Aggregation of the black chafer *Holotrichia loochooana loochooana* (Sawada) (Coleoptera: Scarabaeidae): Function of female pheromone and possible adaptive significance

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Abstract
Attracted by their released pheromone, anthranilic acid, the females of the black chafer, *Holotrichia loochooana loochooana*, form aggregations on their food-plant colonies. To investigate the benefits they derive from joining such aggregations, the process of aggregation formation was observed, and the frequency of males landing on lures with or without anthranilic acid [(+)- or (−)-lure] was studied as a function of the lure positioning. Subsequently, within 30 min after the first females settled on a plant, several females would form a small aggregation. Thereafter, the females would land, near the landing spot of the first females, and the aggregation would expand in both the leeward and windward directions. Since there are very large daily and individual variations in the amount of pheromone released by the females, we conducted our field experiments using two extreme pheromone-release models, i.e., a (+)-lure that releases anthranilic acid in a quantity corresponding to the maximum amount released by a female (major female), and a (−)-lure that releases no pheromone (minor female). While no male was observed to land on a single, solitary (−)-lure, the males would land on a (−)-lure when it was positioned within 20 cm, particularly if it was leeward of the (+)-lure. This means that it is possible for females that had released no pheromone to still get mates provided they land within 20 cm from the females that had released a large amount of the pheromone. When a (+)-lure was surrounded by four (+)- or (−)-lures at 10- and 20-cm intervals, the frequency of male landings on the lure was not significantly different from the case of the solitary (+)-lure, except that the landing frequency on the windward (+)-lure was lower than that on other (+)-lures. Thus, it would be more advantageous for minor females to join female aggregations rather than remaining in a solitary mode.

Key words: Female aggregation; mating behavior; mate location; visual cue

INTRODUCTION
The forming of conspecific aggregations is known to occur in various insects: feeding aggregations of fruit flies, communal roosts of butterflies, sleeping clusters of bees, hibernating associations of coccinellid beetles (Matthews and Matthews, 1978), and harem formations of stink bugs (Hibino, 1985, 1986). Although the advantages of forming aggregations have not been investigated directly but were merely suggested in most cases, they can be grouped four general categories: increase of the efficiency of resource use, mate location, protection from natural enemies, and protection from environmental conditions (Wertheim et al., 2005).

The black chafer *Holotrichia loochooana loochooana* (Sawada) is distributed on Miyako, Iriu, Ishigaki and Iriomote Islands in Okinawa, Japan (Azuma and Oshiro, 1967; Nagamine, 1980a,b;
Mating is observed mainly on the beach naupaka, *Scaevola sericea* Vahl (Campanulales: Goodeniaceae), between 16:00 h and 19:00 h from May to July on Miyako Island (Kawamura et al., 2001). The females that feed on a leaf usually take a calling posture and emit pheromone, identified as a free amino acid, anthranilic acid (Yasui et al., 2003). The males were observed flying toward a female and landing directly on its back to copulate (Kawamura et al., 2001). The anthranilic acid attracts males (Arakaki et al., 2003), and causes them to land directly on a black or dark object, such as a female. In the process, the males switch orientation cues from olfactory to visual when they are around 15 to 20 cm from the pheromone source (Fukaya et al., 2004, 2006).

The fact that the female sex pheromone also attracts females, suggests that it promotes female and male aggregation (Arakaki et al., 2003). We found extremely large daily and individual variations in calling durations and amounts of pheromone released by the females (Yasui et al., 2007). In other words, some females may release little or no pheromone even when assuming a calling posture.

In the present field studies, we observed the process of aggregation formation of *H. l. loochoana*, and measured the direction and distance of the female landings near a single pheromone source. Subsequently, we confirmed that the ratio of the sexes was biased toward females. Since the males change from olfactory to visual orientation cues once they come near the pheromone source (Fukaya et al., 2006), this female-biased sex ratio and the non-uniformity in the female pheromone release led us to draw the following hypotheses: A female that releases little or no pheromone may increase its chances of getting a mate if it takes a position near one or more females that had released a large amount of pheromone. If the late-arriving females also release their pheromone within the pheromone plume from the females that arrived earlier, the total attractiveness of the plume to males will increase (Arakaki et al., 2003). Having examined these hypotheses by using cotton lures treated with anthranilic acid, we will provide a brief discussion of the benefits the females obtain from forming aggregations.

