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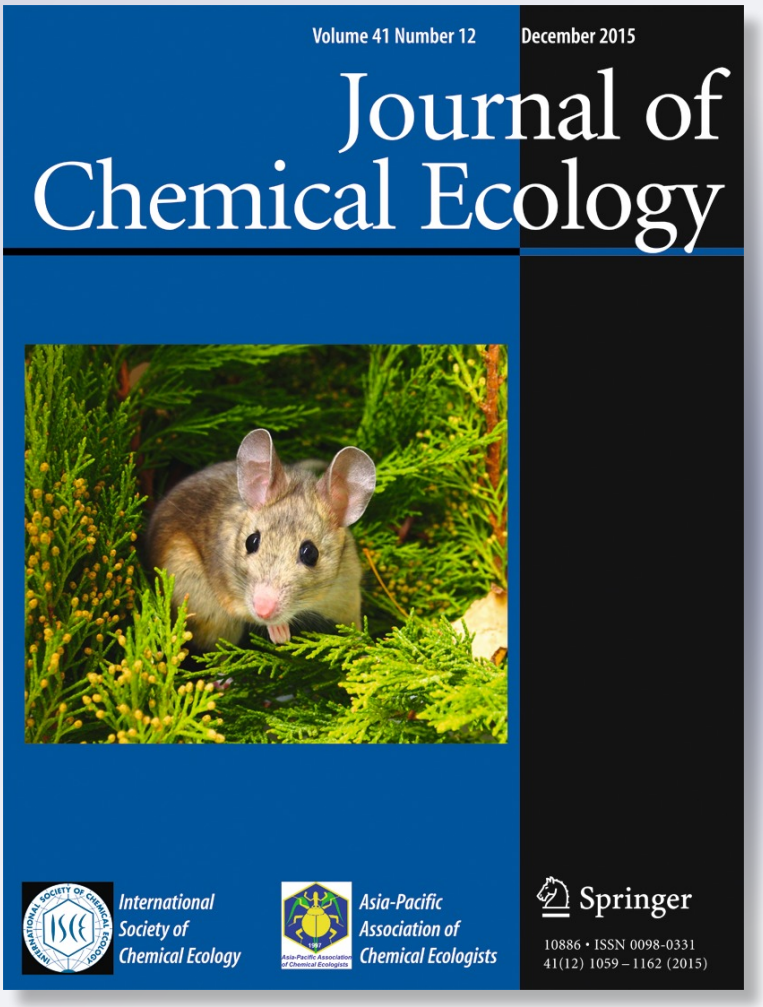

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Isolation of a Female-Emitted Sex Pheromone Component of the Fungus Gnat, *Lycoriella ingenua*, Attractive to Males

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Abstract *Lycoriella ingenua* Dufour (Diptera: Sciaridae) is acknowledged as the major pest species of the white button mushroom, *Agaricus bisporus*, throughout the world. Components of the female-produced sex pheromone of this species were identified previously as C_{15}-C_{18} *n*-alkanes, with the major component *n*-heptadecane, and shown to be attractive to *L. mali*. However, a subsequent report could not repeat this work. We reinvestigated the sex pheromone of this species by confirming that virgin females were attractive to males in a Y-tube bioassay and by collection of extracts from virgin females. Extracts were analyzed by gas chromatography coupled to electroantennographic detection, and by the less widely-used technique of gas chromatography coupled to a behavioral bioassay to detect compounds causing wing-fanning and copulatory abdomen curling in males. A single, behaviorally-active pheromone component was isolated and characterized by gas chromatography coupled to mass spectrometry. This component was definitively not *n*-heptadecane or any of the other C_{15}-C_{19} *n*-alkanes reported previously, but is proposed to be a sesquiterpene alcohol having analytical characteristics that closely matched those of reference germacradienols.

Keywords Mushroom fly · Fungus gnat · Female extract · Y-tube olfactometer · Gas chromatography/electroantennographic detection · Gas chromatography/behavioral bioassays

Introduction

Lycoriella ingenua (Dufour) (Diptera: Sciaridae) (formerly known as *L. mali* Fitch) is acknowledged as the major pest species of commercial mushrooms throughout the world causing severe damage (Erler et al. 2011; Park et al. 2006). Larvae of the flies feed on the compost, mycelium, and sporophores, and tunnel into the caps and stems of mushrooms (Lewandowski et al. 2004; Shamshad 2010). Moreover, adults of *L. ingenua* vector fungus spores of *Trichoderma aggressivum* (Samuels & W. Gams) (Hypocreales: Hypocreaceae), which cause severe epidemics of "green mold" and lead to additional losses (Shamshad 2010). Thus, control of *L. ingenua* is a necessity worldwide, and so far efforts rely primarily on applications of conventional synthetic pesticides (Cantelo 1979, 1983; Shamshad 2010; Shamshad et al. 2008). However, insecticide options are limited because they are subject to label restrictions of the number of applications per season or the total amount of active ingredient applied. Moreover, the efficacy of insecticides is inconsistent because larvae move away from the hatching site to feed inside the caps and stems of mushrooms where they are well protected. In addition, repeated applications produce undesirable side-effects, such as insecticide residues and reduced populations of natural enemies as well as insecticide resistance (Bartlett and Keil 1997; Brewer and Keil 1989). It is, therefore, essential to develop efficient monitoring methods that include damage thresholds, and alternative control strategies such as the use of sex pheromones and other semiochemicals.

Stefanos S. Andreadis and Kevin R. Cloonan contributed equally to this work.

