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ORIGINAL ARTICLE

Electrophysiological identification of 4 macrocyclic lactones as female-specific volatiles of the agarwood tree defoliator *Heortia vitessoides* (Lepidoptera: Crambidae)

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Abstract Agarwood trees (*Aquilaria* spp.) are widely cultivated in tropical Asia for their valuable resin. The defoliator moth *Heortia vitessoides* Moore (Lepidoptera: Crambidae) is a devastating pest that significantly limits the productivity of agarwood plantations. Sex pheromones offer a sustainable and efficient approach for monitoring and controlling this pest. In this study, we report the identification of female-specific volatiles (FVs) as candidate sex pheromones produced by the female of *H. vitessoides*. The FVs were identified and extracted from the 3rd to 5th abdominal segments of the calling females. We combined gas chromatography coupled with mass spectrometry (GC-MS) and GC coupled with electroantennographic detection (GC-EAD) to analyze the components of the FVs in *H. vitessoides*. Four EAD active compounds were specific to the female abdomen, with 2 being the major components. By comparing retention times and mass spectra with synthetic racemic standards, the primary components were determined to be 4 macrocyclic lactones: 14-methyloxacyclotetradecan-2-one (A), oxacyclopentadecan-2-one (B), 16-methyloxacyclohexadecan-2-one (C), and oxacycloheptadecan-2-one (D). Further analysis using chiral standards revealed that compounds A and C possess the *S* configuration. GC-EAD tests on a chiral column demonstrated that all enantiomers of A and C elicited antennal responses in males, with stronger responses to the naturally occurring *S* enantiomers. In the choice wind tunnel assay, synthetic compounds tested individually or in blend attracted or repelled males as calling females. This study represents the first identification of macrocyclic lactones as sex pheromone candidate FVs in Lepidoptera.

Key words chiral GC-EAD; EAG gland identification; female moth specific macrolides; oxacyclopentadecan-2-one; oxacycloheptadecan-2-one; (S)-14-methyloxacyclotetradecan-2-one; (S)-16-methyloxacyclohexadecan-2-one

Introduction

Agarwood, one of the most prized and sought-after natural spices in Asia, is renowned for its rich bioactive compounds, primarily produced by *Aquilaria* tree species in response to fungal infection. All *Aquilaria* species are classified as key protected plants due to their ecological

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and economic significance (Sha *et al.*, 2018). The extensive historical use and continuously growing demand for agarwood have led to the depletion of natural resources, prompting the cultivation of *Aquilaria* trees as a prominent economic forestry activity. However, the success of these plantations has been significantly hindered by a major pest, the defoliator moth *Heortia vitessoides* Moore (Order: Lepidoptera; Family: Crambidae) (Qiao *et al.*, 2013).

The larvae of *H. vitessoides* exhibit explosive population growth and voracious feeding behavior, posing a severe threat to tree health, reducing the quality of agarwood production, and even causing mass mortality of *Aquilaria* plants (Qiao *et al.*, 2013). This pest has inflicted substantial economic losses on agarwood production across Southeast Asia (Sha *et al.*, 2018). Currently, the management of *H. vitessoides* predominantly relies on chemical pesticides (Sha *et al.*, 2018). However, the overuse of these pesticides has resulted in increased environmental pollution and the development of pesticide resistance in pest populations (Witzgall *et al.*, 2010). Alternative biological control methods, such as the application of biopesticides (Zhou *et al.*, 2016) and the use of egg parasitoids (Wang *et al.*, 2022), have been explored but remain impractical due to the labor-intensive requirements for field implementation.

Insect sex pheromones, which elicit innate behavioral responses in conspecific individuals of the opposite sex, represent a highly promising tool for biological pest control. Significant progress has been made in the study of pheromones within the order Lepidoptera (Ando *et al.*, 2004). These pheromones exhibit several advantages, including low application rates, high species specificity, and minimal risk of resistance development (Rizvi *et al.*, 2021), underscoring their considerable potential in integrated pest management (IPM). However, research on the sex pheromones of *H. vitessoides* has lagged. Although sex pheromones of related Crambidae moths have been successfully identified and applied for pest control (Ishikawa *et al.*, 1999; Takanashi *et al.*, 2000; Kawazu *et al.*, 2009; Witzgall *et al.*, 2010; Peng *et al.*, 2012; Ma *et al.*, 2017; Zhang, 2021; Qian *et al.*, 2024b), the sex pheromone of this economically significant pest remains unidentified.

The chemical structures of sex pheromones in Crambidae moths exhibit considerable diversity (Witzgall *et al.*, 2010). For example, phylogenetic relatives of *H. vitessoides* have been reported to produce distinct pheromone compounds, including (*E*)-11-tetradecenyl acetate and (*Z*)-11-tetradecenyl acetate in *Pyrausta machaeralis* (Qian *et al.*, 2024b); (*E*,*E*)-10,12-hexadecadienal and (*E*,*E*)-10,12-hexadecadien-1-ol in *Diaphania angustalis* (Ma *et al.*, 2017); (*Z*)-11-hexadecenyl acetate, (*Z*)-11-hexadecenal, and (*Z*)-11-hexadecenol in *Trichophysetis cretacea* (Peng *et al.*, 2012); (*Z*)-9-tetradecenyl acetate, (*E*)-11-tetradecenyl acetate, (*Z*)-11-tetradecenyl acetate, (*E*)-12-tetradecenyl acetate, (*Z*)-12-tetradecenyl acetate, and (*E*)-11-tetradecenol in *Ostrinia* spp. (Ishikawa *et al.*, 1999; Zhang, 2021); and (*Z*)-11-hexadecenyl acetate and (*Z*)-13-octadecenyl acetate in *Cnaphalocrocis medinalis* (Kawazu *et al.*, 2009).

