

Antennal and behavioural responses of *Heortia vitessoides* females to host plant volatiles of *Aquilaria sinensis*

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Abstract

This study investigated volatiles from *Aquilaria sinensis* (Lour.) Gilg (Thymelaeaceae) leaves that attracted *Heortia vitessoides* Moore (Lepidoptera: Crambidae). Volatiles from young and old *A. sinensis* leaves were identified and quantified by gas chromatography–mass spectrometry and gas chromatography–electroantennogram detection. Both wind tunnel bioassays and field tests were conducted to measure the attraction of adults to synthetic blends of volatiles from leaves of different maturations. Consistent electroantennographic activity was obtained for nine and three compounds from headspace collections of young and old *A. sinensis* leaves, respectively. Qualitative and quantitative differences were found among two odour profiles. In wind tunnel experiments, the fresh young leaves proved to be more attractive to females than old leaves. A nine-component mixture, including hexanal, limonene, 2-hexanol, octanal, (*Z*)-3-hexenyl acetate, (*Z*)-3-hexen-1-ol, nonanal, decanal, and 2,6,10-trimethyl-dodecane (with a ratio of 2:16:9:4:63:100:13:10:5) from young leaves attracted moths significantly more than the three-component mixture of nonanal, decanal, and 2,6,10-trimethyl-dodecane (with a ratio of 11:14:26) from old leaves. Further subtractive bioassays conducted in the wind tunnel showed that both the complete nine-component mixture and a subtracted four-component mixture of hexanal, (*Z*)-3-hexenyl acetate, nonanal, and decanal (with a ratio of 2:63:13:10) elicited equivalent responses in females. All components in the four-component blend were essential for optimal attraction. In a field trial using the nine- and four-component blends, more moths were captured using both blends than in traps baited with hexane only. Our study indicates that the odour blends of young leaves play an important role in *H. vitessoides* host plant recognition. The mechanisms behind host recognition and age-dependent changes in leaf chemistry are discussed.

Introduction

Aquilaria sinensis (Lour.) Gilg (Thymelaeaceae) is an archaic tropical evergreen tree that is native to southern

China, including the provinces Hainan, Guangdong, Guangxi, Fujian, Yunnan, and Taiwan. This plant species is the principal source of Chinese agarwood (also called Chen Xiang in China; Liu, 1999). The resinous part of the injured *A. sinensis* tree forms agarwood, which has been used as a sedative, analgesic, and digestive aide in traditional Chinese medicine since the 16th century (Liu, 1999; Chin Pharmacopoeia Commission, 2010).

Heortia vitessoides Moore (Lepidoptera: Crambidae) is the most serious pest of *A. sinensis*. Its distribution ranges from India, Nepal, China, Sri Lanka, through South-East

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Asia and the East Indies to Queensland, the New Hebrides, and Fiji (Munroe, 1977; Kalita et al., 2002). Like other oligophagous herbivores, *H. vitessoides* has a narrow range of host plants and mainly feeds on plants of the genera *Aquilaria* and *Rhus* (Su, 1994; Kalita et al., 2002). In southern China, the larvae of *H. vitessoides* solely feed on the leaves of *A. sinensis* and occur for up to seven or eight overlapping generations throughout the growing season. In severe infestations, the insects completely eat up the leaves of *A. sinensis*, causing severe economic losses. Management of *H. vitessoides* in the growing areas of *A. sinensis* trees in southern China has mainly relied on the use of conventional insecticides (Su, 1994). However, one important drawback is the severe pesticide residues, which represent a serious threat to the quality of agarwood. It is therefore imperative that alternative control strategies are developed.

Plant-derived volatiles are of paramount importance in guiding female herbivorous insects to their host plants for oviposition (Visser & Avé, 1978; Dethier, 1982; Mustaparta, 2002; Angioy et al., 2003; Anton et al., 2007). There is strong evidence that plant volatiles elicit specific responses in females of several species of insects, both in the laboratory (Hern & Dorn, 2004; Tasin et al., 2005, 2007, 2009; Pinero & Dorn, 2007, 2009) and in the field (Cha et al., 2008a,b; Anfora et al., 2009). In China, *A. sinensis* is the unique host plant of *H. vitessoides*. Therefore, odour mixtures derived from *A. sinensis* should play an important role in host recognition for *H. vitessoides*. In addition, Blüthgen & Metzner (2007) suggested that substantial age-dependent changes in leaf maturation could affect insects' feeding habits differentially, as they demonstrated that young leaves could receive more herbivores. Because the young leaves of *A. sinensis* are the sole attractant to females for oviposition (Su, 1994; HL Qiao, PF Lu & J Chen, unpubl.), the hypothesis in our study was that the volatile signals from the young leaves of *A. sinensis* allowed *H. vitessoides* females to discriminate suitable larval hosts from the background chemical environment and to guide orientation flights towards plants for oviposition. Information on such volatiles may be valuable in improving the efficiency of semiochemical-based monitoring and control techniques of moths in the field. Up to now, little is known about semiochemicals of *H. vitessoides*.

The objectives of this study were: (1) to identify and compare the potential of volatile chemicals from young and old leaves of *A. sinensis* to attract *H. vitessoides*, and (2) to test behavioural responses of *H. vitessoides* to synthetic blends of these chemicals in a wind tunnel and field test. We also examined the relationship between leaf age preferences and host plant recognition in *H. vitessoides*.