**MATERIALS AND METHODS**

**Chemicals.** Anthranilic acid was purchased from the Tokyo Chemical Industry Co., Ltd., Tokyo (purity 99%), and used without further purification since no impurities were detected by high performance liquid chromatographic (HPLC) analyses. Anthranilic acid was dissolved and diluted with methanol of HPLC grade (Wako Pure Chem. Ind., Ltd., Osaka).

**Cotton ball lures.** A wad of absorbent cotton was wrapped in black polyester cloth and tied with plastic coated wire to form a ball of ca. 1.5 cm in diameter. The cotton ball lures were mounted 5 cm above the center and edges of two crossed wooden bars (0.7×0.7×20 or 40 cm length) with wire, and the entire assembly was mounted on a plastic coated, round steel rod (4.2 mm in diam.×60 cm length, Fig. 1). Anthranilic acid (10 mg) was applied as 1 ml methanol solution onto the balls more than 15 min before the experiments (lure). According to the method described by Yasui et al. (2007), the amount of anthranilic acid discharged from this treatment was measured to be almost equal to the maximum level released by a female (ca. 400 ng/30 min). Cotton balls that had not been treated with anthranilic acid (−lure) were used as female models that release no pheromone.

**Field experiments.** All the field experiments were conducted at Nishi-henna-zaki (125°15′E, 24°54′N), on the northern cape of Miyako Island, Okinawa, Japan, from 16:00 h to 19:00 h in late May 2003, 2005 and 2006. Meteorological data during the experiments were calculated from 15:00 h to 19:00 h using the AMeDAS data provided by Japan Meteorological Agency (Table 1).
Experiment 1. To investigate the process of aggregation formation by *H. l. loochooana*, we selected an observation area (ca. 9 m x 5 m) along a pavement path (ca. 2 m wide) between *Pandanus odoratissimus* shrubs of ca. 2.5 m in height, where wind direction was generally constant (Fig. 2a). This area was covered with low colonies (ca. 20 cm ht.) of *S. sericea* and *Ipomoea pes-caprae*, which are food plants for adult *H. l. loochooana* (Fig. 2b).

For recording the positions of the insects, small flags on plastic coated steel sticks were set up as markers at 1-m intervals in the observation area. Pictures of the area were taken in several sections (ca. 3 m x 2 m) and printed on paper sheets. Manually entering data onto these sheets enabled us to easily record times and places of the observed events without disturbing the insects.

Continuous observation of the area under natural conditions was carried out on 24 and 26 May 2005 from 16:00 h until 18:30 h, and data on the landing points, time, sex, and mating of the beetles were recorded. On 27 and 29 May, a cotton ball lure treated with anthranilic acid [(+)-lure] was set at ca. 50 cm above the *S. sericea* leaves at the center of the area (“0” point, Fig. 2b). All the beetles in the area were collected between 18:30 h and 19:00 h on the preceding evening and released at a distance of more than 30 m from the lure.

Experiment 2. In order to measure the distance and direction of the landing points of the females from a pheromone source, (+) - and (-)-lures were individually fixed at ca. 20 cm above the low colonies of *S. sericea* or other food plants. The test area was monitored continuously, and the distance and directions of the landing position and the sex were recorded for chafer that landed within 3 m from the lure. These chafers were collected and soon released at a distance of more than 10 m from the lure. Each lure had a wind streamer of fine fibers attached to the middle of the stick, and wind directions were recorded as twelve azimuths, with 12 o’clock being the windward direction and proceeding clockwise from 1 to 11 o’clock (see Fig. 5). Observations were repeated 5 or 6 times from 16:00 h to 18:30 h on 24, 26 and 27 May 2005.

For statistical analyses, the directions were categorized into four classes: windward (11, 12, and 1 o’clock), rightward (2, 3, and 4 o’clock), leftward (8, 9, and 10 o’clock) and leeward (5, 6, and 7 o’clock). The number of females landed ($X$) was transformed to $(X+0.5)^{1/2}$ and subjected to two-way-layout ANOVA. The means were subsequently ranked by Tukey’s method. In the text, backtransformed means and SEs are presented.

