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Hydrocarbon Components in Contact Sex Pheromone of the White-Spotted Longicorn Beetle, *Anoplophora malasiaca* (Thomson) (Coleoptera: Cerambycidae) and Pheromonal Activity of Synthetic Hydrocarbons

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Abstract. GC-MS analyses revealed more than 40 hydrocarbons in the hexane fraction from silica gel column chromatography of female elytra extract, which evoked mating behavior in male *Anoplophora malasiaca* when blended with the ether fraction. When the hexane fraction was separated into saturated, monoenyl, dienyl and trienyl fractions by column chromatography on silica gel impregnated with silver nitrate, only the fraction that contained saturated hydrocarbon showed pheromonal activity when mixed with the ether fraction. This activity was comparable with that observed when the mixture of the eight major synthetic hydrocarbons was blended. The eight major hydrocarbons were *n*-heptacosane, *n*-nonacosane, 4-methylhexacosane, 4-methyloctacosane, 9-methylheptacosane, 9-methylnonacosane, 15-methylhentriacontane and 15-methyltritriacontane. These hydrocarbons were indicated to be the components of the contact sex pheromone in *A. malasiaca*, which works together with an unknown polar component(s).

Key words: Contact sex pheromone, hydrocarbons, *Anoplophora malasiaca*, Cerambycidae.

Introduction

The white-spotted longicorn beetle, *Anoplophora malasiaca*, is a serious pest of various trees including citrus, apple, pear, maple and willow. This species is distributed in Japan, Korea, China, Taiwan and Malaysia (Ohbayashi, 1992). It is difficult to control with conventional methods since the larvae enter deeply into the tree trunk and adults have potent flight activity (Adachi, 1990). In China, *Anoplophora* beetles have been also known to cause serious damage on various trees. These include *A. grablipennis* on poplar trees and *A. chinensis* on citrus trees (Gao *et al.*, 1993; Wang *et al.*, 1996; Wu & Jiang, 1998).

Mating sequence and evidence for contact sex pheromone in *A. malasiaca* have been reported in the previous paper (Fukaya *et al.*, 1999), while Wang (1998) also showed evidence for contact sex pheromone in *A. chinensis*. The contact sex pheromone in *A. malasiaca* was believed to consist of at least two different compounds, probably hydrocarbon(s) and a more polar compound(s) (Fukaya *et al.*, 1999). In this paper, we focused on hydrocarbon compounds in the elytra extract of female *A. malasiaca* as contact sex pheromone to identify and evaluate their pheromonal activity. This may provide a step toward further understanding of the mating system in this beetle and developing a new technique for pest control.

Materials and Methods

Insects

Anoplophora malasiaca adults were collected in mandarin orange fields at Kunisaki-machi, Oita Prefecture on June 29, 1999. They were individually reared on an artificial diet (wet type of Silkmate 2S, Nihon Nosan Kogyo Co., Ltd.) in plastic cups (ca. 11 cm dia. × 9.5 cm ht.) at 25℃ and 15L : 9D. The diet was provided every 2–3 days.

Extraction

Female elytra were removed and dipped in ether (ca. 1.5 ml/female) for 5 min. The extract was separated from the residue by decantation and the residue

was rinsed twice with the same volume of ether, and the rinses were added to the extracts. Ether was removed from the extracts under reduced pressure below 30℃ and the residue was dissolved with hexane to store below −20℃ untill use. All the solvents used were distilled immediately before use.

Column chromatography

The crude extract of female elytra (38 FE: female equivalent) was applied as hexane solution onto a silica gel column (20 g, Wako gel C-200, particle size 75–150 μm, Wako Pure Chem. Ind., Ltd., Osaka, Japan). Compounds were successively eluted with 200 m*l* each of hexane and ether. The hexane fraction containing hydrocarbons was further separated by column chromatography on 1 g of silica gel impregnated with 16.7% of silver nitrate ($AgNO_3/SiO_2$). Compounds were eluted with 10 m*l* each of hexane and 2, 5, and 50% ether in hexane. Subsequent gas chromatography-mass spectrometry (GC-MS) analyses revealed that saturated, monoenyl, dienyl and trienyl hydrocarbons were eluted with hexane, 2, 5, and 50% ether-in-hexane fractions, respectively.

Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS)

GC analyses were conducted with a Hewlett-Packard (HP) 5890II GC equipped with a cool on-column injector, an HP-1 fused silica column (15 m × 0.25 mm (ID) × 0.25 μm film thickness, HP), and a flame ionization detector (FID). Helium was used as the carrier gas at a column head pressure of 60 kPa. Injection was made directly onto the capillary column through the cool on-column injector and the injector temperature was programmed at oven temperature plus 3℃. Temperature program (TP) in the column oven was 50 to 310℃ at 10℃/min and then held at the final temperature for 20 min [abbreviation for this program: 50(1)-10-310(20)].

GC-MS analyses were achieved with an HP6890 gas chromatograph interfaced to a JEOL JMS SX-102A mass spectrometer (EI mode, 70 eV) and operated with an HP Model 715/64 computer. GC was operated in the same condition as above, but column head pressure was 18 kPa and TP was 50(15)-30-230(0)-10-310(10).

Chemicals

Hydrocarbons with a straight carbon chain, that included heptadocosane (nC27) and nonadocosane (nC29) were purchased from Sigma Chemical Co., USA. 4-Methylhexacosane (4MeC26) was synthesized from 1-docosyne and 2-pentanone by acetylene coupling reaction and subsequent dehydration and hydrogenation reactions. 9-Methylnonacosane (9MeC29) was synthesized from 1-bromohexane, 3-methylpent-2-en-4-yn-1-ol and 1-octadecyne by repeated acetylene coupling and hydrogenation reactions.

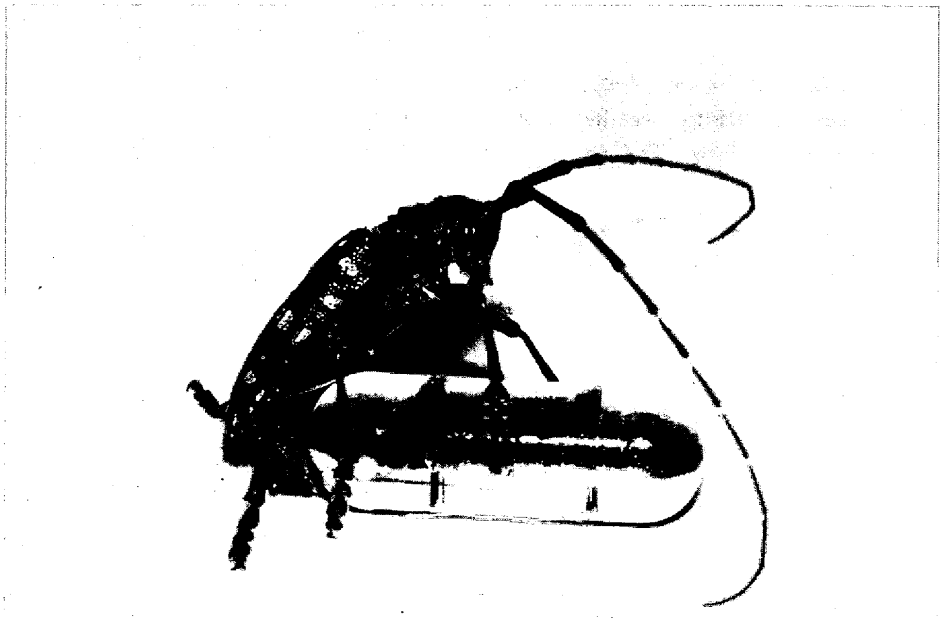

Fig. 1. *Anoplophora malasiaca* male showing abdominal bending response toward glass dummy coated with blend of synthetic hydrocarbons and ether fraction of female elytra extract. See text for synthetic hydrocarbons and ether fraction.

4-Methyloctacosane (4MeC28) and 15-methylhentriacontane (15MeC31) were from 2-bromopentane and tetracosanal, and from 2-hexadecanone and 1-bromohexadecane, respectively, by Grignard reaction and subsequent dehydration and hydrogenation reactions. 9-Methylheptacosane (9MeC27) and 15-methyltritriacontane (15MeC33) were synthesized from 1-bromooctadecane and 2-decanone, and from 1-bromooctadecane and 2-hexadecanone, respectively, via Wittig reaction and subsequent hydrogenation reaction. Chemical purity of these compounds was greater than 99%.

