

Female orientation to the male in the white-spotted longicorn beetle, *Anoplophora malasiaca* (Thomson) (Coleoptera: Cerambycidae) by visual and olfactory cues

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Abstract

White-spotted longicorn beetle, *Anoplophora malasiaca* (Thomson) (Coleoptera: Cerambycidae), females were observed to use both olfactory and visual cues for orientation to males in the laboratory. When a freshly killed male was fixed at ca. 10 cm ahead and 5 cm to the right or left from a starting point on a 75° slope panel, 36% of the females oriented themselves toward the male before direct contact. Similar behavioral responses to a glass-rod model treated with male extract were observed. When a black or white glassrod treated with the extract was presented, the orientation toward the black rod was significantly greater than that to the white one. Few females oriented toward rods without extract. This indicates that the olfactory cue is essential for female orientation, and that a visual cue enhances the effect of the olfactory cue.

Key words: *Anoplophora malasiaca*; female orientation; olfactory cue; visual cue; Cerambycidae

INTRODUCTION

In mate location and recognition in cerambycid beetles, sex pheromone is involved as a key signal (Fukaya and Honda, 1992; Fukaya et al., 1999; Hanks, 1999). The female white-spotted longicorn beetle *Anoplophora malasiaca* (Thomson) (Coleoptera: Cerambycidae, Lamiinae) produces a sex pheromone on her body and evokes sequential copulating behaviors in males through direct contact with antennae, palpi, or tarsi (Fukaya et al., 1999, 2000; Akino et al., 2001; Fukaya, 2003; Yasui et al., 2003). Recently, we reported that a female volatile chemical mediates short distance mate location in *A. malasiaca* by males, and that visual stimuli in association with female substances are also involved (Fukaya et al., 2004).

It has also been reported that females approach males in response to male sex pheromone in some cerambycid species. In *Xylotrechus pyrrhoderus* (Cerambycidae), flying females approach males that emit attractant pheromone (Iwabuchi, 1982). Subsequently, the male responds to the female sex pheromone that is in close range, and attempts to copulate (Iwabuchi, 1985). Female approach to

males was observed in *Monochamus alternatus* (Lamiinae) (Fauziah et al., 1987). Kim et al. (1992) confirmed the existence of male attractant pheromone and female contact pheromone. Recently, male-specific compounds were identified and synthesized in *A. glabripennis* (Zhang et al., 2002). These synthetic chemicals showed attractiveness in a typical two-choice olfactometer test.

In the present study we examined close-range mate location by female *A. malasiaca*, and described the significance of volatile and visual cues.

MATERIALS AND METHODS

Insects. Adult *A. malasiaca* were collected in mandarin orange orchards in Oita Prefecture, Japan in mid-June 2003. For extraction and bioassays, males were individually kept in clear plastic cups (8.5 cm diam., 4 cm high), provided with mandarin orange twigs for a few days, and then killed by freezing at temperatures lower than –20°C. For behavioral studies, 250 females were individually kept in plastic cups (ca. 12 cm diam., 10 cm high) lined with a sheet of filter paper, and provided with a piece (ca. 7×20×20 mm) of artificial silkworm

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diet (Silkmate 2S, Nihon Nosan Kogyo, Yokohama, Japan). The diet and filter paper sheet were replaced every 2–3 d. Insects were kept at 25°C, ca. 50% RH, and L15 : D9.

Extraction of male elytra. Male elytra were removed from 50 frozen bodies and immediately dipped in 75 ml of pure ether for 5 min. The extract was separated from the elytra, the residues were rinsed twice with the same volume of ether, and the rinses were added to the extract. The extract was dried over anhydrous Na_2SO_4 , the solvent was removed under reduced pressure below 30°C, and the concentrate was dissolved with hexane and stored below –20°C until use.

Separation of crude male extract with column chromatography. The male extract was chromatographed on a silica gel column (particle size: 75–150 μm , Wako gel C-200, Wako Pure Chem. Ind., Ltd., Osaka, Japan). The crude extracts of male elytra (50 male equivalents: ME) were applied as hexane solution onto a silica gel column (ca. 5 g SiO_2). Compounds were successively eluted with 50 ml hexane, 10, 20, and 50% ethyl acetate (EtOAc) in hexane, and EtOAc and methanol (MeOH).

Extraction of partial male body. Five frozen male bodies were dissected into head, prothorax, meso+metathorax, elytra, hind wings, and abdomen. Each body part was dipped separately in

7.5 ml of pure ether for 5 min. The extracts were separated from each part, which was rinsed twice with the same volume of ether, and the rinses were added to the respective extracts. The extracts were dried over anhydrous Na_2SO_4 , the solvent was removed under reduced pressure below 30°C, and the concentrate was dissolved in hexane and stored below –20°C until use.

