Formation of aggregations in adults of the brown marmorated stink bug, *Halyomorpha halys* (Stål) (Heteroptera: Pentatomidae): The role of antennae in short-range locations†

Masatoshi Toyama,* Fumio Ihara‡ and Katsuhiko Yaginuma

National Institute of Fruit Tree Science; Tsukuba 305–8605, Japan

(Received 23 March 2005; Accepted 28 February 2006)

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Abstract

We examined whether stimuli among one another are involved in the short-range locations of the *Halyomorpha halys* adult, which often forms aggregations when overwintering. Ten diapausing adults were released into a clear container at 10°C. After a certain time period, their distribution was compared with a random distribution, and a tendency for a strong concentrated distribution with crowding was observed under both light and dark conditions. However, antennectomy prevented bugs from aggregating and this clarified the role of antenna in short-range locations. This finding suggests that overwintering aggregation in *H. halys* is guided by a chemical or tactile interaction among individuals, at least within a narrow range. In addition, the relationship between this aggregation habit and temperature was examined. Although temperatures of 15°C and 20°C caused bugs to become active, the habit of settling close to one another was not reduced. Based on these results, we inferred that the aggregation habit of *H. halys* adults itself is not temperature dependent, and that the overwintering aggregation is caused by an increase in opportunity for contact with each other, such as from a mutual preference for the overwintering environment, and a decrease in behavioral activity resulting from a decrease in temperature.

Key words: Fruit-piercing stink bug; sanitary pest; pheromone; pest management; apple

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INTRODUCTION

The brown marmorated stink bug, *Halyomorpha halys* (Stål), causes damage to soybeans and various fruits in China, Korea, and Japan (Yasunaga et al., 1993; Hoebeke and Carter, 2003; Kang et al., 2003). In northern Japan, it has recently become a particularly serious pest for fruit such as apples (Goto et al., 2002; Susa et al., 2002; Funayama, 2003, 2004). As observed in the case of other fruit-piercing stink bugs, *Plautia corssota stali* and *Glaucias subpunctatus*, this species also occurs in wooded areas and attacks orchards sporadically. The sole practical measure for controlling this pest involves repeated chemical spraying; therefore, the development of a new management strategy that lowers the environmental load is desired. For example, for other pest stink bugs, the application of pheromones has been studied (e.g. McBrien and Millar, 1999; Tsutsumi et al., 2003), and natural enemies such as egg parasitoid wasps and tachinid flies have been assessed as biological control agents (Corrêa-Ferreira and Moscardi, 1996; Coombs and Sands, 2000; Mizutani, 2001). However, we have no resources that would be useful as strategies for an alternative to chemical spraying to control *H. halys*.

The formation of aggregations during diapause is a remarkable habit exhibited by many heteropterans. *H. halys* adults also form aggregations when overwintering. This is often troublesome for homeowners because large numbers of bugs enter into buildings and form aggregations within them (Watanabe et al., 1994b). Consequently, this behavior may also provide us with an opportunity to reduce the population density of this pest. If this behavior can be controlled, we can obtain tools such as a mass trap, which can help to directly suppress...
the population density or to precisely forecast the population level in the following season. However, our knowledge of the behavioral background of aggregate formation is very limited.

A similar aggregation is also observed in coccinellids. Several studies on the process of aggregate formation during overwintering have been conducted on these insects. Results suggest that the behavior of coccinellids is composed of several steps that are influenced by both physiological and biological factors (Obata, 1986; Majerus, 1997; Nalepa et al., 2000; Schaefer, 2003). An olfactory stimulus from one insect to another is also suggested to play an important role in these steps (Majerus, 1997).

To explore the mechanisms of overwintering aggregate formation in *H. halys*, we divided the process of formation into two phases: 1) long-range orientation to overwintering sites such as buildings, and 2) short-range location consisting of movement within the sites and the final decision to settle. In this study, we investigated whether an aggregation habit mediated by mutual stimuli is involved in the formation of overwintering aggregations by limiting the subject to a short-range location.