Given the economic and ecological significance of *H. vitessoides*, the identification of its sex pheromone has garnered considerable attention from researchers. In related Crambidae species, sex pheromones are typically produced by glands located at the tip of the abdominal segments. Consequently, initial investigations focused on the abdominal tips of *H. vitessoides* as the presumed source of pheromone production (Qian *et al.*, 2024a). However, recent studies utilizing gas chromatography-mass spectrometry (GC-MS) and electroantennography (EAG) localization have revealed that the female-specific volatiles (FVs) as sex pheromone candidates of *H. vitessoides* are instead released from the 3rd to 5th abdominal segments (Su *et al.*, 2023). Furthermore, behavioral bioassays have demonstrated that pheromone release peaked in 4−8 h of scotophase, naturally between 10:00 pm and 2:00 am (Zhou *et al.*, 2019).

Preliminary analyses using methodologies including calling insect volatile collection and abdominal tip segment dissection and extract, chemical analysis and National Institute of Standards and Technology library searching identification have identified several candidate pheromone compounds, including (*Z*)-8-dodecen-1-ol and (*E*,*E*)-8,10-dodecadien-1-ol from volatile collections, as well as (*Z*)-8-dodecen-1-ol and (*Z*)-7-dodecenyl acetate from abdominal tip extracts (Zhang *et al.*, 2016). However, the compounds of interest are inconsistent between their 2 types of extraction methods and require further validation through GC coupled with electroantennographic detection (GC-EAD), synthetic standards, and behavioral bioassays.

Based on the identification of the 3rd to 5th abdominal segments as the source of EAG active components (Su *et al.*, 2023), this study aims to comprehensively analyze the chemical composition of *H. vitessoides* FVs as its sex pheromone candidates. To achieve this, we will employ a combination of techniques, including solid-phase microextraction (SPME), synthetic chemistry, EAG, GC with a flame ionization detector (GC-FID), GC-EAD, GC-MS, and choice wind tunnel assays.

Materials and methods

Insects

In June 2024, larvae and egg masses of *H. vitessoides* were collected from *Aquilaria* leaves in Yunnan, China (32°04′ 42″ N, 118°48′ 37″ E). The larvae were transported to the laboratory, where they were reared under controlled conditions until molting and eclosion into adults. Adult pairs were subsequently established to facilitate mating and offspring production. Egg masses on leaves were hatched in a sterile polyethylene (PE) box (14 cm × 10 cm × 6 cm). The box was humidified with wet cotton balls in a Petri dish. Fresh, tender *Aquilaria* leaves were provided as a food source for the newly hatched larvae. Larvae above the 3rd instar were transferred and reared in meshed cages (30 cm × 30 cm × 30 cm). The boxes and cages were cleaned daily by removing dead individuals and feces, then sterilized with 75% ethanol. The mature larvae were transferred to PE boxes lined with 2−3 layers of tissue paper to facilitate pupation. Pupae were sexed and placed in separate containers (14 cm × 10 cm × 6 cm) maintained in complete darkness. After adult emergence, individuals were segregated by sex and age into separate mesh cages (30 cm × 30 cm × 30 cm). Adults were provided with a 15% nectar solution for supplementary feeding. Rearing conditions were maintained at 25 ± 2° C, 70% ± 10% relative humidity, and a photo-/scotophase of L : D = 11 h : 13 h. To induce FV production, females were placed in a completely dark environment synchronized with the photoperiod cycle. Calling females were collected during the 4th to 6th h of the scotophase, corresponding to the peak period of FV attractiveness.

Dissection and female-specific volatile (FV) extraction

To confirm the glandular origin of the FVs, calling females were dissected into body parts: the head (H), thorax (T), abdomen (AB), wings (W), and a whole female body without appendages (FM). Additionally, the abdominal tip, suspected to contain the FV glands, was crushed onto filter paper strips (GE). Following the confirmation of the abdomen as the FV source, the abdomens were further segmented into 3 parts: the forepart (AF), comprising the 1st and 2nd abdominal segments; the middle part (AM), consisting of the 3rd to 5th abdominal segments; and the hind part (AH), which included the terminal segments housing the putative common gland. All dissected samples were subjected to male EAG bioassay.

To identify specific compounds, headspace volatiles from EAG active segments of 4 female and 4 male abdomens, dissected in the same way, were extracted using headspace SPME (HS-SPME). Each extraction was performed in triplicate. For chiral identification, FVs from the AM segments of 10 calling females were collected using SPME and replicated 6 times. A 65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) blue fiber (Supelco, CA) was employed for HS-SPME. Dissected samples were sealed in a clean 4 mL vial, and headspace volatiles were extracted for 1 h in a 45 °C metal bath, which enabled a satisfactory recovery rate of compounds without generating contaminants.

For FV extraction, the AM segments of calling females were dissected and placed into a clean 2 mL amber glass vial, pre-rinsed with bi-distilled hexane. The dissections were immersed in 100 μL of bi-distilled hexane for 30 min at 24 °C. The solvent was then transferred using a cleaned glass pipette to a second vial containing 20 mg of anhydrous $MgSO_4$ for drying. Subsequently, the extract was transferred to a 3rd clean vial. The AM residue and $MgSO_4$ layer were rinsed twice with 100 μL of hexane, and the rinses were combined with the initial extract. The combined extract was concentrated under a gentle stream of dried and filtered nitrogen to a final volume of 100 μL, then further concentrated to 20 μL in a cleaned 150 μL micro-insert for chemical analysis. For GC-MS and GC-EAD identification, 3 replicates were performed, each involving 30 dissected females. For quantification, 2 additional samples were prepared, consisting of 10 and 23 females, respectively. Other dissections were processed similarly for comparative analysis. The crude extracts were transferred to sample vials containing clean liners and stored at 4 °C until further analysis.

