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Methyl 6-methylsalicylate: A Female-Produced Pheromone Component of the Parasitoid Wasp *Spalangia endius* (Hymenoptera: Pteromalidae)

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Abstract Sex-pheromone-related behavior and chemistry were studied in the wasp *Spalangia endius* Walker (Hymenoptera: Pteromalidae), a pupal parasitoid of the house fly, *Musca domestica* L. (Diptera: Muscidae). Males responded behaviorally to female extracts by arrestment, whereas females did not arrest to male extracts. In a comparison of male and female extracts by gas chromatography-mass spectrometry (GC-MS), two female-specific compounds were found. One was identified as methyl 6-methylsalicylate (gas chromatographic retention time and mass spectrum versus an authentic standard), but the chemical structure of the second compound is still unknown. Male antennae were sensitive to both compounds in electrophysiological tests (GC-EAD). Males responded behaviorally to methyl 6-methylsalicylate by arrestment, but they did not arrest to the second compound. Methyl 6-methylsalicylate has been reported previously from some ant and beetle species, but never from the Pteromalidae. Chemical analysis of the extracts and the male behavioral results are consistent with the hypothesis that methyl 6-methylsalicylate functions as a female-emitted pheromone component at short range, but the exact role of both compounds in intersexual interactions in *S. endius* remains to be determined.

Key Words Gas chromatography-mass spectrometry (GC-MS) • Methyl 6-methylsalicylate • Parasitoid wasp • Pteromalidae • Sex pheromones • *Spalangia endius*

Introduction

Pheromones are chemical compounds that are secreted to the outside of insects and elicit responses from conspecifics (Blomquist and Vogt 2003). They are used in many different behavioral contexts, e.g., as attractants or repellants. This study examined pheromone components involved in intersexual interactions in the parasitoid wasp *Spalangia endius* Walker (Hymenoptera: Pteromalidae). Many aspects of male-female behavioral interactions have been studied in *S. endius* (King et al. 2005; King and Fischer 2005; King 2006, 2008; Fischer and King 2008; King and Dickenson 2008a, b); yet information on the chemical basis of the behavior has been lacking.

Spalangia endius is a widely distributed, 2-3 mm long parasitoid wasp. Its natural hosts are the pupal stage of various flies (Diptera) found in manure and rotting organic matter (Rueda and Axtell 1985). In *S. endius*, a male's first obvious sexual response to a female begins when he is less than a few centimeters away from her (King et al. 2005). This suggests that at least some pheromone components of *S. endius* act at short range (King and Dickenson 2008a), although long range components may also exist. Upon detecting a female, the male chases her, often wing fanning (rapidly moving his wings up and down) in the process. Males wing fan to females, but not to males (King 2006), suggesting that males can discriminate between the sexes, probably through the presence of a female-specific pheromone component. Upon contact or near contact, the male either retreats or mounts dorsally. He is especially likely to retreat if she has already mated (King et al. 2005); and retreats appear to be related to females releasing an antiaphrodisiac when a male approaches, rather than from physical aggression from the female (King and Dickenson 2008a). If a male mounts the female, he then continues to court her by vibrating his entire body on her. Fanning, mounting, and vibrating by a male do not require any active solicitation by the female, i.e., even dead females elicit such behaviors.

Pheromone components involved in mating behavior in other pteromalid species include pheromones used in attracting a mate and eliciting male courtship behaviors (Ruther et al. 2000). Among insects generally, female-produced pheromones are more common and better known (Keeling et al. 2004); however, in the parasitoid wasp *Nasonia vitripennis* Walker (Hymenoptera: Pteromalidae), some male pheromones are known to affect a female's response to the male (e.g., Ruther et al. 2007). It is not uncommon for male and female parasitoids to have many of the same pheromones but in different quantities (Keeling et al. 2004; Ruther and Steiner 2008), and this is true of *N. vitripennis* (Steiner et al. 2006). Prior to this study no pheromone components of *S. endius* were known for either males or females. We report the identification of a pheromone component in *S. endius* that is very different from that reported for other pteromalids.

Methods and Materials

Spalangia endius *Colony* The *S. endius* were from a colony established from wasps collected in 1996 from Zephyr Hills, Florida, USA and maintained by using pupae of a natural host, the house fly, *Musca domestica* L. (Diptera: Muscidae). Hosts were produced following techniques described in King (1988), but we used wood shavings in

place of vermiculite as the rearing substrate. Parasitized hosts were isolated individually in glass test tubes prior to emergence of the wasp in order to obtain female virgin wasps. Virgin males were obtained from separate colonies parasitized by virgin females.