Behavioral assay

Pheromonal activity was evaluated as in Fukaya *et al.* (1999). A capsule-shaped glass rod (glass dummy, 12 mm dia. × 35 mm length) was fixed on the center of a filter paper disc (12.5 cm dia., Toyo No. 2, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and coated with test material dissolved in ca. 40 μl of hexane. A male beetle was placed nearby and allowed to make contact with the dummy. Behavioral response of the male was observed for 10 min. During the observation period, the male was gently guided to make contact with the dummy with foreleg(s) at least three times when the male left the dummy. Sexual maturity of males used in the assay was confirmed through the observation of abdominal bending behavior toward intact females before use in the assay.

Behavioral responses were noted as follows: male puts his forelegs on the dummy (=holding), male grasps the dummy with his fore- and midlegs and adjusts his body axis to the dummy (=mounting), and male bends his abdomen downward along the dummy surface (=abdominal bending; Fig. 1).

Results

Pheromonal activity of the fractions of female elytra extract

Elytra extract obtained from 38 female beetles was fractionated on silica gel column chromatography to obtain fractions successively eluted with hexane and ether. When only hexane fraction was treated on the glass dummy, abdominal bending (AB) response by male beetles was very weak in spite of the amount of the hexane fraction, which varied from 1 to 16 FE (Fig. 2). The AB response was also weak when only the ether fraction was treated on the dummy. When the hexane and ether fractions were mixed, in contrast, AB responses apparently became stronger, *i.e.* 45 and 50% of males responded to glass dummies

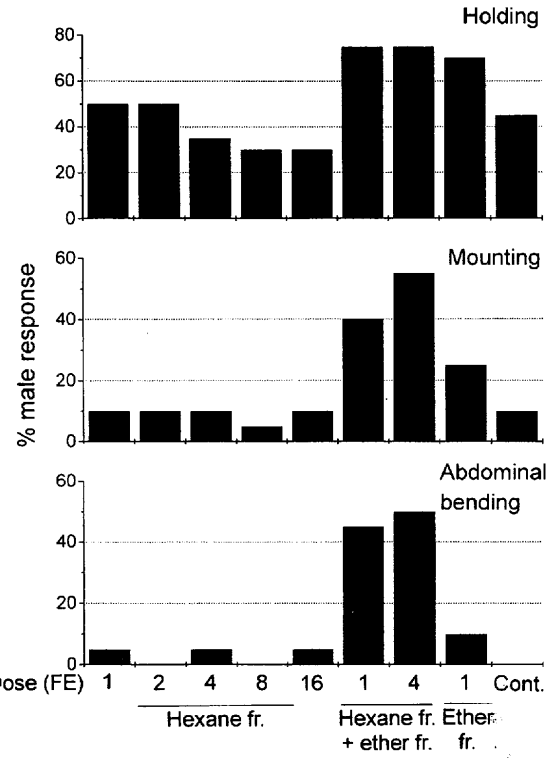

Fig. 2. Behavioral responses of male *Anoplophora malasiaca* to various doses of hexane and ether fractions from silica gel chromatography (N=20). FE: female equivalent.

treated with 1 and 4 FE of the mixture, respectively. These results confirmed the previous conclusion that the contact sex pheromone of *A. malasiaca* consists of a less polar compound(s) and a more polar compound(s) functioning synergistically (Fukaya *et al.*, 1999).

For the identification of the active compound(s) in the hexane fraction, GC-MS analyses were conducted. More than 40 compounds were found when 1/100 FE of the hexane fraction was injected (Fig. 3). Chemical structures of the major compounds were estimated mainly by interpretation of mass spectra (Table 1). All of them were hydrocarbons and consisted of saturated, monoenyl, dienyl and trienyl compounds, which accounted for 69.8, 27.8, 0.9 and 0.3% of the hydrocarbon amount, respectively. The total amount of the hydrocarbons was 1,050 μg/female. Saturated hydrocarbons contained straight-chained (18.5%), monomethyl-branched (70.2%) and dimethyl-branched (11.3%) compounds. As for the major eight saturated hydrocarbons, which are asterisked in Table 1, the identities in mass spectra and retention times to the authentic compounds were confirmed by GC and GC-MS analyses.

