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Hybrid Sex Pheromones of the Hibiscus Flower-bud borer, *Rehimena surusalis*

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Abstract—The sex pheromone of the hibiscus flower borer *Rehimena surusalis* (Walker) (Lepidoptera: Crambidae) was analyzed by gas chromatography with electroantennographic detection (GC-EAD) and GC-mass spectrometry (GC-MS). Three EAD-active components were found in crude pheromone gland extracts of calling females. GC-MS and GC analyses using synthetic chemicals and derivatization of the extracts identified three components as (10*E*,12*Z*)-hexadeca-10,12-dienal (E10,Z12-16:Ald,), (10*E*,12*E*)-hexadeca-10,12-dienyl acetate (E10,Z12-16:OAc) and (3*Z*,6*Z*,9*Z*)-tricosa-3,6,9-triene (Z3,Z6,Z9-23:HC). In field tests, male moths were remarkably attracted to a ternary blend of E10,Z12-16:Ald, E10,Z12-16:OAc and Z3,Z6,Z9-23:HC at a ratio of 1:5:14, but single and binary blend of either compound showed only weak or no attraction activity.

Key Words—Hibiscus flower-bud borer, *Rehimena surusalis*, (10*E*,12*Z*)-10,12-hexadecadienal, (10*E*,12*Z*)-10,12-hexadecadienyl acetate, (*Z3,Z6,Z9*)-3,6,9-tricosatrinene.

INTRODUCTION

Hibiscus flower-bud borer *Rehimena surusalis* (Walker) (Lepidoptera: Crambidae) is widely distributed in Africa, Australia, China, India, Indonesia, Taiwan, Korea and Japan

(Shibuya, 1928, 1929; Inoue et al., 1982; Liu, 1990; Shin, 2001; Ades and Kendrick, 2004; Herbison-Evans and Crossley, 2013) and is a continual pest of Malvaceae garden and street trees including *Hibiscus syriacus* (rose of Sharon), *H. mutabilis* (cotton rose), *H. rosa-sinensis* (Chinese hibiscus) *H. tiliaceus* and *H. glaber* (Sea Hibiscus) (Anonymous 1994, 2006). In Japan and Korea, *H, syriacus* is particularly damaged by *R. surusalis*. *H. syriacus* (mugunghwa in Korean) is authorized as the national flower of Korea, and *R. surusalis* has been reported to eat the seed of this plant (Lee et al., 2005; Kim et al., 2013; Bea 2012). The larvae bore into the developed flowers and flower buds. Because of the larval feeding habit as a typical borer, it is difficult to control this pest with cover sprays of insecticides. To control insects with a perforative lifestyle in the larval stage, pheromones are advantageous to monitor the flying adults, and disrupt their mating, resulting in a reduction in oviposition (Witzgall et al. 2010).

In this study, we identified components of the female sex pheromone of *R. surusalis* and demonstrated sex pheromone activity of synthetics in the field. We also discussed a commonality of the hybrid-type of sex pheromone in Pyraloidea.

MATERIALS AND METHODS

Insects. Colonies of *R. surusalis* were maintained as laboratory cultures. Mated females were allowed to lay eggs in small plastic cylinders that were lined with felt cloth impregnated with methanol extracts of *H. syriacus* flower buds. Because of heavy cannibalism, larvae of *R. surusalis* were individually reared on an artificial diet composed of Insecta® F-II (Nosan Corporation, Japan) and dried leaf powder of *H. syriacus* at a ratio of 8:2. Adults were sexed at the pupal stage and kept separately in cages at 25 ± 2°C, 60–70% relative humidity (RH) and a 15L9D photoperiod, and provided with a 10% sugar solution from cotton pads. A red lamp was used for observations during scotophase.

Extracts and chemicals. For identification of pheromone components, pheromone extracts were obtained from 2 to 7 day old calling females, whose abdominal tips were cut with ophthalmology scissors after half of scotophase by extraction with redistilled *n*-hexane for 20 min. Pooled extracts (60 female equivalents, FE) were stored at -20°C until use for chemical analyses and bioassays. Aliquot of the extracts were subjected into GC analysis for quantitative determination of pheromone candidates in 5 replications. Each four geometric isomers of synthetic 10,12-hexadecadienals (Z10,E12-16:Ald, E10,Z12-16:Ald, Z10,Z12-16:Ald and E10,E12-16:Ald) and 10,12-hexadecadienyl acetates (Z10,E12-16:OAc, E10,Z12-16:OAc, Z10,Z12-16:OAc and E10,E12-16:OAc), and (3Z,6Z,9Z)-tricosa-3,6,9-triene (3Z,6Z,9Z-23:CH) were supplied by coauthors T. A. or S. M. The isomeric purity of all

compounds was confirmed by GC to be ≥ 97%.

