Original Article

Oviposition Marking Pheromone of Two Bean Weevils, *Callosobruchus chinensis* and *Callosobruchus maculatus*

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Uniform egg deposition among available host azuki beans by *Callosobruchus chinensis* and *C. maculatus* is expressed by their behavior that the females prefer to oviposit on the beans less conditioned by eggs and a biological conditioning substance (BCS). BCS is excreted by both males and females, but that from females plays a major role. BCS from one species affects another and is supposed to be a similar substance. The weevils discriminate more BCS-conditioned beans from less conditioned beans, but BCS has no deterring activity for oviposition. Thus BCS from both weevils can be defined as an oviposition marking pheromone.

INTRODUCTION

The cowpea weevils (*Callosobruchus maculatus*) and the azuki bean weevils (*C. chinensis*) are the most destructive insects to stored beans. The oviposition behavior of *C. chinensis* is distinct: the females lay eggs among the available azuki beans as evenly as possible.¹⁾ A female of *C. chinensis* initially oviposits one egg on one bean and continues oviposition on most of available beans, and she then begins the second round of oviposition in the same manner.²⁾ Such a behavior was also found in *C. maculatus*.^{3,4)} Yoshida⁵⁾ suggested the presence of a biological conditioning substance (BCS) which functions to mark the oviposited beans. He also tried to explain interspecific competition between *C. maculatus* and *C. chinensis* in terms of BCS. We have reported isolation and identification of BCS in *C. chinensis*⁶⁾: it consists of a mixture of fatty acids, triglycerides and hydrocarbons, and

each component shows more or less activity. This paper is for further documentation on the subject with *C. maculatus* and *C. chinensis*.

MATERIALS AND METHODS

1. Insects

Both *Callosobruchus maculatus* F. and *Callosobruchus chinensis* L. were reared on azuki beans, *Vigna angularis*. The beans were also used for oviposition experiments. All the rearing and experiments were conducted in a room at a controlled temperature of $26 \pm 2^\circ\text{C}$ and 50–70% relative humidity.

2. Collection of BCS

In order to avoid any possible contamination of bean components upon BCS extraction, glass beads of the size of the azuki bean (about 5 mm i.d.) were used as artificial oviposition substrates. Within 12 hr after emergence from the beans, the males and females were collected and put into plastic cages for 24 hr for mating. In one example, 240,000 adults were sexed and each sex was released on the glass beads at the approximate rate of one adult per bead. Sexing was a time and labor consuming procedure. Therefore, when BCS was to be

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collected regardless of the sex, the bean weevils were released on glass beads within 24 hr after emergence. In both cases, after 10-day-release on glass beads, the beads were separated from the weevils by sieving (15 mesh) and washed three times each with ether of the same volume as that of the beads. The combined ether washing was dried over anhydrous sodium sulfate and the filtrate was concentrated using a rotary evaporator at room temperature.

3. Preparation of BCS-treated Beans

Azuki beans were dipped in the ether solution of BCS one by one with tweezer and air-dried. The amount of BCS loaded on one bean was proportional to the concentration of BCS in the solution. The bean dipped in one percent solution was approximately 150 μg /bean.

4. Preparation of Beans with a Different Number of Eggs

One thousand azuki beans were provided for 20 pairs of the weevils, and after 3 days, beans bearing 1, 2 or 3 eggs per bean were individually grouped. Likewise, beans bearing 4, 5, 6, or 7 and those bearing 7, 8, 9 or 10 eggs were grouped after provision of 100 and 200 pairs, respectively.

5. Bioassay

5.1 Oviposition site preference test

The weevils were collected within 12 hr after emergence and allowed to mate for 24 hr. Then, they were sexed and the females were held in a 100 ml flask for 2 hr prior to the bioassay. Each 10 beans of two different groups were alternately placed apart in equal distance on a filter paper (Toyo No. 2, 18.5 cm i.d.) and covered with a petri dish (15 cm i.d.) (Fig. 1). Then 10 gravid females were released there for 1 hr in darkness. After removing the weevils, the total number of eggs deposited during bioassay on one group (Ns) and the control group (Nc) was counted. Different groups of beans were prepared for the comparison as follows: fresh, oviposited, oviposited and then ether-washed, and oviposited and then egg-removed with a razor.