Glass-rod models. Black and white glass-rod (12 mm diam. \times 35 mm length; similar to *A. malasiaca* body size) models were prepared by painting with black and white acrylic paints (Ivory Black and Titanium White, respectively, Liquitex Color Acrylic Soft Type, Bonny Corporation Co. Ltd.). All painted models were then covered with two layers of a transparent covering medium (Gloss Polymer Medium, Liquitex, Bonny Corporation Co. Ltd.). These were prepared more than 1 d before use in the experiments to fix the medium completely on the glass surfaces.

For the assay, 1 ME of male elytra extract in 20 μl of hexane solution was treated on the upper surfaces of the painted glass-rod models.

Trail analyses of females walking toward male cues. Trail analyses of females were performed in the same manner as Fukaya et al. (2004) (Fig. 1). Either a dead male body or a glass-rod model was horizontally fixed at the center (point M) of a white sheet of paper (21 \times 30 cm). The sheet was pasted

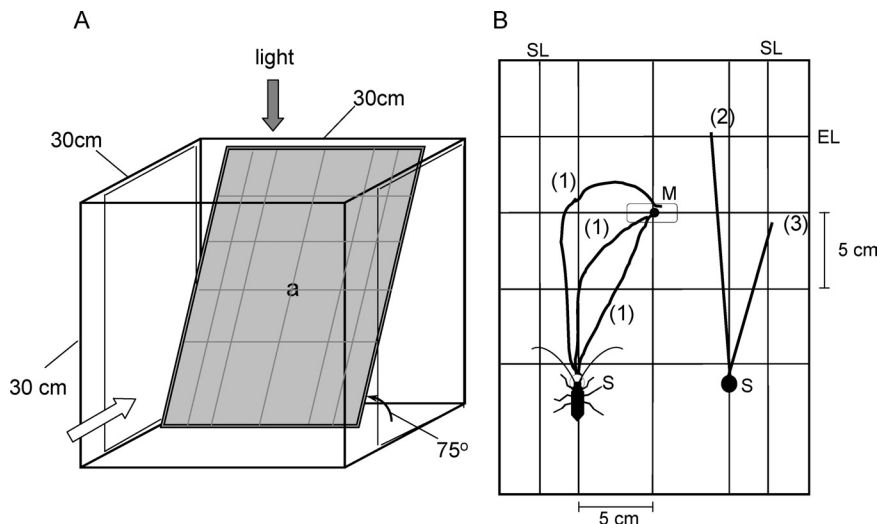


Fig. 1. Behavioral bioassay equipment for female trail analyses. Outline of the equipment (A): Both lateral sides and bottom were covered with white paper sheets, and illumination was through the ceiling of the box. An acrylic plate pasted with an assay sheet (a) was inclined into the box at 75°. In the center of the assay sheet (B), either the dead male body or a glass-rod model was fixed (point M). Test females were introduced at the starting point (S). Typical walking trails were (1) orientation to the model (female curved towards the model), (2) straight walking, and (3) no orientation (see text for details).

on an acrylic plate of the same size, which was inclined into a clear acrylic box (30×30×30 cm) at 75°. The surface of the sheet was illuminated at 1,000 to 1,500 lx by a fluorescent light from the ceiling of the box, the lateral sides and the bottom of which were covered with white sheets of paper. Through the open front side of the box, females were individually introduced at the starting point (S) on the sheet, which was 10 cm ahead and 5 cm either to the right or left of point M.

A trail of the head position of the female was followed and recorded. This was repeated at least 42 times with different beetles. When a female touched a model or a male body with her head, or held it with her forelegs, these behaviors were recorded as “arrival.” When the female stopped for more than 2 min, the observation was cancelled. Dead male bodies were defrosted at room temperature 2 h before the bioassay. The assays were completed within 40 min after treatment of extract on the glass-rod models.

Female walking trails were categorized as follows (see Fig. 1B): (1) Orientation: Female curved towards the model before direct antennal contact; (2) Straight walking: Walking straight along the vertical line until the head passing the end line (EL); (3) No orientation: Female’s head passed over the sideline (SL). In addition to the trails, female arrival at point M was also recorded.

Statistics. For comparisons of values of female responses, when an $n \times 2$ χ^2 -test was significant, a paired χ^2 -test adjusted by Bonferroni inequality was subsequently applied. Values accompanied by the same letter in the figures are not significantly different at $p=0.05$ level. Logistic regression of ANCOVA-type was used in testing the influence of dose and color on the frequency of response.

RESULTS

Female response to male body

When point M was vacant (Fig. 1), 94% of the females were observed to walk straight from the starting point to the upper end, 4% showed orientation and 2% passed point M and were judged as “arrival” (Fig. 2, $N=50$). When a dead male was presented at point M, 36% of the females showed orientation before making contact and 28% arrived at the body (Fig. 2). Females could orient themselves to a male body before making direct contact.