Experiment 3. To determine the distance of visual response of the males within the female

### Table 1. Meteorological data during field experiments

<table>
<thead>
<tr>
<th>Date</th>
<th>Weather</th>
<th>Temperature ($^\circ$C)</th>
<th>Wind velocity (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2003</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28 May</td>
<td>Cloudy/sunny</td>
<td>26.4</td>
<td>5.7</td>
</tr>
<tr>
<td>29 May</td>
<td>Cloudy/drizzly</td>
<td>27.2</td>
<td>5.9</td>
</tr>
<tr>
<td>30 May</td>
<td>Cloudy/sunny</td>
<td>24.8</td>
<td>5.7</td>
</tr>
<tr>
<td>31 May</td>
<td>Sunny/cloudy</td>
<td>25.2</td>
<td>6.1</td>
</tr>
<tr>
<td>2005</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23 May</td>
<td>Sunny/cloudy</td>
<td>29.0</td>
<td>4.1</td>
</tr>
<tr>
<td>24 May</td>
<td>Cloudy/drizzly</td>
<td>27.6</td>
<td>4.7</td>
</tr>
<tr>
<td>25 May</td>
<td>Cloudy/drizzly</td>
<td>24.1</td>
<td>7.0</td>
</tr>
<tr>
<td>26 May</td>
<td>Sunny</td>
<td>26.1</td>
<td>6.5</td>
</tr>
<tr>
<td>27 May</td>
<td>Cloudy/drizzly</td>
<td>26.5</td>
<td>4.2</td>
</tr>
<tr>
<td>28 May</td>
<td>Rainy</td>
<td>24.9</td>
<td>3.3</td>
</tr>
<tr>
<td>29 May</td>
<td>Sunny</td>
<td>26.0</td>
<td>8.3</td>
</tr>
<tr>
<td>2006</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>31 May</td>
<td>Drizzly</td>
<td>24.1</td>
<td>3.1</td>
</tr>
<tr>
<td>1 June</td>
<td>Cloudy</td>
<td>28.3</td>
<td>5.9</td>
</tr>
<tr>
<td>2 June</td>
<td>Cloudy/drizzly</td>
<td>24.4</td>
<td>3.5</td>
</tr>
</tbody>
</table>

*Values indicated are means of values measured at 1-h intervals between 15:00 h and 19:00 h. Original data were measured and provided by the Japan Meteorological Agency (AMeDAS).*
pheromone plume, their landings onto a dummy female placed at different distances from a pheromone source were observed. On the lawn near the *S. sericea* colony, individual (+)-lures on sticks with wind streamers attached were positioned at more than 5-m intervals at a height of 50 cm above the ground. On the leeward side of the (+)-lure, a single (+)- or (−)-lure was placed 5, 10, 20, 50 or 100 cm away from the (+)-lure. The males that landed on the lures were counted, and then removed and released at a distance of more than 30 m from the test area every 10 min, from 16:00 h to 18:30 h. Although the wind direction was generally constant, the position of the leeward lure was checked at every observation time. The experiment had eight replications in total from 31 May to 2 June 2006.

**Experiment 4.** To examine the hypotheses on adaptive significance of aggregation, an experiment was carried out using (+)- and (−)-lures as extreme models of females that release a maximum level of pheromone and of those that release no pheromone at all. A single (+)-lure was surrounded by four (+)-lures or (−)-lures fixed on crossed bars at a distance of 10 or 20 cm from the central (+)-lure (Fig. 1). As a control, a single (+)-lure was affixed to the center of the crossed bars. These sets were placed at more than 3-m intervals unless one was placed on the leeward side of any one of the others. Before the observation, all the chafers within 10 m were removed and released at a distance of more than 30 m from the test area. The males that landed on the lures were counted and removed every 5 min from 16:00 h to 19:00 h. Although the wind direction was generally constant, each crossed-bar set had wind streamers attached and would be adjusted for wind direction at every observation time (Fig. 1). The experiment had 14 replications on 28, 29, and 31 May 2003.

**Statistical analyses.** The number of beetles landed (X) was transformed to \((X+0.5)^{1/2}\) and subjected to two-way-layout ANOVA, where zero data (mean=0) were omitted. The means were subsequently ranked by Tukey’s method, when ANOVA was significant at the \(p=0.05\) level. In the figures, the means accompanied by the same letter were not significantly different, and the same letter was given to the zero data when the confidence range of the mean contained zero \[+(0+0.5)^{1/2}\].

