JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Article

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J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.6b02235 • Publication Date (Web): 05 Aug 2016

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Journal of Agricultural and Food Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

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TITLE

Revisiting the Male-Produced Aggregation Pheromone of the Lesser Mealworm, *Alphitobius diaperinus* (Coleoptera, Tenebrionidae): Identification of a Six-Component Pheromone from a Brazilian Population

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ABSTRACT

The lesser mealworm, *Alphitobius diaperinus* Panzer 1797 (Coleoptera: Tenebrionidae), is a cosmopolitan insect pest affecting poultry production. Due to its cryptic behavior, insecticide control is usually not efficient. Thus, sustainable and effective methods would have an enormous and positive impact in poultry production. The aim of this study was to confirm the identity of the male-produced aggregation pheromone for a Brazilian population of *A. diaperinus,* and to evaluate its biological activity in behavioral assays. Six male-specific compounds were identified: (*R*)-limonene **(1)**, (*E*)-ocimene **(2)**, 2-nonanone **(3)**, (*S*)-linalool **(4)**, (*R*)-daucene **(5)**, all described before in an American population, and a sixth component, (*E,E*)-α-farnesene, which is apparently exclusive to a Brazilian population. Y-Tube bioassays confirmed the presence of a male-produced aggregation pheromone and showed that all components need to be present in a similar ratio and concentration as emitted by male *A. diaperinus* to produce a positive chemotactic response.

KEYWORDS: *Alphitobius diaperinus*, Aggregation pheromone, Lesser mealworm

INTRODUCTION

The lesser mealworm, *Alphitobius diaperinus* Panzer 1797 (Coleoptera: Tenebrionidae), is a cosmopolitan insect pest affecting poultry production.[1-3] Modern broiler facilities offer suitable environmental conditions for insect proliferation, including high temperatures, dark and sheltered sites, moisture and food availability, and consequently, high *A. diaperinus* larvae and adult densities are found, aggregating predominately under feeders and along house edges.[4-6] Bacteria, virus and fungi can infect *A. diaperinus* at all stages of the life cycle, and so these insects are potential disease vectors affecting avine health.[7-11] Control of *A. diaperinus* in poultry houses is currently undertaken using insecticide application, causing potential contamination of poultry and affecting meat quality. Additionally, due to the cryptic behavior of this pest, insecticide control is usually not efficient. Several studies have been conducted with the aim of developing alternative methods for lesser mealworm control, thereby minimizing reliance on the use of insecticides,[12-14] including semiochemicals that modify *A. diaperinus* behavior.[15-18] A five component, male-produced aggregation pheromone for a North American population of *A. diaperinus* was reported.[15] This pheromonal blend was tested in poultry houses, capturing more adults and larvae than control traps,[15,16] indicating effectiveness of these compounds in lesser mealworm management. In view of reported incidences of semiochemical diversity in geographically distinct insect populations,[19-24] the aim of this study was to confirm the identity of the male-produced aggregation pheromone for a Brazilian population of *A. diaperinus,* and to evaluate its biological activity in behavioral assays.

MATERIALS AND METHODS

Chemicals. Hexane for HPLC (≥ 97%) and diethyl ether were purchased from Sigma Aldrich and re-distilled before use. (*R*)-Linalool (95%) was purchased from Sigma-Aldrich (Steinheim, Germany), (*R*)-limonene and (*S*)-limonene (95%) were purchased from TCI-America (Portland, USA). 2-Nonanone (99%) was provided by Jeffrey R. Aldrich Consulting LLC (Santa Cruz, USA). *(E,E)*-α-Farnesene was synthesized in 3 steps from isoprene and sulphur dioxide by modifying the procedure reported by Spicer[25]. Thus, sulphur dioxide (30 ml) was condensed into a pressure flask cooled to -78˚C containing isoprene (10 g, 146.80 mmol) and hydroquinone (0.5 g, 4.68 mmol) before being sealed and stirred for 7 days at room temperature. The reaction flask was cooled

to -78˚C, opened and the sulphur dioxide allowed to evaporate overnight in a fume hood. The residue was dissolved in methanol, filtered and concentrated under vacuum. The crude material was recrystallized from hot methanol to provide 3-methylsulpholene (13.7 g, 71% yield) as a white crystalline solid. ^{1}H-NMR ($CDCl_3$, 500 MHz): 5.70 (m, 1H, $SO_2CH_2C\mathbf{H}$=C), 3.81 (m, 2H, $SO_2C\mathbf{H}_2$=C), 3.69 (m, 2H, $SO_2C\mathbf{H}_2C(CH_3)$), 1.90 (s, 3H, $C\mathbf{H}_3$). To a solution of 3-methylsulpholene (1.32 g, 10.00 mmol) and geranyl bromide (4.34 g, 20.00 mmol) in THF (20 mL), cooled to -98˚C under N_2, was added lithium bis (trimethylsilyl) amide (1.67 g, 10.00 mmol) in THF (7 mL). The resulting solution was stirred for 10 min before being allowed to warm to room temperature over a further 30 min. The reaction was quenched with saturated NH_4Cl and the THF removed under vacuum. The residue was dissolved in EtOAc before being washed with water, dried ($MgSO_4$) and concentrated under vacuum. The crude product was purified on silica gel (20% EtOAc in petroleum ether) to give the product (514 mg, 21% yield) as a colorless oil. ^{1}H-NMR ($CDCl_3$, 500 MHz): 5.70 (m, 1H, $SO_2CH_2C\mathbf{H}$=C), 5.24 (m, 1H, $(CH_3)_2C$=$CHCH_2CH_2C(CH_3)$=$C\mathbf{H}$), 5.09 (m, 1H, $(CH_3)_2C$=$C\mathbf{H}$), 3.69 (m, 2H, $SO_2C\mathbf{H}_2$), 3.53 (m, 1H, $SO_2C\mathbf{H}$), 2.59 (m, 2H, $(CH_3)_2C$=$CHCH_2CH_2C(CH_3)$=$CHC\mathbf{H}_2$), 2.12 – 2.03 (m, 4H, $(CH_3)_2C$=$CHC\mathbf{H}_2C\mathbf{H}_2$), 1.88 (s, 3H, SO_2CH_2CH=$C(C\mathbf{H}_3)$), 1.69 (s, 3H, $(C\mathbf{H}_3)_2C$=CH), 1.68 (s, 3H, $(CH_3)_2C$=$CHCH_2CH_2C(C\mathbf{H}_3)$=CH), 1.62 (s, 3H, $(C\mathbf{H}_3)_2C$=CH). A solution of 2-(*E*-3,7-dimethyl-2,6-octadiene)-3-methylsulpholene (514 mg, 2.02 mmol) in dry pyridine (10 mL) was heated to 150˚C under N_2 for 3 hours. The reaction mixture was cooled, poured into 1M HCl and extracted with petroleum ether. The combined organics were dried ($MgSO_4$) and concentrated under vacuum. The crude product was purified on florisil (100% petroleum ether) to give (*E,E*)-α-farnesene (330 mg, 80%) as a colorless oil (> 95% pure by GC). ^{1}H-NMR ($CDCl_3$, 500 MHz): 6.27 (dd, 1H, J = 17.5, 10.7 Hz, CH_2=$C\mathbf{H}$) 5.49 (t, 1H, J = 7.4 Hz, CH_2=$CHC(CH_3)$=$C\mathbf{H}$)), 5.17 – 5.01 (m, 3H, $(CH_3)_2C$=$C\mathbf{H}CH_2CH_2C(CH_3)$=$C\mathbf{H}CH_2CH$=$C(CH_3)CH$=$C\mathbf{H}_2$), 4.96 (d, 1H, J = 10.6 Hz, $C\mathbf{H}_2$=CH), 2.85 (t, 2H, J = 7.2 Hz, $(CH_3)_2C$=$CHCH_2CH_2C(CH_3)$=$CHC\mathbf{H}_2$), 2.09 (m, 2H, $(CH_3)_2C$=$CHC\mathbf{H}_2$), 2.02 (m, 2H, $(CH_3)_2C$=$CHCH_2C\mathbf{H}_2$), 1.79 (s, 3H, CH_2=$CHC(C\mathbf{H}_3)$), 1.71 (s, 3H, $(C\mathbf{H}_3)_2C$=CH), 1.66 (s, 3H, $(CH_3)_2C$=$CHCH_2CH_2C(C\mathbf{H}_3)$), 1.63 (s, 3H, $(C\mathbf{H}_3)_2C$=CH). (*R*)-Daucene was synthesized from (*R*)-carotol obtained from carrot seeds.[26,27,15] Carrot seeds (15.6 g) of Brasília variety were extracted for 8 hours in *n*-hexane at ambient temperature. The hexane extract was filtered and evaporated under vacuum to provide yellow pale oil