Chemical analysis. Pheromone extracts were subjected to GC-EAD analyses using a HP-5890 series II GS (Agilent Technologies, California, USA) equipped with an HP-5MS capillary column (30 m × 0.32 mm ID, film thickness 0.25 μm; Agilent Technologies, USA) and helium as a carrier gas (37 cm/s). Oven temperature was programmed at 130°C for 2 min, then increased at a rate of 5°C /min to 250°C and held at the final temperature for 10 min. The temperature of the detector and injector was 250°C, and that of the outlet for the EAD was maintained at 300°C. Extracts were injected in splitless mode and chromatographed using helium as a carrier gas (37 cm/s). GC effluent from the column was slit in a 1:1 ratio between the flame inonization detector (FID) and the EAD. The effluent was delivered in humidified air (23°C) to the antennal preparation connected to an EAG probe (Type PRG-2, Syntech, The Netherlands) via Ag-AgCl electrodes with 0.1.M KCl. EAD responses of male antenna were recorded in PC with GC-EAD 2010 software (Ver. 4.60, Syntech) via GC-EAD signal acquisition controller (IDAC-2, Syntech).

Analyses of EAD active components in the extracts by GC-MS employed a MS-600H mass spectrometer (JEOL Ltd., Japan) coupled with HP-6890N GC (Agilent), which was equipped with a DB-5MS (25 m × 0.25 mm ID, film thickness 0.25 μm, Agilent) capillary column, and operated in electron impact ionization mode (70 eV). GC oven temperature was programmed at 100°C for 1 min, then increased at a rate of 10°C /min to 320°C and held at the final temperature for 17 min.

GC analyses were conducted with GC-17A (Shimadzu Co., Ltd., Japan) and GC-6890N (Agilent) fitted with a nonpolar HP-5MS column and a polar DB-23 column (30 m × 0.25 mm ID, film thickness 0.15 μm; Agilent), respectively. GC oven temperature of the nonpolar column was programmed at 130°C for 2 min, then increased at a rate of 5°C /min to 250°C and held at the final temperature for 10 min. GC oven temperature of the polar column was programmed at 80°C for 2 min, then increased at the rate of 3°C /min to 250°C and held at the final temperature for 5 min.

To determine the position of conjugated double bonds, pheromone candidates in the extracts were reacted with 4-methyl-1,2,4-triazoline-3,5-dione (MTAD), followed by GC-MS analysis of the resulting derivatives. Kováts retention indices (KRI) (Kováts, 1958; Dool and Kratz, 1963) of EAD-active components and authentic chemicals were determined with retention times of standard hydrocarbons. The GC peak area of each component on the HP-5MS column was used to determine the ratio of EAD-active components in the pheromone extracts.

Laboratory and field tests. Pheromone activity of candidate components, E10,Z12-16:Ald, E10,Z12-16:OAc and Z3,Z6,Z9-23:HC and their blends were examined by laboratory and field assays. Laboratory cage tests were conducted in a mesh cage (30 cm×25 cm×30 cm) with 10 males at the second half of scotophase that the most of calling by males were observed. Pheromone extracts or synthetics were applied on a filter paper (1cm x 3cm) in 1 μl hexane as solvent. Filter paper was suspended 10 cm from the ceiling with a wire clip. Amounts of synthetics were adjusted to 1 female equivalent (FE)/μl. Crude extracts were concentrated to 1 FE/μl under a gentle N_2 stream. Numbers of males showing orientation flight (OF) by hovering to pheromone source and source contact (SC) were counted for 3 min with 5 ~ 7 replications and the cumulative numbers compared in single, binary and ternary blends of the candidate compounds.