**RESULTS**

**Experiment 1**

Throughout the repeated four days of observations, generally equal numbers of females were observed to land in the area (46, 60, 43, and 47 females, respectively). Observation records were divided into three stages: (1) before 9–13 females had landed to form rather clear aggregations (early stage), (2) before ca. 50% of the remaining females had landed in the area (middle stage), and (3) after that (final stage). These stages are illustrated in Fig. 3a–d.

In the first observation under natural conditions on 24 May, a female was observed to land at point 0-m at 16:25 h, followed, at 17:10 h, by eight females that landed in the observation area, with five of them landing within ca. 1 m from the first female by (Fig. 3a). They appeared to have formed a small aggregation. During this time, one male entered the area from the sideward direction of the first female and oriented itself toward a female to copulate (Figs. 3a and 4). After 17:10 h, 36 females landed one after another at the leeward and windward sides of the first group and 11 males copulated successfully (Figs. 3a and 4). In the second observation on 26 May, when we started the observation, two groups had already formed at the 3-m point and near the −2-m point, and five mating pairs had formed by 16:05 h (Fig. 3b). After that, 35 females landed in the area and 10 of them successfully got a mate (Figs. 3b and 4).

When (+)-lure was set at the 0-m point in the third and fourth observations on 27 and 29 May, the females landed primarily at the leeward side of the (+)-lure in the first stage (Fig. 3c and d). During this stage, three of the females copulated on 27 May, and no males appeared on 29 May. It was sunny but windy during the observation period on 29 May 2005 (Table 1). Subsequently, 33 and 41 females landed in the area, 6 and 3 males entered the area, and 5 and 2 females successfully got a mate on 27 and 29 May, respectively. On 27 May, one male landed on the lure and attempted to copulate with it for a while. After he gave up the attempts, he fed on *S. sericea* leaves but did not attempt to locate a mate on that day.

Local climate conditions are listed in Table 1. Sex ratio (the number of females/number of males) were 45 : 12, 60 : 15, 41 : 11, and 50 : 3 on 24, 26,
27 and 29 May, respectively (Fig. 4). These ratios were significant at \( p=0.001 \) level by chi-square test when sex ratio was supposed to be 1 : 1.

**Experiment 2**

More females were observed to land within 3 m from the (+)-lure than the (−)-lure [4.8±0.22 vs. 0.4±0.16, respectively (backtransformed mean ±SE)], where the difference was significant at \( p<0.001 \) level. The mean distance between (+)-lure and the landing point was 1.33±0.65 m (mean±SD, \( N=22 \)) (Fig. 5). As for the wind direction, there were no significant differences in the number of females landed among the four directions, windward, rightward, leftward, and leeward (chi square test, 1 : 2 : 1, \( p>0.05 \)).

**Experiment 3**

When a pair of black (+)-lures was set along the wind direction at different intervals, there were no significant differences between the windward and leeward in the number of males landing on the lures (Fig. 6a, \( N=8 \)). However, the total number of males that landed on the two (+)-lures were highest when the distance was 20 cm, and the lowest when it was 5 cm (Fig. 6a). The difference between these two extremes is significant at \( p=0.05 \) level by Tukey’s method.

When a (−)-lure was placed in the leeward of the (+)-lure, however, equal numbers of males were observed to have landed on the paired two lures in total (Fig. 6b). Males landing on the (−)-lure were observed as the distance was increased up to 20 cm. However, none were observed at 50 cm.
and 100 cm, while the total numbers of males landing on the (+)-lure and (−)-lure were not significantly different from those with different distances.

**Experiment 4**

When the (+)-lure was surrounded by four (+)-lures, the frequency of male landings differed significantly among the lure positions with respect to the wind direction (Fig. 7, 5P, Tukey’s test, \( p<0.05 \)). Males most frequently chose the leeward lure rather than the other four ones, and the difference from those in the windward side was significant at \( p=0.05 \). By contrast, when a single (+)-lure was surrounded with four (−)-lures, the males landed on the leeward (−)-lure as frequently as on the central (+)-lure (Fig. 7, 1P). When the distance was 10 cm, the males chose the leeward (−)-lure significantly more frequently than the sideward and windward (−)-lures. When the distance was 20 cm, however, the males chose the central (+)-lure significantly more frequently than the sideward and windward (−)-lures.