(571.5 mg), which was subjected to liquid chromatography over silica gel (15 g, Sigma Aldrich, 80/100 mesh). The oil (250 mg) was eluted sequentially with hexane (3×10 mL) and 98:2 diethyl ether:hexane (10×10 mL). The carotol structure was confirmed by NMR and GC-MS analysis and was obtained in 98% purity (by GC analysis). ^{1}H-NMR ($CDCl_3$, 500 MHz): 5.31 (m, 1H, $(CH_3)C=C\mathbf{H}$), 2.25 (br d, 1H, J = 16 Hz, $(CH_3)C=CHC\mathbf{H}_2$), 2.07 (m, 2H, $CH=C(CH_3)C\mathbf{H}_2$), 1.94 (m, 1H, $CH=C(CH_3)CH_2C\mathbf{H}_2$), 1.79 (m, 2H, $(CH_3)_2C\mathbf{H}C\mathbf{H}$), 1.72 – 1.47 (m, 8H), 1.29 (m, 1H, $(CH_3)_2CHCHCH_2C\mathbf{H}_2$), 1.14 (bs, 1H, O**H**), 0.99 (d, 3H, J = 6.7 Hz, $(C\mathbf{H}_3)_2CH$), 0.94 (m, 6H, $(C\mathbf{H}_3)_2CH$ and $C(OH)C(C\mathbf{H}_3)$). $[\alpha]^{20}_D$ = + 27.5 (c. 0.76, $CHCl_3$; literature $[\alpha]^{20}_D$ = + 29.6).[27] Carotol (102.96 mg) was then treated with thionyl chloride in pyridine,[27] with (*R*)-daucene (82.32 mg) obtained with 87% purity (by GC-analysis) following the same procedure of Levissales and Rudler (1967). ^{1}H-NMR ($CDCl_3$, 500 MHz): 5.42 (m, 1H, $(CH_3)C=C\mathbf{H}$), 2.66 (sept, 1H, J = 6.7 Hz, $(CH_3)_2C\mathbf{H}$), 2.39 (m, 1H, $(CH_3)_2CHCC\mathbf{H}_2$), 2.16 (m, 2H, $CH=C(CH_3)C\mathbf{H}_2$), 2.07 – 1.92 (m, 3H, $(CH_3)C=CHC\mathbf{H}_2$ and $(CH_3)_2CHCC\mathbf{H}_2$), 1.82 (m, 1H, $CH=C(CH_3)CH_2C\mathbf{H}_2$), 1.74 (s, 3H, $(C\mathbf{H}_3)C=CH_2$), 1.61 – 1.42 (m, 3H, $CH=C(CH_3)CH_2C\mathbf{H}_2$ and $(CH_3)_2CHCCH_2C\mathbf{H}_2$), 0.97 (d, 3H, J = 6.7 Hz, $(C\mathbf{H}_3)_2CH$), 0.93 (d, 3H, J = 6.7 Hz, $(C\mathbf{H}_3)_2CH$), 0.91 (s, 3H, $C\mathbf{H}_3C$). $[\alpha]^{20}_D$ = + 22.0 (c. 0.53, $CHCl_3$; literature (*S*)-daucene $[\alpha]^{20}_D$ = – 24.9, $CHCl_3$).[28] (*S*)-Linalool was obtained from coriander seed essential oil. Thus, seeds of coriander, *Coriandrum sativum* L. (2.6 kg) were extracted by hydrodistillation providing pale-colored oil (2.2 g). GC-FID analysis showed that the major component (85%) was linalool. The oil (0.6 g) was subjected to liquid chromatography on silica gel (30 g, Sigma-Aldrich, 80/100 mesh) with sequential elution using petroleum ether (5 x 20 mL) and 95:5 petroleum ether: ethyl acetate (20 x 20 mL) to obtain linalool of 98% purity. The enantiomeric purity of the linalool obtained from the coriander seed oil was analyzed using a chiral column β-DEX 325, as described above. The (*S*) to (*R*) ratio was determined to be 86:14 by GC. (*E*)-Ocimene was obtained in high purity from a commercially available sample of ocimene (Sigma Aldrich) containing a 30:65 mix of (*Z*) and (*E)* isomers. A sample of the ocimene (100 mg) was chromatographed on 0.5 g SiO_2 (Sigma—Aldrich, 80/100 mesh) impregnated with 25% $AgNO_3$ (Sigma-Aldrich, St. Louis, USA), with elution using 97.5: 2.5 hexane: diethyl ether (15 x 1 mL). The ratio between both isomers was determined by GC-FID analysis using a DB-5MS column (30 m length, 0.25 mm ID, 0.25 μm film; Supelco, Bellefonte, PA, USA) and a splitless injector, with helium as the carrier gas. The oven temperature program began at

50 °C for 2 min, increased at 15 °C/min to 250 °C and then this temperature was maintained for 20 min. (*E*)-Ocimene was obtained in 98% purity (by GC analysis) and (*Z*)-ocimene in 95 % purity.

Insects. Adults of *Alphitobius diaperinus* were obtained from a commercial poultry farm in PAD/DF, Brasília, DF, Brazil (15°59'40.5"S 47°37'22.8"W). The insects were reared at Embrapa Genetics Resources and Biotechnology, Brasília, DF, Brazil (15°43'48.0"S 47°53'59.5"W) in plastic boxes (40 x 20 cm) containing a fine layer of wood shavings and corrugated cardboard on top[29]. Adults and larvae were fed with a commercial chicken feed three times a week (composition: ground corn, soybeans, wheat, rice meal, meat meal, limestone, salt, vitamin premix, probiotics and minerals) and were provided with water daily. Boxes were kept in environmentally controlled room [(27 ± 2 ^{o}C, 60-80% RH and 16:8 h (D/L) photoperiod]. For bioassays and volatile collections, pupae were separated by sex and placed in new boxes. The genders were distinguished by differences in pupal abdominal appendages.[30]

Semiochemical collection. Two hundred 10-day-old virgin male and female beetles were placed separately in glass chambers (500 mL), containing a fine layer of vermiculite (approximately 1 cm) and a steel mesh (4×10 cm), that were used as shelter. Humidified and purified air (charcoal filter 20-40 mesh) was drawn through the flasks at 300 mL/min using a vacuum pump. Volatiles from the insects were trapped every 24 h using porous polymer adsorbent (100 mg of Porapak-Q 60-80 mesh, Sigma Aldrich®). To avoid contaminants, volatile collections were conducted without food. To guarantee insects were well fed during collections, and minimize release of defense compounds[15,18] each group of 200 insects was replaced every day. Individuals that died were replaced. Volatile collections were performed consecutively for 60 days and the trapped volatiles were eluted from adsorbent using 500 µL of *n*-hexane and pre-concentrated to 100 µL under a gentle flow of N_2. The samples were stored at -20^{o}C until use. The volatiles from the fine layer of vermiculite (approximately 1 cm) and a steel mesh (4×10 cm) (control) were also collected during consecutive 60 days to check for possible contaminants.