Materials and methods

Insects

Heortia vitessoides larvae were collected from the field-infested young leaves of *A. sinensis* in the Chinese Medicinal Material Production Base (CMMPB) of Huazhou Green Life (Guangdong, China; 110°23'58"E, 21°57'30"N) in late May. All insects were reared under controlled conditions at $70 \pm 5\%$ r.h., 25 ± 2 °C, and a L16:D8 photoperiod. Larvae were mass-reared on leaves of *A. sinensis* in glass containers (20- and 30-cm diameter and height, respectively) for four instars and then separately transferred to smaller glass containers (3- and 10-cm diameter and height, respectively) that contained soil for pupation. Newly emerged adults were provided with a sugar–water solution (10% w/vol). Moths were reared in the laboratory for three generations before testing. Virgin females used for electrophysiological tests were 1–2 days old. Wind tunnel bioassays were conducted on 2- to 3-day-old mated females. Mated females were obtained by placing the newly emerged adults into a cage (200 × 200 × 200 cm) with *A. sinensis* at a 2:1 ratio of males:female to ensure mating before the tests were conducted. Only females laying eggs were used in the wind tunnel tests. All females used in the tests had never been exposed to any host plant odour before and were used only once (Tasin et al., 2005).

Chemicals

Blends of synthetic compounds were dissolved in redistilled hexane (>98% after re-distillation, HPLC grade; Sigma–Aldrich, St. Louis, MO, USA). 2-Hexanol (99%) and 2,6,10-trimethyl-dodecane (95%) were purchased from Sigma–Aldrich, (*Z*)-3-hexen-1-ol (98%) and (*Z*)-3-hexenyl acetate (97%) were obtained from Carl Roth (Karlsruhe, Germany). Hexanal (98%), octanal (98%), nonanal (97%), decanal (97%), and limonene (96%) were bought from Fluka Production (Buchs, Switzerland).

Plant material and headspace collection

Ten-year-old *A. sinensis* trees were cultivated in the CMMPB. No insecticides were used on the site during the trials. Young leaves were defined as having an apical position on twigs and a bright green colour and were completely unfolded. Old leaves had a more basal position on twigs, were dark green, and had hardened petioles (Blüthgen & Metzner, 2007). The twigs with leaves were cut from the designated trees, sealed with liquid paraffin (Fluka Production), and transferred to the nearby laboratory in early June 2009 and 2010 (Anfora et al., 2009). Plant materials were picked in the 20 min that preceded the sampling of volatile chemicals or flight tunnel assays. A push–pull system was used to collect headspace volatiles. Leaves were

immediately placed in a 2-l glass jar for extraction. Air aspirated with a vacuum pump (Qianxi Air Company, Beijing, China) was filtered through an activated-charcoal filter, passed through the jar at 300 ml min^{-1} , and finally passed over an adsorbent cartridge (50 mg, Porapak Q, 80/100 mesh; Supelco, Bellefonte, PA, USA). The adsorbents were held between plugs of glass wool in a $0.5 \times 10\text{-cm}$ glass tube. The samples were extracted for 8 h at $25 \pm 2 \text{ }^\circ\text{C}$ and $70 \pm 5\%$ r.h. The adsorbent cartridge was eluted with $500 \text{ }\mu\text{l}$ redistilled hexane at room temperature. For chemical quantification, $0.5 \text{ }\mu\text{g}$ of benzaldehyde (99%; Fluka Production) was added to each sample as an internal standard (Anfora et al., 2009). Extract samples were reduced at room temperature to $50 \text{ }\mu\text{l}$ by a slow stream of nitrogen and then analysed by gas chromatography and mass spectrometry (GC-MS) and gas chromatography-electroantennographic detection (GC-EAD). If not used immediately, extracts were stored in glass vials at $-18 \text{ }^\circ\text{C}$ until analysis. Three samples were collected for young and old leaves.

Chemical analysis

Analysis and identification of headspace collections were conducted on an Agilent Technologies 5973 mass spectrometer (Palo Alto, CA, USA) with electron impact ionization (70 eV), which was interfaced with an Agilent Technologies 6890 N gas chromatograph equipped with a polar DB-Wax or non-polar DB-5 fused silica column (both $30 \text{ m} \times 0.25 \text{ mm} \times 0.25 \text{ }\mu\text{m}$; J&W Scientific, Folsom, CA, USA). The initial oven temperature was kept at $50 \text{ }^\circ\text{C}$ for 1 min and then increased to $120 \text{ }^\circ\text{C}$ at a rate of $3 \text{ }^\circ\text{C min}^{-1}$, which was then followed with $10 \text{ }^\circ\text{C min}^{-1}$ to $240 \text{ }^\circ\text{C}$ for 10 min. Injector temperature was maintained at $250 \text{ }^\circ\text{C}$ with carrier gas at 1.0 ml min^{-1} of helium. The transfer line was set at $280 \text{ }^\circ\text{C}$. Injections ($2 \text{ }\mu\text{l}$) were made in the splitless mode (Tasin et al., 2009). Compounds were identified by comparing the retention time and mass spectra with synthetic standards. Windows NT/MASS Spectral Search Program (Version 1.7) software was used for the data analysis (Condurso et al., 2008).