In comparison with the (+)-lure surrounded by no lures, generally equal numbers of males landed on the solitary (+)-lures and on those surrounded by four (+)- or (−)-lures at distances of 10 or 20 cm \( (p>0.05) \). No males landed on the solitary (−)-lure (Fig. 7).

**DISCUSSION**

In the field, we observed that the first female(s) of *H. l. loochooana* landed and fed on a certain leaf while assuming a calling posture by extruding the pheromone gland. Other females landed individually around the first female, and also fed on leaves, usually assuming calling postures. Subsequently, several females would form small aggregations...
within 30 min after the first females had settled (Fig. 3a, b). After that, the females would land near to each other, one after another, and the aggregation would extend in both leeward and windward directions. These processes also occurred when a \((-/H11001\))-lure was used in place of the first females (Figs. 3c, d, and 5). The females landed not directly on the \((-/H11002\))-lure but around it, and rarely came closer to a \((-/H11002\))-lure (Fig. 5). This indicates that the female pheromone attracts conspecific females, and that it results in formation of female aggregations.

Are there any advantages in male acquisition that depend on the female landing positions? Not all the females taking up a calling posture emit the pheromone (Y asui et al., 2007). As it is impossible to predict the pheromone release by the female calling posture, we conducted field experiments using two extreme pheromone-release models, i.e., a \((+/H11001\))-lure that releases anthranilic acid in a quantity similar to the maximum released by a female, and \((-/H11001\))-lure that releases no pheromone.

When two \((+/H11001\))-lures were placed along the wind direction (Fig. 6) or at a 5-cm interval at right angle to the wind (Fukaya et al., 2006), the males were observed to land equally on the two lures. This suggests that a female releasing a maximum amount of pheromone (hereafter, major female) would have an equal chance to attract a male when she takes a position near another major female. However, the total frequency of male landings on the two \((+/H11001\))-lures showed a peak when the interval was 20 cm (Fig. 6). This is probably because the pheromone concentration and the plume extension were preferable for male orientation. It suggests that a major female has an advantage in male acquisition only when taking a position at an appropriate distance from another major female. By contrast, when the \((+/H11001\)) and \((-/H11002\))-lures were placed side by side in the wind, equal male location was observed until the spacing was extended to 15 cm.
This suggests that a female can get an advantage in male acquisition by landing within 15 cm from a major female even if she releases no pheromone. This might apply to the minor females emitting only small amounts of pheromone, which may represent about half of the total females in the field (Yasui et al., 2007). In cases where many aggregating females each release small amounts of pheromone, the resultant pheromone plume may improve the aggregation members’ chances of attracting males, and joining such an aggregation may be more effective than maintaining a solitary position. Verification of this is an issue for future studies.

When a (+)-lure was surrounded by four (+)-lures at distances of 10 cm, the frequency of male landings decreased by ca. 50%, as compared with a solitary (+)-lure, while the difference was not significant (Fig. 7). When the distances were 20 cm, the center (+)-lure became essentially equivalent to the solitary (+)-lure relative to male landing frequency. The frequency of male landings on each of the five (+)-lures was not significantly larger than that on the single (+)-lure. Therefore, the total attractiveness of the five (+)-lures did not increase in comparison with the single (+)-lure. The hypothesis that the total plume’s attractiveness to males increases (Arakaki et al., 2003) if late arriving females add their pheromone to that of the earlier arrived females, should be revised. The frequencies of male landings on the windward (+)-lure declined significantly more than on the solitary (+)-lure after the additional lures were added (Fig. 7). This suggests that the major female has neither an advantage nor a disadvantage when she is surrounded by major but distant females, but is disadvantaged when major females aggregate leeward of her. In contrast, the increase in male landings on the leeward (−)-lures was significant, especially when the distance was small. It is interesting that a considerable number of females landed at the windward and lateral (rightward and leftward) sides of the (+)-lure or female aggregations (Figs. 3 and 5). It may be true if the females positioned at the windward and lateral side of the (+)-lure, mating chance of them is small but not zero.

With regard to the benefits for the first females, presumably the major females, it is questionable whether they are disadvantaged in acquiring males when surrounded by either minor or major females.
on Miyako Island. Joining an aggregation seems to be beneficial for individual females in getting mates even when they themselves do not secrete sex pheromone.

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