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Sex pheromones involved in intraspecific communication are widely used for population monitoring, and for control by mating disruption, mass trapping, and attract-and-kill (Cardé and Minks, 1995; Leal 2005; Witzgall et al 2008). In the dipteran suborder Nematocera, sex pheromones have been reported to be used by flies in only three families: the Cecidomyiidae, the Psychodidae, and the Sciaridae (Wicker-Thomas 2007). In cecidomyiid species, the sex pheromones are female-produced and cause attraction of males over long distances. They consist of *n*-alkanes with an oxygenated functionality in the 2-position and additional oxygenated functionality and/or unsaturation in most cases reported so far (Hall et al. 2012). On the other hand, the sex pheromone components of sandflies (Psychodidae) are male-produced and consist of sesquiterpenes, as in *Lutzomyia longipalpis* (Lutz & Neiva) (Brazil et al. 2009; Hamilton et al. 1996) and *L. cruzi* (Mangabeira) (Brazil and Hamilton 2002).

In sciarids, only one pheromone has been identified, that of *L. ingenua* (Kostelc et al. 1980), although sex pheromones have been implicated in the mate-finding behavior of several species of *Bradysia* (Alberts et al. 1981; Frank and Dettner 2008; Li et al. 2007, 2008). However, the previous identification of the sex pheromone of *L. ingenua* by Kostelc et al. (1980) has been shown in more recent studies to be questionable (Gotoh et al. 1990). Kostelc et al. (1980) identified the major pheromone component as *n*-heptadecane with the C_{15}, C_{16}, C_{18}, and C_{19} *n*-alkanes. Gotoh et al. (1990) found that *n*-heptadecane was completely inactive; virgin male flies did not make any copulatory responses to doses ranging from 10^{-4} to 10^{-11} g of *n*-heptadecane, and this pheromone blend has never been shown to be effective for any kind of monitoring of *L. ingenua* populations in mushroom houses anywhere in the world.

In this study, we report collection of extract from unmated *L. ingenua* females, and isolation and identification of behaviorally-active fractions of these extracts for inducing courtship behavior in males. We used gas chromatography coupled to electroantennographic detection (GC/EAD) and gas chromatography coupled to a behavioral bioassay (GC/BB) to determine the elution times on two different GC columns of one active compound. Long-distance upwind flights of males in an olfactometer bioassay in response to unmated, mated, or dead unmated females were also characterized.

Methods and Materials

Insect Rearing The insects used were from a 2-yr.-old laboratory colony maintained at the University Park Campus of Penn State University. This colony was initiated in 2012 using gravid adult female flies that had been aspirated from the beds of spawned *A. bisprous* compost in Berks County (PA, USA). Flies were positively identified as *L. ingenua* by Dr. Seunggwan Shin (Department of Entomology, North Carolina State University, Raleigh, NC, USA).

We maintained a robust and healthy colony of *L. ingenua* by using unspawned compost, i.e., compost lacking *A. bisporous* mycelia (unpubl. Data). Nine plastic drinking cups (355 ml) were filled with Phase II mushroom compost with an added nitrogen supplement (100:1, compost:supplement) that were placed into mesh cages (30 × 30 × 30 cm; BioQuip, CA, USA). Approximately 50 male and 50 female *L. ingenua* flies were added to each cage, and the cages were left under colony conditions of 21 °C, 70 % RH, 12:12 L:D photoperiod regime for 2 d to allow the females to oviposit in the compost mixture in the cups. After 2 d, the cages were covered with plastic autoclave bags (30.5 × 60 cm; VWR International, Atlanta, GA, USA) to prevent the compost from drying out. Cages were left under colony conditions until adult flies emerged approximately 21 d later. This process was repeated until we had a continuously-emerging colony.

Because the pupae were difficult to segregate according to sex, in order to obtain virgin adults of both sexes we collected pupae, sorting them from compost by means of a camel-hair brush, and placed them into individual 10 ml disposable culture tubes (15 × 85 mm; VWR International, PA, USA) covered with Parafilm M (Bemis Healthcare Packaging, WI, USA). After adults emerged, they were sexed according to the larger, more pointed abdomen of the female and the more slender abdomen of the male with claspers (Lewandowski et al. 2004). Females were used for obtaining pheromone extract and males for GC/EAD and GC/BB experiments.

Olfactometer Choice Assays A Y-tube olfactometer (70 × 35 × 6 cm i.d.) was used for the behavioral assays under laboratory conditions (23 °C and natural daylight) (Fig. 1a). Charcoal-purified air was pushed through both arms of the Y-tube at a rate of 0.75 l/s via a portable air-pump system (PVAS22, Volatile Assay Systems, NY, USA). Three female *L. ingenua* were placed in a glass cylinder open at both ends (1.5 cm diam × 5.0 cm high), the ends being covered with fine cloth mesh. The vial containing the females then was positioned inside one of the arms of the Y-tube at the upwind end. Choice assays were performed using as treatments starved, live virgin females (1–2-d-old), live mated females, or dead virgin females. Dead females were tested because males had been observed occasionally being attracted to and clasping dead females during abdomen-curling courtship in the laboratory. We thus wanted to quantify this behavioral activity compared to live females in order to determine whether we could obtain sufficient amounts of pheromone from dead virgin females as well as from live females. A blank (empty glass vial) was used as a control in some assays in addition to direct comparisons between live virgin females and dead ones, as

