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Male-Produced Sex Pheromone of the Carrion Beetles, *Oxelytrum discicolle* and its Attraction to Food Sources

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Abstract Carrion beetles are part of the great diversity of insects collected on cadavers. In Brazil, beetles of the genus *Oxelytrum* have great forensic importance in post mortem interval (PMI) estimation. We investigated the system of chemical communication in the attraction of these necrophagous beetles. Gas chromatographic analysis (GC) of female and male aeration extracts revealed the presence of two male-specific compounds, produced in a ratio of 94:6. Bioassays showed that the combination of male produced volatiles and the odor of a food source (carcass volatiles) were attractive to females. Mass and infrared spectral analyses of the male-specific compounds suggested that they were both unsaturated hydrocarbons. Several micro-derivatizations were carried out with the natural products, and the target structures were identified as (*Z*)-1,8-heptadecadiene (major) and 1-heptadecene (minor). The structure of the minor component was assigned by co-injection with a commercial standard. A seven-step synthesis was developed to synthesize (*Z*)-1,8-heptadiene, which co-eluted with the major natural product on three different GC stationary phases. Y-tube olfactometer assays showed that the mixture of synthetic standards in the naturally occurring proportion was slightly attractive to females. The results contribute both to the understanding of the chemical ecology of *O. discicolle* and to its potential to improve the accuracy of PMI estimation.

Keywords Coleoptera · Silphidae · Forensic entomology · Micro-derivatization · (*Z*)-1,8-heptadecadiene · GC/FT-IR · Post mortem interval

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Introduction

Species of Silphidae are known as carrion beetles because they are commonly encountered in vertebrate carcasses (Peck and Anderson 1985). As they seem strictly necrophagous during larval stages, Silphidae are recognized as useful in forensic investigations because they may be indicators of the post mortem interval (PMI) when they are found in corpses (Payne 1965; Smith 1986) The family is recognized as an important forensic indicator; in fact, *Oxelytrum* species already are being used to determine the PMI of corpses in Argentina (Oliva and Di Iorio 2008). Moreover, there are records of occurrences in the initial stages of decomposition of carcasses in Brazil (Mise et al. 2007; Ururahy-Rodrigues et al. 2010). Despite their forensic importance, no prior studies have addressed the chemical ecology of *Oxelytrum* species.

Silphid species of the genus *Nicrophorus* are known to feed and reproduce on small vertebrate carcasses. Haberer et al. (2008) verified that males of *Nicrophorus vespilloides* release sex pheromones that attract females. However, males of *Dermestes maculatus* (Coleoptera: Dermestidae) use a combination of sex pheromones and decomposing odors that attract females (von Hoermann et al. 2011, 2012).

The aim of this paper was to test whether *Oxelytrum discicolle* shows a similar behavior and to investigate the system of carcass invading as well as chemical communication among conspecifics. The study focused primarily on the identification and syntheses of sex pheromones.

Methods and Materials

Insects

A colony of *Oxelytrum discicolle* was started with insects collected from an araucaria forest within the experimental area of UFPR, Paraná, Brazil (25º25′S; 49º14′W). Newly

emerged adults were separated by sex and kept in cages (40×20×20 cm) at 25±1 °C, with 65±15 % relative humidity and a 12/12 h light/dark photoperiod. Insects were fed with ground meat.

Collection of Volatiles

Volatiles were collected by the aeration method (Zarbin et al. 1999, 2009). Seven insects of each sex were placed in aeration chambers (33×3.5 cm ID.) containing daily moistened cottons and a continuous 1 L/min flow of humidified charcoal-filtered air. The collecting apparatus was maintained at the same temperature and photoperiod as the colony. The volatiles were captured in glass traps (11×1 cm ID) containing 20 mg of HayeSep-D adsorbent polymer (Analytical Research systems, Inc., Gainesville, FL, USA). The adsorbed volatile compounds were eluted from the polymer once a day with doubly distilled hexane (400 μl) and concentrated under argon to 160 μl. Extracts were stored at −20 °C for chemical analyses and bioassays. To determine the day cycle, collections were made every 12 h. The carcass volatiles of a laboratory rat (*Rattus norvegicus*) were collected in the bloated phase using the procedures described above.

Chemical Analysis

Extracted volatiles were analyzed with a Shimadzu GC2010 gas chromatograph equipped with an FID detector, a RTX-5 (Restek, 30 m×0.25 m×0.25 μm film thickness) capillary column, and helium as the carrier gas. The GC was operated in splitless mode (250 °C). The temperature began at 100 °C for 1 min, increased at 7 °C/min until reaching 250 °C, and maintained at this temperature for 10 min. To determine the Kovats indices (Lubeck and Sutton 1983) and to examine the co-injection of the natural products with synthetic standards, RTX-WAX (Restek, 30 m×0.25 m×0.25 μm film thickness) and EC^{TM}-1 (Alltech, 30 m×0.25 m×0.25 μm film thickness) capillary columns were employed.