Olfactometry. Behavioral bioassays were conducted using a Y-tube olfactometer manufactured in a square acrylic block (26.0 x 23.0 cm) with a Y-shaped cavity (1.5 cm thickness) sandwiched between two glass plates. The apparatus trunk measured 12.0 cm and each arm measured 10.5 cm of length.[31] Bioassays were carried out in a controlled

environment room at 25 ± 1 ºC and 60 ± 10% RH, on a white bench under red artificial lighting (514 Lux). Charcoal-filtered and humidified air was pushed into the system at 0.6 L/min and pulled out at 0.3 L/min. This 'push-pull' system prevents entry of contaminating volatiles from the exterior. A single virgin *A. diaperinus* (15 days old) was introduced individually at the olfactometer trunk base, and observed for 300 sec. Both sexes were assayed at the same time in two olfactometer sets, until a total of 40 males and 40 females had responded. If no choice was made in 4 minutes, the bioassay was aborted and the insect recorded as non-responding.[32] The following observations were recorded: (1) first choice, considered when beetles entered 2.0 cm into the arm; (2) residence time, measured as the mean of total bioassay time spent in each arm of the olfactometer. Each individual was sampled only once and the filter papers were replaced after every five repetitions. At this time the Y-tube olfactometer was exchanged, cleaned with detergent, distilled water and acetone. Silicone tubing, filter papers and glass syringes were baked in an oven for at least 12 h at 45 ºC prior to use. In the first experiment, the chemotaxis behavior of *A. diaperinus* towards the odor of live conspecifics was assessed. Each olfactometer arm was connected to a 20 mL glass syringe containing either 20 virgin sexually mature females, 20 virgin sexually mature males, 10 of each sex, or air (control). The insects in the syringe were allowed to acclimatize for 30 minutes before experiments started, and were replaced after every 10 repetitions. In the second experiment, the chemotaxis behavior of *A. diaperinus* towards collected male volatiles, synthetic solutions or hexane (control) was recorded. The following treatments were evaluated as odor stimuli: male extract at a concentration of 1 insect equivalent/µL (IE/µL) and synthetic solution (SS) containing all compounds produced by males at 0.1, 1 and 10 IE/µL [(*R*)-limonene, (*E*)-ocimene, 2-nonanone, (*S*)-linalool, (*R*)-daucene and (*E,E*)-α-farnesene)]. Each olfactometer arm was connected to a 10 mL glass syringe containing a filter paper (0.5 cm width, 1.0 cm length, 205 µm thicknesses) treated with 10 µL of each treatment. In order to determine whether all compounds were necessary to modify *A. diaperinus* behavior, a third experiment was conducted using synthetic solutions missing one of the identified components. A fourth experiment was undertaken to evaluate the influence of non-naturally occurring isomers, and for this, four different blends containing the six components (mix), with one of the components present as a different isomer, were evaluated. The isomers evaluated were: (*S*)-limonene, (*Z*)-ocimene, (*R*)-linalool, and a mixture of farnesene isomers. (*S*)-Daucene was not tested because it was unavailable.

Chemical Analyses. GC-FID analyses of collected volatile extracts were performed using a gas chromatograph (Shimadzu 17A) equipped with a DB-5MS column (30 m length, 0.25 mm i.d., 0.25 μm film thickness; Supelco, Bellefonte, PA, USA). The carrier gas was helium. The oven temperature program was programmed to start at 50 °C for 2 min, increase at 5 °C/min to 180 °C, and then increase at 10 ºC/min to 250 °C, with the final hold time of 20 min. One microliter of each selected sample was injected in splitless mode; the injector temperature was 250 °C, and the detector temperature was 270 °C. Compounds were quantified by comparing GC peak areas with the peak area of the internal standard (IS) (*n*-tetracosane, IS was prepared at a final concentration of 1 μg/mL).

For qualitative analysis, selected volatile extracts were analyzed by coupled GC-MS using an Agilent MSD 5975C quadrupole mass spectrometer coupled to a gas chromatograph (GC-MS Agilent, 7890A) equipped with a DB-5 column (30 m length, 0.25 mm ID, 0.25 μm film; Supelco, Bellefonte, PA, USA) and a splitless injector, with helium as the carrier gas, using the same temperature program described for GC-FID analysis. Ionization was performed by electron impact (70 eV; source temperature 200°C). Data were collected using ChemStation software (Agilent Technologies). Tentative identifications were made by comparison of the target spectra with library databases (NIST and Wiley 2008), with published spectra and the retention indices (RI).[33,34] Confirmation of the identifications was done by GC peak enhancement with authentic standards. The absolute configuration of limonene and linalool produced by males was determined by enantioselective gas chromatography using a chiral GC column (30 mm×0.25 mm i.d., 0.25 μm, β-DEX 325 matrix non-bonded with 25% 2,3-di-*O*-acetyl-6-*O*-TBDMS-β-cyclodextrin in SPB-20 poly (20% phenyl/ 80% dimethylsiloxane phase) (Supelco, USA). The oven temperature was programmed as follows: 50 °C for 2 min, increase at 2 °C/min until 210 °C and hold for 10 min. Injections were made in splitless mode with helium as the carrier gas (1.5 mL/min), injector temperature at 250 °C, and detector temperature at 270 °C. To confirm the identity of (*E*,*E*)-α-farnesene, male volatile extracts and authentic standards were also analyzed and co-injected using a DB-WAX column (30 m length, 0.25 mm i.d., 1.0 μm film thickness; Supelco, Bellefonte, PA, USA). The carrier gas was helium. The oven temperature program began at 50 °C for 2 min, increased at 5 °C/min to 180 °C, and then increased at 10 ºC/min to 250 °C; this temperature was maintained for 20 min. One

220 microliter of each selected sample was injected in splitless mode; the injector
221 temperature was 250 °C, and the detector temperature was 270 °C.

222 **Statistical analysis.** The choices made by the insects in the bioassays were analyzed by
223 chi-square test and the residence time was analyzed by Wilcoxon's matched-pairs test,
224 by using the statistical program R 2.14.0,[35] with 95% of reliability.

225

226 **RESULTS**

227 **Chemical analyses.** Chemical analysis of the headspace volatiles collected from both
228 genders of *A. diaperinus* indicated that the males produced six volatile organic
229 compounds that were not present in the volatile collections from females or from
230 vermiculite and steel mesh (control) (Figure 1). GC-MS analysis of the male volatile
231 extracts, comparison of the spectra with NIST, GC peak enhancement and
232 enantioselective GC with authentic standards confirmed the identity of the compounds
233 to be (*R*)-limonene (**1**), (*E*)-ocimene (**2**), 2-nonanone (**3**), (*S*)-linalool (**4**), (*R*)-daucene
234 (**5**) and (*E,E*)-α-farnesene (**6**) (Table 1). The mean production of all six compounds was
235 consistent across all samples analyzed ($N = 60$) (Table 1).