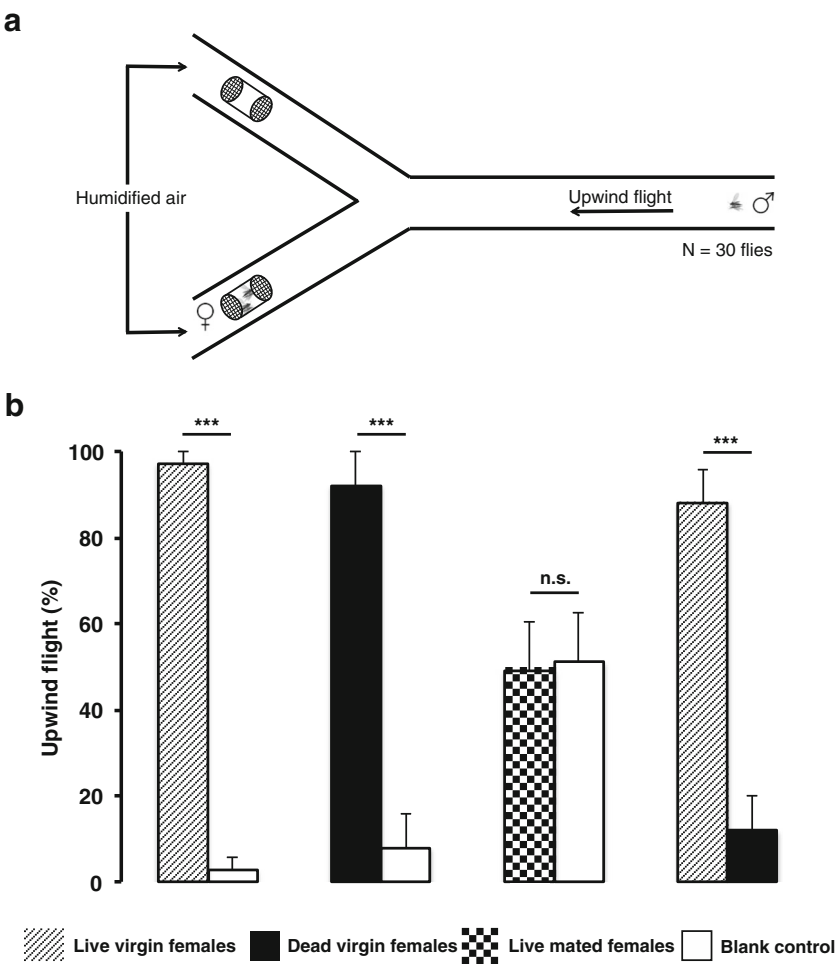

Fig. 1 **a** Schematic drawing of the Y-tube olfactometer used in these studies. **b** Mean percentages of male *Lycoriella ingenua* (± S.E.) that were attracted in the Y-tube olfactometer choice assays to (i) live virgin female flies vs. a blank control, (ii) dead virgin female flies vs. a blank control, (iii) live mated female flies vs. a blank control, and iv) live virgin female flies vs. dead virgin female flies. Asterisks denote significantly different levels of attraction (χ-square 2 × 2 test of independence; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $N = 30$)

well as between live virgin females and live mated females. All dead virgin females that were used in these experiments had died within the 24 h before assay.

Male *L. ingenua* were assayed individually for their ability to perform upwind flight and landing on or near the glass vial containing females. Typically, males flew all the way up the tube with little touching or landing on the glass walls before they landed on, or within a few centimeters downwind of, the females while walking, and with wing fanning for the last few centimeters of their approach. Males had to progress all the way up the tube to a position level with the vial containing the females in the tube in order to achieve a positive score in terms of successful "upwind flight". Each male was given 3 min to respond, and males that did not initiate upwind flight within the 3 min assay period were discarded and not used for the statistical analyses. These non-responders totaled fewer than three individuals per treatment over the entire course of the experiments, and no males went only part way up the tube. Responders were removed from their location near the females using an aspirator and were not re-used. The orientation of the arms of the Y-tube was reversed 180° after five individual flights to avoid lighting-related bias.

In total, six replicates of five individuals were performed for each treatment combination ($N = 30$). A χ^2-test was used to test whether the distribution of the total number of male flies that chose the one arm or the other one across all replicates differed from a 50:50 distribution at $\alpha = 0.05$. Beforehand, a heterogeneity χ^2 test was conducted to ensure that data from each replicate were homogenous (Zar 2009). Statistical analyses were done using SPSS Ver. 22 (IBM Corp Release 2013, Armonk, NY, USA).

Extraction of Sex Pheromone Virgin female *L. ingenua* (0–1 d post-emergence) were collected from their individual emergence vials and transferred into 2 ml clear glass vials (12 × 32 mm; Supelco, PA, USA) in groups of five to ten females in a single vial. These females were held for 24 to 48 h in an environmental chamber maintained at 21 ± 1 °C, 70 % RH, on a 12:12 L:D photoperiod regime before both the female bodies and the glass vial were washed with 50 μl distilled hexane (*n*-hexane, OmniSolv, Germany) for approximately 5 min. The supernatant was transfered into a new clear micro-test-tube that was placed inside a 1-dram vial with a Teflon-lined cap and stored at −20 °C. For GC analyses, the

extract was concentrated under a stream of nitrogen to obtain a concentration of approximately 1 female equivalent (FE) per microliter (1 FE/μl).