Hydrocarbons in the hexane fraction were further separated by column chromatography on $AgNO_3$/

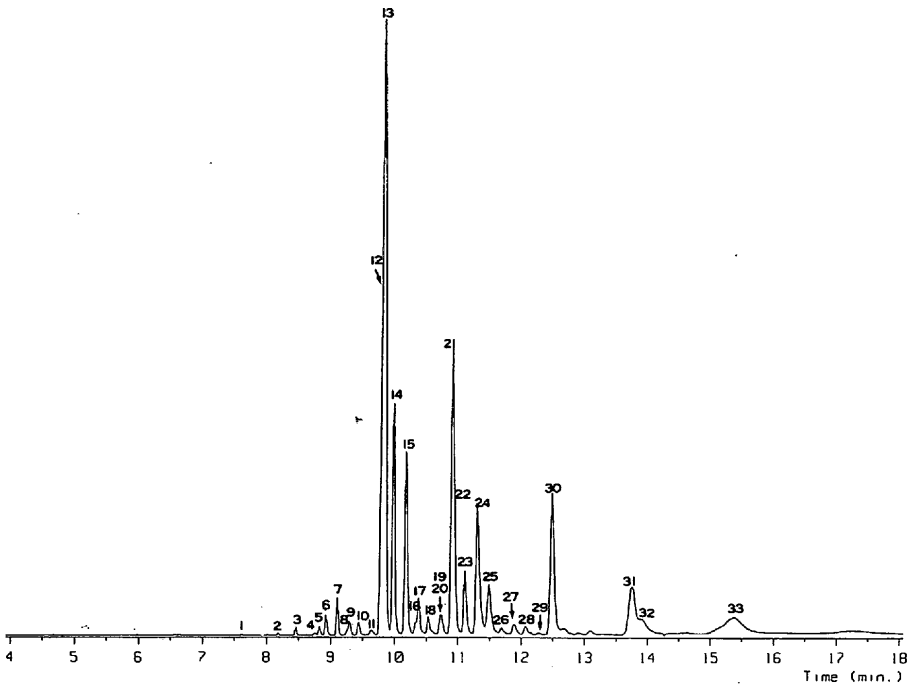

Fig. 3. Gas chromatogram of hydrocarbons in hexane fraction of female *Anoplophora malasiaca* elytra extract. GC conditions; Column: HP-1 15 m×0.25 mm i.d.×0.25 μm film thickness, Temperature program: 50℃ for 1 min, 50℃ to 310℃ at 10℃/min and 310℃ for 20 min. Cool on-column injection. See text for further detailed GC conditions.

SiO_2. Each fraction was assayed by blending with ether fraction that contained a polar active compound(s). Among the hydrocarbons, the fraction that contained saturated hydrocarbons showed clear pheromonal activity when mixed with the ether fraction (Fig. 4). In contrast, the fraction containing monoenes, dienes, or triene(s) was less active.

Pheromonal activity of synthetic hydrocarbons

No method has been convenient for the separation of saturated hydrocarbons to prepare sufficient material for behavioral assays, in which a single test needs at least 4 FE of extract. Therefore, eight major hydrocarbons, which are asterisked in Table 1, were synthesized to evaluate the pheromonal activity of hydrocarbon components. Each assay was performed with mixtures of the synthetic hydrocarbons and the ether fraction obtained from the female elytra extract.

Pheromonal activity of synthetic hydrocarbons was then evaluated as mixtures with the ether fraction from the silica gel column chromatography of female elytra extracts. AB response was induced in 60% of males (Fig. 5) with a mixture of all eight synthetic hydrocarbons, and a similar level of response (50%) was with the natural saturated hydrocarbon mixture (hexane fraction from $AgNO_3/SiO_2$ chromatography) (Fig. 5). For further evaluation of pheromonal activity of the hydrocarbons, the eight major hydrocarbons were divided into four groups according to chemical similarity: 1) hydrocarbons with straight carbon chain (nHCs: nC27 and nC29), 2) 4-methyl-branched hydrocarbons (4MeHCs: 4MeC26 and 4MeC28), 3) 9-methyl-branched hydrocarbons (9MeHCs: 9MeC27 and 9MeC29), and 4) 15-methyl-branched hydrocarbons (15MeHCs: 15MeC31 and 15MeC33).