Field experiments were conducted in fields with *H. syriacus* plantations on the campus of University of Tsukuba (36.1°N, 140.1°E) during June and August in 2013. Similar sets of synthetic blends with those used in the laboratory assays were loaded on gray rubber septa (West Corp., Singapore) at 500 μg / trap. In addition to the regular blend, blends with two and five times excessive Z3,Z6,Z9-23:HC (750 μg and 1750 μg/ trap) were also tested. Each rubber septum was placed on a sticky board trap with a triangle roof (SE-trap, 30 cm in length x 27 cm in width x 10 cm in height; Sankei Chemical Co., Ltd., Kagoshima, Japan). Traps were hung ca. 1.5 m above the ground on tree branches with at least 10 m intervals, and were set in a completely randomized design, and the lure were renewed once a week. Positions of traps were rotated one position every three days to avoid positional effects. As a control, empty traps were also tested. Numbers of captured males in each trap were counted every 3 days.

Statistical analyses. Results of laboratory and field assays were analyzed using one-way analysis of variance (ANOVA), followed by a Tukey-Kramer's honestly significant difference (HSD) test. Numbers of captured males (x) in field tests were transformed $\sqrt{(x+0.5)}$ prior to ANOVA. Software package R 3.0.1 (R Core Team 2013), was used for the statistical analyses.

RESULTS

Chemical analysis. GC-EAD analyses of crude pheromone gland extracts of female *Rehimena surusalis* showed three active components **A** (Rt 11.28 min) **B** (Rt 14.66 min) and **C** (Rt 18.52 min) on FID chromatogram (Fig. 1). In GC-MS analyses, spectra of the active component **A** showed putative parental ion at *m/z* 236 (M^+, 36 %), and fragment ions at *m/z*

67 ($[C_5H_7]^+$, base peak), *m/z* 95 ($[C_7H_{11}]^+$, 41 %), *m/z* 96 ($[C_7H_{12}]^+$, 42 %) and *m/z* 109 ($[C_8H_{13}]^+$, 28 %). The ion peaks spaced by *m/z* 14 and peaks at *m/z* 96 and 109 suggested the double bonds at the 10- and 12- (ω4, ω6) positions in a straight carbon chain (Ando et al. 1998). From these spectral data, the

structure of compound **A** was consistent to 10, 12-hexadecadienal ($C_{16}H_{28}O$). Relatively high intensity of molecular ion peak (*m/z* 236) also supported this identification for component **A**.

GC-MS analysis of component **B** showed ion peaks at *m/z* 280 (M^+, 38 %), *m/z* 61 ($[CH_3COOH+2H$, 5%], *m/z* 67 ($[C_5H_7]^+$, base peak), *m/z* 95 ($[C_7H_{11}]^+$, 48 %), *m/z* 96 ($[C_7H_{12}]^+$, 58 %), *m/z* 109 ($[C_8H_{13}]^+$, 29 %), and *m/z* 220 ($[M-CH_3COOH]^+$, 16%). Mass spectra with ion peaks spaced by *m/z* 14 and two prominent peaks at *m/z* 96 and 109 suggested a straight carbon chain and double bond positions at 10, 12- (ω4, ω6) positions in $C_{16}H_{32}O_2$. Two diagnostic ion peaks at *m/z* 61 and *m/z* 220 predicted structure of compound **B** to be 10, 12-hexadecadienyl acetate. Relatively high intensity of molecular ion peak at *m/z* 280 also indicated conjugated double bonds in compound **B**.

In GC-MS analysis, component **C** showed ion peaks at *m/z* 318 (M^+, 6 %), *m/z* 79 ($[C_6H_7]^+$, 79%), *m/z* 93 ($[C_7H_9]^+$, 33%), *m/z* 107 ($[C_8H_{11}]^+$, 15%), *m/z* 108 ($[C_8H_{12}]^+$, base peak), *m/z* 121 ($[C_9H_{13}]^+$, 18%) and *m/z* 262 ($[M-C_4H_8]^+$, 19%). The fragmentation pattern indicated an unsaturated straight-chain compound, with possible molecular formula of $C_{23}H_{42}$, consistent with a tricosatriene (3,6,9-23:HC). In addition, three conspicuous diagnostic ion peaks at *m/z* 79, *m/z* 108 and *m/z* 262 indicated three double bonds at 3, 6 and 9-position of compound **C** (Ando et al. 2004).

The position of double bonds in **A** and **B** were further confirmed by derivatization with MTAD, which reacts specifically with conjugated dienyl structures. The mass spectra of MTAD reaction products exhibited ions at *m/z* 349 (M^+, $[C_{19}H_{31}O_3N_3]^+$, 17 %), *m/z* 208 ($[C_{10}H_{12}O_2N_3]^+$, base peak) and *m/z* 306 ($[C_{16}H_{24}O_3N_3]^+$, 57%) for compound **A**, and at *m/z* 393 (M^+, $[C_{21}H_{35}O_4N_3]^+$, 17 %), *m/z* 208 ($[C_{10}H_{12}O_2N_3]^+$, base peak) and *m/z* 350 ($[C_{18}H_{28}O_4N_3]^+$ for compound **B** supporting two conjugated double bonds at either 3- and 5-positions or 10- and 12-positions in hexadecadienal and hexadecadienyl acetate, respectively.