Gas Chromatography (GC) Analyses GC analyses were performed in splitless mode on a Hewlett-Packard 5890 Series II gas chromatograph with ChemStation software (version D.01.00 Build 75, Agilent Technologies, Wilmington, DE, USA). Analyses were performed using either a relatively non-polar EC-5 column (30 m × 0.320 mm; 1.0 μm; Agilent Technologies) or a more polar DB-225 column (30 m × 32 mm; 0.25 μm; Agilent Technologies). Extract (1–2 μl) was injected to give ca. 1 ng of pheromone component per injection for each GC run, because the concentration of pheromone component in the 1FE/μl extract varied slightly between different cohorts of females that had been used to obtain extract on different days. With the EC-5 column, the carrier gas (He) linear velocity was 45 cm/s before being equally split via a fused silica "Y GlasSeal" connector (Supelco, Bellefonte, PA, USA) 1 m before the FID and EAD sensor outlets. The injector temperature was 250 °C with split opening 1 min after injection. The initial column temperature was 100 °C and was increased to 220 °C at 10 °C/min and held for 25 min. With the DB-225 column, carrier gas linear velocity was 45 cm/s, and injector temperature was 250 °C with the split opened 1 min after injection. The initial column temperature was 80 °C and was increased to 200 °C at a rate of 15 °C/min and held for 25 min.

Kovats Retention Indices (Kovats 1965; Robards et al. 1994) of compounds were calculated relative to the retention times of *n*-alkanes (C_7 - C_{30}).

Gas Chromatography/Mass Spectrometry (GC/MS) Analyses GC/MS analyses were performed in electron impact (EI) mode using a Hewlett-Packard 6890 gas chromatograph equipped with an HP-5MS bonded phase capillary column (0.25 mm × 0.25 μm × 30 m; Agilent Technologies, Little Falls, DE, USA) or the above DB-225 column interfaced to a Hewlett-Packard 5973 mass spectrometer (Hewlett-Packard, Palo Alto, CA, USA). An injection of 20 FE of virgin female extract that had been concentrated to 2 μl of hexane was made with the inlet in splitless mode at 250 °C, a split time of 0.75 min, and helium carrier gas flow rate of 0.7 ml/min. For the HP-5MS column, the oven temperature was programmed from 100 °C at 10 °C min^{-1} to 220 °C with a 5 min hold time. For the DB-225 column, the oven temperature was programmed from 80 °C at 10 °C/min to 200 °C and held for 5 min. EI analyses used the default settings (ion source: 230 °C, quadrupole: 150 °C, and with spectra generated at 70 eV). Tentative identification of compounds was performed using the NIST 08 library as well as published retention indices and spectra of sesquiterpene alcohol standards.

Gas Chromatography/Electroantennographic Detection (GC/EAD) Analyses A portable, Quadroprobe recording system (Syntech®, The Netherlands) (Park et al. 2002) was used for simultaneous EAG recordings from eight male antennae placed in parallel (Fig. 2). The stainless-steel probes consisted of a single, common reference electrode and four independent recording electrode channels. However, only one of the four channels was used, whereby we placed four males between the reference and the recording electrodes without cutting the antennal tips (Fig. 2). A salt-free hypoallergenic electrically conductive gel (Spectra 360, Parker Laboratories Inc., USA) was used to establish electrical contact between antennae or abdomens of the flies and the electrodes (Fig. 2). The gel was essential in restraining the positions of the males' bodies and antennae with high stability between the recording and reference electrodes. The eight-antennae EAG preparation was positioned on the inside of, and in the middle of, a stainless steel air tube (0.8 cm inner diam × 14 cm long) that carried a humidified air stream (charcoal-filtered house-air; ca. 20 cm/s) over the antennae. This airstream carried the GC effluent emanating from the GC column (EC-5 or DB-225 as above) via the heated EAG transfer line (250 °C) from the GC. The EAG signals from the Quadroprobe were amplified and monitored with a four-channel portable preamplifier unit (Syntech B, The Netherlands) and digitized and stored on a PC using custom, in-house software. The stored signals were analyzed further with custom, in-house PC-based data processing software designed for four-channel EAG recording analysis (Park et al. 2002).

Gas Chromatography/Behavioral Bioassay (GC/BB) Analyses The GC/BB technique was used for behavioral assays of compounds continuously eluting from the GC, as previously described (Hummel 1984; Leal et al. 1992, 1994). GC/BB was carried out using the effluent-splitter and airstream delivery tube used for GC/EAD analyses. However,

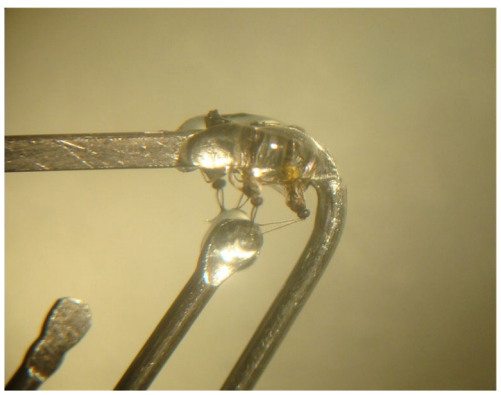

Fig. 2 Close-up image of the setup used for GC/EAD recordings. Four *Lycoriella ingenua* males' abdomens formed an electrical connection with one of the stainless steel electrodes on the Quadroprobe using electroconductive gel, and their eight antennae contacted the second, recording electrode such that the eight antennae were placed in parallel

for GC/BB analyses, the exit of the airstream delivery tube flowed into a cylindrical glass vial (1.5 cm i.d. × 5.0 cm long) open at both ends. Each vial contained three virgin starved males aged 1–2 d-old and was covered with fine mesh at both ends to contain the males. One of the meshed ends was positioned to be touching the end of the airstream delivery tube so that the GC effluent could flow over the males in the vial. For each GC/BB run, the vial containing the males was not placed in the airstream until immediately after the solvent peak had eluted. Males were used for only one GC/BB run and then discarded. GC/BB analyses were performed on the EC-5 and DB-225 columns using the same flow rates and temperature programs as were used for the GC/EAD investigations above.

