Studies on the Scent of Stink Bugs (Hemiptera : Pentatomidae)

II. Aggregation Pheromone Activity

Taketoshi ISHIWATARI¹

Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183, Japan

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Aggregate formation of the 1st instar larvae of *Eurydema rugosa* was induced by both olfactory and tactile responses rather than visual perception. The chemical stimuli responsible for the aggregation were derived from scent components produced by the stink bugs themselves. These chemical substances were considered as "aggregation pheromone." *trans*-2-Hexenal, one of the major components of the scent of *E. rugosa*, had roles of "attractant" and "arrestant". The same aldehyde was reported to have an alarm effect for aggregating larvae of *R. rugosa* (ISHIWATARI, 1974), therefore, *trans*-2-hexenal was considered to be not only the alarm pheromone component but also the aggregation pheromone component.

INTRODUCTION

Most of the pentatomid bugs discharge a disagreeable scent which includes unsaturated aldehydes as the characteristic component. This scent has been considered as a "defensive substance" because ants were apparently repelled and paralyzed by it. (WATERHOUSE et al., 1961; REMOLD, 1962). The scent has also been described as an "alarm pheromone" on the basis of the observation that dispersion of bug aggregates occurs following scent discharge of some of its own kind (CALAM and YOUDEOWEI, 1968; ISHIWATARI, 1974). Many kinds of stink bugs lay eggs in a batch, and the newly hatched larvae have a strong tendency to aggregate. If the aggregating larvae are dispersed, they begin to aggregate in different places and settle down within one hour. Although some mechanisms and functions of aggregation phenomena were studied in several non-social insect species, only a few works have been reported on a scent which plays an important role in the formation of aggregation [e.g. *Lycus loripes* (EISNER and KAFATOS, 1962), *Blaps sulcata* (KAUFMAN, 1966), *Blattella germanica* (ISHII and KUWAHARA, 1967) and *Cimex lectularis* (LEVINSON and BAR ILAN, 1971)].

The cabbage bug, *Eurydema rugosa*, deposits egg masses which are normally composed of twelve eggs, and the 1st instar larvae will aggregate even on a sheet of filter paper in the laboratory. A series of experiments using the 1st instar larvae were designed mainly to elucidate mechanisms of aggregate formation and a possible role of the scent of the bugs as attractant and/or arrestant.

MATERIALS AND METHODS

Insects. First instar larvae of the cabbage bug, *Eurydema rugosa*, were primarily

¹ Present address: Otsuka Pharmaceutical Co., Ltd. 463-10 Kagasuno Kawauchi-cho, Tokushima 771-01, Japan

used. They were reared on wild cruciferous plants such as *Rorippa indica*, *R. pulstris* and *Tharaspis arvense*, at 23°C under 14-hour-light photoperiods. Two other species, *Eurydema pulchra* and *Nezara viridula* were reared on cruciferous plants and sunflower seeds, respectively, at 23°C under 15-hours-light photoperiods. Tested insects were removed from the host plants just before each test and used once a day.

Scent source. The scent of stink bugs was extracted with dichloromethane. Whole bodies of 200 larvae (2nd and 3rd instar) of each of the three species were immersed in dichloromethane for two days, filtered, and washed with the same solvent. *trans*-2-Hexenal (Tokyo Kasei Co., Ltd., reagent grade), one of the major components of the scent discharged from the larvae of *E. rugosa*, was also used in solution with dichloromethane.

Antennectomy. Amputation of antenna(e) was made under CO₂ anesthetization with a pair of forceps. (1) Partial unilateral, (2) total unilateral, (3) partial bilateral, and (4) total bilateral antennectomy were conducted. When the whole antenna was amputated, the larva was greatly disturbed and activity was suppressed.

Other special methods employed are described under each subject heading.

RESULTS AND DISCUSSION

Illustration of aggregate formation

Fifteen 2- or 3-day-old 1st instar larvae were gently shaken from the host plant onto a sheet of filter paper (9 cm in diameter) which was horizontally held at a height of 3 cm with a line supporter in a glass pot (12 cm in diameter, 9 cm in depth). The glass pot was kept at 23°C under illumination at 1000 lux. When a larva encountered another individual, both would make physical contact with their antennae and usually settle upon the filter paper in this position. Recording of the reaggregations in this experiment was made by counting the total number of the solitary individuals and the number of reagggregates (composed of at least two individuals) during a 40 minute period. When the experiments were carried out in complete darkness, observation was made by weak red light.

Fig. 1 The shift to the aggregate state of the 1st instar larvae of *E. rugosa* on a sheet of filter paper. Forty replicates were tested under illumination, while 15 were tested under darkness. Tests using each type of antennectomized larvae involved more than 4 replicates, respectively. Ordinate: Total number of solitary individuals and reagggregations.