236 **Olfactometry bioassays.** Behavior bioassays using live *A. diaperinus* as the odor
237 source showed that both genders were attracted to the odor from live adult males
238 compared to air (control) (Female, $\chi^2 = 12.80$, $P < 0.001$; Male, $\chi^2 = 9.80$, $P = 0.03$)
239 (Figure 2A and C), and spent more time in the arm containing odor from live male
240 adults (Female, W = 705, $P < 0.001$; Male, W = 681, $P < 0.001$) (Figure 2B and D). The
241 same behavior was observed when the odors of both live genders were compared to air
242 [first choice (Female, $\chi^2 = 9.81$, $P = 0.002$; Male, $\chi^2 = 9.72$, $P = 0.002$) (Figure 2A and
243 C); residence time (Female, W = 70, $P < 0.001$; Male, W = 681, $P < 0.001$)] (Figure 2B
244 and D). When the odor from live females was evaluated, females were not attracted (χ^2
245 = 0.81, $P = 0.37$, n = 40) (Figure 2A), and males were attracted to air ($\chi^2 = 5.03$, $P =$
246 0.02, $n = 40$) (Figure 2C), while for the residence time, both females and males showed
247 no preference between odor from live females and air (Female, W = 421, $P = 0.85$, n =
248 40; Male W = 431, $P = 0.74$, n = 40) (Figure 2B and D).
249 Both genders preferred odor from male volatile collections when compared to the
250 control *n*-hexane (Female, $\chi^2 = 9.05$, $P = 0.003$; Male, $\chi^2 = 4.68$, $P = 0.03$) (Figure 3A
251 and C) and spent more time in the arm containing the odor from male volatile

collections (Female, W = 703.5, $P < 0.001$; Male, W = 674, $P < 0.001$) (Figure 3B and D). Males and females were attracted to odor emitted from a synthetic solution containing the all six male-specific components [(*R*)-limonene, (*E*)-ocimene, 2-nonanone, (*S*)-linalool, (*R*)-daucene, and (*E,E*)-α-farnesene] in the same ratio produced by males and at concentration of 1 IE/μL compared to odor from hexane (Table 2, Figure 3A and C), and spent more time in the arm containing the odor from the synthetic solution (Table 2, Figure 3B and D). However, insects showed no preference for the synthetic solution when tested at 0.1 IE/μL or 10 IE/μL (Table 2, Figure 3A and C) over the solvent control ($P > 0.05$), and did not spend more time in the arm containing the odor from the synthetic solution ($P > 0.05$) (Table 2, Figure 3B and D). Usually when each male-specific compound was tested individually at 1 IE/μL males and females showed no significant behavior activity, except for (*S*)-linalool and (*R*)-daucene (Table 2, Figure 4). Males and females were not significantly attracted to synthetic pheromone blends with one component missing (Table 2, Figure 5), or when one of the six-component blend was incorporated as an incorrect isomer (Table 2, Figure 6).

DISCUSSION

Males of the Brazilian population of the lesser mealworm, *A. diaperinus*, have been shown to produce and emit six male-specific volatile compounds. Five of the six compounds identified were also described as components of the aggregation pheromone of this species in North America,[15] i.e., (*R*)-limonene, (*E*)-ocimene, 2-nonanone, (*S*)-linalool and (*R*)-daucene. The sixth component identified in this study, (*E,E*)-α-farnesene, is apparently exclusive to a Brazilian population. Y-Tube bioassays confirmed the presence of a male-produced aggregation pheromone, with all six identified compounds required for pheromone activity. Furthermore, components need to be present in a similar ratio and concentration as emitted by male *A. diaperinus* to produce a positive chemotactic response in laboratory conditions. The bioassays conducted with odor from live females corroborated the chemical analysis, showing that live females do not produce the attractant that males produce to attract males and females, and that their odor does not interfere with the male-produced aggregation pheromone. Males and females were not attracted to synthetic blends with concentration 10 times more or less compared to the amount produced naturally by males. *A.*

diaperinus, as well as for other Coleoptera species *Phyllotreta cruciferae* Goeze (Chrysomelidae)[36] and the boll weevil *Anthonomus grandis* Boheman (Curculionidae)[37] did not show attraction to enantiomers of aggregation pheromone components. For pheromone-mediated behavior in most species, and particularly by aggregation pheromones, a naturally occurring pheromone enantiomer is more attractive than the non-naturally occurring enantiomer.[38] The effect of racemic blends differs between species.[38] In some instances, the unnatural enantiomer does not interfere with the response to the active enantiomer, but in other species the presence of the enantiomer can reduce or eliminate the response.[38] The stereochemistry of optically active pheromone components was elucidated in this work and matched those reported.[15] (*E,E*)-α-Farnesene has been reported as an important semiochemical that acts as an attractant and kairomone for Coleoptera[39,40] and Hymenoptera.[41] This compound is also present in pheromone blends of Diptera, Hemiptera, Hymenoptera, Isoptera and Lepidoptera,[42-48] and here, we report the first instance of its appearance as an aggregation pheromone component for a member of the Coleoptera.

In addition to the composition difference, the ratio between the components produced by males from the Brazilian population was different from the North American population, i.e., for the Brazilian population the major component was (*S*)-linalool, whilst for the North American population, the major component is (*E*)-ocimene. Wind tunnel bioassays showed that only three components of the *A. diaperinus* aggregation pheromone are necessary to attract both genders of North American populations,[17] whereas our results showed that Brazilian populations need all six male-specific compounds for pheromone-mediated behavior.

Differences in pheromonal blend might be due to geographical isolation. For Coleoptera (beetles) this phenomenon was reported for pine bark beetles, *Ips pini* Say (Scolytidae), where eleven years after the first pheromone identification, a new component was isolated from another population.[19,20,49,50] For Hawaiian and Australian populations of cane weevil borers, *Rhabdoscelus obscurus* Boisduval[22,51] and also for bark beetles, *Ips subelongatus* Motschulsky, where population divergence in aggregation pheromone responses was reported.[24] This phenomenon is not exclusive to Coleoptera. Differences between populations also occur, for example, in the sex pheromone of the European Corn Borer, *Ostri nia nubilalis* Hübner (Lepidoptera: Pyralidae)[21] and a trail pheromone present in stingless bees, *Trigona corvina* Cockerell (Hymenoptera: Apidae).[23]

In the United States, the pheromonal blend of North American *A. diaperinus* showed promising results in trapping experiments in poultry houses, with pheromone traps attracting three times more adults and larvae than control traps.[15,16] Our results suggest that if the North American pheromone blend were used in Brazil, the mixture would probably not be effective for *A. diaperinus* management. Problems with deployment of sex pheromones for control of insect pests across different geographical regions have been reported elsewhere. For fall armyworm, *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae), interpopulational differences in sex pheromone components between sympatric regions presents difficulties in the application of this technology.[52,53] Thus, for control of Brazilian populations, careful consideration for use of the six components blend must be applied, whereas for other populations outside of North America and Brazil, pheromone composition must be verified prior to deployment in trapping systems.

One of the major problems in poultry production in Brazil is to keep poultry litter free of *A. diaperinus.* Since extensive chemical control is unaffordable and often involves replacement of poultry litter, sustainable and less expensive methods to control and manage *A. diaperinus* populations in poultry houses would have an enormous and positive impact in Brazil. Further studies are required to investigate the pheromone composition in different populations of *A. diaperinus* around the world in order to provide control of this cosmopolitan pest. For Brazilian populations, the next step is to test the feasibility of using pheromone based traps in poultry houses and quantify the economic and social impact of reduced chemical control upon poultry production.

ACKNOWLEDGMENTS: The authors express their special thanks to Anildo Cunha Jr., and Gerson Neudi Scheuermann, for providing information about *A. diaperinus*.

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FUNDING SOURCES

We thank the Fitotecnia Post-Graduate Program of the Federal University of Rio Grande do Sul for the use of their facility. This work received financial support from the Coordination of Superior Level Staff Improving (CAPES) through a grant to Marla J. Hassemer and from the National Council for Scientific and Technological Development (CNPq), the Brazilian Corporation of Agricultural Research (EMBRAPA) and Research Support Foundation of the Federal District (FAP-DF). Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council (BBSRC) of the United Kingdom.