Electrophysiological recording

Headspace collections were also analysed by an Agilent Technologies 7890N GC with a flame ionization detector (FID) coupled with an electroantennogram detector (Syntech, Hilversum, The Netherlands). Column and oven temperature programs were as described elsewhere. Nitrogen was used as the carrier gas (1.0 ml min^{-1}). The antenna of a *H. vitessoides* female was cut using microscissors. A few segments from the tips of the antennae were clipped off and mounted on the antenna holder with two metal electrodes using conductive gel (Spectra 360; Parker

Laboratories, Fairfield, NJ, USA), and the holder was then inserted into the EAG probe. The electrode was linked with a micromanipulator (MP-15; Syntech). All signals were amplified with a high-impedance amplifier (IDAC-4; Syntech). Splitless injection ($2 \text{ }\mu\text{l}$) was used. The outlet of the GC column was split with a specific Electronic Pressure Control splitter (Agilent) to obtain the requested flow accuracy in a ratio of 1:2 for the FID and the cut antenna. Volatile compounds eluting from the GC column were led to the mounted antenna through a glass tube ($12 \times 0.8 \text{ cm}$) with a charcoal-filtered and humidified air stream aspirated with a stimulus controller (CS-5; Syntech). The EAG and FID signals were amplified and recorded simultaneously using the GC-EAD software developed by Syntech. Each sample was tested four times. Each tested antenna was derived from a different female and used only once. In addition, blends of nine EAD-active synthetic compounds (10 ng of each) were analysed by GC-EAD (Tasin et al., 2005).

Wind tunnel bioassays

Behavioural bioassays were carried out in a Plexiglas wind tunnel (flight section: $200 \times 60 \times 60 \text{ cm}$). Charcoal-filtered air was blown by a horizontal fan, which was installed at the upwind end to create a steady airflow into the tunnel. The wind speed was set at 0.3 m s^{-1} at the point of release of the moths. The upwind and downwind ends of the tunnel consisted of two layers of gauze to prevent escape of the moths (Cha et al., 2008a,b). Light was provided from above at 0.6 lux, and the room was kept at $25 \pm 2 \text{ }^\circ\text{C}$ and $75 \pm 5\%$ r.h.

We first tested the response of *H. vitessoides* females to fresh plant materials (young and old leaves of *A. sinensis*). Freshly cut twigs with young or old leaves (ca. 200 g) were wrapped with liquid paraffin, and then placed in the center of the upwind end of the flight tunnel, covered with gauze to prevent orientation towards visual cues (Tasin et al., 2006). Secondly, we tested the responses of *H. vitessoides* females to headspace collections ($10 \text{ }\mu\text{l}$) from the plant materials (young and old leaves of *A. sinensis*). Finally, we tested the activity of two synthetic blends, which were based on consistent GC-EAD responses to young and old leaves, respectively. A blend of nine components found in young leaves, including hexanal, limonene, 2-hexanol, octanal, (*Z*)-3-hexenyl acetate, (*Z*)-3-hexen-1-ol, nonanal, decanal, and 2,6,10-trimethyl-dodecane (with a 2:16:9:4:63:100:13:10:5 mass ratio), and a blend of three components found in old leaves, including nonanal, decanal, and 2,6,10-trimethyl-dodecane (11:14:26 mass ratio), were compared to determine which attracted the moths the most in the wind tunnel. Synthetic blends were prepared according to the natural ratios in the headspace collections (Table 1).

Table 1 Composition and quantities of volatile compounds in headspace collections from young and old leaves of *Aquilaria sinensis*

Compounds	Young leaves (n = 3)		Old leaves (n = 3)		Retention time (min)
	Concentration (ng g ⁻¹ ± SD)	Relative amounts ¹	Concentration (ng g ⁻¹ ± SD)	Relative amounts ¹	
Alcohols					
1-Hexanol	1.279 ± 0.206	4			13.50
2-Hexanol	2.521 ± 0.138	9			8.79
2-Hexadecanol	0.304 ± 0.040	1			32.24
(Z)-3-Hexen-1-ol	29.289 ± 0.435	100			14.62
<i>E</i> -2-Tetradecen-1-ol	0.750 ± 0.008	3			11.58
Trans-2-undecen-1-ol	0.285 ± 0.041	1			17.04
2-Methyl-1-hexadecanol	0.052 ± 0.005	<1	0.371 ± 0.025	4	28.27
4-(1-Methylethyl)-benzenemethanol	1.007 ± 0.037	3			30.78
Aldehydes					
Hexanal	0.547 ± 0.029	2			5.03
Octanal	1.063 ± 0.023	4			10.96
Nonanal	3.753 ± 0.295	13	1.024 ± 0.054	11	14.82
Decanal	2.725 ± 0.264	10	1.309 ± 0.071	14	18.86
Dodecanal	0.057 ± 0.013	<1			23.82
Benzaldehyde	0.529 ± 0.039	2			19.43
4-Ethyl-benzaldehyde	1.388 ± 0.052	5			26.71
Hydrocarbons					
Decane	0.168 ± 0.007	<1	0.341 ± 0.044	4	3.71
Tridecane			0.310 ± 0.061	4	11.49
Tetradecane	1.409 ± 0.087	4	1.401 ± 0.020	14	15.25
Hexadecane	0.782 ± 0.021	3	1.728 ± 0.103	19	22.95
Heptadecane			0.750 ± 0.043	8	26.25
Heneicosane	2.317 ± 0.161	8	3.784 ± 0.360	41	35.13
Docosane			1.472 ± 0.079	16	33.28
Heptacosane	2.501 ± 0.145	9	4.505 ± 0.336	49	35.99
Octacosane	1.588 ± 0.006	5	3.048 ± 0.529	34	34.23
2,6,10-Trimethyl-tetradecane	0.235 ± 0.010	<1	0.344 ± 0.035	4	26.22
2,6,10-Trimethyl-dodecane	1.564 ± 0.034	5	2.436 ± 0.078	26	19.14
6-Methyl-octadecane	0.368 ± 0.030	1			5.49
Octamethyl-cyclotetrasiloxane	0.666 ± 0.015	2	0.146 ± 0.011	2	3.42
Ketones					
6-Methyl-5-hepten-2-one	1.932 ± 0.053	7			12.69
1-(4-Ethylphenyl)-ethanone	1.700 ± 0.042	6	2.464 ± 0.108	26	29.20
1-(2,4-Dimethylphenyl)-ethanone	1.862 ± 0.051	6			28.60
Esters					
(Z)-3-Hexenyl acetate	18.394 ± 0.517	63			12.01
Butyl acetate			0.329 ± 0.062	4	4.90
Benzenoids					
Methyl benzene			4.492 ± 0.352	49	4.31
Ethyl benzene	0.347 ± 0.022	1	5.756 ± 0.457	64	6.03
<i>o</i> -Xylene	0.180 ± 0.014	<1			6.23
<i>p</i> -Xylene	0.138 ± 0.003	<1	4.641 ± 0.444	51	7.52
1,3-Dimethyl-benzene	0.713 ± 0.033	2	9.345 ± 0.614	100	6.37
1,2,3-Trimethyl-benzene			1.626 ± 0.108	18	10.55
1,3,5-Trimethyl-benzene	0.559 ± 0.019	2			10.70
1-Ethyl-3-methyl-benzene			2.977 ± 0.362	33	8.80
Ethenyl benzene	8.436 ± 0.241	29			9.75
1-Phenyl-1-butene			0.615 ± 0.044	6	16.36