Results

Olfactometer Choice Assays *Lycoriella ingenua* males exhibited significant upwind flight responses in the olfactometer assays (Fig. 1a), flying quickly upwind and landing on or near the glass vial containing the females. In most cases, males took less than 10 s to land on the glass vial and displayed characteristic courtship behavior of wing fanning and abdomen curling.

A greater number of male flies was attracted to live virgin females compared to the blank control arm ($\chi^2 = 26.13$, $df = 1$, $P < 0.001$, $N = 30$) (Fig. 1b). In response to dead virgin females, more male flies flew upwind to the dead females compared to the blank control arm ($\chi^2 = 14.72$, $df = 1$, $P < 0.001$, $N = 30$) (Fig. 1b). The percentage of males flying to the live, mated females was not different from the percentage flying into the blank control arm ($\chi^2 = 0.03$, $df = 1$, $P > 0.05$, $N = 30$) (Fig. 1b). In the comparison between live vs. dead virgin females, a greater percentage of males flew upwind towards and landed on live females compared to dead ones ($\chi^2 = 11.63$, $df = 1$, $P < 0.001$, $N = 30$) (Fig. 1b). It would appear that there is a greater amount or higher quality of pheromone emanating from live virgin females compared to that remaining in or on dead virgin females, and this result guided our decision to optimize collection of pheromone by using live virgin females only.

GC/EAD Analyses GC/EAD analyses of extracts of virgin female *L. ingenua* using the Quadroprobe electrode arrangement with eight male antennae in parallel (Fig. 2) revealed one EAG-active compound eluting from the EC-5 column at 6.94 min on more than 10 different GC/EAD runs using several different aliquots of extract obtained from different batches of virgin females (Fig. 3a). The amounts extracted from different groups of females over the many months of this study never exceeded 1 ng per FE. For instance, the amounts extracted from three different representative batches of 50 or more females in different months were 1.0 ng/FE, 0.6 ng/FE, and 0.25 ng/FE, respectively. The Retention Index of the EAD-active component on the EC-5 column was 1659 (Fig. 3b). No detectable amounts of *n*-heptadecane were observed in our extracts, and no EAD activity was observed during any GC runs at the corresponding elution time.

On the DB-225 column, there was a single GC/EAD-active compound that eluted consistently at 7.54 min (Fig. 4a) on more than 10 different GC/EAD runs from different batches of female extract. Other possible GC/EAD-active compounds were present but not consistently so (Fig. 4a). The amount of compound present in this peak from a 1FE injection was consistently ca. 1 ng or less, just as on the EC-5 column, and its Retention Index was 2065 (Fig. 4b). As with the analyses on the EC-5 column, no significant amounts of *n*-heptadecane were detected from our extract during any GC runs, nor was any EAD activity observed at the elution time of *n*-heptadecane.

GC/BB Analyses When 1 FE of female extract was injected on the EC-5 column on ten different GC/BB replicate runs, 90 % of the males (27/30) exhibited immediate wing fanning, abdomen curling, and clasper extension while trying to copulate with each other as soon as the compound at 6.93 min began eluting (Fig. 5a) (Movie S1-video file). Males were quiescent or exhibited only occasional, slow walking in the holding tube during the minutes before the compound at 6.93-min eluted. The courtship and copulatory behavior then persisted for ca. 30–45 s (Fig. 5a), and males then became quiescent again, with occasional slow walking. We had expected that there would be long-lasting behavioral excitation for tens of seconds, not only because this long-duration wing fanning is typical of male insects excited by pheromone, but also because the airflow through the bioassay cylinder will have been slowed considerably by the fine-mesh gauze coverings at both ends of the cylinder that kept the males from escaping.

On ten different GC/BB injections on the DB-225 column, the same courtship and copulatory behaviors as above were exhibited by 80 % of the males (24/30), now in response to the compound eluting at 7.54-min on this column (Fig. 5b). These wing fanning, abdomen-curling, and clasper extension behaviors did not occur before 7.54 min, and they persisted for ca. 45 s after it eluted before returning towards baseline quiescence levels (Fig. 5b).

GC/MS Analyses The EI mass spectrum of the compound eluting at the Kovats Index closest to our GC/EAD-active compound on the EC-5 column is shown in Fig. 6. It has a molecular ion of *m/z* 222, which is consistent with a sesquiterpene alcohol. The base peak is *m/z* 82 (Fig. 6) with strong signals for fragments at *m/z* 67, 93, 107, 121, 149, and 161. This fragmentation pattern is similar to published mass spectra of germacradienols (Cornwell et al. 2001; He and Cane 2004).

In GC/MS analyses using the DB-225 column, the compound eluting at the Kovats Index corresponding to that of the