Fig. 1 shows the shift to the aggregate state of the test insects in light and dark conditions. The ordinate indicates the total number of the solitary individuals and the reaggregations. The number started from fifteen (the solitary individuals only) and decreased to about six after two hours. The experiments were replicated 22 times, and no significant difference was found between the shift to the aggregation under illumination and complete darkness. These results suggest that the aggregation in the 1st instar larvae of *E. rugosa* depends on olfactory and/or tactile responses rather than on visual senses.

Roles of antennae in aggregate formation

Since visual communication was not responsible for aggregate formation, the role of the antennae was examined. At first, it was confirmed that each type of antennectomized larvae never aggregated even after two hours (Fig. 1). When antennectomized larvae were put together with intact larvae on a sheet of filter paper, the aggregation took place among the intact larvae within one hour. No aggregations were found among the heterogeneous individuals and among the antennectomized larvae.

When a solitary larva was released on a sheet of filter paper on which small pieces of cork, rubber, and glass, or freeze-killed larvae of the 1st instar were placed, it drummed these materials with its antennae but never rested in contact with them. This phenomenon suggests that aggregate formation of the 1st instar larvae is not induced by tactile senses only.

Attractiveness of the extracted scent and *trans*-2-hexenal was tested with the following apparatus [1] Y-form glass tube (6 cm in diameter), [2] plastic case olfactometer (23 cm × 33 cm × 6 cm) modified from HIRAO and ISHIKAWA (1964), and [3] two choice tests of the conditioned filter paper (1 cm × 2 cm) modified from ISHII (1970). The scent source was a sheet of filter paper (7 cm in diameter) permeating 1 ml diluted dichloromethane solution of the extracted scent or *trans*-2-hexenal. But the results of the experiments suggest that the scent source, which was the most attractive dose, had very weak appeal for the 1st instar larvae (Table 1). On the other hand, 3rd and 4th instar larvae were tested in a modified McINDOO's olfactometer using a diluted water emulsion of *trans*-2-hexenal. Fig. 2 shows that the tested insects were attracted to as little as 0.001% *trans*-2-hexenal.

Although aggregate formation of the 1st instar larvae depends on an antennal

TABLE 1. ATTRACTIVENESS OF 1/100 INDIVIDUAL EQUIVALENT OF THE EXTRACTED SCENT (E) OR 100 μ g OF *trans*-2-HEXENAL (H) FOR THE 1ST INSTAR LARVAE OF *E. rugosa*

Method or apparatus	Scent source	No. of test insects	No. of attracted insects (%)
Y-form tube	1/100 (E)	40	22 (55.0)
Plastic case olfactometer	100 μ g (H)	122	71 (58.1)
Two choice test of the conditioned filter paper ^a	1/100 (E)	240	142 (59.1)
Two choice test of the conditioned filter paper ^b	100 μ g (H)	250	139 (55.6)

^a Modified method of HIRAO and ISHIKAWA (1964).

^b Modified method of ISHII and KUWAHARA (1967).

Fig. 2. Response of the 3rd and 4th instar larvae of *E. rugosa* to diluted water emulsions of *trans*-2-hexenal. The experiment was conducted in a modified McIndoo's olfactometer.

response, results of these experiments indicate that aggregation behavior is not induced solely by a tactile or olfactory stimuli, but instead is dependent upon both.

Mechanisms of aggregate formation

In order to examine the possibility that both tactile and olfactory senses might be necessary in the formation of aggregation, a series of experiments was designed. Twenty-five pieces of cork of the same size as the 1st instar larvae were fixed with agar solution on a sheet of circular laminated paper (9 cm in diameter). The scent was permeated through 25 small holes made near each piece of cork. The scent source was a sheet of filter paper (7 cm in diameter) in which the extracted scent or *trans*-2-hexenal had been absorbed; this was placed under the perforated laminated paper. Five 1st instar larvae were released on the paper. The total number of individuals which came in contact with the cork bits was recorded for 10 minutes as well as their total resting periods near the cork bits.

Results of the experiments are given in Table 2. The average frequency of

TABLE 2. REACTION OF THE 1ST INSTAR LARVAE OF *Eurydema rugosa* TO THE EXTRACTED SCENT (E) AND *trans*-2-HEXENAL (H), AND TO 25 PIECES OF CORK WITH OR WITHOUT THE SCENT SOURCE

Scent source	No. of cork bits	Replication ^a	Contact frequency (C. F.)	Resting periods (R. P.)	(R. P.)/(C. F.)
None	—	3	0.7 ^b	1.5 sec.	2.1
None	25	6	2.8	17.3	6.2
1/10 (E)	—	2	1.6 ^b	6.5	4.1
1/1000 (E)	—	2	1.4 ^b	12.9	9.2
1/10 (E)	25	5	3.9	50.4	12.9
1/1000 (E)	25	7	4.8	28.0	5.8
50 μ g (H)	—	4	1.8 ^b	3.5	1.9
5 μ g (H)	—	4	1.4 ^b	6.5	4.6
50 μ g (H)	25	4	2.3	15.2	6.6
5 μ g (H)	25	5	4.8	62.0	12.8

^a Each test was conducted by using five larvae for 10 minutes under illumination at 1000 lux.