Table 1 Continued

Compounds	Young leaves (n = 3)		Old leaves (n = 3)		Retention time (min)
	Concentration (ng g ⁻¹ ± SD)	Relative amounts ¹	Concentration (ng g ⁻¹ ± SD)	Relative amounts ¹	
Benzothiazole			1.743 ± 0.144	19	30.48
Terpenoids					
(+)- α -Pinene	0.391 ± 0.022	1	1.524 ± 0.099	17	4.05
Limonene	4.788 ± 0.333	16			8.01

Compounds in bold elicited responses from antennae of female *Heortia vitessoides* in GC-EAD experiments (Table 2).

¹Amounts relative to the most abundant compound (set at a value of 100). The average amount of the most abundant compound collected from 200 g of young leaves [(*Z*)-3-hexen-1-ol] was 732.27 ± 10.88 ng h⁻¹ and that of the most abundant compound from 200 g of old leaves (1,3-dimethyl-benzene) was 233.63 ± 15.36 ng h⁻¹.

Chemicals were diluted with hexane and compounds were mixed according to the respective ratios. The amount of the most predominant substance in the blend was 0.5 mg. Preliminary flight-tunnel experiments showed that these concentrations were adequate to elicit moth responses in the wind tunnel. A septum initially treated with solvent only, without a lure, served as a control (Anfora et al., 2009).

To determine which compounds were crucial for female attraction, the synthetic nine-component blend (blend A), which resembled the active compounds from young leaves, was further evaluated by subtractive bioassays in the wind tunnel. Blend A was arbitrarily subtracted to produce blends B–F. One by one, the components of the five-component blend F were subtracted to produce the four-component blends G–J (see the horizontal axis in Figure 3 for the precise blend compositions). A series of subtractive behavioural bioassays was studied in the wind tunnel. If one compound in the nine-component blend was removed without reducing the attractiveness relative to the complete blend, this compound was omitted; if not, this compound was added to the original blend and deemed as a key component.

Headspace collections, synthetic blends, and hexane were loaded in green rubber septa 1–2 h prior to the bioassays, respectively (Najar-Rodriguez et al., 2010). Septa were placed in the laboratory for 2 h to permit solvent evaporation (Anfora et al., 2009). Each septum loaded with one of the test samples was placed on a holder at the upwind end of the tunnel, above 30 cm from the floor. After each treatment, the flight section of the wind tunnel was scrubbed with hexane, and then dried with an electric hair drier (HP 8200; Philips, Zhuhai, China).

The mated females were transferred to the wind tunnel room and allowed to acclimate to the conditions for 2 h prior to the bioassays. The bioassays began 2 h after the start of the scotophase and lasted 3 h (from 20:00 to

Table 2 Antennal responses of *Heortia vitessoides* females to 10 ng of synthetic compounds identified from young leaves of *Aquilaria sinensis* as analysed by gas chromatography-electroantennographic detection (GC-EAD)

Compound	Antennal response (mV) Mean ± SD (n = 4)	Relative response (%) vs. (<i>Z</i>)-3-hexen-1-ol
Alcohols		
2-Hexanol ¹	0.116 ± 0.012	103
(<i>Z</i>)-3-Hexen-1-ol ¹	0.113 ± 0.026	100
Aldehydes		
Hexanal ¹	0.058 ± 0.006	51
Octanal ¹	0.095 ± 0.005	84
Nonanal ^{1,2}	0.147 ± 0.011	130
Decanal ^{1,2}	0.132 ± 0.031	117
Esters		
(<i>Z</i>)-3-Hexenyl acetate ¹	0.203 ± 0.008	180
Hydrocarbons		
2,6,10-Trimethyl-dodecane ^{1,2}	0.144 ± 0.008	125
Terpenoids		
Limonene ¹	0.057 ± 0.011	50

¹Compound from collections of young leaves (n = 4).