Semiochemical Extraction and Gas Chromatography-Mass Spectrometry Analyses Gas chromatography-mass spectrometry (GC-MS) was used to compare semiochemical extracts from males and females to determine if sex-specific compounds were detectable. The semiochemical extracts from males and females were obtained by two different methods, solvent extraction of whole insects and solid phase micro extraction (SPME). A third method (Super-Q trapping of volatiles) was used to prepare extracts from females alone. The purpose of solvent extractions was to remove all soluble compounds from the wasp's exoskeleton, whereas SPME was used specifically to collect emitted volatiles. For each solvent extraction, ten wasps were killed by freezing at -17^{o}C, transferred to a microtube and extracted in dichloromethane (30 min, 60 µl). Extract volume was reduced to 20 µl under a gentle stream of nitrogen. For SPME, collections were made by exposing the fiber [65 µm polydimethylsiloxane/divinylbenzene blend (Supelco Inc., Bellefonte, PA)] for 20 min to the static headspace of a 1.5 ml vial with a Teflon-lined septum (National Scientific, Rockwood, TX) containing ten insects.

Headspace volatiles were collected from females by using Super-Q porous polymer traps (Alltech Associates, Deerfield, IL). This technique permitted a larger quantity of volatiles to be collected than the SPME method. For the trap collection of the volatiles a Magnetek Universal Ser. 41ZF vacuum pump was used to pull the headspace of a 125 ml side-arm flask through 5-mm-diam. glass tubing that contained a 5 to 8-mm plug of Super-Q porous polymer). The Super-Q was held in place by a fine stainless steel screen (fused into trap glass wall) and glass wool. A variable number of 0-d-old females were placed in the flask. Females were replaced with new females every 2 d. Volatiles from a total of 200 females were collected over a 2-mo period. The flask opening was plugged with a cork with a hole, which was filled with glass wool to allow the free inflow of air. To recover the collected volatiles, the Super-Q traps were rinsed weekly with dichloromethane (2 ml). This was concentrated to 400 µl or 2 µl/ female equivalent. No food or water was fed to the insects during the collection period.

All three types of extracts were analyzed by a Hewlett Packard 5973 mass selective detector, interfaced with a Hewlett Packard 6890 GC (Agilent Technologies Inc., Santa Clara, CA). A split/splitless inlet was used in splitless mode with either a 15-m DB-1 (0.25 mm i.d., 0.1 µm film thickness) or a 30-m DB-5MS (0.25 mm i.d., 1.0 µm film thickness) capillary column (J & W, Agilent Technologies Inc., Santa Clara, CA). The oven temperature started at 50^{o}C for 1 min and increased to 280^{o}C at a rate of 10^{o}C/min. Carrier gas was He set at constant pressure (6 psi) and injector temperature was set at 280^{o}C.

GC-Electroantennographic Detection (GC-EAD) Analysis Male antennal responses to female-specific compounds from the SPME and Super-Q extracts were examined to determine how sensitively the female-specific compounds were detected. GC-EAD connections were made by inserting the base of an excised male antenna into a saline-filled Ag/AgCl glass pipet grounding electrode. The suspended male antenna was maneuvered by a micromanipulator, into a stream of purified, humidified air (20 ml/sec)

that emerged from a 20-cm-long L-shaped glass tube (7 mm i.d.) that ended 1 cm from the antenna. A second glass pipet Ag/AgCl-recording probe (Syntech, Hilversum, The Netherlands), fitted onto a second micromanipulator, was placed in contact with the distal cut end of the antenna. Stimuli were introduced into the air stream from the GC effluent (split between FID and EAD, GC-EAD interface temperature set at 280°C) through a hole in the glass tube positioned 10 cm from the antenna. The GC-EAD responses were amplified (500×) with an AC/ DC UN-6 amplifier (Syntech). Acquisition and analysis of the responses were performed by a computer equipped with an analog to digital conversion board (IDAC, Syntech) running GC-EAD software (Syntech). The GC was a Hewlett Packard 6890 GC (Agilent Technologies Inc., Santa Clara, CA). A split/splitless inlet was used in splitless mode with 15-m DB-1 (0.25 mm i.d., 0.1 µm film thickness) capillary column (J & W, Agilent Technologies Inc., Santa Clara, CA). The oven temperature started at 50°C for 1 min and increased to 150°C at a rate of 10°C/min, followed by 30°C/min till 280°C. Carrier gas was He set at a flow rate of 24 cm/sec, and injector temperature was set at 250°C.