Components **A** and **B** had KRIs similar to those of each four isomers of 10, 12-16: Ald and 10,12-16: OAc on both nonpolar and polar GC columns. The 3, 5-dienes would have been expected to elute much more earlier than 10, 12-dienes on GC (Ando et al., 2004). As shown in Table 1, KRIs of components **A** and **B** corresponded well to those of (10*E*,12*Z*)-hexadeca-10,12-dien-1-al (E10,Z12-16:Ald,) and (10*E*,12*Z*)-hexadeca-10,12-dien-1-yl acetate (E10,Z12-16:OAc), respectively, on both HP-5MS and DB-23 columns. KRI of component **C** was compared with only that of Z3,Z6,Z9-23:HC, because 3,6,9-tricosatrienes as insects

pheromones are considered to be biosynthesized from (9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoic acid with elongation of the carbon chain (Ando et al. 2008). The geometric configuration of component **C** was confirmed to be 3Z,6Z,9Z–isomer from agreement with the RI.

The amounts of these three components (**A**, **B** and **C**) in the extracts were determined to be 0.77 ±0.08 ng, 3.60 ± 0.56 ng and 11.1 ± 0.96 ng per female, respectively, at ratio of 1:5:14.

Laboratory and field tests. In the laboratory test, pheromone activities of the crude pheromone extract and all of possible combinations of synthetic E10,Z12-16:Ald, E10,Z12-16:OAc and Z3,Z6,Z9-23:HC are summarized in Fig. 2. Three one-component baits and binary blends of E10,Z12-16:Ald and E10,Z12-16:OAc, and Z3,Z6,Z9-23:HC with E10,Z12-16:Ald or E10,Z12-16:OAc showed no pheromone activity in both activity criteria, orientation flight and source contact by male moths, whereas significantly higher activity in orientation flight was observed with binary combination of E10,Z12-16:Ald and E10,Z12-16:OAc though it was still lower than that of the extract. Highest activity in orientation flight was observed with the ternary blend of the above synthetics in natural amounts, and it corresponded well to activity of the extract. In source contact by male moths, only the ternary blend showed significantly different activity from that of the crude extract.

In the field tests, the ternary blend of E10,Z12-16:Ald, E10,Z12-16:OAc and Z3,Z6,Z9-23:HC attracted the highest number of male moths in all treatments tested, whereas single and binary blends attracted fewer or no male moths (Fig. 3). Similar to the results of the laboratory tests, the binary blend of E10,Z12-16:Ald and E10,Z12-16:OAc showed also relatively high activity in male attraction. When the amount of Z3,Z6,Z9-23:HC was increased, trap catches somewhat decreased at 700 μg, and significantly decreased at 1750 μg (Fig. 3).

DISCUSSION

Three GC-EAD active components were identified as E10,Z12-16:Ald, E10,Z12-16:OAc and Z3,Z6,Z9-23:HC by GC and GC-MS analyses. The ternary blend of these compounds in a ratio of 1:5:14 showed pheromone activity to male moths of *R. surusalis* in laboratory and field bioassays. These results show that the sex pheromone of *R. surusalis* consists of three components in this ratio. 10,12-Hexadecadienals are widely known as major or minor components of sex pheromones of several moth families including Noctuidae (Cork et al., 1988), Sphingidae (Starratt et al., 1979; Bestmann et al., 1992; Uehara et al., 2012, 2015), Pyralidae or Crambidae (Klun et al., 1986; Raina et al 1986; Honda et al.,1994), Saturniidae

(Dai et al. 1988; McElfresh and Millar, 1999a.b) and also Bombycidae (Daimon et al., 2012). E10,Z12-16:Ac was also identified as a sex pheromone in Bombycidae (Daimon et al,. 2012) and Saturniidae (Dai et al., 1987; McElfresh and Millar 1999a,b,c; 2001).