When these four groups of hydrocarbons were coated on glass dummies together with the ether fraction, 9MeHCs evoked AB response in 57% of males. This activity was comparable with the mixture of all eight of the hydrocarbons (60%) (Fig. 6), which was significantly higher than that of the ether fraction ($P<0.05$). The activity of 4MeHCs (30%) was lower than that of the eight-hydrocarbon mixture but slightly higher than nHCs (23%) or 15MeHCs (17%), although the differences were not significant ($P>0.05$). Responses to nHCs and 15MeHCs were almost the same as that of the ether fraction (20%).

To confirm the activity, mixtures lacking single groups of hydrocarbons were also tested (Fig. 6). When nHCs or 4MeHCs were lacking, male response (53% and 47%) was almost comparable to those to whole mixtures (60%) or 9MeHCs (57%). When 9MeHCs were absent, male response decreased slightly to 40% ($P>0.05$). This value was higher than for single constituent groups, although the difference was not statistically significant ($P>0.05$). When

Table 1. Hydrocarbons found in the hexane fraction eluted from silica gel column chromatography of elytra extract of *Anoplophora malasiaca*.

Peak	t_R (min)	Suspected compounds (approx. ratio)	μg/female	%
1	7.61	*n*-Docosane	0.3	0.03
2	8.18	9-Methyltricosane	0.5	0.04
3	8.46	*n*-Tetracosane	2.0	0.19
4	8.76	4-Methyltetracosane	0.6	0.06
5	8.83	9-Pentacosene[1]	2.6	0.24
6	8.93	*n*-Pentacosane	6.7	0.63
7	9.11	11- and 13-Methylpentacosanes (5 : 1)	11.4	1.08
8	9.29	3-Methylpentacosane	3.6	0.34
9	9.29	9-Hexacosene[1]	2.4	0.23
10	9.44	*n*-Hexacosane	4.8	0.46
11	9.64	Heptacosadiene	2.4	0.22
12	9.83	9-Heptacosene[1]	280	26.60
13	9.83	4-Methylhexacosane*	70	6.65
14	9.99	*n*-Heptacosane*	88	8.37
15	10.18	9-*, 11-, and 13-Methylheptacosanes (3 : 2 : 1)	77	7.32
16	10.36	Dimethylheptacosane(s)	5.9	0.56
17	10.38	3-Methylheptacosane	13.4	1.27
18	10.53	*n*-Octacosane	6.5	0.61
19	10.73	Nonacosatriene	3.1	0.30
20	10.73	Nonacosadiene	7.3	0.70
21	10.93	4-Methyloctacosane*	139	13.21
22	10.93	9-Nonacosene[1]	7.4	0.70
23	11.11	*n*-Nonacosane*	26	2.49
24	11.31	9-*, 11-, 13- and 15-Methylnonacosanes (3 : 1 : 1 : 5)	73	6.94
25	11.49	11,17-Dimethylnonacosane	29	2.76
26	11.69	*n*-Triacontane	1.7	0.10
27	11.89	unidentified hydrocarbon(s)	5.8	0.55
28	12.07	unidentified hydrocarbon(s)	4.4	0.42
29	12.16	unidentified hydrocarbon(s)	0.5	0.04
30	12.49	13- and 15-*Methylhentriacontanes (1 : 5)	80	7.62
31	13.76	13-, 15-* and 17-Methyltritriacontanes (3 : 5 : 1)	48	4.59
32	13.87	13,17-Dimethyltritriacontane	16.5	1.57
33	15.37	13,19-Dimethylpentatriacontane	32	3.05
		Total	1050	100

* Major hydrocarbons with evaluation of biological activity in this study.

[1] Positional isomerism was determined by GC-MS analyses of dimethyl disulfide (DMDS) derivative, but geometric isomerism was not.

15MeHCs were absent, male response was reduced to 30%. No groups of hydrocarbons without blending with the ether fraction evoked any apparent response in males (Fig. 7).