Chemicals Methyl 4-methylsalicylate was purchased from Frinton (Vineland, NJ). Methyl 3- and methyl 5-methylsalicylate were purchased from Aldrich (St. Louis, MO). Methyl 6-methylsalicylate was synthesized from ethyl 6-methylsalicylate (APIN, Abingdon, Oxon) according to Castracani et al. (2003), except that the reaction ran for 5 h. Reaction completion was checked by GC-MS.

Bioassays All behavioral assays were conducted in a small blue plastic dish (1.5 cm diameter, 1 cm height) filled three quarters with dry sand and covered by a glass cover slip. A wasp was tapped from its glass tube into the dish. The dish contained a single treatment or control on filter paper (6 mm diam. Whatman No. 1), which was centered on top of the sand. Arrestment time of a test wasp was recorded for 5 min (adapted from Steiner et al. 2006). Arrestment time was the time that the wasp spent with a body part touching the filter paper (or carcass in the first experiment). No wasp was tested more than once and all were between 0-1 d old. Prior to data analyses, arrestment times were log transformed to improve normality and homoscedasticity. Log-transformed arrestment times were compared among treatments by using analyses of variance (ANOVA) or independent *t*-tests.

Response to Extracts of the Opposite Sex In the first behavioral assay, male responses to female extract were examined to determine whether pheromones were involved in sexual attraction. Steiner et al. (2006) showed that responses to extract can be greater when it is applied to insect cuticle than to filter paper, so tests were done with both. There were four treatments ($N = 15$ per treatment): 1) extract on a filter paper disc; 2) extract applied to a male carcass situated on top of a filter paper disc; 3) solvent on a filter paper disc; and 4) solvent on a male carcass on top of a filter paper disc. Extraction was with dichloromethane as described above. Extract treatments consisted of two female equivalents (4 µl); the solvent treatments consisted of 4 µl of pure dichloromethane. Male carcasses for the assays were prepared by using the same method as for extracting females, i.e., by soaking males for 30 min in 60 µl of dichloromethane and then letting them dry for 5 min. The second behavioral assay was the same as the first, but used male

extracts, female carcasses, and live virgin females. Carcasses of the same sex as the responder were used to avoid traces of compounds from the opposite sex that might cause any arrestment during the first and second experiments.

Responses to Fractionated Female-specific Compounds The third bioassay experiment compared male behavioral responses to fractions containing one of the female-specific compounds or their combination. First a female extract was obtained by soaking 150 females in 900 µl of dichloromethane for 30 min. Ninety female equivalents of the extract were blown dry under nitrogen and resuspended in 200 µl hexane prior to column chromatography on silica gel. The column was made with 5 cm of silica gel (70-230 mesh) in a Pasteur pipette with glass wool blocking the tapered part of the pipette. The female extract was loaded on the silica column and three fractions were collected. Elution solvents were hexane, 1:1 diethyl ether: hexane, and diethyl ether (2 ml of each, in that order). The fractions were analyzed by GC-MS. One female-specific compound (previously identified as **A**) was present in the 1:1 diethyl ether/hexane fraction, and the second compound (previously identified as **B**) was found in the diethyl ether fraction. These two fractions were blown dry under nitrogen and resuspended in dichloromethane prior to behavioral analysis. The experiment compared the male behavioral responses to four treatments: 1:1 diethyl ether:hexane fraction (**A**), diethyl ether fraction (**B**), 1:1 blend of the two fractions (**A**+**B**), and solvent (dichloromethane) control (N = 15 males each). Each treatment consisted of two female equivalents (4 µl) applied to a 6 mm piece of filter paper. The solvent control was applied in the same volume.