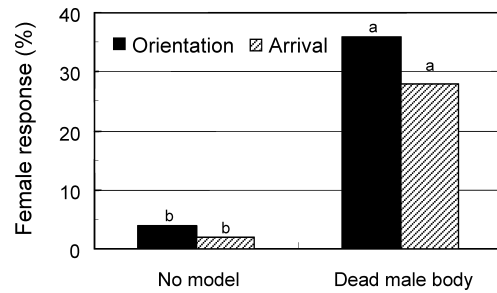


Fig. 2. Responses of *A. malasiaca* females to a dead male body ($N=50$). The differences between female responses to the dead male and no model were significant by paired χ^2 -tests ($p<0.01$).

This suggests that male cues (probably visual and/or chemical) guide females to the males.

Effect of model color and male extract on female orientation

When various doses of the male elytra extracts (1/16, 1/8, 1/4, 1/2 and 1 ME) were treated on black and white glass-rod models (Fig. 3, $N=45$), the frequency of female orientation significantly increased with increasing doses (likelihood ratio test: $\chi^2=27.7$, $df=1$, $p<0.001$; Fig. 3A), indicating that the female orientation is stimulated by a volatile chemical cue. The frequency of female response to the black model was significantly greater than that to the white one (likelihood ratio test: $\chi^2=17.5$, $df=1$, $p<0.001$; Fig. 3A). This indicated that a visual attribute (model color) also guided the female to the model, in association with male substances that provide the olfactory signal.

Frequency of arrival at the model significantly increased with increasing doses (likelihood ratio test: $\chi^2=30.4$, $df=1$, $p<0.001$; Fig. 3B). The black color caused a significantly increase in the frequency of arrival (likelihood ratio test: $\chi^2=5.0$, $df=1$, $p=0.025$; Fig. 3B).

Distribution of the volatiles in male body parts

To examine the distribution of the olfactory cue on the male body, black glass rods were treated with the extracts of male body parts at 1 ME. Female orientation was greatest to the head extract. Rates of orientation were not significantly different between extracts of the prothorax, elytra, hind wings, and abdomen (Fig. 4), but were significantly lower to the meso+metathorax extract. Therefore, it is most likely that the active volatiles are distrib-

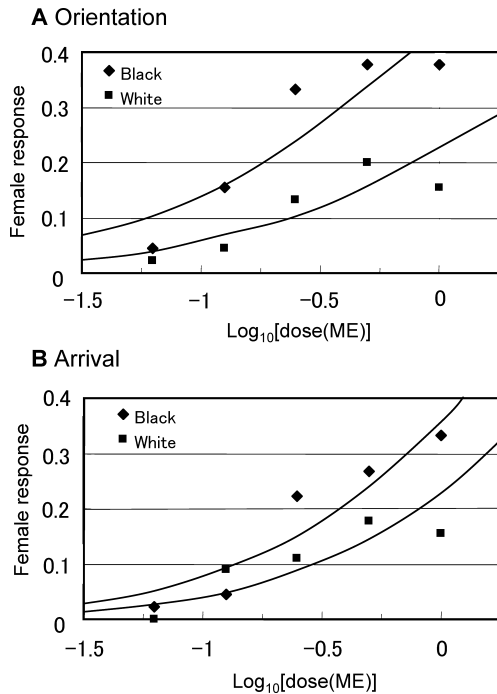


Fig. 3. Effect of model color and male extract on *A. malasiaca* female response ($N=45$). A: orientation. Curves indicate the estimated frequency; black: $\ln(p/(1-p)) = -0.20 + 1.62 \log_{10}(\text{doseME})$, white: $\ln(p/(1-p)) = -1.28 + 1.62 \times \log_{10}(\text{doseME})$. B: arrival at the model. Curves indicate the estimated frequency; black: $\ln(p/(1-p)) = -0.59 + 1.90 \times \log_{10}(\text{doseME})$, white: $\ln(p/(1-p)) = -1.22 + 1.90 \log_{10}(\text{doseME})$.

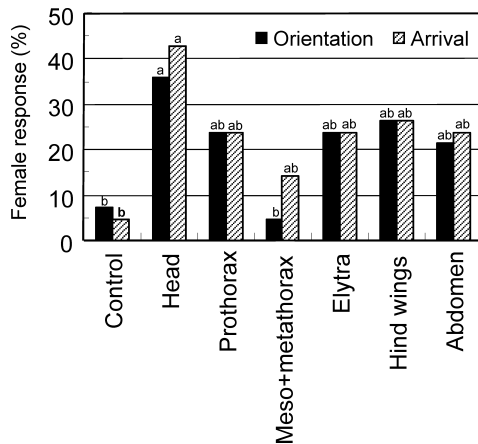


Fig. 4. Percentage of female *A. malasiaca* response on black glass-rod models treated with extracts of male body parts ($N=42$). Dose: 1 ME/glass rod. Values accompanied by the same letters in the same category are not significantly different ($p < 0.05$; paired χ^2 -tests adjusted by Bonferroni inequality).

uted over the entire male body except for the meso+metathorax. The meso+metathorax may possibly possess deterrent.