Chemical standards

Commercially available oxacycloheptadecan-2-one (Chemical Abstracts Service number109-29-5, 97% purity by GC, Meryer) was purchased for use in the study. Other FV compounds were synthesized. The synthesis of 14-methyloxacyclotetradecan-2-one commenced with 11-bromoundecanoic acid as the starting material. Methyl esterification of the acid yielded methyl 11-bromoundecanoate, which was subsequently condensed with ethyl acetoacetate in the presence of sodium ethoxide under nitrogen protection. The condensation products were hydrolyzed and decarboxylated to produce 13-oxo-tetradecanoic acid. Reduction of the keto acid with sodium borohydride ($NaBH_4$) yielded 13-hydroxytetradecanoic acid. Lactonization was

then performed using 2,2′-dipyridyl disulfide and triphenylphosphine in highly diluted methylbenzene, resulting in a racemic mixture of 14-methyloxacyclotetradecan-2-one (99.5% purity by GC). For the synthesis of oxacyclopentadecan-2-one, 14-hydroxytetradecanoic acid was used as the starting material. The final lactonization step, identical to that described for 14-methyloxacyclotetradecan-2-one, yielded high-purity oxacyclopentadecan-2-one (99.5% purity by GC). The synthesis of 16-methyloxacyclohexadecan-2-one began with 15-hydroxypentadecanoic acid. Methyl esterification produced methyl 15-hydroxypentadecanoate, which was subsequently oxidized with pyridinium chlorochromate (PCC) to yield methyl 15-oxo-pentadecanoate. A Grignard reaction was then employed to produce methyl 15-hydroxyhexadecanoate. Hydrolysis of the ester with sodium hydroxide (NaOH) was followed by lactonization, yielding a high-purity racemic mixture of 16-methyloxacyclohexadecan-2-one (99.8% purity by GC). To prepare (*S*)-enantiomer-enriched chiral standards of compounds A and C, the corresponding keto acids were reduced using a borane-tetrahydrofuran (BH3/THF) solution catalyzed by the (S)-CBS reagent. Although pure chiral standards were not obtained, (*S*)-enantiomer-enriched hydroxy acids were successfully synthesized. These chiral hydroxy acids were lactonized following the same procedure as described above, yielding chiral macrocyclic lactones with an enantiomeric excess (ee) of approximately 10%. All synthetic compounds were purified using silica gel chromatography to ensure high chemical purity.

Instrumental analysis

Solvent extracts and HS-SPME samples were analyzed using GC-FID. An HP-8890 GC system was used (Agilent, USA). Samples were injected in splitless mode at 250 °C. Two different capillary columns were employed: an HP-5 column (30 m × 320 μm × 0.25 μm, Agilent) and a chiral CYCLOSIL-B column (30 m × 250 μm × 0.25 μm, Agilent). Nitrogen was used as the carrier gas at a flow rate of 37 cm/s. For solvent samples analyzed on the HP-5 column, the oven temperature program was initiated at 50 °C for 2 min, then 10 °C/min to 280 °C, held for 20 min. For HS-SPME samples analyzed on the CYCLOSIL-B column, the oven temperature was initially held at 40 °C for 4 min, then 8 °C/min to 220 °C held for 10 min. Solvent extracts were further analyzed by GC-MS using an HP 7890A-5975C system (Agilent) equipped with an HP-5ms capillary column (30 m × 250 × 0.25 μm, Agilent). The injector was operated in pulsed splitless mode at 250 °C. A volume of 2.0 μL of AM extract, equivalent to one female, was injected for each analysis. Helium was used as the carrier gas at 37 cm/s. The oven temperature program was set as follows: initial hold at 50 °C for 4 min, then 10 °C/min to 280 °C, maintained for 10 min. The quadrupole mass spectrometer was operated with a 70 eV electron ionization (EI) source at 230 °C. The mass range scanned was *m*/*z* 28.5 to *m*/*z* 300 at a rate of 2 × 4 scans/s, with a detection abundance threshold set to 10. Total ion chromatograms (TIC) and mass spectra were analyzed using Chemstation software (Agilent Technologies) and the AMDIS software with NIST17 MS library. Linear retention indices (LRI) were calculated based on the retention times of the C21 increased C8–C40 *n*-alkanes series analyzed under the same GC and GC-MS conditions. Quantification of compounds in the extracts was performed using calibration curves constructed from pure synthetic standards with quantities matching the peak areas observed in the samples.

Electrophysiological analysis

Two-day-old male insects, conditioned in darkness for 4 h, were used for EAG and GC-EAD experiments. Under a stereomicroscope, the head of each male was excised using a scalpel blade. The mouthparts and connecting membranes between the head and thorax were carefully trimmed, and the distal end flagella of both antennae were cut using iris scissors. Two of the male antennae were tested in pairs, with each pair recorded using 2 separate probes. Glass electrodes filled with Ringer's solution containing 130 mmol/L NaCl, 6 mmol/L KCl, 4 mmol/L $MgCl_2$, and 5 mmol/L $CaCl_2$ were used. Platinum wires (30 mm, 0.4 mm inner diameter) linked the saline to the input terminals of the probes and the grounding reference. The head capsule was connected to the reference electrode, while the 2 antennae were connected to the glass electrodes of the probes. Within the EAG probes, the antennal potential was amplified using an ADA4530-1 operational amplifier (National Instruments, USA), characterized by an ultra-low bias current of 1.0 fA and a high input impedance of 10^{14} Ω. The probes were powered with interference-free batteries. The DC signals of 2 probes, filtered between 0.1 and 20 Hz, were recorded using 2 HP 34465A digital multimeters (Keysight, USA) separately for complete channel separation (120 dB). Odor stimuli were delivered either as pulsed odor (2 s) from samples deposited in pipettes for EAG or as a continuous flow from the GC column