²Compound from collections of old leaves (n = 4).

23:00 hours). *Heortia vitessoides* was active during the time in preliminary experiments. Batches of 10 females were placed in a cylindrical gauze cage (10- and 15-cm diameter and height, respectively), which had a solid lid on one side. The cage with moths was kept on a holder at a height of 30 cm in the centre of the downwind end of the wind tunnel. At the beginning of the bioassays, the lid facing the odour source was opened, allowing the females to leave the cage. The behaviours of individual females were scored for flying upwind in the centre of the tunnel over 120 cm and approaching the odour source within 5 cm. Females

responding to the odour sources were removed from the tunnel. Each batch of 10 mated females was observed for 20 min, and six batches of females were used per day. Each odour source was tested with nine batches of moths on different days. Females were used only once in the bioassays.

Field experiment

On the basis of results of the GC-EAD and wind tunnel bioassays, compounds from the young leaves that elicited antennal responses in female *H. vitessoides* were formulated in blends for the field tests. Nine EAD-active compounds, which were the same as used in the wind tunnel, were prepared in the ratio emitted by the natural volatile mixture of young leaves. In addition, based on the results of subtractive bioassays in the wind tunnel, a four-component mixture of hexanal, (*Z*)-3-hexenyl acetate, nonanal, and decanal (ratio 2:63:13:10) was also applied as an odour source. The formulations contained 100 mg of the most abundant compound and the others were in the same proportion as in the natural volatiles. Preliminary field experiments showed that such concentrations can provide an adequate emission under field conditions and avoid early evaporation. The lures were prepared 2 h before the beginning of the field tests and then attached to the trap with an insect pin (Cha et al., 2008b). Unbaited traps (hexane solution) served as controls.

The field test was conducted in August 2010 in the *A. sinensis* plant yard at CMMPB. White delta traps (35 × 20 × 20 cm) with sticky bottoms (Geruibiyuan Technology, Beijing, China) were used in the field test. The trial was carried out in a randomized complete-block design. Each treatment was repeated five times in five blocks. The blocks were 800-m apart. In each block, traps were hung ca. 2 m from the ground and set up at intervals of at least 20 m. The traps were monitored four times weekly. The number and sexes of moths trapped were recorded. Preliminary experiments had shown that the moths could be attracted during the 1st week. Therefore, the trial lasted for 1 week. The data of the captures over a 7-day period were pooled.

Statistical analysis

The mean numbers of females responding in the wind tunnel and both sexes captured in traps in the field were analysed by one-way analysis of variance (ANOVA). The means were separated by Tukey's multiple range tests ($\alpha = 0.01$). Data were analysed with SPSS, version 13.0 (IBM, Armonk, NY, USA).

Results

Identification of volatiles from *Aquilaria sinensis*

Many compounds belonging to various chemical classes, such as hydrocarbons, alcohols, aldehydes, esters, ben-

zene derivatives, ketones, and terpenoids, were identified from young and old *A. sinensis* leaves. The composition and relative content of compounds released by young and old leaves were different (Table 1). Most alcohols [1-hexanol, 2-hexanol, 2-hexadecanol, (*Z*)-3-hexen-1-ol, *E*-2-tetradecen-1-ol, trans-2-undecen-1-ol, and 4-(1-methylethyl)-benzenemethanol], aldehydes (hexanal, octanal, dodecanal, benzaldehyde, and 4-ethyl-benzaldehyde), ketones [6-methyl-5-hepten-2-one, 1-(2,4-dimethylphenyl)-ethanone], and esters [(*Z*)-3-hexenyl acetate] were characteristic of young leaves and were completely absent in old leaves. In particular, (*Z*)-3-hexen-1-ol was the most abundant compound that was present only in young leaves, whereas the concentrations of other volatiles, such as nonanal and decanal, decreased as the leaves matured. In old leaves, hydrocarbons, benzene derivatives, and terpenoids represented the dominant compounds. The most abundant compounds that were released from old leaves were 1,3-dimethyl-benzene, methyl benzene, and ethyl benzene (Table 1), which were also found in much smaller amounts in young leaves.

Antennal responses to volatiles from *Aquilaria sinensis* leaves

The EAD-active odour profiles presented large differences between the young and old leaves. However, nonanal, decanal, and 2,6,10-trimethyl-dodecane were common components of both plant materials, whereas 2-hexanol, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate, hexanal, octanal, and limonene were present only in young leaves. The antennae of *H. vitessoides* females not only responded to the most abundant compounds, such as (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate but also to the less-abundant compounds, such as hexanal, and octanal (Table 2, Figure 1). Synthetic (*Z*)-3-hexenyl acetate elicited the strongest antennal response in *H. vitessoides* females (Table 2).