522 FIGURE CAPTIONS

523 Table 1. Mean quantity ± standard error (SE) (ng/insect/day) and retention index of each
524 male specific compound using DB-5MS and DB-WAX GC columns.

525

526 Table 2. Statistical analysis of the first choice and residence time data for female and
527 male *Alphitobius diaperinus* in Y-tube olfactometer bioassays with different synthetic
528 solutions, at 0.1, 1 or 10 Insect Equivalent (IE)/µL, containing components of male
529 aggregation pheromone against *n*-hexane (control). Analyses were carried out using chi-
530 square test for first choice and Wilcoxon's matched-pairs test for residence time.
531 **P*<0.05, ***P*<0.01 and ****P*<0.001.

532

533 Figure 1. Gas chromatograms of volatile collections of glass chambers containing
534 vermiculite (control), *Alphitobius diaperinus* females plus vermiculite, and *A.*
535 *diaperinus* males plus vermiculite. (*R*)-limonene (**1**); (*E*)-ocimene (**2**); 2-nonanone (**3**);
536 (*S*)-linalool (**4**); (*R*)-daucene (**5**) and (*E*,*E*)-α-farnesene (**6**).

537

538 Figure 2. First choice and residence time of female (A and B) and male (C and D)
539 *Alphitobius diaperinus* in Y-tube olfactometer bioassays with the odor of live insects
540 against air as control. Analyses were carried out using chi-square test for first choice
541 and Wilcoxon's matched-pairs test for residence time. Bars indicate the number of
542 responsive insects and the residence time (%) to each olfactometer arm. **P*<0.05 and
543 ****P*<0.001. Numbers in brackets in the figure represent the number of insects that did
544 not respond to the treatment tested.

545

546 Figure 3. First choice and residence time of female (A and B) and male (C and D)
547 *Alphitobius diaperinus* in Y-tube olfactometer bioassays in response to collected male
548 volatile extracts at 1 Insect Equivalent (IE)/µL, and synthetic solutions (SS) containing
549 all male specific compounds [(*R*)-limonene, (*E*)-ocimene, 2-nonanone, (*S*)-linalool, (*R*)-
550 daucene and (*E*, *E*)-α-farnesene] at 0.1, 1 and 10 IE/ µL against *n*-hexane as control.
551 Analyses were carried out using chi-square test for first choice and Wilcoxon's
552 matched-pairs test for residence time. Bars indicate the number of responsive insects
553 and the residence time (%) to each olfactometer arm. * *P*<0.05 and ***P*<0.01. Numbers

554 in brackets in the figure represent the number of insects that did not respond to the
555 treatment tested.

556

557 Figure 4. First choice and residence time of female (A and B) and male (C and D)
558 *Alphitobius diaperinus* in Y-tube olfactometer bioassays in response to individual
559 components of male-produced aggregation pheromone at 1 Insect Equivalent (IE)/ μL
560 against *n*-hexane as control. Analyses were carried out using chi-square test for first
561 choice and Wilcoxon's matched-pairs test for residence time. Bars indicate the number
562 of responsive insects and the residence time (%) to each olfactometer arm. $^{**}P<0.01$
563 and $^{*}P<0.05$. Numbers in brackets in the figure represent the number of insects that did
564 not respond to the treatment tested.

565

566 Figure 5. First choice and residence time of female (A and C) and male (B and D)
567 *Alphitobius diaperinus* in Y-tube olfactometer bioassays in response to synthetic
568 solutions (SS) of aggregation pheromone minus (w/o) a single component, at 1 Insect
569 Equivalent (IE)/ μL against *n*-hexane as control. Analyses were carried out using chi-
570 square test for first choice and Wilcoxon's matched-pairs test for residence time. Bars
571 indicate the number of responsive insects and the residence time (%) to each
572 olfactometer arm. n.s. = no significance. Numbers in brackets in the figure represent the
573 number of insects that did not respond to the treatment tested.

574

575 Figure 6. First choice and residence time of female (A and B) and male (C and D)
576 *Alphitobius diaperinus* in Y-tube olfactometer bioassays in response to synthetic
577 solutions (SS) of aggregation pheromone, prepared with one component present as an
578 incorrect isomer, at 1 Insect Equivalent (IE)/ μL against *n*-hexane as control. Analyses
579 were carried out using chi-square test for first choice and Wilcoxon's matched-pairs test
580 for residence time. Bars indicate the number of responsive insects and the residence
581 time (%) to each olfactometer arm. Numbers in brackets in the figure represent the
582 number of insects that did not respond to the treatment tested.

583

TABLES

Table 1.

Compounds	Quantity ± SE ng/insect/day	Retention index DB-5MS	Retention index DB-WAX
(*R*)-limonene	49.0 ± 10.4	1030	1188
(*E*)-ocimene	31.3 ± 6.9	1050	1249
2-nonanone	7.0 ± 1.4	1091	1386
(*S*)-linalool	50.0 ± 12.9	1103	1551
(*R*)-daucene	18.4 ± 1.2	1378	1487
(*E,E*)-α-farnesene	44.5 ± 10.8	1508	1735

Table 2.

	Amount (IE/µL)	Males response		Females response	
		First choice	Residence Time	First choice	Residence Time
Synthetic solution (SS) composition					
(*R*)-limonene, (*E*)-ocimene, 2-nonanone, (*S*)-linalool, (*R*)-daucene, (*E,E*)-α-farnesene	0.1	$\chi^2 = 0.07$ $P = 0.79$	W = 250, $P = 0.48$	$\chi^2 = 1.14$, $P = 0.28$	W = 251.5 $P = 0.46$
(*R*)-limonene, (*E*)-ocimene, 2-nonanone, (*S*)-linalool, (*R*)-daucene, (*E,E*)-α-farnesene	1	$\chi^2 = 3.48$ $P = 0.03$*	W = 689 $P < 0.001$***	$\chi^2 = 7.49$ $P = 0.003$**	W = 688 $P < 0.001$***
(*R*)-limonene, (*E*)-ocimene, 2-nonanone, (*S*)-linalool, (*R*)-daucene, (*E,E*)-α-farnesene	10	$\chi^2 = 0.29$ $P = 0.59$	W = 140 $P = 0.66$	$\chi^2 = 1.14$ $P = 0.28$	W = 290 $P = 0.12$
Individual compounds					
(*R*)-limonene	1	$\chi^2 = 0.28$ $P = 0.59$	W = 220.5 $P = 0.95$	$\chi^2 = 0.06$ $P = 0.79$	W = 235 $P = 0.71$
(*E*)-ocimene	1	$\chi^2 = 0.78$ $P = 0.38$	W = 131 $P = 0.83$	$\chi^2 = 0.08$ $P = 0.77$	W = 97 $P = 0.13$
2-nonanone	1	$\chi^2 = 0.08$ $P = 0.78$	W = 16 $P = 0.90$	$\chi^2 = 3.00$ $P = 0.08$	W = 189 $P = 0.48$