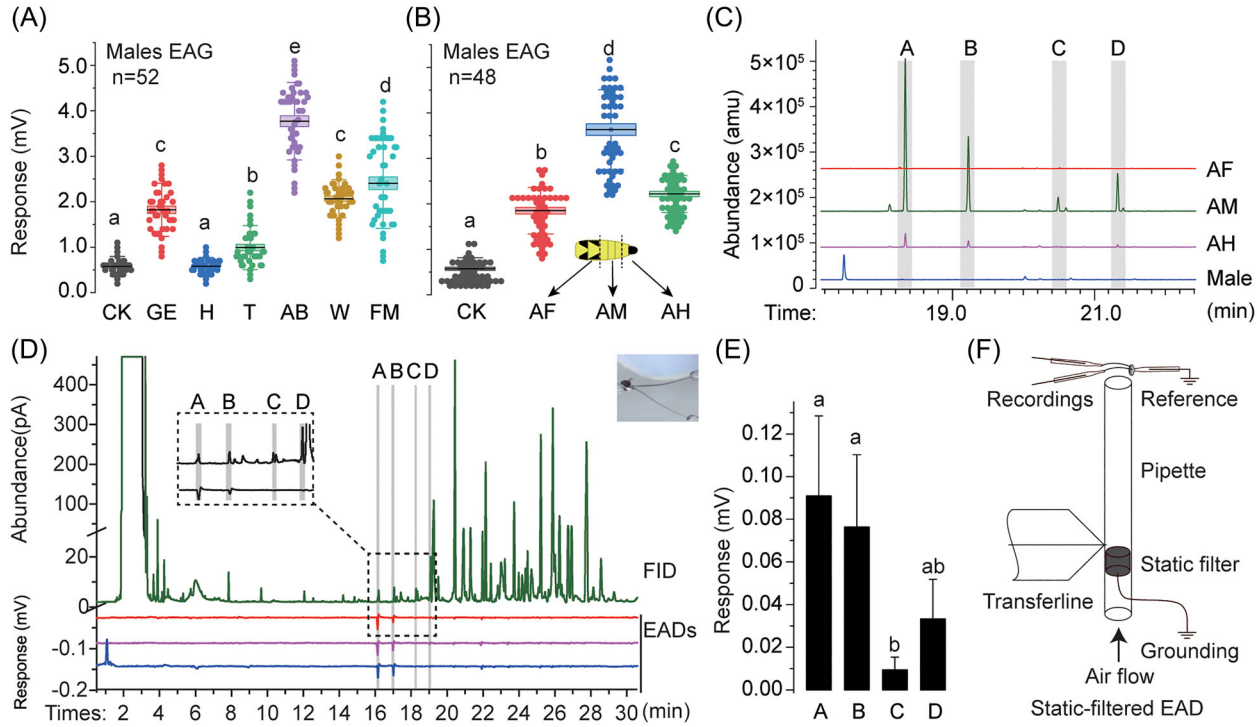

Fig. 1 EAG and GC-EAD identification of FV source and compounds. (A) Male EAG responses to calling female whole body dissection. H is short for head, T for thorax, AB for abdomen, W for wings, and FM for the whole female body without appendixes, GE for abdominal tip with supposed sex pheromone glands smashed on a filter paper strip. (B) Male EAG responses to calling female abdominal dissections. The middle abdominal segments induced the highest EAG response. A diagram of segment positions is shown. AF is short for abdominal fore parts with 1−3 segments, AM for abdominal middle parts with 3−5 segments, and AH is for abdominal hind parts. (C) Comparison of the TICs of the headspace solid-phase microextraction sample from female abdominal dissections and male abdomens. (D) Male GC-EAD response to AM solvent extracts. (E) Comparison of male response to reproducible EAD active peaks. Peaks A and B induced the highest responses. Columns marked without the same small letters were significantly different ($P < 0.05$). GC and EAD peaks marked in the same shaded box were the same in retention times. (F) Partial diagram for the static filtered EAD setup. The photos show the antennal preparation. AM, middle abdominal segments; EAG, electroantennography; FV, female-specific volatile; GC-EAD, gas chromatography coupled with electroantennographic detection; GE, filter paper strips; TIC, total ion chromatograms.

for GC-EAD. The odor stream was diluted into a clean, moist, and static-free airflow (40 cm/s, room temperature, relative humidity > 95%) passing through a polytetrafluoroethylene odor pipette (15 cm, 1.5 cm inner diameter). Prior to the odor pipette, the carrier airflow was directed through a grounded porous copper filter (1.0 cm, 8 mm inner diameter) to eliminate static electricity generated by systematic flow turbulence (Fig. 1F). This step was critical to ensure signal stability, as static interference could significantly compromise the quality of electrophysiological recordings. The antennal preparations were positioned 2.0 mm from the outlet of the odor pipette.

To identify the FV source, dissected abdominal segments of calling females were placed in the odor pipette for EAG tests. One female equivalent dissection was used as the odor source for each test, and the odor source was discarded after a single use. To minimize inter-individual variability, a one-way repeated measures design was employed, with males exposed to a series of samples in each test. Empty, clean odor pipettes served as blank controls (CK). A total of 52 and 48 males were tested in the 2 independent experiments, respectively, to localize the glandular origin of the FVs.

For GC-EAD identification of active compounds, the EAG setup was coupled to the HP 8890A GC system (Agilent). First, solvent extracts were analyzed for FID chromatograms. Then, the end of the HP-5 column was connected to the EAD odor pipette through a custom-made 40 cm heated transfer line (260 °C), allowing compounds separated by the chromatography column to be directly delivered to the EAD system. EAD and FID signals were aligned in data processing. GC-EAD

tests using female extracts on the HP-5 column were repeated 11 times, while tests using racemic standards of compounds A and C on the CYCLOSIL-B column were repeated 6 times.

