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OVIPOSITIONAL BEHAVIOR OF *Bracon mellitor* SAY (HYMENOPTERA: BRACONIDAE), A PARASITOID OF BOLL WEEVIL (*Anthonomus grandis* Boh.) I. ISOLATION AND IDENTIFICATION OF A SYNTHETIC RELEASER OF OVIPOSITOR PROBING[1]

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Abstract—Female *Bracon mellitor* Say responded to the frass of diet-reared boll weevil larvae by probing with the ovipositor. Similar responses were elicited by the hexane and chloroform–methanol fractions after differential extraction of boll weevil feces. The active component of the chloroform–methanol fraction was identified as methyl *p*-hydroxybenzoate (methyl parasept). Because the methyl parasept is an artificial component of the diet, the results suggest that the parasitoid response may be associatively learned. The response to methyl parasept decreased with time in the absence of reinforcement. Results demonstrate that certain chemicals may release behavior normally evoked by kairomones, and may interfere with the isolation of natural kairomones.

Key Words—boll weevil, cotton insects, parasitoid behavior, *Bracon mellitor*, *Anthonomus grandis*, ovipositor probing behavior, kairomone, methyl *p*-hydroxybenzoate.

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INTRODUCTION

Host-searching, location, and recognition by entomophagous parasitoids involve a sequence of behavioral responses triggered by various stimuli. The types of clues utilized by parasitoids in host selection have been reviewed by Vinson (1975). Chemicals appear to play an important role as the primary stimuli for host-finding by parasitoids. These transpecific chemical messengers have been referred to as *kairomones* (Brown et al., 1970; Whittaker and Feeny, 1971).

Bracon mellitor Say is an ectoparasitoid (Pierce, 1910; Willard, 1927) of many coleopterous larvae of the family Curculionidae and several species of Lepidoptera (Cross and Chesnut, 1971). The biology (Adams et al., 1969) and ovipositional behavior (McGovern and Cross, 1974) of *B. mellitor* have been described. *Bracon mellitor* has been referred to as the most important parasite of the boll weevil in the southwestern United States (Cross and Chesnut, 1971; Cross, 1973). However, very little work has been done toward elucidating the factors important in host selection by *B. mellitor*. Adams et al. (1969) suggested that females detected the presence of hosts by IR radiation. Folsom (1936) indicated that the parasitoid responded with tactile sensitivity.

Oviposition of boll weevil females in flower buds (squares) and fruits (bolls) of cotton has been described by Cushman (1911). In squares, immature weevils continue to develop until about the molt from second to third larval instar, at which time the squares either completely abscise and fall to the ground or incompletely abscise and "hang" on the plants. Supposedly, the parasitoids use some chemical stimulus elicited by the plant during this abscission to locate areas housing potential hosts. Hunter and Hinds (1905) and Pierce (1908) were among the first to note the significance of these "hanging squares" in consistently containing a higher percentage of parasitized weevil larvae.

Bracon mellitor females must locate boll weevil–susceptible stages inside these plant structures. Realistically, such host location has to be considered in light of the female parasitoid's response to the cotton plant. Bottrell and Walker (unpublished data) indicated a significant response of *B. mellitor* females to freshly abscised cotton squares independent of weevil infestation.

The current study was not intended to mimic the complexity of the cotton ecosystem or to determine long-range host-finding cues used by *B. mellitor*. We were interested in determining whether contact chemicals are a factor important in host selection by *B. mellitor*.

METHODS AND MATERIALS

Insects

The host, *Anthonomus grandis* Boheman, was reared according to the method of Vanderzant and Davich (1958), modified by the addition of 0.2% methyl *p*-hydroxybenzoate in the diet to retard fungal growth. A modification of the procedure of Adams and Cross (1967) was used to rear the parasitoid, *B. mellitor*.

Bioassays

From 5 to 10 third-instar boll weevil larvae were placed in the lid of a 9-cm plastic petri dish and covered with a 9-cm circle cut from a Kim Wipe.® The Kim Wipe was forced into contact with the larvae for 20 min by placing the petri dish bottom on top of the Kim Wipe. The Kim Wipe–covered larvae were then exposed to 20 adult female and male parasitoids for 2 or 3 hr in a 30 × 30-cm-high Plexiglas cage. Two 4-cm holes at each end of the cage for air flow were covered with organdy cloth to prevent the escape of the parasitoids. Adults had access to a solution of 1 part honey and 4 parts water. All insects were held at 26–28°C on a 14:10, L:D circadian cycle and a relative humidity of about 45%.

