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) 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
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$_2$SO$_4$, the solvent was removed under reduced pressure below 30°C, and the concentrate was dissolved in hexane and stored below −20°C until use.

**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$_2$SO$_4$, 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. × 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 (21×30 cm). The sheet was pasted...
on an acrylic plate of the same size, which was in-
clined 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 tempera-
ture 2 h before the bioassay. The assays were com-
pleted within 40 min after treatment of extract on
the glass-rod models.

Female walking trails were categorized as fol-
lows (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, fe-
male arrival at point M was also recorded.

Statistics. For comparisons of values of female
responses, when an $n \times 2 \chi^2$-test was significant, a
paired $\chi^2$-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 orienta-
tion 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 them-
selves to a male body before making direct contact.

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

Effect of model color and male extract on fe-
male 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 re-
sponse 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 sub-
stances 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 fre-
quency 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. Fe-
male 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. 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 $\chi^2$-tests
($p<0.01$).
uted over the entire male body except for the meso+metathorax. The meso+metathorax may possibly possess deterrent.

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 pheromones 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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