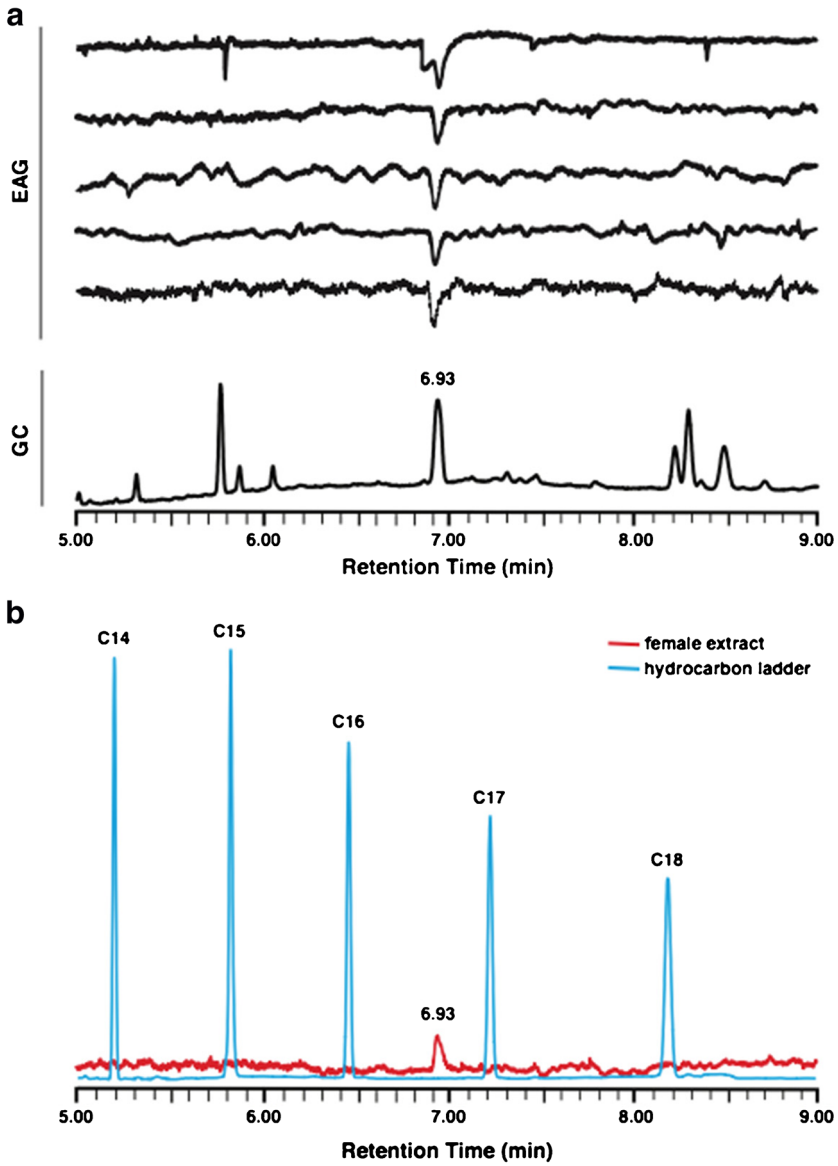

Fig. 3 **a** Five representative GC/EAD recordings (top) from male antennae in response to virgin female *Lycoriella ingenua* extract injected on the EC-5 column. Bottom trace shows a representative FID output from the extract on this column. **b** The elution time, 6.93 min, of the GC/EAD-active compound on the EC-5 column compared to elution times of *n*-alkanes (Kovats Index 1659)

component active in GC/EAD analyses exhibited a mass spectrum identical to the mass spectrum from the HP-5MS column shown in Fig. 6.

Discussion

Using GC/EAD analyses and GC/BB assays on two different types of GC column, we have isolated a behaviorally-active sex pheromone component from virgin female *L. ingenua.* The Kovats Retention Index of this compound on EC-5 is 1659 and thus its molecular weight is likely to be between those of C_{16} and C_{17} *n*-alkanes. The Kovats Index of the active compound on DB-225 is 2065 and indicates it is much more polar than a simple hydrocarbon.

This compound is clearly not the *n*-heptadecane previously stated to be the sex pheromone of this species by Kostelc et al. (1980), and furthermore we could not detect n-heptadecane in extracts of virgin female *L. ingenua* or register an EAG response from male *L. ingenua* at the corresponding retention times in GC/EAD analyses of the female extract. Gotoh et al. (1990) could not demonstrate any pheromonal activity for *n*-heptadecane in *L. ingenua*, although these authors did not investigate the pheromone further and the compounds identified by Kostelc et al. (1980) have still been referred to as components of the female sex pheromone of *L. ingenua* in subsequent reviews (e.g., Wicker-Thomas 2007).

The EI mass spectrum of the proposed pheromone component has a fragmentation pattern consistent with that of a sesquiterpene alcohol, and is an especially close match to mass spectra of germacradienols (Cornwell et al. 2001; He and Cane 2004). The mass spectrum shows a likely molecular ion at *m/z* of 222, with a base peak at *m/z* 82. Other sesquiterpene alcohols such as β–eudesmol and its