Sex pheromone components can be categorized into Type I and Type II groups depending on whether they have or don't have terminal functional groups in the molecules, and compounds such as E10,Z12-16:Ald, and E10,Z12-16:OAc belong to the Type I group but polyenyl hydrocarbons such as Z3,Z6,Z9-23:HC belong to Type II group (Ando et al. 2004). Recently so-called hybrid type of pheromone systems consisting of Type I and Type II compounds such as that of *R. surusalis*, are reported mainly in Crambid and Pyralid species (Cabrera et al 2001; Millar et al. 2005; Leal et al. 2005; Gibb et al. 2007; Miller et al. 2010; Löfstedt et al. 2012; EI-Sayed et al. 2013; Yan et al. 2014).

Rehimena surusalis male moths showed low but significant orientation flight responses to a binary blend of E10,Z12-16:Ald and E10,Z12-16:OAc, although neither component was active as a single component, in the laboratory cage test and field tests (Fig. 2 and 3), indicating a crucial synergistic function of E10,Z12-16:Ald and E10,Z12-16:OAc in male attraction from a long distance. Z3,Z6,Z9-23:HC significantly increased male catches in the field traps, suggesting synergistic effect to E10,Z12-16:Ald and E10,Z12-16:OAc. However, trap catches decreased when Z3,Z6,Z9-23:HC was mixed with these dienyl components at 1:5:70 (25. 125, 1750 μg), showing an optimal ratio of the trienyl hydrocarbon component for the pheromone system in this species.

In the laboratory tests, the numbers of source contacts by male moths significantly increased when Z3,Z6,Z9-23:HC was added to the binary blend. In some lepidopteran species, hydrocarbons of body waxes have critical effects, such as a releaser for copulation (Grant et al. 1987) or stimulator for contact to pheromone source (Schlamp et al. 2005; Xiao et al 2010; 2011; 2012), over short range behaviors such as synergistic effects with other high volatile pheromone components. Xiao (2011) showed the possibility that although their actual functions are unknown, homologous polyene hydrocarbons including Z3,Z6,Z9-23:HC also widely exist in body wax of moths other than Crambidae, because similar synergistic activity was observed when body wax extracts of some Noctuidae and Sphingidae species were mixed with the two aldehydes as sex pheromone components.

The four families, Noctuidae, Arctiidae, Lymantriidae and Geometridae use Type II compounds as female sex pheromones (Ando, 2014; El-Sayed, 2014). However, Zahiri et al (2010) reconstructed Noctuidae *sensu lato* by molecular phylogeny, and showed traditional Arctiidae and Lymantriidae *sensu* Miller (1991) were included in Erebidae with various Type II-pheromone-using noctuids. This indicated that only Geometroidea and Noctuoidea, which

show sister linkages in recent molecular phylogenetic trees (Regier et al 2009) use Type II sex pheromones and also that the origin of Type II pheromones may be from a common ancestor of the two taxa. However, recently hybrid type pheromone system has been reported in several Pyraloidea species (Cabrera et al 2001; Millar et al. 2005; Leal et al. 2005; Gibb et al. 2007; Miller et al. 2010; Lofstedt et al. 2012; EI-Sayed et al. 2013; Yan et al. 2014). In Pyraustinae *sensu lato*, *R. surusalis* is the 4th species that has a hybrid type pheromone system as shown in two *Conogethes* species (Xiao et al 2010, 2011b, 2012; El-Sayed et al 2013) and *Omphisa anastomosalis* (Yan et al 2014). These results suggest that the hybrid type pheromone system is at least common in Pyraloidea, and the origin of Type II pheromones may be a common ancestor of Pyraloidea and Geometroidea + Noctuoidea. However, the Pyraloidea + (Geometroidea + Noctuoidea) clade include some taxa, e.g., Bombycoidea, Lasiocampoidea or Drepanoidea, that have no reports of Type II pheromones (Regier et al 2009). To reveal the origin of Type II pheromones, we must carefully reinvestigate some species which use only Type I compounds for their female sex pheromones, included into the Pyraloidea + (Gemoetridea + Noctuoidea clade), by physiological or molecular biological methods.

Three Crambidae species, *Haritalodes derogate*, *H. basipunctalis* and *R. surusalis* use E10,Z12-16:Ald as a sex pheromone component, and occur sympatrically in hibiscus plantations. This sympatric reproductive biology may be allowed by their species-specific pheromone systems, which consist of binary mixtures of E10,Z12-16:Ald and E10,E12-16:Ald at different ratios in the two *Haritalodes (Notracha)* species (Honda et al., 1994), and addition of E10,Z12-16:OAc and Z3,Z6,Z9-23:HC in *R. surusalis*.