**MATERIALS AND METHODS**

**Insects.** *H. halys* adults were collected in Tsukuba, Japan in 2003, and their eggs were used for establishing a laboratory colony. The colony was maintained at 23±2°C under 16L–8D (L: 7:00–23:00) conditions and fed dry soybeans and raw peanuts. The bugs used for the study were isolated 2 wk after hatching and were reared under 12L–12D (L: 8:00–20:00) conditions; a photoperiod leading the bug into diapause; the condition of which was confirmed by checking the development of gonads as they are inhibited in diapausing adults (Niva and Takeda, 2003). One week after adult emergence, they were transferred to a chamber controlled at 15±1°C under a 12L–12D regime. More than 1 wk after the transfer, they were used for experiments (<5 wk old).

**Mutual influence on location of diapausing bugs.** To investigate the mutual influence on the short-range location of diapausing *H. halys*, we observed the distribution of 10 diapausing adults released into a clear plastic container, which was 290 mm long, 215 mm wide and 55 mm high. A sheet of filter paper was placed on the floor of the container, and a water box was kept at the center, to supply test bugs with water and to prevent the interior of the container from drying. Bugs were subjected to a temperature of 5°C for approximately 30 min to immobilize them and were then released into the prepared box. Subsequently, they were provided a recovery period of 1 h at 18°C, followed by a trial period at 10°C. To record the distribution of bugs in the container after the trial period, the container space was divided into quarters, and the number of bugs in each division was counted (Fig. 3); bugs that settled across the border between divisions were counted according to the position of the head. Bugs were considered to have aggregated when they touched each other and showed no or only slight movement. The aggregation size was recorded. In trials where no aggregation occurred, one bug was regarded as having formed a size 1 aggregation, and this was recorded as the largest aggregation size in that trial.

The observed distributions were compared with those under the assumption of random distribution. If bugs locate in a container without any interactions with each other, it is expected that the frequency of the number of bugs within a quarter division will be given by successive terms of the expansion of the binomial, \((1/4 + 3/4)^{10}\). The \(\chi^2\) test was applied for this comparison, in which the number of bugs in a division was classified into six categories (0, 1, 2, 3, 4, and 5–10) to ensure that the expected frequency of each category was kept above five (Sokal and Rohlf, 1969).

The trial was conducted under conditions of both illumination and complete darkness. Illumination was provided by a fluorescent light and was maintained at a low intensity, ranging from 330 to 620 lx, throughout the recovery and trial periods. Two batches, each comprised of 30 males and 30 females, were prepared for trials under illumination, and one batch was subjected to trials under darkness. Each batch was divided into six test groups, which were each comprised of five males and five females; then the test groups were regrouped after each group had undergone one trial. When test insects were deemed unfit for any reason, they were replaced with new ones. Recordings were made 5 h after the start of the trial period, and an additional recording was made after 24 h only.
for trials under darkness. Data after 24 h were used as the control in the comparison of the largest aggregation size and the number of solitary bugs in the following experiments.

Effect of antennectomy. To examine the function of antennae in determining the location of the bugs in the container, we conducted the following three antennectomized treatments: (1) excision of the entire antenna, (2) excision of two terminal segments and (3) excision of the terminal antennal segments. A batch of 30 males and 30 females was assigned to each treatment. Antennae of bugs assigned to (1) were cut at the basal scape. Amputations of antennae were performed under anesthesia by refrigeration at 5°C. The procedure for this experiment was the same as that for the above experiment; all trials were carried out in complete darkness and test bugs underwent a trial period of 24 h. In the comparison between observed distributions and those under the assumption of random distribution, the number of bugs in a division was classified into one of five categories (0–1, 2, 3, 4 and 5–10) to ensure that the expected frequency of each category was kept above five.

Effect of temperature. To examine the effects of temperature on the behavior of the stink bug, the same experiment was conducted at 15°C and 20°C. The procedure was the same as that followed in the experiment at 10°C, which was mentioned above, except for the temperature of the trial period, and test bugs underwent a trial period of 24 h in complete darkness. For this experiment, 30 males and 30 females were assigned to each temperature group.

In the experiment, the number of bugs in a division was classified into one of five categories (0–1, 2, 3, 4 and 5–10) in the comparison between observed distributions of bugs and those under the assumption of random distribution.

RESULTS

Mutual influence on location of diapausing bugs

Diapausing bugs that were released into a container were usually still at the time of recording. They showed a conspicuous tendency to come close to each other under both light and dark conditions.