Initial studies were designed to identify the source of an odor or contact chemical that would result in ovipositor probing by *B. mellitor*. From 6 to 10 small (0.5 cm) squares of diet, frass caps produced by the diet-reared larvae, third-instar larvae, pupae, or legless adults (legs removed to prevent their escape) were placed in a petri dish. In some tests, the samples were covered with a 9-cm Kim Wipe circle, which was contaminated by placing it in contact with the samples. In several tests, the Kim Wipes were elevated 2 cm above the samples, thus preventing their contamination.

Bioassays to monitor the active components during isolation were carried out by applying 10 μl of the frass extract or the purified and isolated chemicals to the center of one half of a 9-cm Whatman® No. 1 filter paper disc. Ten μl of the appropriate solvent was applied to the other half of the filter paper disc as a control. The extracts resulted in a 0.5-cm-diameter spot, which was outlined lightly in pencil. After solvent evaporation, the filter paper disc was placed in a cage with 20 females and 20 males. The responses of females were recorded in two ways: In tests used to monitor the isolation and identification of the active material, the number of females probing the treated spot with their ovipositors at 5-min intervals for 25 min was recorded. In later tests, which examined female response to different concentrations of the identified material, the activity was scored by recording the number of ovipositor probing responses of females and the total number

of female contacts with the test material during 30 min of constant observation. The females used in all tests, with the exception of those in Table 4, cage B, were laboratory-reared, and had been exposed to diet-reared weevil larvae. Some of the females used to obtain the data in Table 4, cage B, were field-collected, and were presumably exposed to field hosts. All tests were replicated and the results expressed as the percentage of contacts by females that resulted in ovipositor probing.

Chemical Isolation and Characterization

From 1 to 2 g of frass from diet-reared third-instar boll weevil larvae were extracted in a solvent series of hexane, chloroform, methanol, and water. In later extractions, the frass was homogenized in cold chloroform:methanol (2:1 vol/vol), and the homogenate was extracted according to Folch et al. (1957). The chloroform lower phase was dried with Na_2SO_4 applied to a florisil column, and eluted with 12 25-ml volumes of chloroform, followed by an increasingly more polar series of solvents. Chloroform fractions 6–8 were the most active, and were further fractioned on silica gel TLC plates developed with chloroform:acetone (90:10 vol/vol). The various bands were scraped off and eluted with methylene chloride. The active band after additional purification by TLC was subjected to mass spectral analysis.

RESULTS AND DISCUSSION

The response of female *B. mellitor* to the diet and various stages of the boll weevil revealed that the artificial diet was not active, while the frass or exudates from boll weevil larvae were active in eliciting ovipositional probing (Table 1). Although a large number of antennal responses to naked boll weevil larvae were observed, very few ovipositional attempts were made. However, female parasitoids were observed to probe the areas around the larvae. Kim Wipes contaminated with boll weevil exudate elicited ovipositor probing by a number of females, while uncontaminated Kim Wipe held 2 cm above the larvae did not elicit such response.

Bioassays of the boll weevil frass extracts (Table 2) revealed the major activity to be present in the hexane, methanol, and chloroform–methanol fractions. Further separation of the chloroform layer of the chloroform–methanol fraction on a florisil column disclosed several active fractions (Table 2). Fractions 6–8 were further separated by TLC. Since the greatest activity was present in a discrete band at R_f 0.06, this band was subjected to further analysis. Thin-layer chromatography of the active band in several systems yielded a single spot. Mass spectral analysis of this fraction yielded a mass spectral fragmentation pattern with M+ 152, *m/e* 121, 39, 65, 93,

TABLE 1. RESPONSE OF FEMALE *B. mellitor* TO DIFFERENT MATERIALS ASSOCIATED WITH BOLL WEEVILS

Material	Total number of females responding at 5-min intervals for 30 min		
	Antennal touching	Ovipositional probing	Control[a]
Media	3	1	0
Frass	27	18	2
Third-instar BW[b] larvae	36	8	1
BW pupae	0	0	3
BW adults	0	0	2
KW[c]-covered media	8	2	3
KW-covered larvae	23	21	1
KW-covered BW pupae	2	0	3
KW-covered BW adults[d]	0	0	1
KW-covered BW larval frass	13	13	2
KW 2 cm above BW larvae[e]	3	3	0