Choice wind tunnel assay

A choice wind cuboid tunnel (104 cm × 60 cm × 53 cm) (Fig. 4A) was used to test the attraction of FVs. The apparatus consisted of a main box made of aluminum foil-covered cardboard, with both ends covered by cotton net (2 mm pore size). The intake end of the tunnel was separated in the middle by the same cardboard. Air flow at 20 cm/s was drawn by a fan at the outlet end. Activities of moths were recorded from the outlet with an infrared camera under infrared LED light illumination. Rubber septa containing 10 equivalents of synthetic racemic A (50.8 ng) and racemic C (56.4 ng), B (26.2 ng), and D (58.5 ng), separately or combined in one blend as 5 samples, were tested. In the synthetic blend, the ratio of racemic compounds was 2 times higher than the *S* configuration-only compounds in the natural ratio based on the quantification and chiral identification results. A sample with 3 caged calling females was tested for positive comparison. For blank contrast, the blank control was hexane-treated septa. After evaporation of the solvent, the dispensers were attached to the entrance mesh on either side of the selection chamber. Then, 20 2-d-old males, conditioned in darkness for 4 h, were released for selection in each test. After each trial, the positions of the septa were swapped, the mesh was washed with deionized water, and the system was ventilated for at least 8 h to eliminate residual odors. The number of males selected in each sample was recorded. The assays for each sample were replicated 3 times. Although the variation of replicates was not significant, the data for each sample were pooled together for analysis.

A choice wind cuboid tunnel (104 cm × 60 cm × 53 cm) (Fig. 4A) was employed to evaluate the attraction of FVs. The apparatus comprised a main chamber constructed from aluminum foil-covered cardboard, with both ends enclosed by cotton mesh. One-third of the intake end of the tunnel was partitioned into 2 sections using the same material. Airflow at a velocity of 2 cm/s was generated by a drawing fan positioned at the outlet end. Moth behavior was recorded using an infrared charge-coupled device camera and PC under infrared LED illumination at the outlet.

Five test samples were prepared: rubber septa loaded with synthetic racemic A (50.8 ng), B (26.2 ng), racemic C (56.4 ng), and D (58.5 ng), either individually or combined into a single blend. The synthetic blend was formulated with racemic compounds at twice the amount of the (*S*)-enantiomers, based on the natural ratio determined through quantification and chiral identification. A positive control sample was prepared using 3 caged calling females. For the blank control, rubber septa treated with hexane were used. After solvent evaporation, the dispensers were affixed to the centers of the entrance mesh on either side of the selection chamber.

In each trial, 20 2-d-old male moths, conditioned in darkness for 4 h, were released into the tunnel for selection. After each trial, the positions of the septa were alternated, the mesh was rinsed with deionized water, and the system was ventilated for at least 8 h to remove residual odors. The number of males attracted to each sample was recorded. Each sample was tested in triplicate. As the variation among replicates was not statistically significant, the data for each sample were pooled for analysis.

Statistics

For EAG and EAD response data, dissections were analyzed with one-way repeated measure analysis of variance (one-way analysis of variance, ANOVA). Means were compared with Turkey's Honestly Significant Difference methods. The ratio of compounds and selection data in bioassays was analyzed using Chi-square tests. Male selection ratios in bioassay were analyzed with generalized linear models with binary probit as the link function. The estimated marginal means of the ratio were compared with the sequential Bonferroni correction methods. Statistical software, including SPSS (IBM, US) and the R platform, were used for data analysis and plotting.

Results

Female middle abdominal segments produced 4 EAD active compounds

By using calling female whole body dissections as odor sources, the EAG tests showed that the abdomen (AB) induced the highest response from male antennae (one-way ANOVA: $F_{(1, 51)} = 896.00$, $P < 0.001$) (Fig. 1A). By using dissected abdominal segments as middle abdominal segments (AM), we produced the major EAG active odor to males (one-way ANOVA: $F_{(1, 47)} = 1621.63$, $P < 0.001$) (Fig. 1B). When comparing the TICs of HS-SPME sample from female abdomen dissections and male abdomen dissections in vials, 4 compounds were found specific to female AM (major amount) and AF

Table 1 Male electroantennographic detection active compounds in the female abdominal extracts of *Heortia vitessoides*.

Peaks	LRI*	Chemical Abstracts Service numver	Compounds	Male antennal responses (mV, ± SE)	Quantities (ng/insect, ± standard error)
A	1504	27198-63-6	14-Methyloxacyclotetradecan-2-one	0.09 ± 0.04	2.54 ± 0.01
B	1603	3537-83-5	Oxacyclopentadecan-2-one	0.08 ± 0.03	2.62 ± 0.02
C	1703	4459-57-8	16-Methyloxacyclohexadecan-2-one	0.01 ± 0.01	2.82 ± 0.23
D	1802	109-29-5	Oxacycloheptadecan-2-one	0.03 ± 0.02	5.85 ± 0.26

*Linear retention indices.

(trace amount) segments (Fig. 1C). Thus, the FV sources were identified as the AM segments with specific compounds.

By using hexane extract of AM in GC-EAD tests, 4 compounds, labeled as A, B, C, and D, elicited reproducible responses of male antennae (Fig. 1D). The 4 compounds were secreted steadily in ratio (A: B: C: D = 1: 1: 1.1: 2, $n = 3$ with 10 females in each sample). Although compound D is the major compound with the most quantities secreted by females, the minor compounds A and B induced major EAD responses (1-way ANOVA: $F_{(1, 10)} = 5.03$, $P = 0.05$) (Fig. 1E, Table 1).