Response to Methyl Methylsalicylate Isomers The fourth behavioral assay compared the male behavioral responses to authentic standards of four methyl methylsalicylate positional isomers vs. vacuum-collected female volatiles. The two female-specific compounds were present in the vacuum collected volatiles. All methyl methylsalicylate isomers were applied at two female equivalents (about 300 ng). An arrestment time bioassay of virgin males was completed by using six different treatments, methyl 3-methylsalicylate, methyl 4-methylsalicylate, methyl 5-methylsalicylate, and methyl 6-methylsalicylate, along with vacuum-collected female volatiles (two equivalents) and a solvent control, all in 4 µl of dichloromethane. The temperature and relative humidity during these four behavioral experiments ranged from 21 to 24^{o}C and 35 to 55%, respectively.

Results

Gas Chromatography-Mass Spectrometry Analyses Comparison of SPME collection (N=4) from adult *S. endius* males and females revealed two female-specific peaks (**A** and **B** in Fig. 1). The most abundant, **A,** was also present in all Super-Q collections (N=8); but only trace amounts of **B** could be detected in these collections. The two peaks were always detected in female body washes (N=5) in a 98:2 ratio, respectively. These two compounds were never detected in male volatiles collections or body extracts. The mass spectrum of compound **A** (Fig. 1) suggested an isomer of methyl methylsalicylate; no MS library (Wiley 1995) match was found for compound **B** (Fig. 1). Compounds **A** and **B**

eluted from the silica column in 1:1 diethyl ether:hexane and diethyl ether, respectively, suggesting that both compounds are oxygenated.

The library mass spectra of the four possible isomers of methyl methylsalicylate were very similar; thus authentic standards were acquired for all four for comparison to the natural product. The standard isomers were all separable by GC. Only methyl 6-methylsalicylate had an identical GC retention time and mass spectrum compared to compound **A** (Fig. 2).

Gas chromatography-Electroantennographic Detection (GC-EAD) Analysis The GC/EAD analysis showed that male antennae (N=5) responded to the two female-specific compounds (Fig. 3 top) and that the male antennal response to natural **A** was identical in GC retention time compared to the response to synthetic methyl 6-methylsalicylate (Fig. 3 bottom).

Response to Extracts of the Opposite Sex A 2-way ANOVA of arrestment time by extract (extract, solvent) and carcass (present, absent) was done for males and females separately. For males, there was not a significant interaction between extract and carcass (F=0.09, df=1, 56, P=0.77), meaning the effect of each does not depend on the other. There were significant individual effects for both extract and carcass (F=45.79, df=1, 56, P=<0.001; F=21.62, df=1, 56, P=<0.001, respectively). Male arrestment was increased by the extract as can be seen by comparing solvent to extract and by comparing carcass with solvent to carcass with extract (Table 1). Male arrestment was also increased by the carcass being present as can be seen by comparing extract alone to carcass with extract and solvent alone to carcass with solvent. For females, there were no significant effects of extract, carcass, or the interaction between them (F=0.31, df=1, 56, P=0.58; F=0.52, df=1, 56, P=0.47; F=1.66, df=1, 56, P=0.20). Thus, females did not appear to arrest to extract or carcass.

Responses to Fractionated Female-specific Compounds A 2-way ANOVA of arrestment time by fraction-containing-**A** (present, absent) and fraction-containing-**B** (present, absent) was performed (Table 2). There was no significant interaction (F=0.013, df=1, 56, P=0.91) and no significant effect of the fraction containing **B (**F=0.071, df=1, 56, P=0.79), but there was a significant effect of the fraction containing **A** (F=20.09, df=1, 56, P<0.001). Thus, this experiment showed that relative to solvent control, males arrest to the fraction containing the **A** compound, but not to the fraction containing the **B** compound, and that the response to one fraction is not affected by the presence of the other fraction. Males periodically fanned during trials that included the **A** containing fraction.

Response to Methyl Methylsalicylate Isomers To preserve statistical power, planned comparisons were used (Ruxton and Beauchamp 2008). The first comparison of arrestment times in the bioassay (Table 3) was between the female-specific volatiles and methyl 6-methylsalicylate. Arrestment times did not differ (t = 0.061, df= 28, P=0.95), and so their times were combined into group 1. The next comparison was among the other three synthetic isomers (methyl 3-methylsalicylate, methyl 4-methylsalicylate, methyl 5-methylsalicylate), which did not differ either (F=0.57, df=2, 42, P=0.57); so the

isomers' arrestment times were grouped and became group 2. Groups 1 and 2 were then compared, which showed that arrestment time was greater for female-specific volatiles and methyl 6-methylsalicylate than for other isomers (t =3.76, df=73, P< 0.001; untransformed means: 20.27 ± 8.01 s versus 4.07 ± 0.71 s, respectively). The last comparison was group 2 and the solvent control, which showed that males do not arrest significantly to the methyl 3, methyl 4, and methyl 5 isomers (t =0.87, df=58, P=0.39). Although males did not significantly arrest unless methyl 6-methylsalicylate was present, occasional courtship-fanning to methyl 6-methylsalicylate and other isomers was seen.