When bugs were released under illumination, the frequency of a specific number of bugs in a quarter division after 5 h was significantly different from that under the assumption of random distribution, and they located within the container without any interactions with each other (Fig. 1; \( \chi^2 = 528.0, \text{df}=5, p<0.001 \)). This was apparently due to the fact that divisions containing more than four adults were often observed despite the expectation that such divisions would be very rare. At the same time, along with the increase in such divisions containing many bugs, there was a considerable increase in the number of divisions in which no adults were settled and a decrease in the frequency of divisions with 2–4 adults, which occur most frequently with random distribution.
Such concentrations of bugs resulted from their formation of aggregations (Table 1, Fig. 3a). In many cases, the bugs were settled very close to one another at the corner of the container. More than 80% of the trials (39/48) had aggregations comprising more than four bugs, and there was no trial in which bugs did not form any aggregations.

The bugs also formed aggregations in the dark. Aggregations were observed in most of the trials after 5 h and the distribution of concentrations was significantly different from random distribution (Fig. 2; 5 h after: $\chi^2=47.0$, df=5, $p<0.001$). Although more than half of the bugs were solitary after 5 h, and the number of solitary bugs and the largest aggregation size was significantly different from that in the trials under illumination, the tendency to aggregate was accelerated with time (Table 1). After 24 h, the number of solitary bugs was significantly decreased and aggregation of more than four bugs formed in 62.5% of the trials (Fig. 2; comparison between the observed distribution and random distribution 24 h after: $\chi^2=84.6$, df=5, $p<0.001$).

### Effect of antennectomy

Antennectomy prevented bugs from forming aggregations in the container. The distribution of bugs without antennae did not differ significantly from the expectation that they would independently locate in the container (Fig. 3b; $\chi^2=1.72$, df=4, NS). Most of the bugs were solitary (Table 2); no aggregation of more than three bugs was observed in any trial, and there was no aggregation at all in 13 out of 19 trials.

Excision of the two terminal flagella, which possess many hair-shaped sensilla, had the same effect of inhibiting aggregate formation as that of antennectomy. No significant difference was noted between the observed frequency and the expectation under random distribution in terms of the number of bugs in a division ($\chi^2=1.50$, df=4, NS). No trial resulted in an aggregation of more than three bugs,

### Table 1. Aggregation response of diapausing *H. halys* under light and dark conditions

<table>
<thead>
<tr>
<th>Lighting condition</th>
<th>Recording time</th>
<th>No. trials</th>
<th>Largest aggregation (mean±SD)</th>
<th>No. solitary bugs (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Illumination</td>
<td>5 h after</td>
<td>48</td>
<td>6.92±2.26***</td>
<td>2.10±1.94***</td>
</tr>
<tr>
<td>Darkness</td>
<td>5 h after</td>
<td>24</td>
<td>4.00±1.91**</td>
<td>5.12±2.38***</td>
</tr>
<tr>
<td>Darkness</td>
<td>24 h after</td>
<td>24</td>
<td>5.08±2.19 NS</td>
<td>3.46±2.45**</td>
</tr>
</tbody>
</table>

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* Ten adults (five males and five females) were released in each trial, and kept at 10°C.
* *** $p<0.001$, ** $p<0.01$: comparisons by Wilcoxon rank sum test.

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**Fig. 2.** Distribution of diapausing *H. halys* in darkness. Bars show the frequency of a specific number of bugs in a quarter division 5 h and 24 h after the start of the trial. The dots in the figure represent the expectation that 10 bugs would be randomly distributed without any interaction with each other.
and most of the bugs were settled alone (Table 2). In 15 out of 20 trials there were no aggregations of any size.

The excision of only the terminal flagellum did not completely inhibit aggregate formation. A majority of the bugs was observed to be solitary, and the largest aggregation size and the number of solitary bugs were significantly different from those of bugs with intact antennae (Table 2). However, there was a significant difference from the expected frequency by random distribution ($\chi^2 = 18.6$, df=4, $p < 0.01$), and a portion was recognized to form aggregations similar to the bugs with intact antennae; in four out of 20 trials aggregations of more than four bugs had formed, and one of those aggregations consisted of seven bugs.