[a] A solution of 1 part honey and 4 parts water on a sponge served as a control.
[b] BW: boll weevil.
[c] KW: A Kim Wipe that covered and touched the material, thus being contaminated.
[d] Legs were removed to prevent movement.
[e] Kim Wipe was held 2 cm above the boll weevil larvae, so that only odors or sound would pass through, eliminating contact chemicals.

and 152. This suggested the methyl ester of *p*-hydroxybenzoic acid (methyl parasept) as the component. Cochromatography of the active material with an authentic sample of methyl parasept yielded a single spot. When authentic samples of methyl parasept were exposed to females, the characteristic ovipositor probing was elicited.

The responses of female parasitoids to different amounts of methyl parasept are shown in Table 3. The maximal percentage of females responding occurred at 100 ng/spot. The range of responses by *B. mellitor* to methyl parasept declined rapidly below 10 ng/spot and above 1 μg/spot. Although many females did not respond by ovipositor probing at the lowest amount, a number of females did respond briefly with antennal examination of the treated spot. The amount of methyl parasept that elicited a maximal response by *B. mellitor* was slightly greater than the amounts of kairomone necessary to maximally stimulate *Orgilus lepidus* (Hendry et al., 1973) and *Microplitis croceipes* (Jones et al., 1971).

Methyl parasept is a component of the host diet; thus, its isolation as an active factor eliciting ovipositor probing by *B. mellitor* suggested a number of interesting questions. Methyl parasept is often added to insect diets to

TABLE 2. OVIPOSITIONAL PROBING RESPONSE OF FEMALE *B. mellitor* TO VARIOUS FRACTIONS OF THE FECES OF DIET-REARED BOLL WEEVIL LARVAE

Feces fraction	Number of females responding at 5 5-min intervals
Hexane extract	10
Chloroform extract	18
Methanol extract	23
Water extract	8
Chloroform-methanol homogenate	17
Chloroform layer	16
Methanol layer	6
Chloroform fractions 1–5[a]	0
Chloroform fractions 6–8	8
Chloroform fractions 9–12	1
Chloroform:methanol (90:10)	5
Chloroform:methanol (80:20)	2
Chloroform:methanol (70:30)	0
Chloroform:methanol (50:50)	1
Methanol	5

[a] Eluates from a florisil column.

TABLE 3. RESPONSE OF FEMALE *B. mellitor* TO DIFFERENT AMOUNTS OF METHYL PARASEPT APPLIED IN ACETONE

Amount of methyl parasept applied (ng)[a]	Contacts	Percentage responding[b]
10^6	24	16.6
10^5	24	25.0
10^4	18	33.3
10^3	26	84.6
10^2	22	90.9
10	20	30.0
1	14	14.2
0.1	18	11.1

[a] Applied to Whatman® #1 filter paper, resulting in a 0.5-cm circular spot.

[b] Only those that raised their abdomens and began ovipositor probing were recorded as responding.

prevent fungal growth (Singh, 1974), but has rarely been reported in nature. One exception is the production of methyl parasept by the giant water beetle, *Dytiscus latissemus*, and several related species (Schildknecht, 1968). The function of methyl parasept in the giant water beetle was presumed to be antimicrobial (Schildknecht et al., 1964).

Because females did not respond to the diet containing methyl parasept, but did respond to frass (Table 1), the levels of methyl parasept in the diet and frass were determined by gas chromatography (3% QF-1). There was essentially no difference in the percentages of water in the diet (average of 71.7%) and in the frass (average of 73.7%). There was also little difference in the amount of methyl parasept in the diet or frass, as the methyl parasept ratio of frass to diet equaled 1.05.

These results were surprising in view of the observation that females probed the frass, did not probe the diet, and yet responded to the methyl parasept fraction. However, in our rearing procedure, the parasites were exposed to boll weevil larvae removed from the diet, placed in a petri dish, and covered with a Kim Wipe. As the Kim Wipe above the weevil larvae become contaminated with frass containing methyl parasept, the methyl parasept would act as an orienting cue. The concentration of methyl parasept in the diet and frass was determined to be 0.4 μg/mg wet weight, which was close to the amount that gave a maximal response in our bioassays (Table 3).