Discussion

The study showed that female *S. endius* emitted two female-specific compounds, methyl 6-methylsalicylate and an unidentified second compound. The analyses of the extracts and the male behavioral results are consistent with the idea that methyl 6-methylsalicylate functions as a female-emitted pheromone component. Both female-specific compounds were biologically active when analyzed by GC-EAD. However, males arrested only to the silica fraction containing methyl 6-methylsalicylate and to the same degree as they arrested to the complete natural blend. In other words, the silica fraction with the unidentified material did not arrest males and did not increase arrestment to the silica fraction containing methyl 6-methylsalicylate.

The exact roles of methyl 6-methylsalicylate and the unidentified compound in intersexual interactions in *S. endius* remain to be determined. This study only evaluated male arrestment responses; it is possible that these two female-specific compounds, individually or as a blend, have long-range behavioral effects. Methyl 6-methylsalicylate might be described as a sex pheromone component because males fanned to the compound periodically, and fanning is only seen during male attraction to females (King et al. 2005; King 2006). However, why only some males fanned is unclear. Given that males arrested to it, the compound might also be important in eliciting or maintaining the courtship that males perform once mounted, as an attraction pheromone, or both.

Methyl 6-methylsalicylate has been identified before in some ants (e.g., Torres et al. 2001; Schönrogge et al. 2008) and some beetles (Moore and Brown 1979; Gnanasunderam et al. 1984). It has never been identified before in the Pteromalidae or from any other wasps or bees. Although methyl 6-methylsalicylate has been identified from more than twenty species of ants, its function is known for only a fraction of them. It acts as a sex pheromone in *Polyergus breviceps* and *P. rufescens* (Greenberg et al. 2004; Castracani et al. 2005); a trail pheromone in *Tetramorium impurum* and *Mayriella overbecki* (Morgan and Ollett 1987; Kohl et al. 2000); and as an alarm pheromone in *Gnamptogenys pleurodon* (Duffield and Blum 1975). Sometimes methyl 6-methylsalicylate is mixed with other compounds to elicit a response, such as in *P. breviceps* and *P. rufescens* (Greenberg et al. 2004; Castracani et al. 2008); or it is the only compound needed to elicit a sexual response, such as in *P. rufescens* (Castracani et al. 2005). In *Camponotus* spp., *Bothroponera soror*, *P. breviceps, P. rufescens,* and *G. pleurodon* methyl 6-methysalicylate comes from the mandibles (Brand et al. 1973; Longhurst et al. 1980; Blum et al. 1987; Castracani et al. 2005, Greenberg et al. 2004). In *T. impurum* and *M. overbecki*, methyl 6-methylsalicylate is found in the poison gland

(Morgan and Ollett 1987; Kohl et al. 2000). The location of methyl 6-methylsalicylate in *S. endius* is currently unknown, though preliminary experiments suggest it may be distributed over the entire cuticle.

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Table 1 Arrestment times of males and females to solvent and to extract of the opposite sex, with or without a carcass of same sex present[a]

	Solvent	Extract	Solvent with carcass	Extract with carcass
Male	0.29 ± 0.09 (0-1.00)	0.97 ± 0.14 (0-1.63)	0.75 ± 0.10 (0-1.20)	1.49 ± 0.06 (0-1.87)
Female	0.61 ± 0.12 (0-1.26)	0.83 ± 0.12 (0-1.53)	0.85 ± 0.13 (0-1.59)	0.77 ± 0.10 (0-1.18)

[a]Table entries are log-transformed mean ± s.e. (minimum-maximum), in log sec, N =15 per treatment per sex.