Wind tunnel bioassays

Fresh young leaves proved to attract females the most in the wind tunnel compared with all other odour sources. Females became active from rest, and 43.8% of females flew upwind over 120 cm, and 23.4% arrived within 5 cm of the fresh young leaves. Synthetic young leaf blends [hexanal, limonene, 2-hexanol, octanal, (*Z*)-3-hexenyl acetate, (*Z*)-3-hexen-1-ol, nonanal, decanal, and 2,6,10-trimethyl-dodecane, with a ratio of 2:16:9:4:63:100:13:10:5] were ranked second with regard to attracting females. In comparison, the number of females flying upwind and approaching the young leaves headspace was significantly different from the female response to fresh young leaves and synthetic young leaves blends. However, fewer females flew upwind and arrived at odour sources made of blends

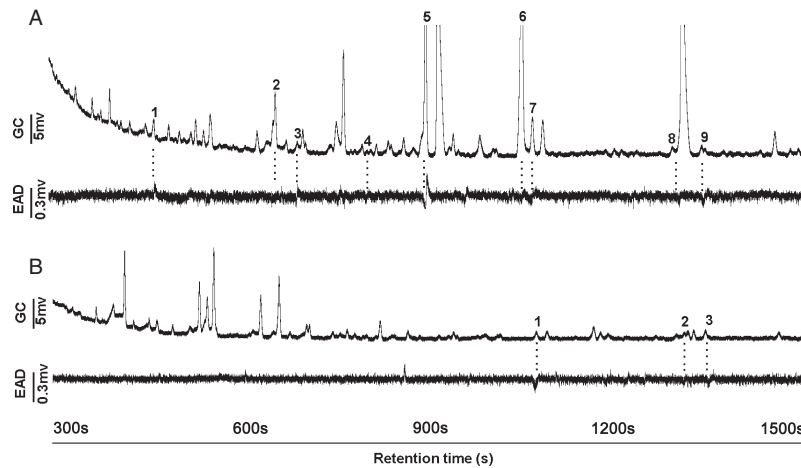


Figure 1 Simultaneously recorded gas chromatography-electroantennographic detection (GC-EAD) using the antennae of *Heortia vitessoides* females in response to volatiles collected from (A) young and (B) old *Aquilaria sinensis* leaves. The upper trace represents the flame ionization detector response and the lower trace represents the female-consistent antennal response (EAD). For young leaves (A): (1) hexanal, (2) limonene, (3) 2-hexanol, (4) octanal, (5) (*Z*)-3-hexenyl acetate, (6) (*Z*)-3-hexen-1-ol, (7) nonanal, (8) decanal, and (9) 2,6,10-trimethyl-dodecane; for old leaves (B): (1) nonanal, (2) decanal, and (3) 2,6,10-trimethyl-dodecane.

of fresh old leaves or headspace collections of old leaves, compared with those made of young leaves (Figure 2).

In the further subtractive bioassays in the wind tunnel, 37.8% of the test females flew upwind over 120 cm from the release cage towards the source of the blend consisting of the nine synthetic GC-EAD active compounds, and 16.7% of the females arrived within 5 cm of the source (see blend A in Figure 3). Upwind flight and landing responses of *H. vitessoides* towards B, C, E, and F were not significantly different from those towards blend A. In particular, omission of (*Z*)-3-hexen-1-ol, which was the most abundant compound in the complete blend, from blend B did not cause any behaviour that was significantly different from behaviour elicited by blend A (see blend C in Fig-

ure 3). Fewer females flew upwind and approached the source after subtraction of either (*Z*)-3-hexenyl acetate, nonanal, or decanal in comparison with blend A. The differences between blends G, H, and I were not significant. Omission of 2,6,10-trimethyl-dodecane (blend J) did not give a significant reduction of the number of females responding. Female attraction to the four-component blend J was not significantly different from the nine-component blend A. We concluded that hexanal, (*Z*)-3-hexenyl acetate, nonanal, and decanal in blend J were essential compounds for attracting *H. vitessoides* females.

Field experiment

High numbers of *H. vitessoides* were present in the season, based on the results of the light traps. The traps that were

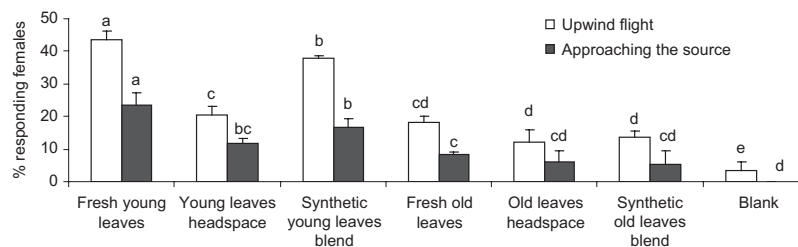


Figure 2 Attraction of mated *Heortia vitessoides* females (mean % responding females + SD; $n = 90$) to fresh plant material (young and old leaves), headspace collections, and synthetic blends from the same plant material in the wind tunnel. Synthetic blends from young and old leaves eliciting consistent antennal responses in female *H. vitessoides* were prepared according to the natural ratios of each compound to the headspace collections (Table 1). Synthetic young-leaf blends included hexanal, limonene, 2-hexanol, octanal, (*Z*)-3-hexenyl acetate, (*Z*)-3-hexen-1-ol, nonanal, decanal, and 2,6,10-trimethyl-dodecane (with a ratio of 2:16:9:4:63:100:13:10:5). Synthetic old-leaf blends consisted of nonanal, decanal, and 2,6,10-trimethyl-dodecane (ratio 11:14:26). Females were scored for upwind flights over 120 cm (white columns) and for approaching the source within 5 cm (black columns). Bars with the same colour and different letters were significantly different (Tukey's multiple range test: $P < 0.01$).