Discussion

We had already reported that male mating behavior of *Anoplophora malasiaca* is evoked by contact sex pheromone distributed on the female body surface, and that the pheromone consists of a less polar compound(s), probably hydrocarbon(s), and a more polar compound(s) (Fukaya *et al.*, 1999). In this study, we focused on the former, and conducted identification and evaluation of the major hydrocarbon components of contact sex pheromone in the extract of female elytra.

Ether extract of female elytra was fractionated by column chromatography on silica gel to obtain hexane fraction containing less polar compounds that included hydrocarbons, and to obtain ether fraction that contained polar compounds. Male mating behavior

was definitely evoked when these two fractions were blended and coated on a glass dummy, but the activity was apparently reduced when single fractions were placed on the dummy (Fig. 2). The hexane fraction was further chromatographed on silica gel impregnated with silver nitrate for the separation into saturated, monoenyl, dienyl and trienyl hydrocarbons. Pheromonal activity was obtained when the fraction that contained saturated hydrocarbons was blended with the ether fraction (Fig. 4). Saturated hydrocarbon component(s) were therefore considered to contain pheromonal active component(s).

More than 40 hydrocarbons were found in the hexane fraction by the GC-MS analyses (Fig. 3) and chemical structures of eight major saturated hydrocarbons were elucidated, which are asterisked in Table 1. Saturated hydrocarbons accounted for 71% of the quantity of the hydrocarbon complex, and the eight major hydrocarbons accounted for 60% of the satu-

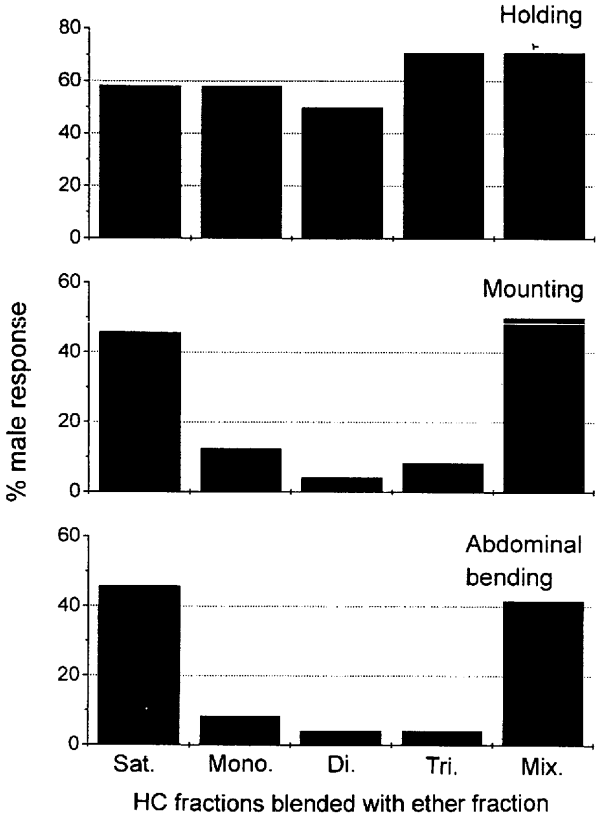

Fig. 4. Behavioral responses of male *Anoplophora malasiaca* to blends of ether fraction and fraction containing saturated (Sat.), monoenyl (Mono.), dienyl (Di.) or trienyl (Tri.) hydrocarbons from $AgNO_3$/SiO_2 column chromatography of hexane fraction (Dose: 4 FE, N=24). Mix.: mixture of all the fractions. See text for ether and hexane fractions.