Table 2 Arrestment times of males and number of males that fanned to column fractions containing methyl 6-methylsalicylate and/or unknown compound and to solvent control[a]

Fraction content	Arrestment time	# Males that fanned
Solvent[b]	0.71 ± 0.11 (0-1.49)	0
Methyl 6-methylsalicylate (A)	1.24 ± 0.12 (0.30-2.19)	4
Mixture (A+B)	1.19 ± 0.14 (0-2.39)	2
Unknown compound (B)	0.69 ± 0.09 (0-1.40)	0

[a]Table entries are log-transformed mean ± s.e. (minimum-maximum), in log sec, N =15 per treatment.
[b]Dichloromethane.

Table 3 Arrestment times of males and number of males that fanned to isomers of methyl methylsalicylate, as well as to solvent and to volatiles of females[a]

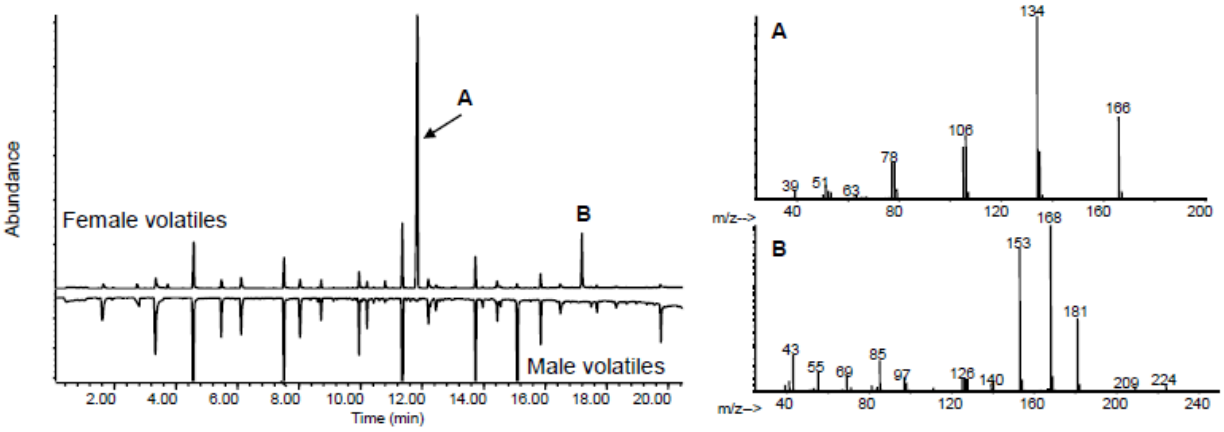
Isomer or control	Arrestment time	# Males that fanned
Solvent[b]	0.61 ± 0.10 (0-1.36)	0
3	0.44 ± 0.11 (0-1.18)	1
4	0.47 ± 0.12 (0-1.23)	3
5	0.60 ± 0.11 (0-1.28)	2
6	0.95 ± 0.12 (0-1.84)	5
Volatiles	0.93 ± 0.17 (0-2.38)	1