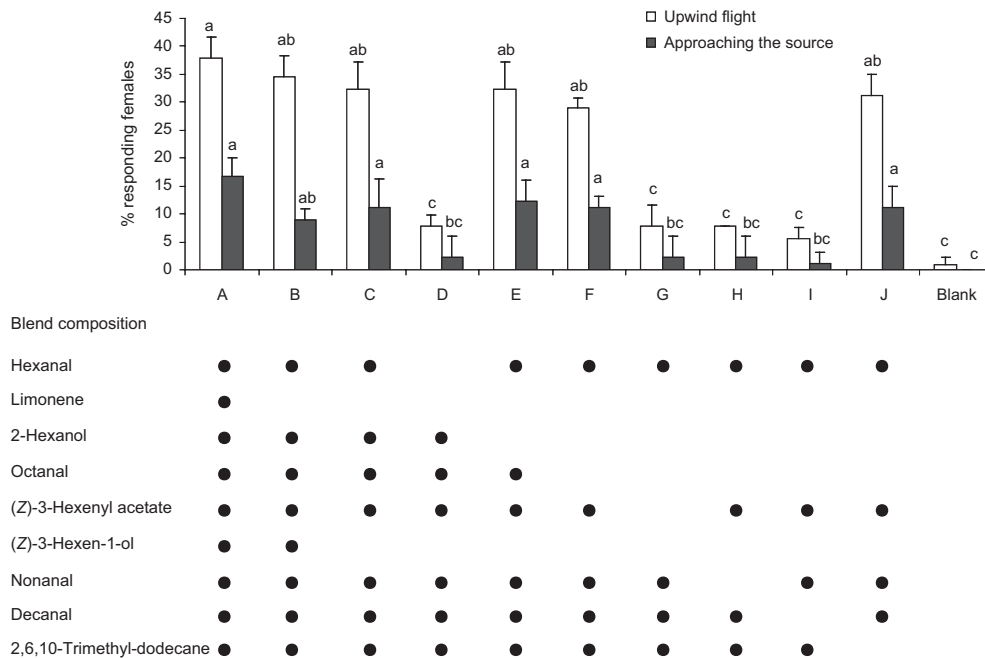


Figure 3 Behavioural response of mated *Heortia vitessoides* females (mean % responding females + SD; n = 90) to synthetic blends identified from the headspace of young *Aquilaria sinensis* leaves eliciting consistent antennal responses. Treatment A was a mixture of all nine compounds; treatments B–J were subsets. Low female upwind attraction was recorded to a blank stimulus. Females were scored for upwind flights over 120 cm (white columns) and for approaching the source within 5 cm (black columns). Bars within a colour with different letters were significantly different (Tukey's multiple range test: $P < 0.01$).

baited with the nine-component blends caught more females and males than the four-component blends, but there were no significant differences between the traps. No moths were caught in the control treatment. Overall, the number of moths that were caught was low. During the 7-day field test period, the nine-component blend trapped, on average, 3.4 females and 1.2 males per trap.

Discussion

Volatile plant secondary metabolites provide important cues for herbivorous insects to locate suitable plants as host and to avoid plants, which are unsuitable (Bruce & Pickett, 2011). Consequently, the identification of the particular types and amounts of volatiles emitted by host plants is crucial for better understanding of patterns of host plant use and for the development of a semiochemical-based control strategy. In the present study, nine EAD-active compounds identified from young *A. sinensis* leaves [i.e., hexanal, limonene, 2-hexanol, octanal, (Z)-3-hexenyl acetate, (Z)-3-hexen-1-ol, nonanal, decanal, and 2,6,10-trimethyl-dodecane, in a ratio of 2:16:9:4:63:100:13:10:5] were significantly attractive to *H. vitessoides* females in the wind tunnel assay and field test. Our study indicates that

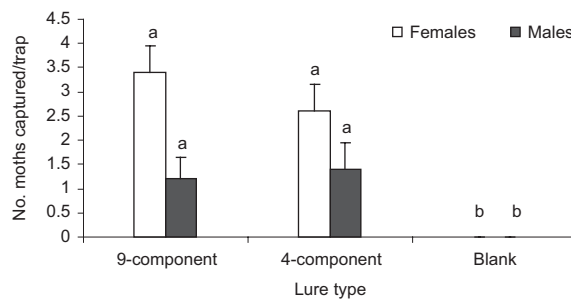


Figure 4 Mean (+ SD) total number of *Heortia vitessoides* females and males captured per white delta trap (n = 5) at the *Aquilaria sinensis* experimental base of the Chinese Medicinal Material Production Base over a 7-day period in August 2010. Each lure with green rubber septum was impregnated with a nine-component blend, a four-component blend, or a hexane control. Nine-component blend: 2-hexanol, (Z)-3-hexen-1-ol, hexanal, octanal, nonanal, decanal, (Z)-3-hexenyl acetate, limonene, and 2,6,10-trimethyl-dodecane; 4-component blend: hexanal, nonanal, decanal, and (Z)-3-hexenyl acetate. Bars within a colour with different letters were significantly different (Tukey's multiple range test: $P < 0.01$).

olfactory cues from *A. sinensis* young leaves are pronounced and important in host recognition by *H. vitessoides*.

Further subtractive bioassays conducted in the wind tunnel showed that both the complete nine-component mixture and a subtracted four-component mixture of hexanal, (*Z*)-3-hexenyl acetate, nonanal, and decanal (with a ratio of 2:63:13:10) elicited equivalent responses in females. Moth numbers captured in the field using either blend were not significantly different. Our results indicated that there was redundancy in the composition of host odour blends. Not all components were essential for recognition of a host blend, but all components in the subtracted four-component blend were essential for optimal attraction. Bruce & Pickett (2011) indicated that often certain components in the blends of host plants volatiles are more important than others and their omission from mixtures results in loss of attraction, whereas others are not essential for eliciting attraction and can be omitted. For example, Tasin et al. (2007) found that, after having identified 10 components of a blend of volatiles from grape that attracted grapevine moth, *Lobesia botrana* (Denis & Schiffermüller), a three-compound blend elicited equivalent level response to the complete 10-component blend. Similar results were reported for other species (Nojima et al., 2003; Cha et al., 2008b).