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Figure legends

Fig.1 GC/EAD analysis of a crude pheromone extract from *R. surusalis* on HP-5MS GC column (upper trace EAD, lower trace GC)

Fig.2 Cumulative number of male exhibiting orientation flight (OF) to pheromone source and source contact (SC) in laboratory assays. The amount of the synthetic components in the respective baits are shown under the bars. Bars with the same letters are not significantly different at P<0.05 by Tukey–Kramer's HSD test after ANOVA (OF: N=5, F=56.75, P<0.01; SC: 21.31, P<0.01). The number of trapped males was transformed to $\sqrt{(x+0.5)}$ prior to the test.

Fig.3 Field catches of male *R. surusalis* in traps baited with synthetic E10,Z12-16:Ald(Ald), E10,Z12-16:OAc(OAC) and Z3,Z6,Z9-23:HC(HC) and their mixtures. Bars with the same letters are not significantly different at P<0.05 by Tukey–Kramer's HSD test after ANOVA (*N*=9, F=5.838, *P*<0.01). The number of trapped males was transformed to $\sqrt{(x+0.5)}$ prior to the test.

Fig.4 Type of female sex pheromone and molecular phylogenetics in the crade Ditrysia (Lepidoptera). Type II pheromone was identified from 3 taxonomic groups (Geometroidea, Geometridae and Noctuoidea: Erebidae and Pyraloidea). Papilionoidea etc. indicates a crade ((((Nymphalidae + Pieridae) + (Hesperioidea + Hedyloidea)) + Thyridoidea) + (Papilionidae + Calliduloidea)) + (Copromorphoidea + Hyblaeoidea). Alucitoidea, Urodoidea and Choreutoidea were omitted from the phylogenetic tree that was modified from Regier et al (2009).

Fig.1 Honda et al.