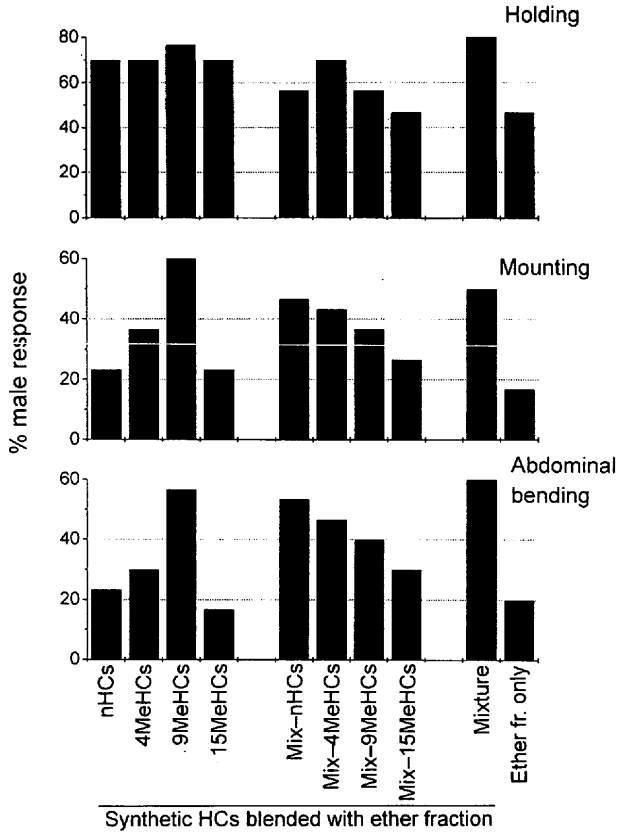

Fig. 6. Behavioral responses of male *Anoplophora malasiaca* to synthetic hydrocarbons (HCs) blended with ether fraction (Dose: 4 FE, N=30). nHCs: nC27+nC29, 4MeHCs: 4MeC26+4MeC28, 9MeHCs: 9MeC27+9MeC29, 15MeHCs: 15MeC31+15MeC33, Mix: mixture of eight hydrocarbons. Mix–: HC mixture lacking of the indicated HCs.

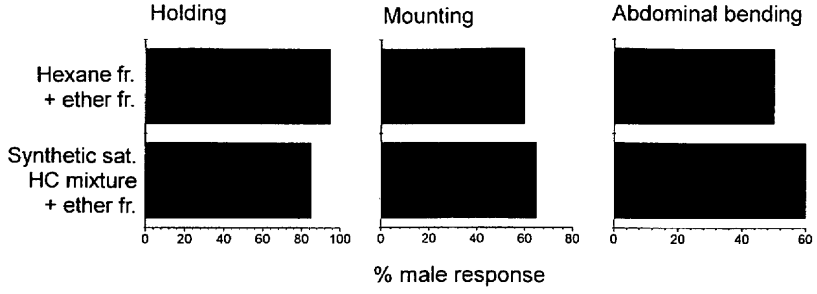

Fig. 5. Behavioral responses of male *Anoplophora malasiaca* to blends of ether fraction and hexane fraction or mixture of synthetic hydrocarbons (Dose: 4 FE, N=20).

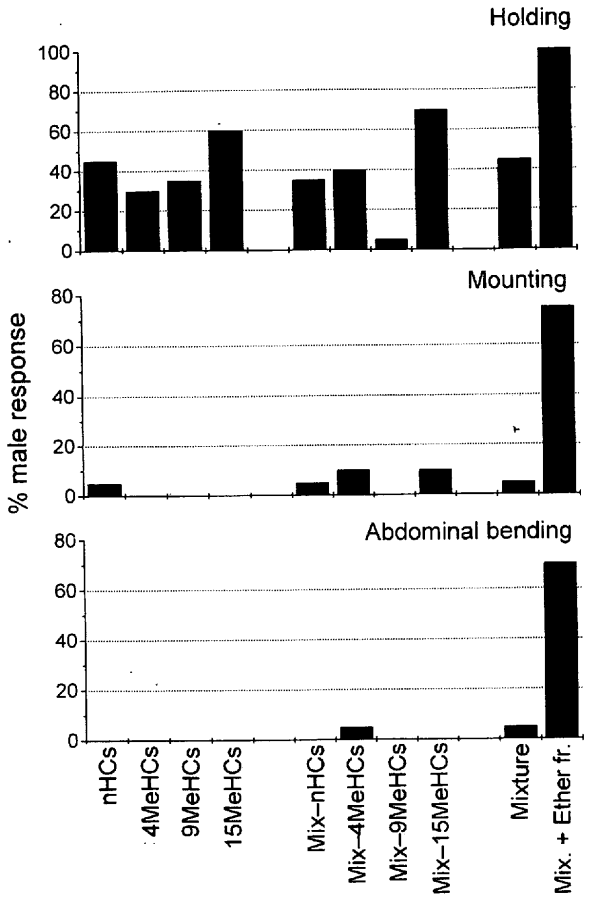

Fig. 7. Behavioral responses of male *Anoplophora malasiaca* to blends of synthetic hydrocarbons (Dose: 4 FE, N=20). See footnote of Fig. 6 for abbreviations for HC.