5.2 Oviposition deterring test

Each batch of 300 beans treated with a

Fig. 1 Oviposition preference test.

different amount of BCS in ether was provided for 20 pairs of adults within 12 hr after emergence in a petri dish (9 cm i.d.). In a separate petri dish, 300 beans treated with only ether were provided for 20 adult pairs. After 10 days in darkness, the total number of eggs on each batch was counted. For the time course study, 200 beans treated with BCS (75 μg /bean) were used.

RESULTS AND DISCUSSION

1. Factors Affecting Conditioning of Beans during Oviposition

Oviposition site preference tests were designed for four comparisons: Expt. 1-1, oviposited beans vs. fresh beans; Expt. 1-2, oviposited beans vs. ether-washed oviposited beans; Expt. 1-3, egg-removed beans vs. fresh beans; Expt. 1-4, ether-washed oviposited beans vs. fresh beans. The results are shown in Table 1 for *C. chinensis* and Table 2 for *C. maculatus*. The consequences were similar in both species.

In Expt. 1-1 in both Tables 1 and 2, when one-egg-oviposited beans were compared with fresh beans, Ns/Nc was 15/47 and 22/45 (two replications) for *C. chinensis*, and 31/49 and 25/44 for *C. maculatus*, indicating that the females could recognize even one egg. Discrimination became more distinct when more eggs had been initially deposited. In an example where the initial number of egg/bean was 5 (Table 1), one group was deposited 50 eggs in total ($=5 \times 10$) at the beginning of the experiment. During the one hour experiment, the females deposited eggs almost exclusively on the side of initially fresh beans, resulting in 49 or 53 eggs, while

Table 1 Host preference of *C. chinensis* over oviposition.

Expt.	Host	Ns/Nc (two replications)									
		Initial number of eggs/bean									
		1	2	3	4	5	6	7	8	9	10
1-1)	Oviposited bean/fresh bean	15/47	7/45	1/50	0/54	1/49	0/41	1/57	2/57	1/46	0/51
		22/45	16/45	3/55	0/53	1/53	0/43	0/56	1/67	2/47	0/59
	Mean	0.40	0.26	0.04	0	0.02	0	0.01	0.03	0.03	0
1-2)	Oviposited bean/ether-washed oviposited bean	17/38	8/38	3/30	0/39	0/27	0/26	0/42	0/30	0/23	0/35
		15/40	0/30	5/44	1/40	1/34	2/24	3/29	0/22	2/18	0/35
	Mean	0.41	0.11	0.11	0.01	0.02	0.04	0.05	0	0.06	0
1-3)	Egg-removed bean/fresh bean	21/33	20/41	23/51	25/37	18/45	13/46	16/51	12/42	5/43	18/55
		26/34	21/44	22/43	24/40	7/50	12/40	17/45	10/43	4/40	18/55
	Mean	0.70	0.48	0.48	0.64	0.27	0.29	0.35	0.26	0.11	0.33
1-4)	Ether-washed/fresh bean oviposited bean	35/34	25/48	11/47	15/57	13/46	4/51	12/48	3/52	4/37	11/57
		26/40	16/50	16/52	10/52	20/54	1/44	17/54	2/42	3/50	6/49
	Mean	0.84	0.42	0.27	0.24	0.33	0.05	0.28	0.05	0.08	0.16

Table 2 Host preference of *C. maculatus* over oviposition.

Expt.	Host	Ns/Nc (two replications)									
		Initial number of eggs/bean									
		1	2	3	4	5	6	7	8	9	10
1-1)	Oviposited bean/fresh bean	31/49	19/46	20/59	12/58	12/56	6/68	4/56	4/54	4/50	4/61
		25/44	22/66	32/56	10/66	7/65	1/63	1/56	4/48	3/49	0/50
	Mean	0.60	0.39	0.46	0.19	0.16	0.05	0.01	0.08	0.07	0.03
1-2)	Oviposited bean/ether-washed oviposited bean	33/38	30/35	23/24	15/44	9/47	10/42	17/41	7/33	7/40	5/46
		30/33	36/44	27/36	12/39	15/33	18/46	12/61	9/42	8/42	5/40
	Mean	0.87	0.84	0.85	0.32	0.32	0.31	0.30	0.21	0.18	0.12
1-3)	Egg-removed bean/fresh bean	29/39	29/41	24/30	23/42	10/46	13/33	25/47	13/46	13/48	9/39
		31/41	28/40	31/43	25/39	23/34	43/55	11/33	13/45	12/47	27/52
	Mean	0.75	0.70	0.76	0.59	0.46	0.59	0.45	0.29	0.26	0.37
1-4)	Ether-washed/fresh bean oviposited bean	38/39	30/47	29/42	19/48	19/47	18/42	25/51	11/55	10/49	7/49
		34/48	31/44	24/46	18/47	11/48	23/44	16/52	7/45	7/45	6/50
	Mean	0.84	0.67	0.61	0.39	0.32	0.48	0.40	0.19	0.18	0.14