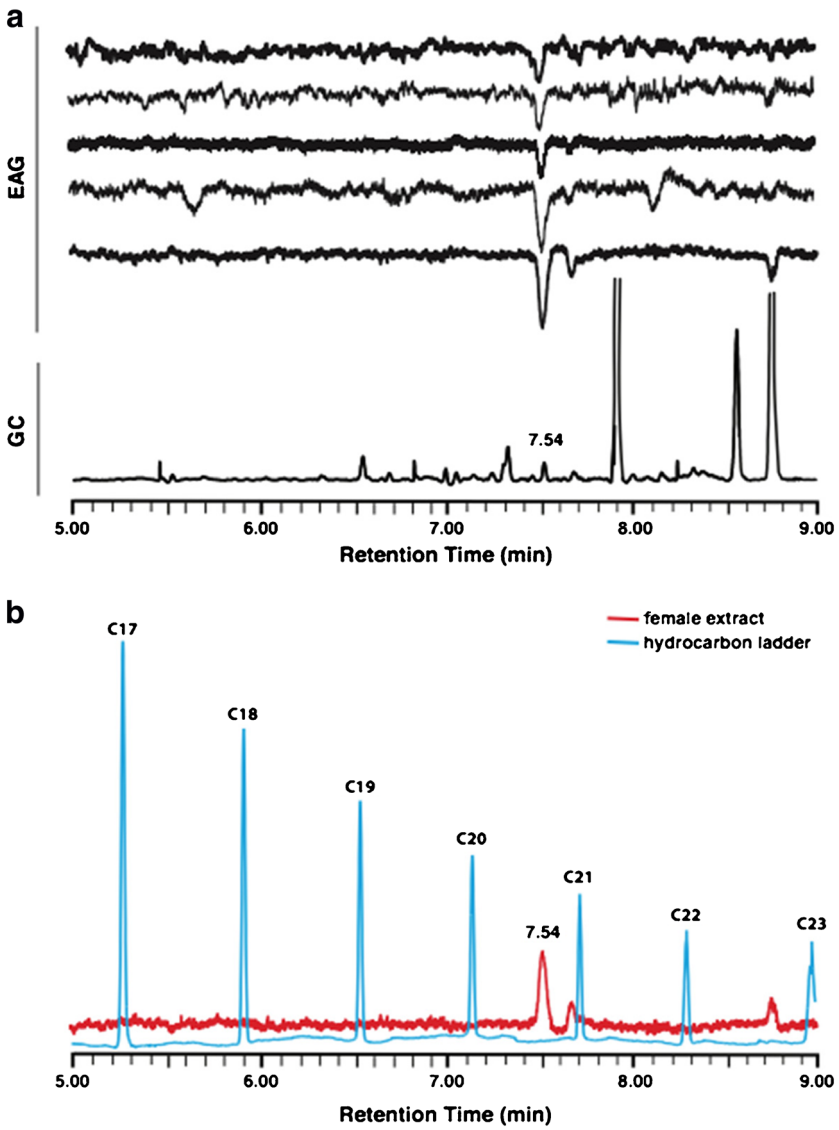

Fig. 4 **a** Five representative GC/EAD recordings (top) from male antennae in response to virgin female *Lycoriella ingenua* extract injected on the DB-225 column. Bottom tracing shows a representative FID output from the extract on this column. **b** The elution time, 7.54 min, of the GC/EAD-active compound on the DB-225 column compared to elution times of *n*-alkanes (Kovats Index 2065)

related stereoisomers (Yu et al. 2008), α-cadinol (Cornwell et al. 2001), or zingiberenol, a sex pheromone component of the rice stink bug, *Oebalus poecilus* (Borges et al. 2006; Oliveira et al. 2013), do not have base peaks in the *m*/*z* 80 - *m*/*z* 90 region in their mass spectra.

4-β-Hydroxy-1(10),5-germacradiene (β-germacrenol) and 6-α-hydroxy-1(10),4-germacradiene (kunzeaol) have mass spectra similar to that of the proposed pheromone component but their base peaks are at *m*/*z* 81 or *m*/*z* 84, respectively, rather than *m*/*z* 82 (Cornwell et al. 2001). He and Cane (2004) isolated 11-hydroxy-1(10),5-germacradiene from the volatiles of *Streptococcus coelicolor* bacteria that had a base peak at *m*/*z* 82 in the mass spectrum (He and Cane 2004 – see their Supplementary Fig. S1B, Structure 2). Other ions in the mass spectrum of this germacradienol were present in nearly identical proportions to those in the spectrum of the proposed pheromone component, except for small differences in the ratio of *m*/*z* 161 to 164 and the intensity of the ion at *m*/*z* 59.

The pheromone components of other nematoceran flies, the psychodid sandfly species, *L. cruzi*, and *L. longipalpus*, also include a germacrene-related sesquiterpene, 9-methyl-1(10), 5-germacradiene ((±)-9-methylgermacrene-B) (Brazil and Hamilton 2002; Brazil et al. 2009).

Our Y-tube bioassays showed that the *L. ingenua* sex pheromone is emitted only by virgin females and attracts males from at least a distance of 70 cm via upwind flight to the females followed by landing, wing fanning, abdomen-curling, and clasper extension. The pheromone is retained in or on the dead females for at least 24 h at sufficient concentrations to evoke attraction in males, but mated, live females did not attract males. The compound proposed to be a pheromone component of *L. ingenua* is highly active itself in eliciting these courtship and copulatory behaviors, and it may represent the complete pheromone. It is possible that one or more additional minor components may be required, but no other consistent responses were observed in our GC/EAD analyses.