(*S*)-linalool	1	$\chi^2 = 2.57$ $P = 0.11$	W = 284.5 $P = 0.15$	$\chi^2 = 4.57$ $P = 0.03$*	W = 251.5 $P = 0.46$
(*R*)-daucene	1	$\chi^2 = 0.06$ $P = 0.79$	W = 328.5 $P = 0.01$**	$\chi^2 = 4.57$ $P = 0.03$*	W = 282 $P = 0.16$
(*E,E*)-α-farnesene	1	$\chi^2 = 3.33$ $P = 0.07$	W = 93 $P = 0.06$	$\chi^2 = 1.20$ $P = 0.27$	W = 68 $P = 0.65$
SS minus one component					
SS w/o (*R*)-limonene	1	$\chi^2 = 1.80$ $P = 0.18$	W = 420 $P = 0.68$	$\chi^2 = 0.20$ $P = 0.65$	W = 440 $P = 0.68$
SS w/o (*E*)-ocimene	1	$\chi^2 = 3.20$ $P = 0.07$	W = 414 $P = 0.96$	$\chi^2 = 1.80$ $P = 0.18$	W = 475 $P = 0.24$
SS w/o 2-nonanone	1	$\chi^2 = 0.80$ $P = 0.37$	W = 352 $P = 0.99$	$\chi^2 = 0.20$ $P = 0.65$	W = 496.5 $P = 0.39$
SS w/o (*S*)-linalool	1	$\chi^2 = 1.80$ $P = 0.18$	W = 470.5 $P = 0.42$	$\chi^2 = 0.20$ $P = 0.65$	W = 441.5 $P = 0.30$
SS w/o (*R*)-daucene	1	$\chi^2 = 0.20$ $P = 0.65$	W = 431.5 $P = 0.77$	$\chi^2 = 0.20$ $P = 0.65$	W = 422 $P = 0.87$
SS minus (*E,E*)-α-farnesene	1	$\chi^2 = 0.20$	W = 447.5	$\chi^2 = 0.80$	W = 461.5

		$P = 0.65$	$P = 0.62$	$P = 0.37$	$P = 0.69$
SS with incorrect isomers					
(*S*)-limonene, (*E*)-ocimene, 2-nonanone, (*S*)-linalool, (*R*)-daucene, (*E,E*)-α-farnesene	1	$\chi^2 = 3.20$ $P = 0.07$	W = 131 $P = 0.33$	$\chi^2 = 0.80$ $P = 0.37$	W = 102 $P = 0.78$
(*R*)-limonene, (*Z*)-ocimene, 2-nonanone, (*S*)-linalool, (*R*)-daucene, (*E,E*)-α-farnesene	1	$\chi^2 = 0.20$ $P = 0.65$	W = 142.5 $P = 0.16$	$\chi^2 = 3.20$ $P = 0.07$	W = 99.5 $P = 0.86$
(*R*)-limonene, (*E*)-ocimene, 2-nonanone, (*R*)-linalool, (*R*)-daucene, (*E,E*)-α-farnesene	1	$\chi^2 = 1.80$ $P = 0.18$	W = 112.5 $P = 0.78$	$\chi^2 = 3.20$ $P = 0.07$	W = 106 $P = 0.97$
(*R*)-limonene, (*E*)-ocimene, 2-nonanone, (*S*)-linalool, (*R*)-daucene, farnesene isomers	1	$\chi^2 = 3.20$ $P = 0.07$	W = 110.5 $P = 0.84$	$\chi^2 = 1.80$ $P = 0.18$	W = 133 $P = 0.29$

FIGURES

Figure 1.

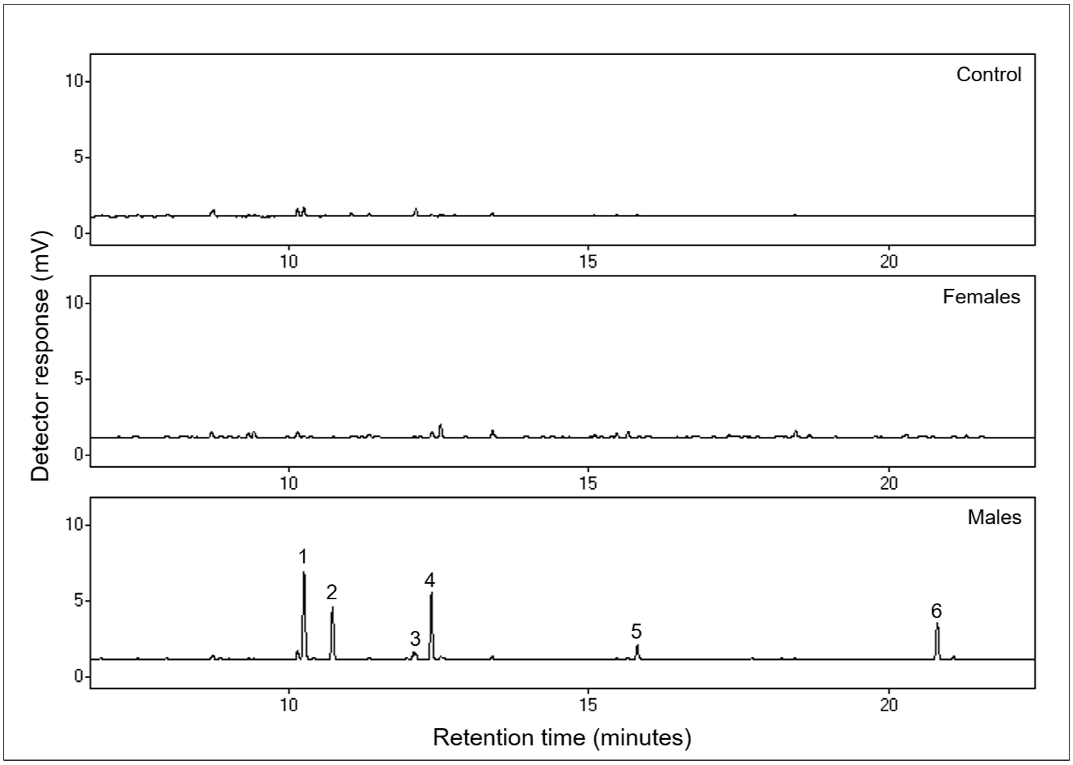


Figure 2.

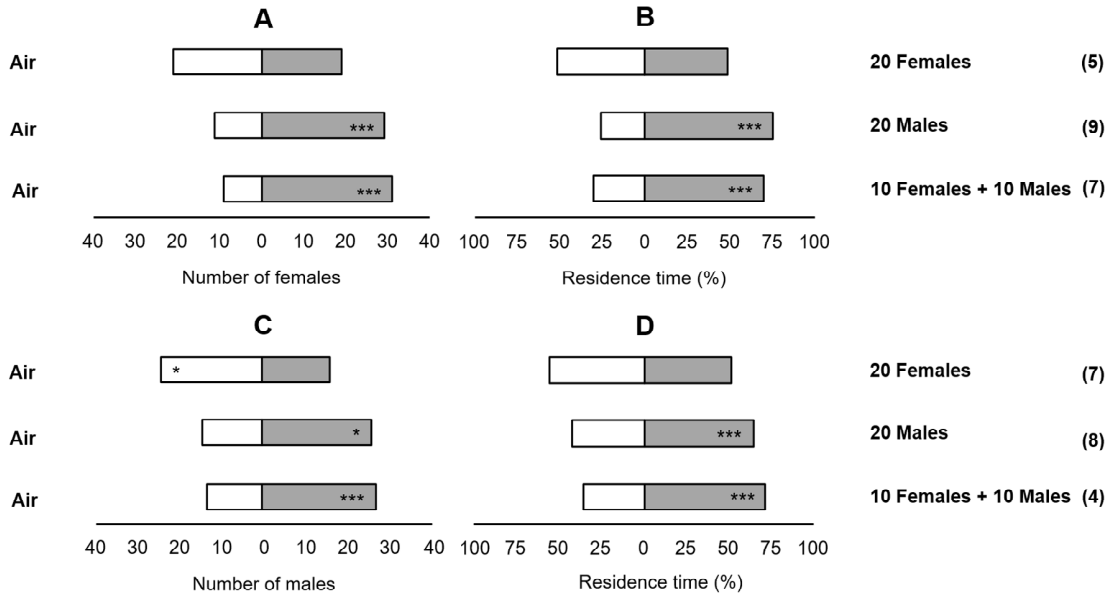

A
B
Air
Air
Air
***
***
***
20 Females (5)
20 Males (9)
10 Females + 10 Males (7)
40 30 20 10 0 10 20 30 40
100 75 50 25 0 25 50 75 100
Number of females
Residence time (%)
C
D
Air
Air
Air
*
*
***
***
***
20 Females (7)
20 Males (8)
10 Females + 10 Males (4)
40 30 20 10 0 10 20 30 40
100 75 50 25 0 25 50 75 100
Number of males
Residence time (%)

Figure 3.