Retention times and mass spectra of active compounds were identical to synthetic standards

In comparison, these 4 compounds showed identical retention indices (Table 1), retention times (Fig. 2A), and EI-mass spectra (Fig. 2B) with synthetic standards, respectively. On HP-5ms column based GC-MS and HP-5 based GC-FID comparison, the retention indices and the EI-mass spectrum of compound A (LRI 1504) were identical to those of 14-methyloxacyclotetradecan-2-one (LRI 1504), those of B (LRI 1603) were identical to those of oxacyclopentadecan-2-one (LRI 1603), those of C (LRI 1703) were identical to 6-methyloxacyclohexadecan-2-one (1703), and those of D (LRI 1802) were identical to those of oxacycloheptadecan-2-one (LRI 1802) (Fig. 2A,B). Traces of differences in EI-mass spectra between extracted compounds and synthetic standards were caused by the dose-response effect of EI and some trace instrumental pollution.

Configuration of A and C in females was (S) confirmed by chiral GC and GC-EAD

The results indicated compounds A (24.34 min) and C (26.74 min) in HS-SPME extracts of female AM segments showed the same retention times as synthetic (*S*)-14-methyloxacclotetradecan-2-one (Fig. 3A) and (S)-16-methyloxacyclohexadecan-2-one (Fig. 3B), respectively. No (*R*) enantiomers of compounds A and C were observed in female extracts. The chiral GC-EAD tests with synthetic racemic standards showed that males exhibited reproducible antennal responses to all the enantiomers of compounds A and C (Fig. 3C,D), suggesting that while the (*R*)-enantiomers of compounds A and C were not produced by female *H. vitessoides*, they were perceived by the male antennae. For both compounds, the antennal response to the natural (*S*)-enantiomer was higher than that to the (*R*)-enantiomer. The (*S*)-14-methyloxacclotetradecan-2-one induced higher EAD response than (*R*)-14-methyloxacclotetradecan-2-one (1-way ANOVA: $F_{(1, 5)} = 63.15$, $P < 0.001$), and (*S*)-16-methyloxacyclohexadecan-2-one induced higher response than (*R*)-14-methyloxacclotetradecan-2-one (1-way ANOVA: $F_{(1, 5)} = 11.77$, $P < 0.05$).

Synthetic FVs attracted males in the wind tunnel assay

Among the 60 males tested, 43 insects showed selection landing on the end mesh where the samples were positioned; other individuals were retained in front of the flying chamber. In contrast to blank solvent controls, males significantly selected the septa containing racemic A ($\chi^2 = 6.11$, $df = 1$, $P = 0.013$), racemic C ($\chi^2 = 4.48$, $df = 1$, $P = 0.034$) and blends ($\chi^2 = 5.23$, $df = 1$, $P = 0.022$), as well as 3 calling females ($\chi^2 = 13.44$, $df = 1$, $P < 0.001$). However, in selection among solvent blanks, compound B showed repellence to males ($\chi^2 = 7.71$, $df = 1$, $P = 0.005$), and no preference to compound D was observed ($\chi^2 = 0.36$, $df = 1$, $P = 0.549$) (Fig. 4B). In comparison of the ratio of males selecting FVs, there was a significant effect from FV sample types ($G^2{}_5 = 28.14$, $P < 0.001$). However,

