances seemed responsible for the marked decrease in response for later specimens.

Test Compound- 20-Hydroxyecdysone

Specimen #	Antennule Twitches	Large Amplitude Movements	Main Antenna Sweeping	Large Claw Presentation	Feeding Motion	Searching Motion
1	Y	Y	Y	N	Y	Y
2	Y	N	N	N	N	N
3	Y	Y	N	N	Y	N
4	Y	N	N	N	Y	N
5	Y	N	N	N	N	N
6	Y	Y	N	N	N	N
7	Y	N	N	N	N	N

Y indicates a recorded positive increase in behavioral type.

Test Compound- α–Hydroxyecdysone

Specimen #	Antennule Twitches	Large Amplitude Movements	Main Antenna Sweeping	Large Claw Presentation	Feeding Motion	Searching Motion
1	Y	Y	Y	Y	Y	Y
2	Y	Y	Y	Y	Y	Y
3	Y	Y	Y	N	Y	Y
4	Y	Y	N	N	Y	N
5	Y	Y	Y	Y	Y	Y
6	Y	Y	Y	N	Y	N
7	Y	Y	Y	Y	Y	Y
8	Y	Y	Y	Y	Y	Y

Y indicates a recorded positive increase in behavioral type.

Test Compound- Sucrose Solution

Specimen #	Antennule Twitches	Large Amplitude Movements	Main Antenna Sweeping	Large Claw Presentation	Feeding Motion	Searching Motion
1	Y	Y	Y	Y	Y	Y
2	Y	Y	Y	Y	Y	N
3	Y	Y	Y	N	Y	Y
4	Y	Y	N	N	Y	Y
5	Y	Y	Y	Y	Y	Y
6	Y	Y	Y	N	Y	N

Y indicates a recorded positive increase in behavioral type.

Test Compound- Glucuronamide

Specimen #	Antennule Twitches	Large Amplitude Movements	Main Antenna Sweeping	Large Claw Presentation	Feeding Motion	Searching Motion
1	Y	N	Y	N	Y	Y
2	Y	Y	N	N	N	N
3	Y	Y	N	N	N	N
4	Y	N	N	N	Y	N
5	Y	Y	N	N	N	N
6	Y	Y	N	N	N	N
7	Y	N	Y	Y	Y	Y

Y indicates a recorded positive increase in behavioral type.

Test Compound- Ethyl Alcohol

Specimen #	Antennule Twitches	Large Amplitude Movements	Main Antenna Sweeping	Large Claw Presentation	Feeding Motion	Searching Motion
1	Y	N	Y	N	Y	Y
2	Y	Y	N	N	N	N
3	Y	Y	N	N	N	N
4	Y	N	N	N	Y	N
5	Y	Y	N	N	N	N
6	Y	Y	N	N	N	N
7	Y	N	Y	Y	Y	Y

Y indicates a recorded positive increase in behavioral type.

Test Compound- Ethyl Alcohol

Specimen #	Antennule Twitches	Large Amplitude Movements	Main Antenna Sweeping	Large Claw Presentation	Feeding Motion	Searching Motion
1	Y	Y	Y	Y	Y	Y
2	Y	Y	N	Y	Y	Y
3	Y	Y	Y	Y	Y	Y
4	Y	Y	Y	N	Y	N
5	Y	Y	Y	Y	Y	Y
6	Y	Y	Y	N	Y	Y

Y indicates a recorded positive increase in behavioral type.