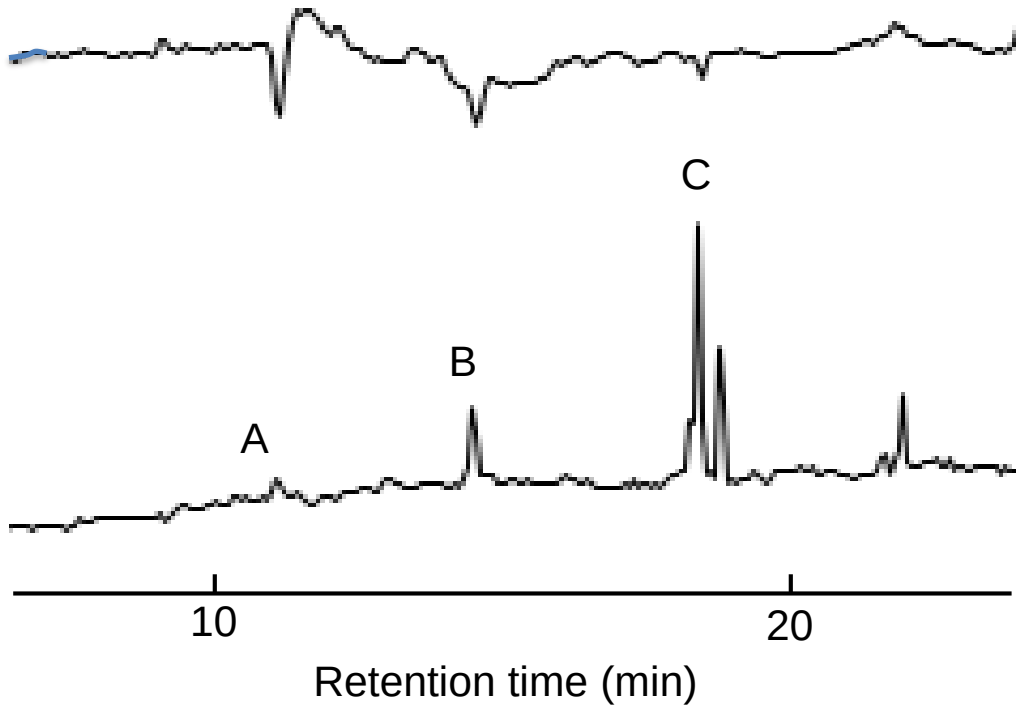
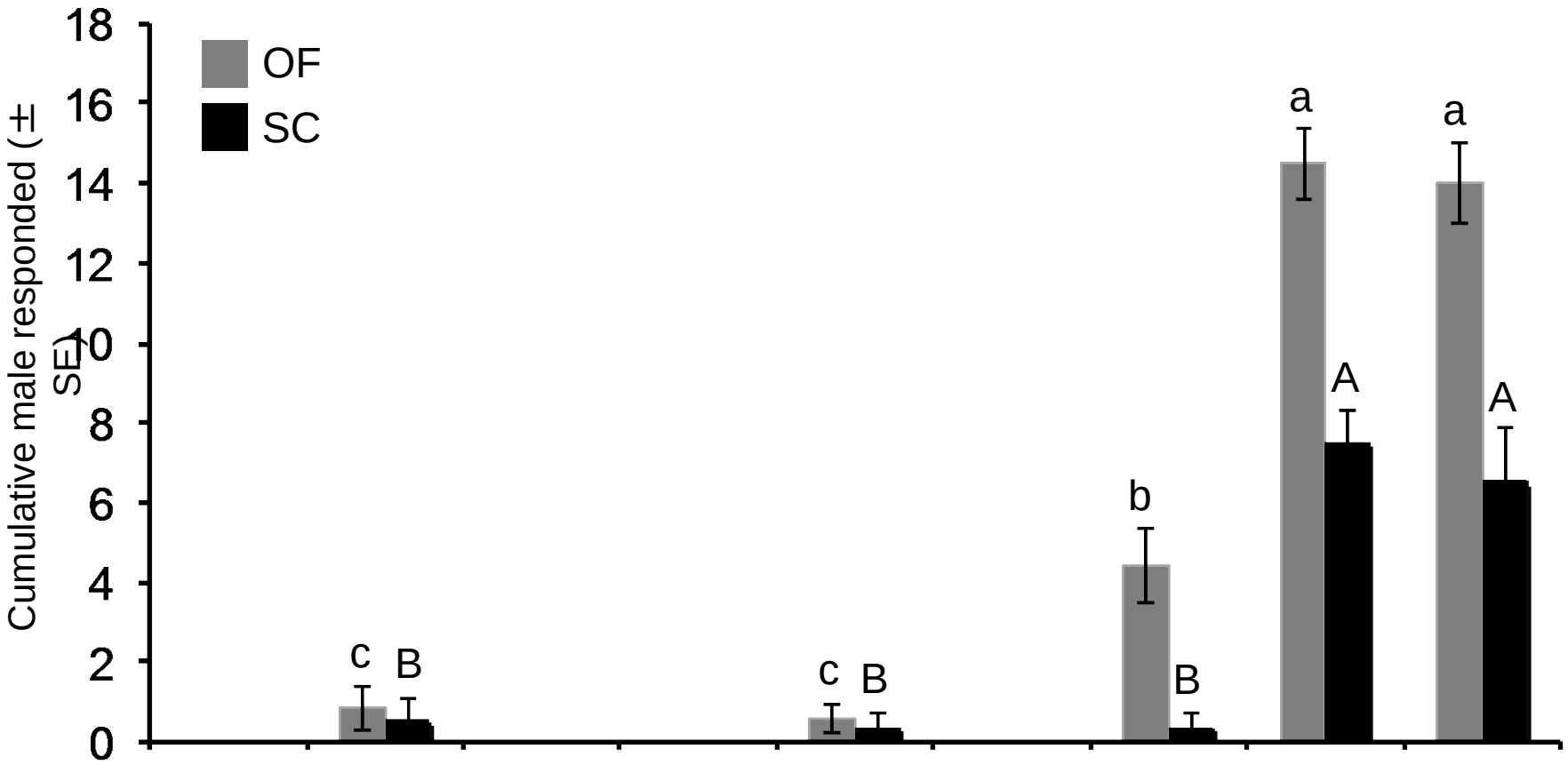


Table 1

Retention indices of EAD-active components and synthetic compounds on GC columns with different polarities

	Kovats Retention Index (KRI)	
Compounds	HP-5MS	DB-23
Compound A	1862	2252
Compound B	2051	2369
Compound C	2276	2391
Z10,E12-16:Ald	1853	2243
E10,Z12-16:Ald	1862	2252
Z10,Z12-16:Ald	1874	2254
E10,E12-16:Ald	1880	2257
Z10,E12-16:OAc	2039	2360
E10,Z12-16:OAc	2051	2369
Z10,Z12-16:OAc	2063	2372
E10,E12-16:OAc	2069	2374
Z3,Z6,Z9-23:HC	2276	2390