In our study, nine and three EAD-active compounds eliciting *H. vitessoides* female antennal responses were identified from young and old leaves of *A. sinensis*, respectively. These compounds were only partly overlapping. In the wind tunnel assay, the nine-component blend from young leaves was significantly more attractive to *H. vitessoides* females than the three-component blend from old leaves. Many herbivorous insects can distinguish young from old leaves of their host plants from a distance (Blüthgen & Metzner, 2007). *Heortia vitessoides* also has the physiological and behavioural capacity to detect active compounds from various stages of leaf maturation.

Why are *H. vitessoides* more attracted to young leaves than to old leaves of host plants? First, plant volatiles play an important role in host recognition. Young leaves of *A. sinensis* emitted more volatiles that elicited consistent antennal response and behavioural activity than old leaves did. In our study, 37 and 26 headspace volatile components, and nine and three EAD active compounds, were identified from young and old leaves, respectively. Four green leaf volatiles [hexanal, 2-hexanol, (*Z*)-3-hexenyl acetate, and (*Z*)-3-hexen-1-ol], three aldehydes (octanal, nonanal, and decanal), a terpenoid (limonene), and a hydrocarbon (2,6,10-trimethyl-dodecane) were essential substances in young leaves that attracted *H. vitessoides* females. However, most of these substances, especially the green leaf volatiles, were completely absent in old leaves. Second, non-volatile substances are also crucial for host recognition. It is generally assumed that young foliage is

chosen because it provides tender food for freshly hatched, delicate young larvae (Bergström et al., 1994). Young leaves are easier to digest and contain higher nutrient quality and water concentrations than old leaves (Strauss & Zangerl, 2002; Lambdon & Hassall, 2005; Blüthgen & Metzner, 2007). Third, the defensive systems that are present in young and mature leaf tissues are an important factor in host recognition. Many reports suggest that there are two basic chemical plant-defensive systems, associated with leaf age (Cates, 1980). Mature leaves often contain high concentrations of quantitative defenses. Some digestibility-reducing substances, such as tannins, resins, and silica, are often found in mature plant tissue. In contrast, young leaves often contain higher concentrations of qualitative defenses, such as alkaloids, mustard oils, pyrethrins, and other toxic chemicals, than old leaves (Cates, 1980). Because of the limited number of host plant species that make up the diet of monophagous-oligophagous herbivores, they are more likely to be adapted to the qualitative defenses and toxins that exist in one or a few closely related plant species. Monophagous-oligophagous herbivores have evolved stronger detoxification systems. In China, *H. vitessoides* should be classified as a typical oligophagous insect. *Heortia vitessoides* were more attracted to young than to old leaves of their host plant, suggesting that the adults select young leaves of *A. sinensis* for oviposition to improve nutritional conditions for their offspring.

The minor components in volatile host plant blends have been shown to be essential in attracting insects to their host plants (Pinero & Dorn, 2007; D'Alessandro et al., 2009). It has been reported that minor constituents in a blend might interact synergistically with major components to affect fruit moth attraction (Pinero et al., 2008). In our study, hexanal was a minor constituent of young *A. sinensis* leaves, but it played a key role in behavioural discrimination. The removal of the minute amounts of hexanal resulted in a significant decrease in the number of *H. vitessoides* females responding (blend D in Figure 3). Although minor constituents of blends are often overlooked, they may be determinant for successful recognition and behavioural discrimination of suitable host plants by herbivorous insects (Pinero et al., 2008).

There was no apparent correlation between the magnitude of antennal response to each component in the GC-EAD analysis and the component's behavioural activity. Our results showed that (*Z*)-3-hexen-1-ol, 2-hexanol, and 2,6,10-trimethyl-dodecane evoked particularly strong EAG responses (Table 2), but the females of *H. vitessoides* were not obviously attracted to these three compounds in wind tunnel bioassays. A higher level of attraction was obtained when any one compound was omitted (see blends C, E, and J in the subtraction experiments;

Figure 3). In contrast, hexanal, which elicited weak EAG responses, evoked high behavioural activity in the wind tunnel (see blend D in Figure 3). The difference between antennal response and behavioural activity was also shown in other insects. For example, allyl isothiocyanate was effective in the wind tunnel, despite the lack of an antennal response to *Mamestra brassicae* L. females (Rojas, 1999). Williams et al. (2010) reported that EAG response to (S)-(-)- α -pinene was weak for both sexes of *Lygus hesperus* Knight, but a strong behavioural response to this monoterpene was observed for both male and female bugs.

Although the synthetic mixture of prominent volatiles of young leaves caught more *H. vitessoides* than the traps baited with the control, the use of synthetic blends in the field resulted in low trap catches. Several factors may be responsible. First, background odour from host and non-host volatiles interferes with host recognition. Randlkofer et al. (2010) suggested that arthropod orientation in natural environments is strongly determined by the relationship between plant volatile and plant species diversity. The low attraction of insects to the synthetic blends could be due to strong competition with the natural odours in the field. Synthetic blends may not stand out to moths from the complex and diverse background odours (Cha et al., 2008b; Knudsen et al., 2008; Anfora et al., 2009; Tasin et al., 2009).

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