^b Frequency of approach to the holes.

approach to the 25 holes without scent source was 0.7/larva for 10 minutes. For the holes which emitted the scent, the approach frequency was about twice this value. The average frequency at which contact was made with the 25 bits of cork without the scent source was 2.8/larva 10 minutes. For the cork bits associated with the scent, contact frequency was nearly twice this value. A similar tendency was obtained on the resting period of the larvae near the cork bits. It should be noted that 0.1 individual equivalent of the extracted scent and 5 μ g of *trans*-2-hexenal showed the same potency for the behavioral responses. However, Table 2 suggests that aggregate formation of the 1st instar larvae of *E. rugosa* is induced by both chemical and tactile stimuli.

Species specificity

Aggregation of the 1st instar larvae of *Eurydema pulchra* and *Nezara viridula* was examined respectively under illumination and complete darkness by the same method described for *E. rugosa*. The shift to the aggregate state in the two species indicated a pattern similar to the aggregation of *E. rugosa* (Figs. 3 and 4).

Fig. 3. The shift to the aggregate state of the 1st instar larvae of *E. pulchra* on a sheet of filter paper. Twenty-seven replicates were tested under illumination, while 54 were tested under darkness. Ordinate: Total number of solitary individuals and the reaggregations.

Fig. 4. The shift to the aggregate state of the 1st instar larvae of *N. viridula* on a sheet of filter paper. Twenty-five replicates were tested under illumination, while 27 were tested under darkness. Ordinate: Total number of the solitary individuals and reaggregations.

TABLE 3. COMPONENT SPECIES IN THE AGGREGATED GROUPS BY THE MIXING OF TWO SPECIES^a

Combination	No. of aggregated group			
	<i>E. rugosa</i> alone	<i>E. pulchra</i> alone	<i>N. viridula</i> alone	Two species
<i>E. rugosa</i> + <i>N. viridula</i>	25 (7)	—	21 (8)	1 (0)
<i>E. pulchra</i> + <i>N. viridula</i>	—	6	5	0
<i>E. rugosa</i> + <i>E. pulchra</i>	6	4	—	33

^a First instar larvae of each three species were used. Numbers in parentheses show the results under conditions of total darkness.

The 1st instar larvae of *E. rugosa* were released on the filter paper together with either *E. pulchra* or *N. viridula*. When most of the tested insects reaggregations on the filter paper after 3 hours, each component species was recorded. Since it was difficult to distinguish *E. rugosa* and *E. pulchra* in their larval stage, all of the reaggregations were separately reared until adult stage to identify the species. The tests on combination of *E. rugosa* and *N. viridula* were also conducted in complete darkness. Table 3 shows the proportion of the component species in the reaggregations after 3 hours. Numbers in parentheses indicate the results of the experiments under dark conditions. Aggregate formation in the two species of the same genus does not appear to reflect species-specificity. On the contrary, aggregation among the 1st instar larvae of the two species representing different genera is species-specific under both the illuminated and dark conditions.

Gas chromatograms of the scent extracted from the young instar larvae of *E. rugosa* and *E. pulchra* showed similar patterns, but in that of *N. viridula*, no peak of *trans*-2-hexenal was reported (ISHIWATARI, 1974). This difference in scent components between *E. rugosa* and *N. viridula* may be considered a cause for species-specificity of the aggregate formation.

The chemical stimuli responsible for aggregate formation are derived from the volatile substances produced by stink bugs themselves. These chemical substances inclusive of *trans*-2-hexenal are considered as "aggregation pheromone". On the other hand, Fig. 2 shows that the tested insects are obviously repelled by more than 0.05% water emulsion of *trans*-2-hexenal. ISHIWATARI (1974) reported that the same aldehyde had an alarm pheromone activity for the larvae of *E. rugosa* and other pentatomid bugs. Therefore, the aggregating and dispersing behaviour of the 1st instar larvae of *E. rugosa* is mediated by the identical substance discharged from the bugs themselves.

This bifunctional effect of a particular pheromonal compound in hemipterous insects is concluded as follows. When large quantities of *trans*-2-hexenal are suddenly sprayed, other members of the same species in the vicinity of the predator disperse. When the secretion is exuded gradually and in a very small quantity, the scent plays an important role in the formation of aggregation. Accordingly *trans*-2-hexenal is not only a component of the alarm pheromone but also a component of the aggregation pheromone.

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