deposited only one egg, on the side of initially oviposited beans, ending in total 51 eggs. Thus, the number of eggs on both sides became almost equal. In another example where the initial number of egg/bean was 10 (Table 1), one group was deposited 100 eggs in total ($=10 \times 10$) at the beginning of the experiment. The females deposited eggs exclusively on the side of initially fresh beans, resulting in 51 or 59 eggs within 1 hr, which further increased with the elapse of time (not shown in

Tables 1 and 2).

In Expt. 1-2, both sides had been oviposited equally, but one side was washed with ether leaving eggs on the beans. The females preferred the ether-washed side for oviposition, which indicated that an ether-soluble BCS must be involved in oviposition preference. The experiment was for the comparison of egg+BCS vs. egg. When a one-egg-oviposited bean was compared with ether-washed one, there was a tendency to discriminate as indi-

cated by Ns/Nc: 17/38 and 15/40 for *C. chinensis* (Table 1), suggesting that BCS associated with one egg was enough for discrimination. In case of *C. maculatus*, BCS corresponding to 4 or more eggs showed distinct discrimination. Discrimination became more distinct when more eggs had been initially deposited, suggesting that more BCS was present on more densely oviposited beans.

In Expt. 1-3, designed to compare egg-removed beans with fresh ones, the females preferred oviposition on fresh beans. The two experiments, 1-2 and 1-3, indicate that the oviposition behavior is affected by BCS and the females prefer less marked beans for further oviposition.

However, in Expt. 1-4 on the comparison of egg vs. none, the females preferred oviposition on fresh beans, indicating that the egg was also a factor for oviposition. More numbers of eggs, more discrimination.

2. Effect of Isolated BCS on Oviposition

Following three experiments were conducted: Expt. 2-1, collection of BCS; Expt. 2-2, effect of BCS on oviposition site preference; Expt. 2-3, effect of BCS on oviposition deterring.

Table 3 shows the result of Expt. 2-1: the amount of BCS per insect. A female gave two (for *C. maculatus*) to three (for *C. chinensis*) times more BCS than a male, and a female *C. chinensis* gave 1.7 times more BCS than a *C. maculatus* counterpart. In Expt. 2-2 (Table 4), gravid females were given a choice to oviposit either on BCS-treated beans or untreated beans. They preferred untreated beans to beans treated only with *ca.* 20 $\mu\text{g}/\text{bean}$. BCS from the male had some activity, but BCS from the female was several times more active.

Expt. 2-3 was designed to see whether or not BCS had any deterring effect on oviposition. All the beans were treated with BCS and the treated beans were compared with untreated ones in a separate petri dish. As shown in Table 5, there was no significant difference by statistical analysis in the number of deposited eggs after 10 days regardless of the amount of BCS used for the treatment. However, by observation there was a difference: gravid females hesitated to oviposit on BCS-treated beans at the beginning wandering around with-

Table 3 The amount of BCS released from weevils.

Species	BCS $\mu\text{g}/\text{insect}$			
	Sex	Male	Female	Mixed sex
<i>C. chinensis</i>		11.42	36.92	29.70
<i>C. maculatus</i>		9.87	21.26	28.80

Average of over fifty thousand adults during the life span.

Table 4 Effect of BCS on oviposition preference.

Origin of BCS	Ns/Nc ^{a)}			
	Dose ($\mu\text{g}/\text{bean}$)			
	150	75	38	19
<i>C. maculatus</i>				
Female	0	0.09	0.21	0.26
Male	0.13	0.32	0.54	0.66
Mixed sex	0	0.04	0.22	0.41
<i>C. chinensis</i>				
Female	0.05	0.39	0.45	0.49
Male	0.10	0.70	0.81	0.85
Mixed sex	0.11	0.04	0.13	0.15

^{a)} Ratio of deposited eggs on treated and untreated beans.