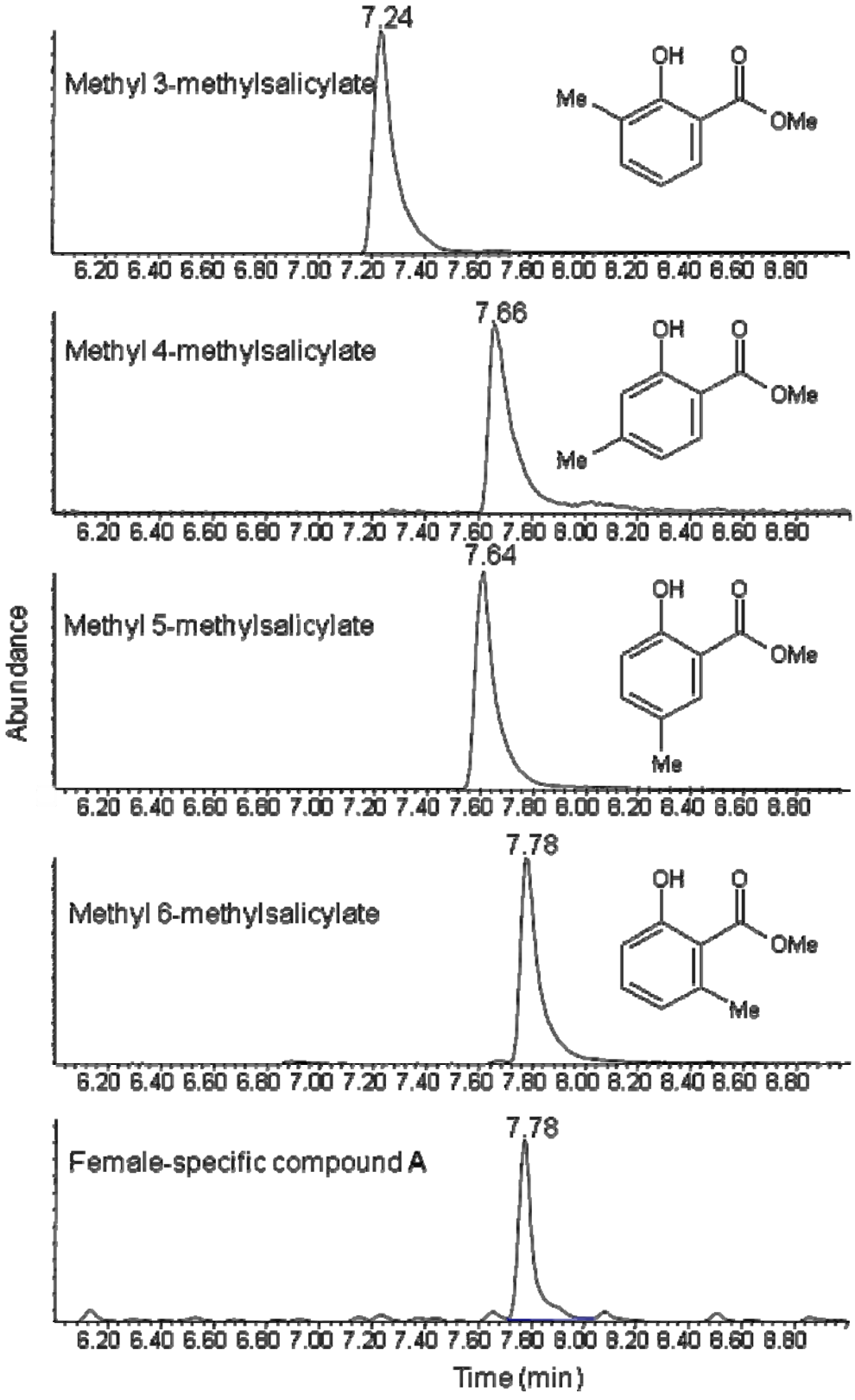
[a]Table entries are log-transformed mean ± s.e. (minimum-maximum), in log sec, N =15 per treatment.
[b]Dichloromethane.