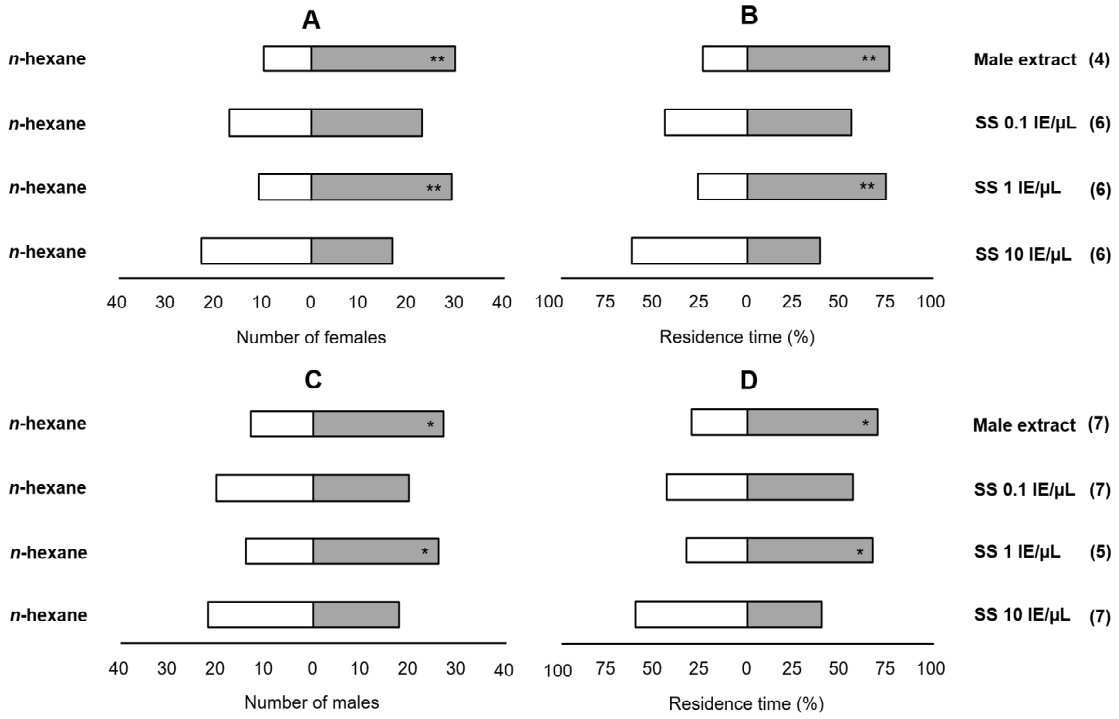

A
B
C
D
n-hexane
Male extract (4)
SS 0.1 lE/µL (6)
SS 1 lE/µL (6)
SS 10 lE/µL (6)
Male extract (7)
SS 0.1 lE/µL (7)
SS 1 lE/µL (5)
SS 10 lE/µL (7)
Number of females
Residence time (%)
Number of males
Residence time (%)

Figure 4.

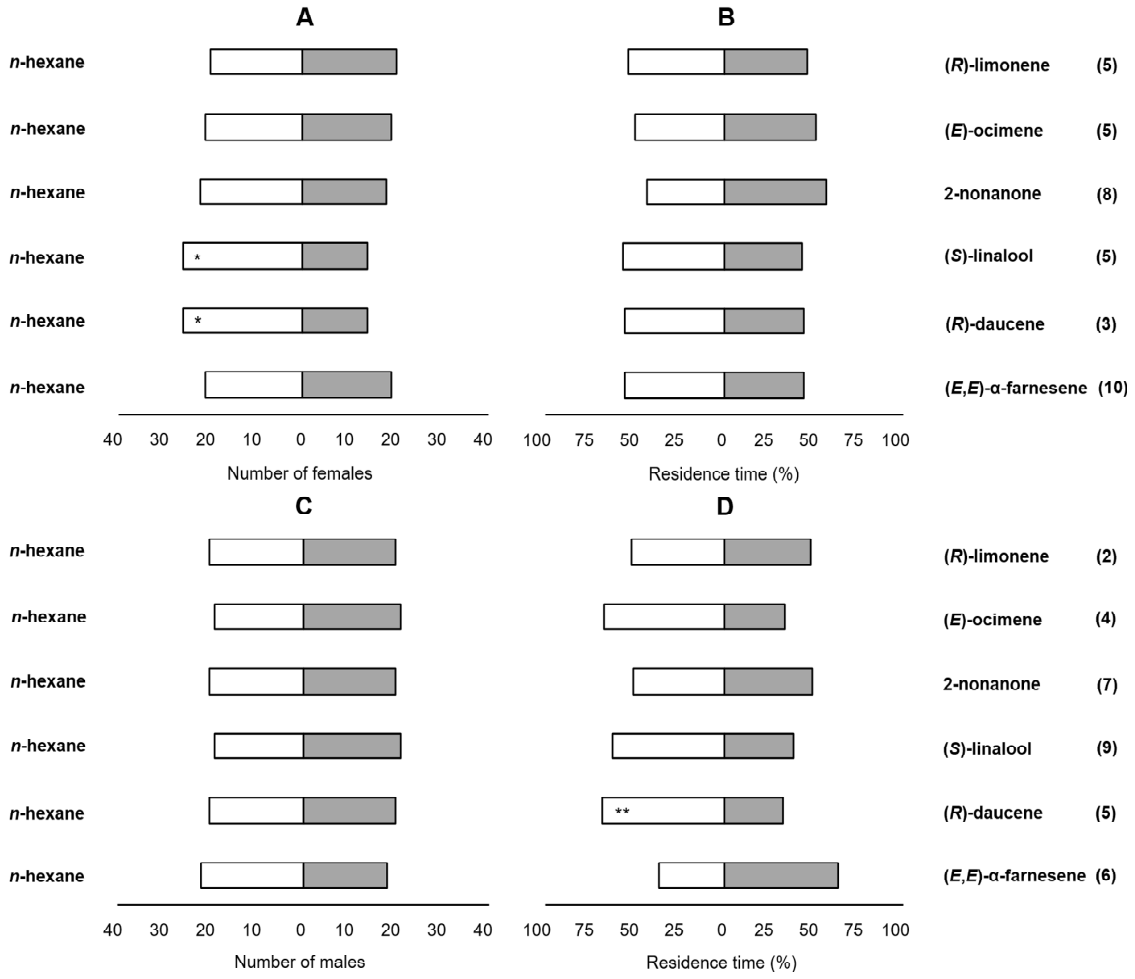

A
B
n-hexane
n-hexane
n-hexane
n-hexane
n-hexane
n-hexane
*
*
(R)-limonene (5)
(E)-ocimene (5)
2-nonanone (8)
(S)-linalool (5)
(R)-daucene (3)
(E,E)-α-farnesene (10)
40 30 20 10 0 10 20 30 40
Number of females
100 75 50 25 0 25 50 75 100
Residence time (%)
C
D
n-hexane
n-hexane
n-hexane
n-hexane
n-hexane
n-hexane
**
(R)-limonene (2)
(E)-ocimene (4)
2-nonanone (7)
(S)-linalool (9)
(R)-daucene (5)
(E,E)-α-farnesene (6)
40 30 20 10 0 10 20 30 40
Number of males
100 75 50 25 0 25 50 75 100
Residence time (%)

Figure 5.