Table 5 Effect of BCS on oviposition.

Origin of BCS	Total number of oviposited eggs				
	Dose ($\mu\text{g}/\text{bean}$)				
	150	75	38	19	0
<i>C. maculatus</i>					
Female	1015	1039	1080	854	1012
Male	793	820	993	859	
Mixed sex	951	1062	1162	951	
<i>C. chinensis</i>					
Female	1140	1251	1341	1350	1324
Male	888	1181	1212	1199	
Mixed sex	1356	1415	1309	1080	

Twenty adult pairs were allowed to oviposit on 300 azuki beans for 10 days.

out ovipositing, but they eventually oviposited. How the number of deposited eggs per female of *C. maculatus* changed with time was watched. There was a delay of oviposition on BCS (mixed sex)-treated beans (Fig. 2).

Fig. 2 Effect of BCS on oviposition of *C. maculatus* on azuki beans.

Twenty adult pairs were allowed to oviposit on 200 azuki beans untreated (○) or treated (□) with BCS (75 µg/bean).

The females of both *C. maculatus* and *C. chinensis* discriminate between fresh beans and beans conditioned by either eggs or BCS, preferring further oviposition on the less conditioned beans. This is a possible cause of the uniform egg deposition among available beans. Each of eggs and BCS shows such conditioning activity as analyzed, but under natural conditions both of them coexist and contribute for the conditioning. The female BCS, larger in amount and higher in activity, plays a major role in both species, but under natural conditions, both *C. chinensis* and *maculatus* release the same amount of BCS per insect of mixed sex origin. However, to a lower dose of own BCS, *C. chinensis* seems more sensitive than *C. maculatus*. This may be the cause of more distinct discrimination shown by *C. chinensis* than *C. maculatus* in Expt. 1-2 and 1-3 (Tables 1 and 2), where the effect of BCS on oviposition preference is clear.

Effect of BCS (mixed sex) from one species on oviposition by another species is shown in Table 6. BCS from *C. maculatus* was effective on oviposition by *C. chinensis* and *vice versa*, although BCS from *C. chinensis* seemed more effective than that from *C. maculatus* to both species, suggesting a similarity of BCS from two species. Chemistry of BCS from *C. macula-*

Table 6 Effect of BCS from one species on oviposition by another species.

Origin of BCS	Ns/Nc				
	Dose (µg/bean)				
	300	150	75	38	19
Oviposition by <i>C. maculatus</i>					
<i>C. maculatus</i>	0	0	0.04	0.22	0.41
<i>C. chinensis</i>	0.03	0.01	0.03	0.13	0.26
Oviposition by <i>C. chinensis</i>					
<i>C. maculatus</i>	0.14	0.15	0.12	0.48	0.40
<i>C. chinensis</i>	0	0.11	0.04	0.13	0.15
BCS from mixed sex.					

tus will be discussed in detail elsewhere in comparison with that of *C. chinensis*.

There have been reports on oviposition deterring pheromones in several *Tephritidae* species.⁷⁻¹⁴⁾ However, BCS from two species of *Callosobruchus* has no distinct deterrent activity, although a delay of oviposition was noticed. The weevils seem to reduce competition among larvae to maximize the utilization of host beans by using their BCS as well as eggs. Pheromone, by definition, is a substance secreted by an animal that affects the behavior of other animals of the same species. Therefore, BCS, from *C. maculatus* and *C. chinensis* can be defined as an oviposition marking pheromone.

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要 約

アズキゾウムシおよびヨツモンマメゾウムシの 産卵目印フェロモン

酒井案理, 本田 博, 大島康平, 山本 出
アズキゾウムシ, ヨツモンマメゾウムシは寄主アズキ
へ均等に産卵するが, これは卵と BCS (生物的条件づけ
物質) による条件づけが少ないほうのアズキを選び, 雌
が産卵していくためである. この BCS は雌雄が分泌し
アズキに付着するが, 雌からのものが主な役割を果たし
ている. これらのマメゾウムシは BCS の多少を判別す
るが, 産卵阻止活性はなかった. また両種からの BCS は
相互に働き, 類似の物質と考えられる. 以上より BCS
は産卵目印フェロモンといえる.