Because methyl parasept gave a response, we field-collected several grams of both weevil-infested squares and weevil larvae. These specimens were extracted and carried through the same procedure used to isolate and identify methyl parasept in the diet. No methyl parasept was detected. The absence of methyl parasept in the field-collected boll weevil larvae and infested squares suggests that *B. mellitor* females may learn to cue on certain chemicals associated with the host. Several species of parasitoids have been reported capable of associative learning (Arthur 1966, 1971; Taylor 1974). Because females exposed to diet in earlier studies had not responded, the responses of females to weevil larvae covered with a Kim Wipe and to methyl parasept were recorded during their repeated exposure to weevil larvae in the diet (Table 4). Females responded to methyl parasept following exposure to Kim Wipe–covered weevil larvae, but did not respond to the diet or frass. However, after repeated exposure of females to plates of diet containing weevil larvae, responses to the frass were observed. When females that were responding to the frass in media plates were reexposed to methyl parasept, the response to the methyl parasept greatly decreased (Table 4). The initial low response of females to the media even though it contained methyl parasept may be due to the presence of components that are slight repellent. Further, because the contraction of methyl parasept was approximately equal in the diet and the frass, the methyl parasept would not serve

TABLE 4. PROBING RESPONSE OF FEMALE *B. mellitor* TO A SEQUENCE OF MATERIALS OVER A PERIOD OF DAYS

Exposure sequence	Exposed materials	Cumulative number of females probing at 5-min intervals for 25 min in each cage		
		A	B	C
1	Kim Wipe–covered larvae	25	19	31
2	Methyl parasept[a]	16	14	17
3	Diet plate[b]			
	Diet	1	0	1
	Frass	2	1	4
10	Diet plate[c]			
	Diet	2	3	1
	Frass	14	9	18
11	Methyl parasept[a]	1	2	2
12	Kim Wipe–covered larvae	12	16	9

[a] Methyl parasept, 100 ng, applied to 9 spots equidistant from one another on a 9-cm filter paper disc.
[b] Responses by females to frass and diet were recorded independently.
[c] Responses recorded as in sequence 3 after 6 previous 8-hr exposures/day to diet plates.

in orienting the female parasitoid to the host or as a cue to host presence. As can be seen in Table 4, the response of females to the frass after being exposed to diet infested with weevil larvae increased after a number of exposures, but the response to methyl parasept decreased. However, the response to boll weevil larvae covered with the Kim Wipe continued. These results, while speculative, can be interpreted if the response to methyl parasept is a learned response in association with a second compound, possibly an unidentified component in the hexane fraction from the frass or weevil larvae.

To determine whether the response to methyl parasept would continue if female parasitoids were not continually reinforced by being exposed to methyl parasept–contaminated frass, a test was designed to expose females only to methyl parasept. Female parasitoids were first exposed to Kim Wipe–covered weevil larvae for 8 hr a day for several days to ensure a strong response to methyl parasept. The females were then held for 2 days with no exposure. On the third day, they were exposed to Kim Wipe–covered weevil larvae for 1 hr, then exposed at different times to filter paper discs containing 9 approximately equally placed 0.5-cm spots, each containing 100 ng methyl parasept. The results (Table 5) show that females responded to methyl parasept through the first 8 hr, with a drop-off in response in 24–36 hr.

TABLE 5. PROBING RESPONSE OF FEMALE *B. mellitor* TO METHYL PARASEPT AT DIFFERENT TIMES AFTER INITIAL EXPOSURE TO DIET-REARED BOLL WEEVIL LARVAE

Time after initial exposure	Cummulative number of females probing at 5-min intervals[a]		
	A	B	C
1 hr	10	11	16
4 hr	9	7	18
8 hr	2	1	5
24 hr	1	1	3
36 hr	0	0	1

[a] Exposed to 100 ng methyl parasept applied to 9 equally placed spots on a 9-cm filter paper disc and placed in cage for 25 min.

The results suggest that the response to methyl parasept decreases in the absence of reinforcement, and is similar to the loss of avoidance behavior exhibited by *Drosophila melanogaster* to certain odors after removal of shock reinforcement (Quinn et al., 1974) or habituations.

The results of this study suggest that *B. mellitor* may associatively learn certain chemical cues, although a more detailed study is necessary before such conclusions can be reached. The study also points up a serious problem in the isolation and identification of kairomones involved in host location and selection by insect parasitoids with a wide host range. Although a very specific bioassay may be developed, it can lead to the isolation of chemicals other than naturally occurring kairomones that are capable of eliciting the behavior under conditions of rearing or the bioassay procedure.

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