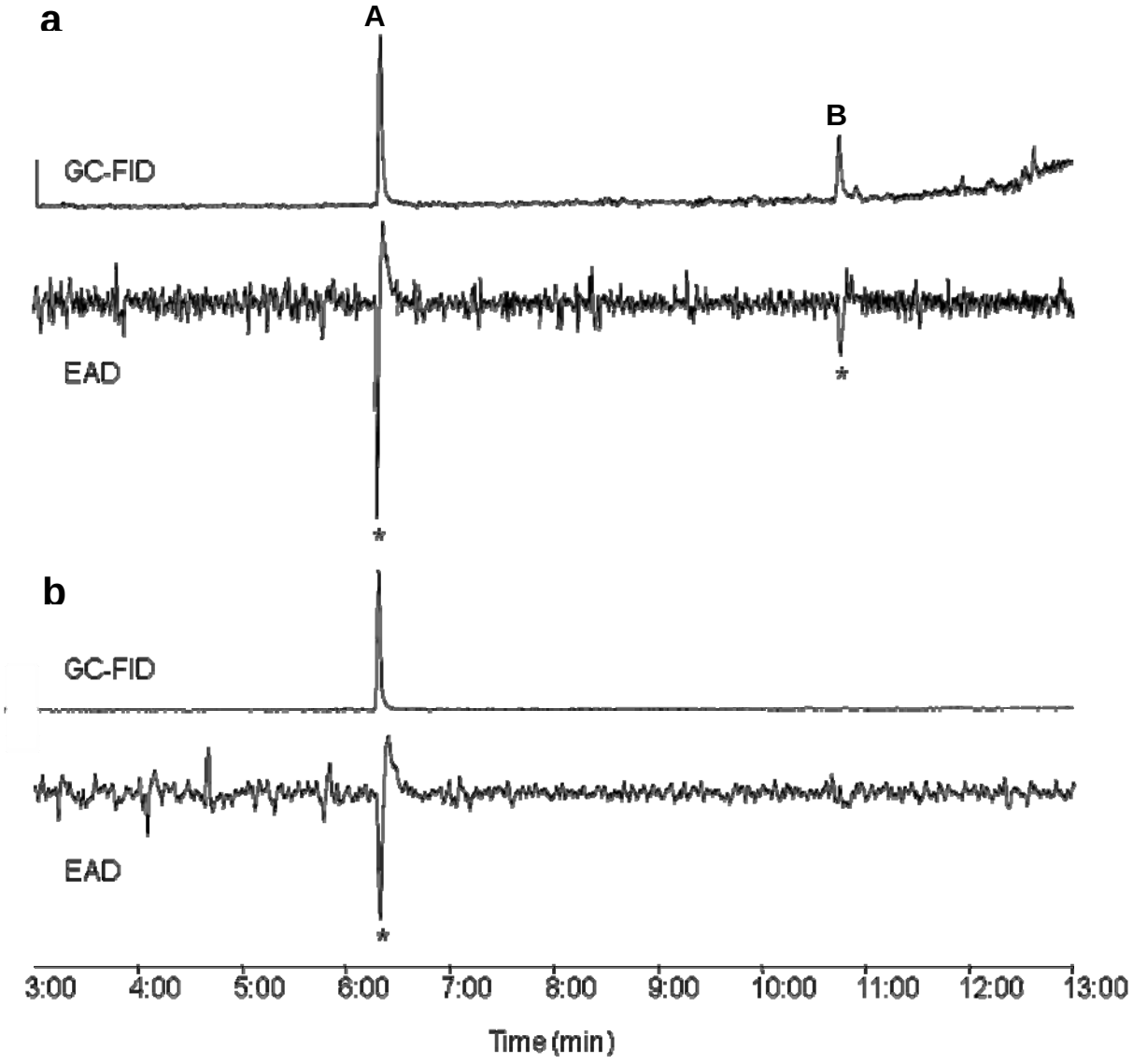
Fig. 1 Comparison of total ion chromatograms of SPME samples from male and female *Spalangia endius*. This illustrates the two female-specific compounds, **A** and **B**, and their mass spectra.

Fig. 2 Comparison of total ion chromatograms of authentic standards of four possible isomers of methyl methylsalicylate and female-specific *Spalangia endius* compound **A** (retention times from analysis on DB1 capillary column)**.**

Fig. 3 Antennal response of male *Spalangia endius* to **a**) female-specific compounds **A** and **B** from the SPME sample and **b**) synthetic methyl 6-methylsalicylate; ***** indicates a positive response.


Abundance
Female volatiles
Male volatiles
A
B
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Time (min)
A
134
166
106
78
39
51
63
m/z-->
40
80
120
160
200
B
168
153
181
43
55
69
85
97
126
140
209
224
m/z-->
40
80
120
160
200
240


7.24
Methyl 3-methylsalicylate
OH
O
Me
OMe
6.20 6.40 6.60 6.80 7.00 7.20 7.40 7.60 7.80 8.00 8.20 8.40 8.60 8.80
7.66
Methyl 4-methylsalicylate
OH
O
OMe
Me
6.20 6.40 6.60 6.80 7.00 7.20 7.40 7.60 7.80 8.00 8.20 8.40 8.60 8.80
7.64
Methyl 5-methylsalicylate
OH
O
OMe
Me
Abundance
6.20 6.40 6.60 6.80 7.00 7.20 7.40 7.60 7.80 8.00 8.20 8.40 8.60 8.80
7.78
Methyl 6-methylsalicylate
OH
O
OMe
Me
6.20 6.40 6.60 6.80 7.00 7.20 7.40 7.60 7.80 8.00 8.20 8.40 8.60 8.80
7.78
Female-specific compound A
6.20 6.40 6.60 6.80 7.00 7.20 7.40 7.60 7.80 8.00 8.20 8.40 8.60 8.80
Time (min)


a
A
B
GC-FID
EAD
*
*
b
GC-FID
EAD
*
3:00
4:00
5:00
6:00
7:00
8:00
9:00
10:00
11:00
12:00
13:00
Time (min)