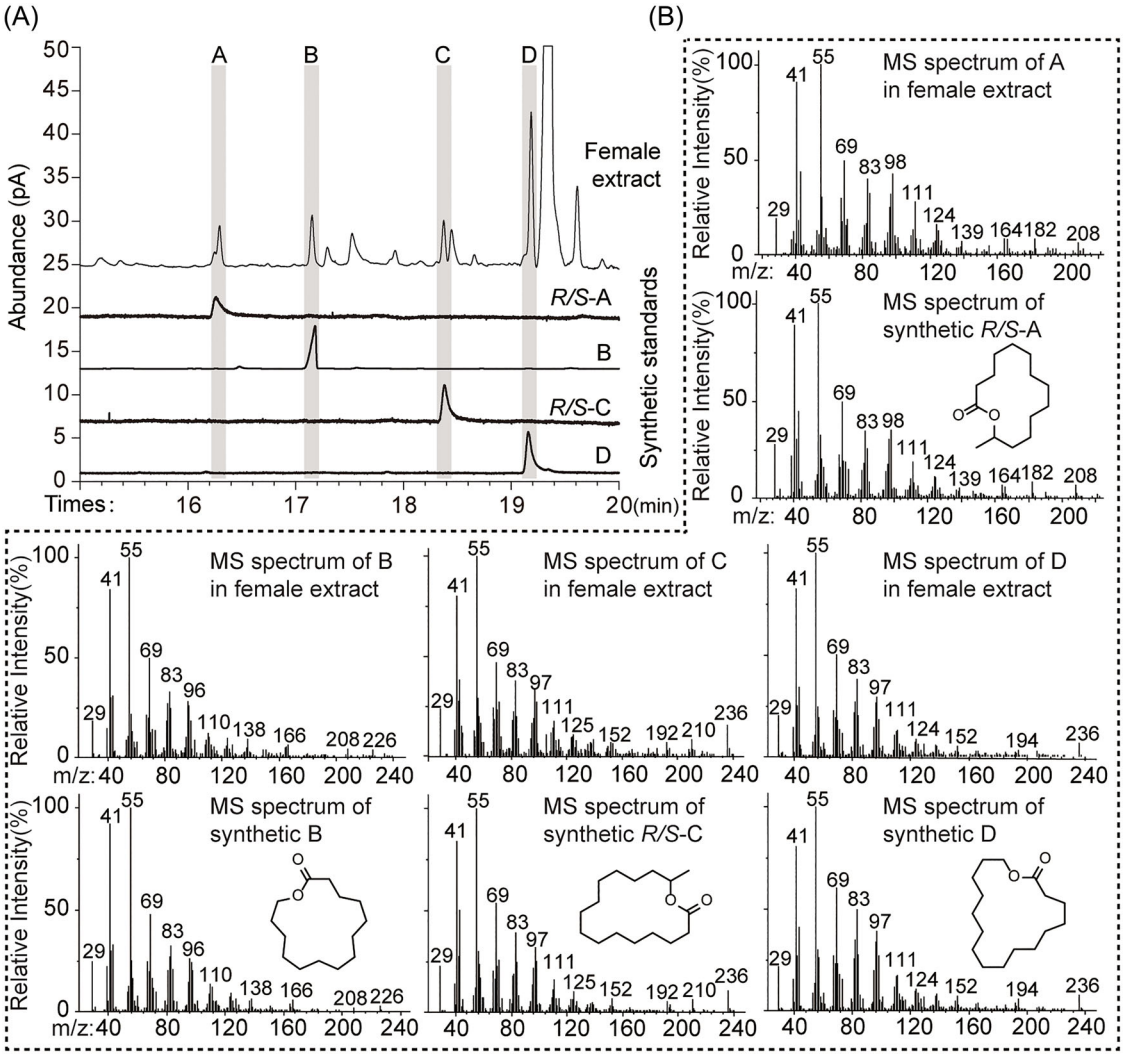

Fig. 2 Comparison of retention times and mass spectra of EAD active female gland compounds. (A) Retention time comparison between peaks in extract and synthetic standards on the HP-5 column in GC-FID analysis. GC peaks marked in the same shaded box were the same in retention times. (B) Electron ionization-mass spectra comparison between compounds and synthetic extracts in GC-MS analysis. EAD, electroantennographic detection; GC, gas chromatography; GC-FID, gas chromatography with a flame ionization detector; GC-MS, gas chromatography coupled with mass spectrometry.

the samples showing attraction were not significantly different ($P > 0.05$) (Fig. 4C).

Discussion

Conclusion of this FV identification

In this study, we identified 4 macrolide compounds as the FVs of *H. vitessoides*: (*S*)-14-methylox acyclotetradecan-2-one (A), oxacyclopentadecan-2-one (B), (*S*)-16-methyloxacyclohexadecan-2-one (C), and oxacycloheptadecan-2-one (D), which were isolated from the AM of calling females as sex pheromone candidates. The identification was supported by multiple lines of experimental evidence. First, EAG analysis of female dissections confirmed the AM as the source of pheromone production. And in moth species without defensive volatiles, the EAG method is applicable for pheromone glandular source localization, for example, in *Conogethes punctiferalis* (Stanley *et al.*, 2018). Second, thermal desorption GC-MS analysis of HS-SPME samples revealed 4 specific compounds unique to the female AM segments. Third, GC-EAD analysis of solvent dissections

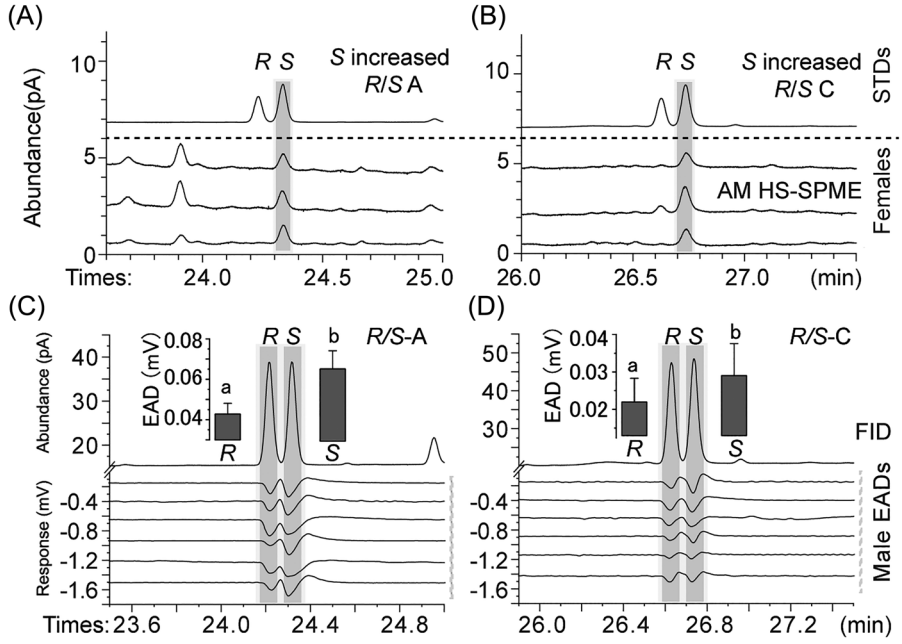

Fig. 3 Chiral GC and GC-EAD analysis for compounds A and C. (A, B) Comparison of retention times between synthetic chiral (*S* configurations increased chiral standards) A (A) and C (B) and corresponding A and C peaks from headspace solid-phase microextraction extracts of dissected female AM. (C, D) Comparison of male EAD responses to the 2 enantiomers of synthetic racemic standards A (C) and C (D). GC and EAD peaks marked in the same shaded box were the same in retention times. AM, middle abdominal segments; EAD, electroantennographic detection; GC, gas chromatography; GC-EAD, gas chromatography coupled with electroantennographic detection.