rated hydrocarbons. The pheromonal activity of these eight hydrocarbons was evaluated using synthetic hydrocarbons. This was because further separation of natural compounds was impeded by the difficulty of obtaining a sufficient amount of single compounds for the behavioral assay.

The blend of the eight major hydrocarbons at natural ratio and dose evoked abdominal bending response in male *A. malasiaca*, and this activity was comparable to that of hexane fraction from female elytra extracts (Fig. 5). In contrast, neither certain blended hydrocarbons nor single hydrocarbons evoked significant male response when not mixed with the ether fraction (Fig. 7). This confirmed that hydrocarbons certainly contain pheromonally active compound(s) that work synergistically with the unknown polar component in the ether fraction.

Pheromonal activity of the individual hydrocarbons, however, is not easy to interpret (Fig. 6). 9MeHCs seem essential for pheromonal activity since males showed high response to single 9MeHCs, and to mixtures lacking nHCs or 4MeHCs but containing 9MeHCs. The mixture containing 9MeHCs but lacking 15MeHCs, however, released AB response in only 30% of males. The reason is unclear, although this reduction might be due to possible experimental error. The contributions of nHCs and 4MeHCs to pheromonal activity do not seem important since single combinations evoked less response and the absence of these combinations did not reduce male response. The behavior of 15MeHCs seems curious, since the activity of the single combination was not recognized but lack of this combination considerably reduced the activity. Further detailed evaluation is necessary to understand the contribution of the individual hydrocarbons to pheromonal activity. Nevertheless, this is probably the first documented case in which synthetic hydrocarbons successfully evoked a comparable response to natural contact hydrocarbon pheromone in Cerambycidae.

Certain methylalkanes found in female *A. malasiaca* have been identified in various insects (Lockey, 1976, 1982; Howard, 1993). These alkanes have often been found in cuticular wax of many insects. Some of them have been reported to show biological activities as sex pheromones, kairomones, and possible cues for nestmate recognition in social insects, etc. (Nelson, 1993; Howard, 1993). Although biological activity was examined in naturally occurring hydrocarbons, neither reconstruction of the hydrocarbon complex nor evaluation of the biological activity through use of synthetic compounds has ever been conducted.

In our previous report (Fukaya *et al.*, 1999), we considered that hydrocarbon(s) functions as a contact sex pheromone and polar substance(s) acts as a synergist in *A. malasiaca*. We now partially revise this view. The polar compound(s) may be an essential component of the contact sex pheromone, since the ether fractions evoked weak but stable response to males in comparison to the response to hexane fractions (Figs. 2 and 6).

During the behavioral assay of the ether fraction, we observed that males responded very near the dummy before making direct contact with antennae or palpi. In contrast, a careful search with palpi was observed before the mounting on the dummy when hexane fraction or hydrocarbons were applied. This suggests that unknown polar substance(s) might be weakly volatile and used for short-range orientation to a female.

In the yellow-spotted longicorn beetle, *Psacothea hilaris* (Pascoe), (*Z*)-21-methyl-8-pentatriacontene was identified as a contact sex pheromone component

(Fukaya *et al.*, 1996) but a different component(s) was demonstrated to stimulate the male to dash toward the female (Fukaya & Honda, 1992, 1995). In the Japanese pine sawyer beetle, *Monochamus alternatus* Hope, Kim *et al.* (1992) demonstrated both volatile and contact sex pheromones. Volatile sex pheromone is released by the male and attracts the female. Contact sex pheromone on the female body stimulates the male to copulate after direct contact, although chemical identification was not made.

For further understanding of the chemical communication by contact sex pheromone in *A. malasiaca*, chemical elucidation of the polar component(s) is essential, and is now in progress. This will also provide a new method to control this severe pest species.

Acknowledgments

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