**Effect of temperature**

It appeared that the bugs became active at higher temperatures; bugs in motion were often observed at 15°C and 20°C. However, as for their location in containers, there were significant differences between observations and the expectation under random distribution at 15°C ($\chi^2 = 79.1$, df=4, $p < 0.001$) and 20°C ($\chi^2 = 93.2$, df=4, $p < 0.001$) because of the higher frequency of divisions with
more than four bugs. Aggregations of more than 4 bugs were observed in half of trials (9/18) at 15°C and in 55.6% of trials (10/18) at 20°C; there were also no significant differences in the number of solitary bugs and the largest aggregation size from trials in comparison with those of the trial at 10°C (Table 3).

**DISCUSSION**

This study showed that *H. halys* adults are in the habit of staying close to one another. This fact suggests that the formation of overwintering aggregations in *H. halys* is not a mere result of independent site selection by individuals, but is at least partially mediated by some mutual interaction among them.

At present, our knowledge of the nature of the stimulus and the manner in which it facilitates the formation of aggregations is limited. However, our observations imply that the interaction is limited to a very narrow range. When bugs were released inside containers, they were observed to walk around within the container and contact among the bugs appeared to occur casually. Bugs that encountered aggregations frequently contacted settled bugs with their antennae and then participated in the aggregations. It is probable that the aggregate formation observed in this study was primarily due to the function of arrestant stimuli rather than attractants.

The fact that this behavior was completely eliminated in antennectomized adults and the fact that darkness did not prevent the bug from forming aggregations suggests that olfactory and/or tactile cues play an important role in the formation and maintenance of aggregations whereas visual information does not. Although bugs’ reactions in forming aggregations in the dark were less frequent in comparison with those under illumination, this is probably due to their tendency to stay more still in the dark. The partial-antennectomy experiment showed that hair-shaped sensilla on the antennae, most of which occur on the two terminal segments, are key receptors of the stimuli; however, this study could not distinguish their sensory functions. To explore interactions in aggregates in greater detail, examination from two perspectives is necessary; i.e., in terms of the chemical sensory function and the tactile function of the antennae.

In this study, no influence by temperature on the aggregation habit was found. Although temperature affected behavioral activity, warm conditions did not always prevent bugs from aggregating; conversely, cold conditions did not always prompt bugs to form aggregations. This indicates that the aggregation habit itself is not influenced by temperature.

It is necessary to include a reference to the relationship between temperature and the formation and maintenance of overwintering aggregations in fields. Watanabe (1994a) observed that diapausing bugs did not completely retreat and form aggregations immediately after arriving at overwintering sites, and suggested that a fall in temperature is responsible for keeping themselves still in retreats. In addition, disintegration of the overwintering aggregation in spring is accompanied by an increase in temperature. However, our laboratory result and this suggestion from field observations contradict each other. Temperature must directly affect the behavioral activity, and high behavioral activity will increase behavioral variations. In contrast, low temperature reduces the behavioral activity of bugs, and keeps them still. Thus temperature indirectly influences the formation and maintenance of overwintering aggregations through behavioral activity. It is probable that this indirect effect hardly appeared under conditions in the narrow experimental space and within the limited time.

The formation of overwintering aggregations is mediated by complicated combinations of various factors (Watanabe et al., 1994a; Nalepa et al., 2000). This study indicates that a mutual stimulus plays an important role in the last process of this aggregate formation as a cue that induces bugs to remain there. However, it also suggests that some other mechanism for assembling bugs in the same place is necessary to enhance contact among them. For example, a mutual preference for the overwintering environment may take part in the mechanism; it must be responsible for their induction into a specific microhabitat and the increase in opportunities to directly interact with one another or at short distances. In addition, we cannot ignore the possibility that a volatile attractant functions in leading bugs to conspecific aggregations. Exploration of the process of search for retreats, including long-range orientation, is a subject for future study.
ACKNOWLEDGEMENTS

We thank K. Funayama of the Akita Fruit-Tree Experiment Station for helpful suggestions, M. Fukuda for her assistance in management of the bugs, and T. Sato for offering the opportunity for this study. We are also thankful to two anonymous referees for their critical reading of the earlier drafts of this manuscript.

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