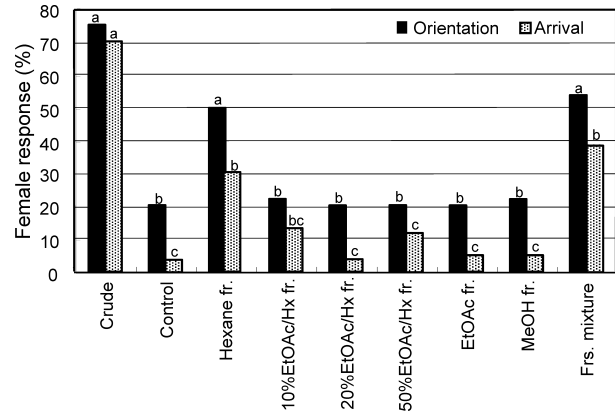


Fig. 5. Percentage of female *A. malasiaca* response on black glass-rod models treated with fractions of silica gel column chromatography of male elytra extract ($N=60$). Dose: 1 ME/glass rod. Values accompanied by the same letters in the same category are not significantly different ($p < 0.05$; paired χ^2 -tests adjusted by Bonferroni inequality).

Separation of the male volatiles that induce orientation by females

To confirm the chemical nature of the male volatiles, six fractions obtained from male elytra extract through silica gel column chromatography were individually treated on the black models (Fig. 5, $N=60$ each). All the fractions were mixed together and applied to the models for comparison with the crude extract. Female orientation response was significantly higher toward the hexane fraction (50%) than toward the other fractions. Rate of orientation was not significantly different among the models treated with crude extract (75%), hexane fraction (50%), and the recombination of the six fractions (mixture: 53%). This indicates that the active substances were eluted in the hexane fraction. Arrival response toward the hexane fraction (30%) and the mixture (38%) was lower than that toward the crude extract. The crude extract was supposed to include contact pheromone components that ensure the females' "arrival" at the model (Fukaya et al., 1999; Akino et al., 2001; Fukaya, 2003).

DISCUSSION

As we previously reported, male *A. malasiaca* can orient to females at close range using both olfactory and visual cues derived from females, and the former is essential (Fukaya et al., 2004). In this study, we reveal that the female also locates males

at close range before making direct body contact (Fig. 2). The olfactory cue derived from males is essential for females in mate location, and it works synergistically with the visual cue derived from males (Fig. 3). Thus, both sexes in *A. malasiaca* can locate the opposite sex at a short distance by means of the respective olfactory and visual cues. This suggests the existence of mutually volatile pheromones for short-range mate location in this species.

In most cerambycid beetles, it has been considered that mate location depends on males encountering females by chance, and that recognition of females is by antennal contact or in response to very short-range pheromones that only operate over distances of several centimeters (Hanks, 1999). In some cerambycids, evidence of volatile pheromone that acts over short and/or long distances for mate location has been shown (Iwabuchi, 1982, 1985; Sakai et al., 1984; Wang et al., 1991; Fauziah et al., 1992; Kim et al., 1992; Zhang et al., 2002). In addition to such olfactory cues, visual orientation is likely to be involved in mate location in some cerambycids (Iwabuchi, 1985; Fukaya et al., 2004). Nevertheless, there have been only a few studies clarifying the importance of visual cues associated with olfactory cues in mate location, even in other insects (Shorey and Gaston, 1970; Hidaka, 1972; Wall, 1989). Our study is presumably the first report to clarify the importance of visual cues cooperating with olfactory cues in female mate location in cerambycids.

The active substance was demonstrated to occur in the extract of the cuticle throughout the whole body surface (Fig. 4). It is probably a hydrocarbon(s), since the activity was found at the fraction which eluted with hexane from silica gel column chromatography (Fig. 5). Female attractant for males also showed similar distribution and nature (Yasui et al., unpublished). It is of interest to determine if the chemicals are identical.

Some cerambycids have been reported to seek or stay on a particular type of host plant to improve their chances of mate encounter (Shibata et al., 1995; Wang et al., 1996; Hanks, 1999). However, *A. malasiaca* adults were observed neither to show a preference for particular trees nor to aggregate in clumps in the field (Adachi, 1990). The density of both sexes is usually low, so it is unlikely that wandering males can often encounter females by

chance. It will be interesting to discover how olfactory and visual cues function in the field, and over long distances, between sexes of this species.

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