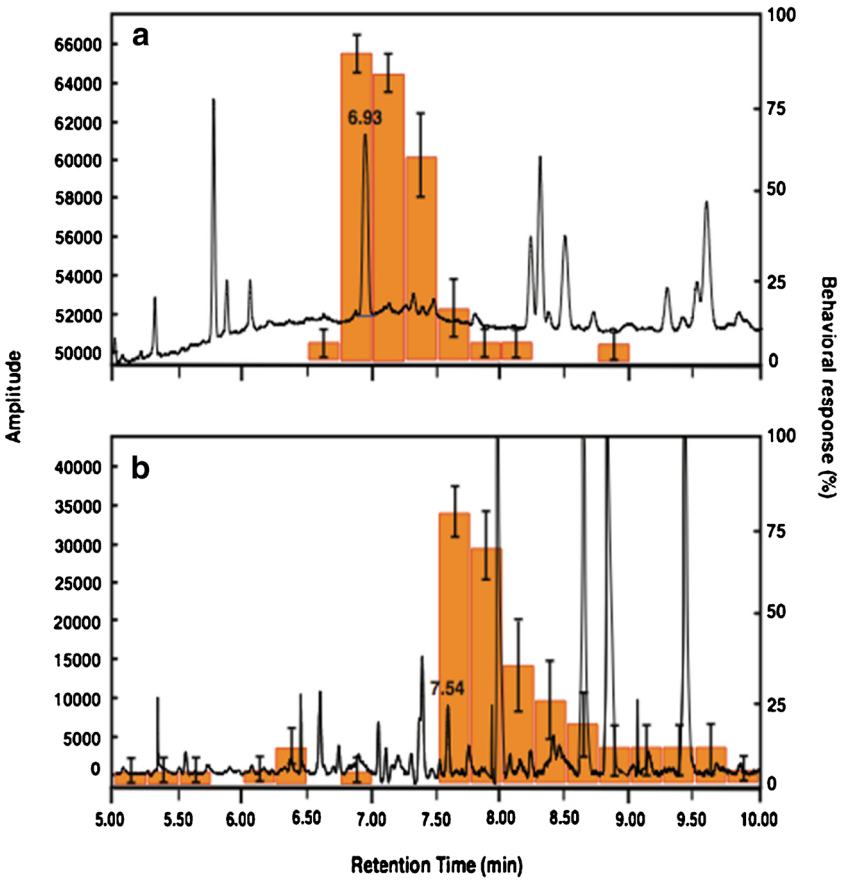

Fig. 5 **a** GC/BB analyses ($N = 30$ males) of extracts of female *Lycoriella ingenua* sex pheromone using an EC-5 column showing maximum behavioral activity (wing-fanning and abdomen curling) from conspecific males at the same retention time as occurred for the GC/EAD-active compound (6.93 min) on this column. **b** GC/BB analyses ($N = 30$ males) of extracts of *L. ingenua* sex pheromone using a DB-225 column showing maximum behavioral activity from males at the same retention time as occurred for the GC/EAD-active compound (7.54 min) on this column

The GC/BB technique (Hummel 1984; Leal et al. 1992, 1994) was helpful in our study in confirming the behavioural activity of the compound eliciting an EAG response in GC/EAD analyses on two different GC columns. The GC/BB technique couples the sensitivity and specificity of insect behavior with the analytical accuracy and reproducibility of gas chromatography (Hummel 1984) but has been used only in identifications of pheromone components in a limited number of moth and beetle species. This limited usage is probably due to the fact that, in most cases, single components of an insect pheromone blend are not behaviorally active, and so GC/EAD is generally more useful for indicating which compounds in an extract have the potential to be pheromone components.

Our knowledge of the pheromone chemistry of sciarid flies (Diptera: Sciaridae) is limited to just this one species, *L. ingenua* (Wicker-Thomas 2007), but evidence for sex pheromone communication is strong for sciarids in another sciarid genus, *Bradysia*. Studies have shown that females or female

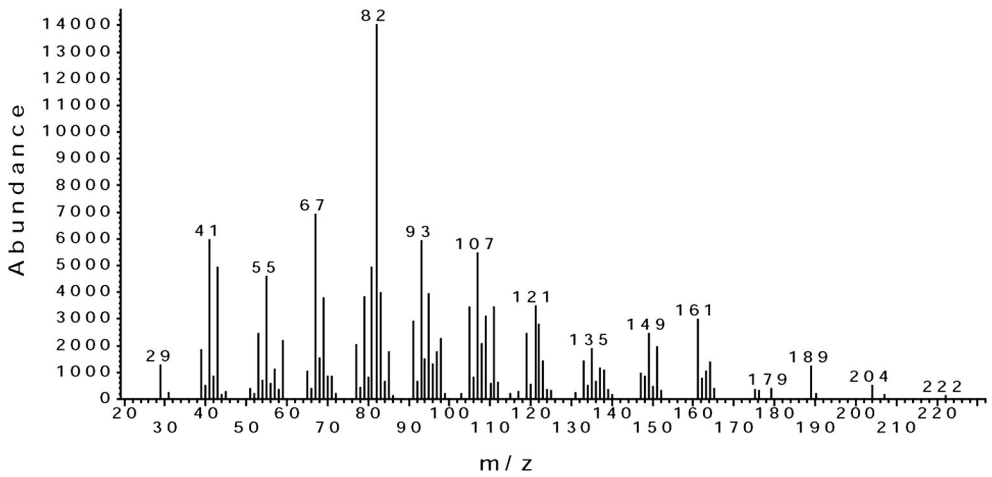

Fig. 6 Electron-Impact mass spectrum, from 20 FE of female extract, of the GC/EAD-active and GC/BB-active compound on an HP-5MS column

extracts evoke male attraction in *B. impatiens* (Alberts et al. 1981), *B. difformis*, *B. optata*, and *B. tilicola* (Frank and Dettner 2008) and *B. odoriphaga* (Li et al. 2007, 2008). These pheromones also evoke characteristic courtship behaviors including wing fanning, abdomen curling, and males grasping females' abdominal tips with their claspers (Frank and Dettner 2008; Li et al. 2007, 2008). The pheromones of some of these species appear to be comprised of several components, as indicated by thin layer chromatography (TLC) separation of the pheromone constituents and male bioassays of active compounds eluting on the TLC plates (Frank and Dettner 2008). Further isolation and identification of any of the pheromone components of these *Bradysia* species has not been reported.

If such studies were to be undertaken on other sciarid species such as those in the genus *Bradysia*, the use the GC/EAD techniques with multiple antennae connected in parallel as in our study might be useful in optimizing the signal-to-noise ratio during GC/EAD runs (Park and Baker 2002). The GC/BB setup that we used might also be another useful technique, if, as in our species, single components have significant behavioral activity. Our current work is aimed at characterizing the structure and stereochemistry of the germacradienol that we have demonstrated to be the major pheromone component of *L. ingenua*.

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Compliance with Ethical Standards

Conflict of Interest The authors have no conflicts of interest, financial or otherwise, that bias our work in any way.

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