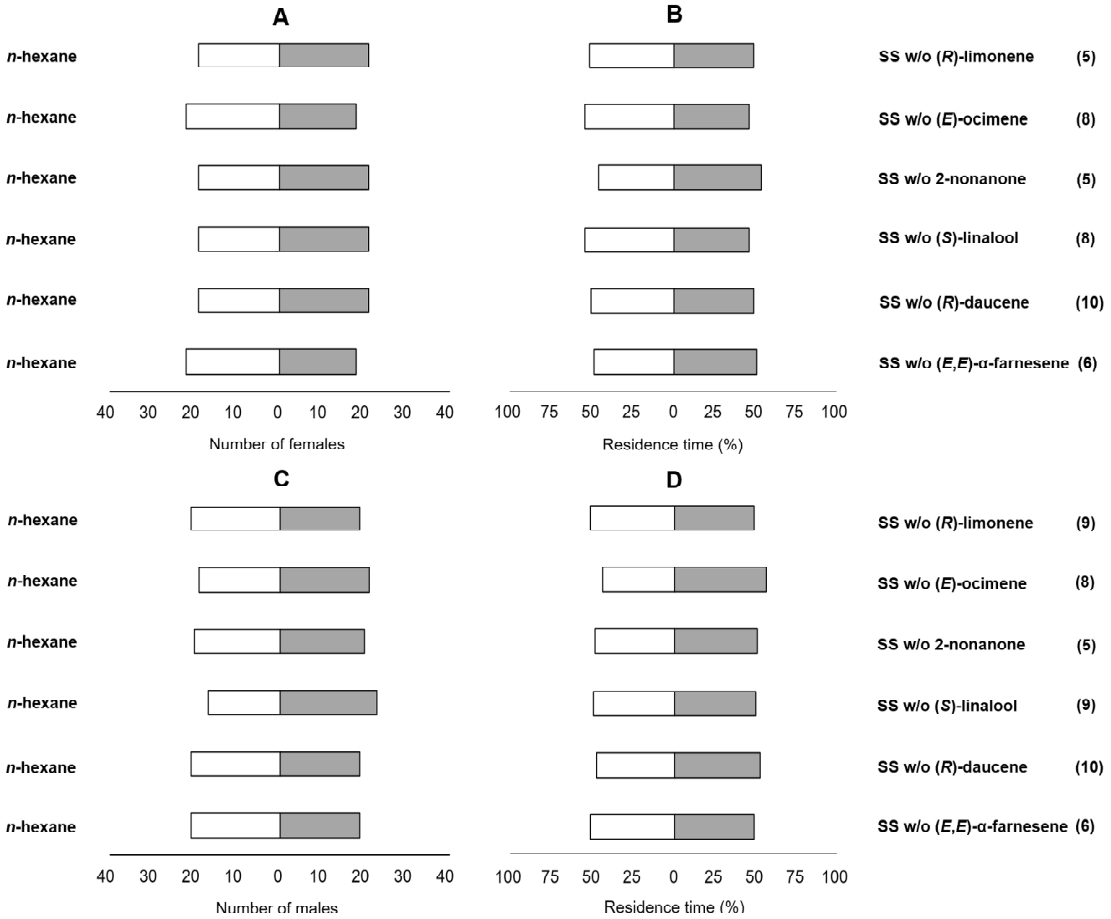
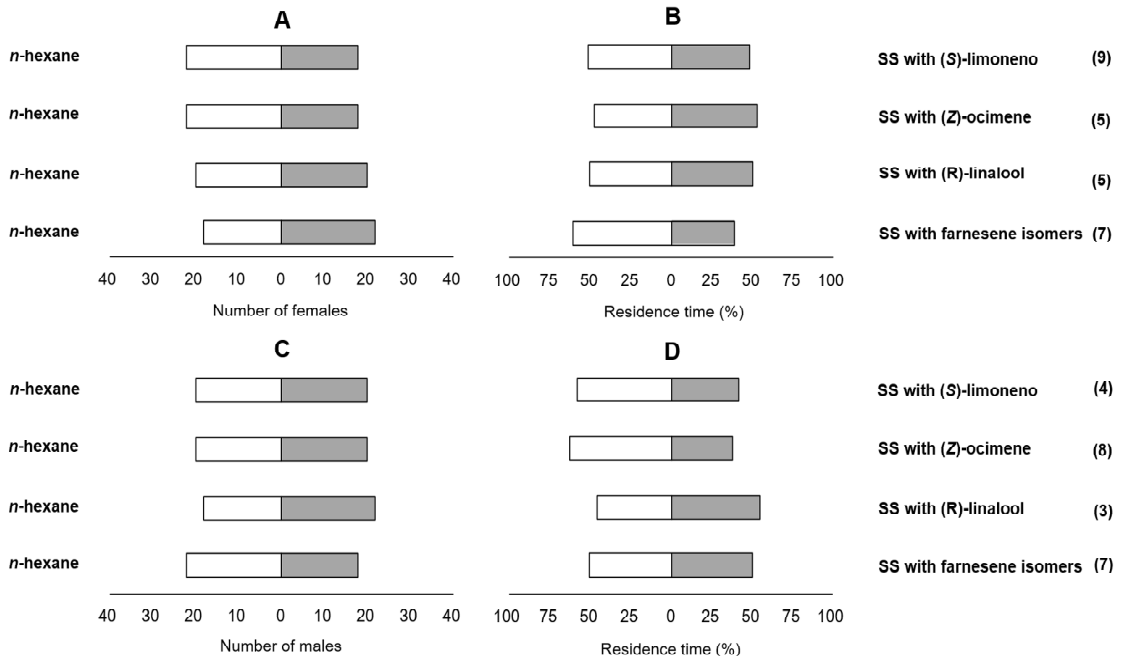
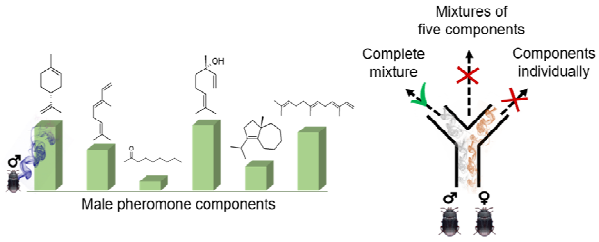

A
B
n-hexane
SS w/o (R)-limonene (5)
SS w/o (E)-ocimene (8)
SS w/o 2-nonanone (5)
SS w/o (S)-linalool (8)
SS w/o (R)-daucene (10)
SS w/o (E,E)-α-farnesene (6)
40 30 20 10 0 10 20 30 40
Number of females
100 75 50 25 0 25 50 75 100
Residence time (%)
C
D
SS w/o (R)-limonene (9)
SS w/o (E)-ocimene (8)
SS w/o 2-nonanone (5)
SS w/o (S)-linalool (9)
SS w/o (R)-daucene (10)
SS w/o (E,E)-α-farnesene (6)
Number of males
Residence time (%)

Figure 6.


A
B
n-hexane
n-hexane
n-hexane
n-hexane
SS with (S)-limoneno (9)
SS with (Z)-ocimene (5)
SS with (R)-linalool (5)
SS with farnesene isomers (7)
40 30 20 10 0 10 20 30 40
Number of females
100 75 50 25 0 25 50 75 100
Residence time (%)
C
D
n-hexane
n-hexane
n-hexane
n-hexane
SS with (S)-limoneno (4)
SS with (Z)-ocimene (8)
SS with (R)-linalool (3)
SS with farnesene isomers (7)
40 30 20 10 0 10 20 30 40
Number of males
100 75 50 25 0 25 50 75 100
Residence time (%)

TOC GRAPHIC


Mixtures of
five components
Complete
mixture
Components
individually
OH
Male pheromone components