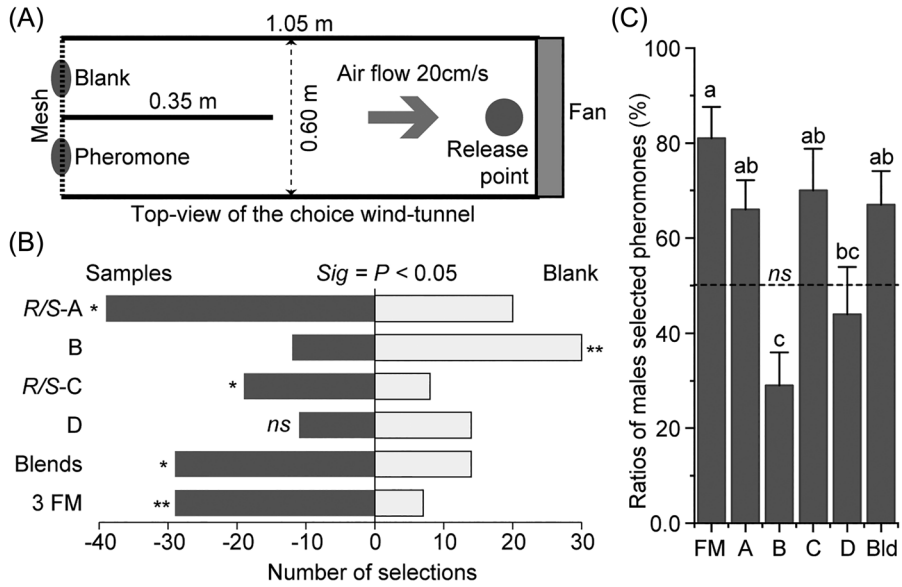

Fig. 4 Selection of male *Heortia vitessoides* to blend of synthetic standards. (A) The setup of the choice wind tunnel assay. (B) The selection of males for the hexane blank dispenser and blend dispenser. The asterisk shows a significant difference in the selection compared to 1 : 1 ratio of expected selection (ns, not significant, $*P < 0.05$, $**P < 0.01$). (C) Comparison of the ratios of male selections. Columns marked without the same letter are significantly different. The dotted line marked with "ns" showed the separation of attraction (>50%) and repellence (<50%).

demonstrated the bioactivity of these compounds. Fourth, LRI and mass spectral comparisons with synthetic standards confirmed their chemical structures. Finally, chiral GC and chiral GC-EAD analyses further validated the stereochemistry and bioactivity of the natural compounds (Mori, 2007). These findings provide a foundation for developing pheromone-based trapping and mating disruption strategies for *H. vitessoides*. Although we were unable to synthesize pure (*S*)-enantiomers of compounds A and C, bioassays using racemic standards did not reveal complete repellence from the unnatural (*R*)-enantiomers. Future preparation of high-purity chiral compounds will be essential to elucidate the role of chirality in bioactivity through field assays. In our choice wind tunnel bioassay, a significant number of males entered the blank control chamber. This behavior may be attributed to the limited space and linear design of the chamber, which facilitated the entry of males flying upwind in an excited state.

Notably, the repellent effect of compound B can be explained by its widespread presence in the environment (Lopes *et al.*, 2004), for example, in fungi (Huang *et al.*, 2012) and plants (Lopes *et al.*, 2004), which likely disrupts species-specific recognition. As single compounds do not encode complete species information, they may induce avoidance behavior to ensure accurate conspecific recognition. Since the choice tunnel assay only tested responses in a short distance, we cannot exclude the possibility that they may function in long-distance attraction, especially compound B, which elicited many upwind flights. For compound D, it is less volatile, but it is present in a high amount. It can be hypothesized that its synergistic effect with host agarwood plants may be responsible for short distance communication. All these behavioral ecology works are complex and deserve more emphasis in future studies for these specialist insects.

Based on the importance of chirality in pheromone bioactivity (Mori, 2007), Compound A, identified as the major EAD active pheromone component with (*S*)-configuration, is likely critical for species-specific mate finding. The importance of chirality in pheromone bioactivity (Mori, 2007) underscores the need for further studies using pure chiral compounds to fully understand the mating behavior ecology of *H. vitessoides*.

Macrolide compounds in chemical communication

Macrolides in non-insect organisms have been widely studied for their antibiotic properties (Xu *et al.*, 2012). Macrolides with low molecular weight, small numbers of ring members, and low polarity are usually volatile, sometimes with musky odor, and can be used as pheromones for chemical communication (Schulz & Hötling, 2015). Related to our research, oxacyclopentadecan-2-one was found in the mushroom *Dictyophora echinovolvata* (Huang *et al.*, 2012), 16-methyloxacyclohexadecan-2-one was detected in the mammal *Hystrix cristata* as a possible sex pheromone (Massolo *et al.*, 2009), and oxacycloheptadecan-2-one was found in the orchid as a musky odor (Hirose *et al.*, 1999). In insects, volatile macrolide compounds have been identified as pheromones. For example, the aggregation pheromones of grain beetles *Cryptolestes* spp contain (3*Z*)-dodecen-12-olide (Hötling *et al.*, 2014). The anti-sex pheromone in the butterfly *Heliconius erato phyllis* is (2*R*,6*E*,10*R*)-2,6-dimethyl-6-undecen-10-olide (Melo *et al.*, 2022). The sex pheromone of halictine bee *Lasioglossum zephyrum* contains macrocyclic lactone (Smith *et al.*, 1985). The queen pheromone of the sweat bee, *L. malachurum*, contains macrolides (Steitz & Ayasse, 2020). However, prior to our study, no Lepidoptera sex pheromone had been identified as a macrolide. In Crambidae moths, sex pheromones typically consist of unsaturated alcohols, acetates, aldehydes, and acids, which are biosynthesized in glands located at the abdominal tip segments. These compounds are produced through a conserved fatty acid-based pheromone biosynthesis pathway (Jurenka, 2021). In contrast, the biosynthesis of macrolides in insects remains poorly understood. In *H. vitessoides*, macrolide formation may involve lactonization following the initial steps of the common fatty acid-based pathway (Vanderwel *et al.*, 1992). The unique pheromone components and glandular origin identified in *H. vitessoides* provide hints for the phylogenetic position of the genus *Heortia* (Law *et al.*, 2022).

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Disclosure

The authors have no relevant financial or nonfinancial interests to disclose.

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