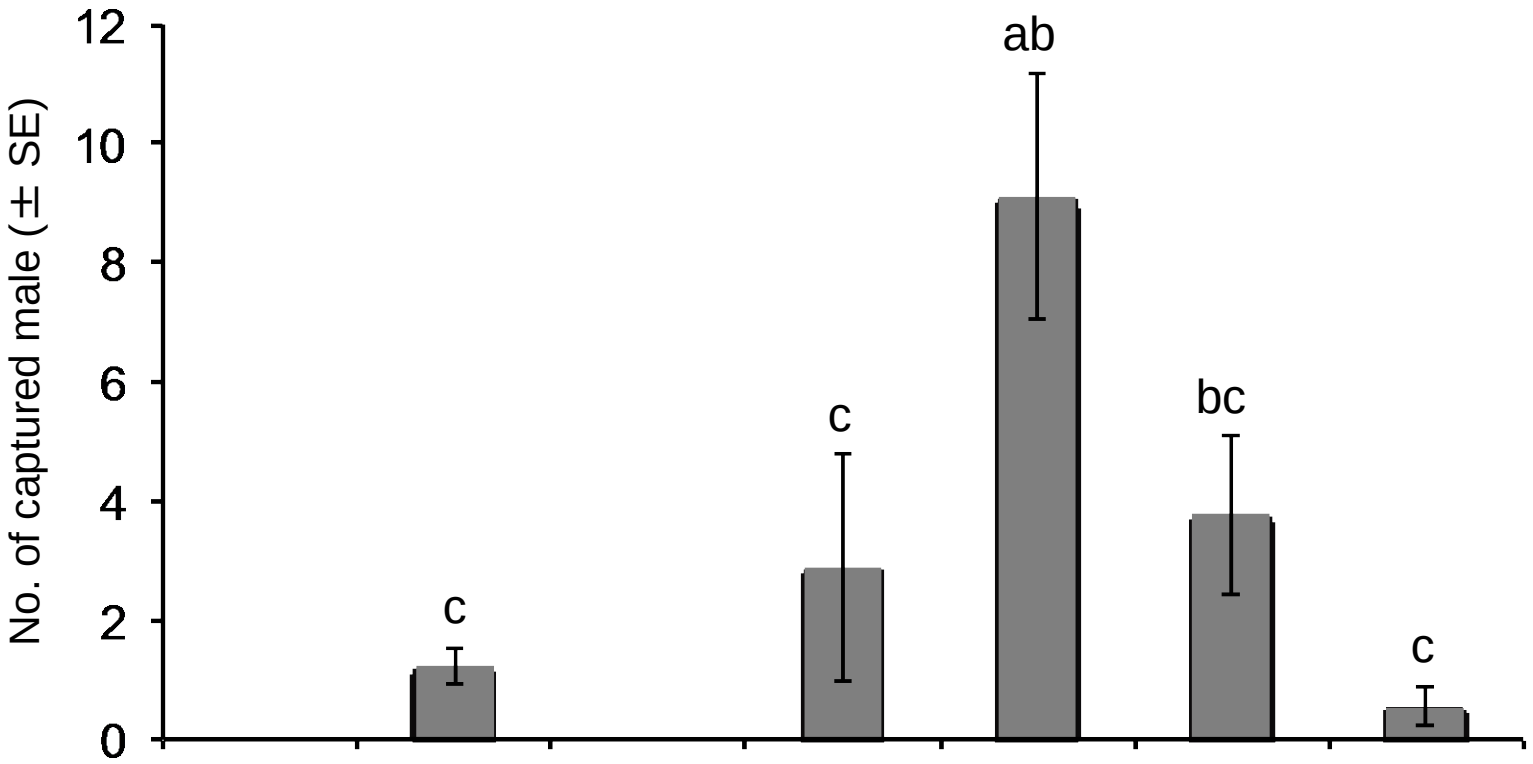
Fig.2 Honda et al.



Ald	0	0.77	0	0	0.77	0	0.77	0.77	Crude Extract (1 FE)
OAc	0	0	3.60	0	0	3.60	3.60	3.60	
HC	0	0	0	11.1	11.1	11.1	0	11.1	

Composition (ng)

Fig.3 Honda et al.



Ald	0	83	0	33	25	25	25
OAc	0	417	132	0	125	125	125
HC	0	0	368	467	350	700	1750

Composition (μg)

Fig.4 Honda et al.