Gas chromatography–mass spectrometry (GC/MS) data were acquired using a Shimadzu QP2010-Plus electron ionization mass detector operating in electron impact mode (70 eV) with an RTX-5 (Restek, 30 m×0.25 m×0.25 μm) capillary column. The injector mode and program temperature were the same as described above.

Volatiles also were analyzed by coupled gas chromatography - Fourier transform infrared spectroscopy (GC/FT-IR) with a Shimadzu GC2010 gas chromatograph linked to a DiscovIR/GC infrared detector (4000–750 cm^{-1}, resolution of 8 cm^{-1}, Spectra Analysis, Marlborough, MA, USA). The capillary column, injector mode and program temperature were the same as those described above.

^{1}H- and ^{13}C-NMR spectra of the synthetic compounds were recorded on a Bruker ARX-200 spectrometer (200 and 50 MHz, respectively) as $CDCl_3$ solutions. Chemical shifts were expressed in ppm relative to TMS.

Microderivatization

Catalytic Hydrogenation with Palladium on Charcoal (Pd/C)

Extracts of aerations from males in hexane were added to ~0.5 mg of Pd/C (5 % Pd) in a glass vial. A balloon filled with hydrogen was attached to the vial, and the reaction was stirred for approximately 2 h. The product solution was filtered and analyzed by GC/MS (Attygalle 1998).

Partial Reduction with Diimine

The extract (10 μl) containing insect volatiles was evaporated to near dryness and then diluted with 20 μl of absolute ethanol in a vial. Subsequently, 10 μl of hydrazine hydrate (10 % in ethanol) and 10 μl of hydrogen peroxide (0.6 % in ethanol) were added. The vial was heated to 60 °C for 20 min. After cooling to room temperature, the reaction was quenched with 20 μl of HCl (10 % in water) and extracted with three portions of hexane (40 μl each). The organic extract was dried over anhydrous sodium sulfate and evaporated under nitrogen to the initial volume of about 10 μl, of which 1 μl was analyzed by GC/MS (Attygalle 1998).

Thiomethylation with Dimethyl Disulfide (DMDS)

A 9-μl portion of the product of partial reduction was added to 10 μl of dimethyl disulfide (DMDS) and 10 μl of iodine solution (5 % in carbon disulfide). The reaction mixture was maintained at 60 °C for 24 h in small-volume sealed vials. Excess iodine was reduced with sodium thiosulfate (10 % in water) and extracted with three 20-μl portions of hexane. The organic extract was dried over anhydrous sodium sulfate, evaporated under nitrogen to the initial volume of about 10 μl, and 1 μl was analyzed by GC/MS (Attygalle 1998; Jham et al. 2005).

For microderivatization of the extract obtained from aerations, the reaction mixture was heated to 90 °C for 48 h. Extraction procedures were identical to those performed with the partially reduced product.

Syntheses

*7-Bromoheptan-1-ol (**4**)*

To a mixture of 1,7-heptanediol (**3**) (4.00 g, 30.30 mmol) and toluene (40.0 ml), 48 % aqueous HBr (2.0 ml) was added. The heterogeneous mixture was stirred and heated at reflux for 6 h

while trapping the water by using a Dean-Stark apparatus. TLC analyses indicated that substantial amounts of diol **3** still remained. Thus, an additional quantity of HBr (2.0 ml) was added, and the mixture was heated at reflux for a further 12 h, at which time TLC analyses showed no diol **3** remaining. The reaction mixture was allowed to cool to room temperature, and the phases were separated. The organic layer was diluted with ethyl acetate and washed with 1 M NaOH and brine. Then, it was dried over anhydrous sodium sulfate, and the solvent was evaporated. The crude product was purified by flash chromatography (hexane/EtOAc: 7/3), yielding compound **4** with an 84 % yield (4.98 g, 25.54 mmol) (Chong et al. 2000). IR Max, cm^{-1}: 983, 1031, 1061, 1227, 1355, 1468, 2860, 2936, 2974, 3349, 3415. ^{1}H NMR (200 MHz, $CDCl_3$, ppm): δ 1.28–1.66 (m, 8H); 1.79–1.95 (m, 2H); 3.41 (t, *J* 6.8 Hz, 2H); 3.65 (t, *J* 6.4 Hz, 2H). ^{13}C NMR (50 MHz, $CDCl_3$, ppm): δ 62.9; 33.9; 32.7; 32.6; 28.5; 28.1; 25.6. MS: *m/z* (%): 176 (1); 150 (32); 148 (33); 97 (33); 81 (11); 70 (9); 69 (83); 68 (15); 67 (19); 56 (17); 55 (100); 43 (18); 42 (14); 41 (48).

*2-(7-Bromoheptyloxy)-tetrahydro-2H-pyran (**5**)*

Dihydropyran (1.7 ml) and α-toluene sulfonic acid (0.1 g) were added to compound **4** (4.33 g, 15.52 mmol) in 3.0 ml of dichloromethane. The reaction was stirred for 22 h at room temperature, diluted with dichloromethane, washed with H_2O and a saturated aqueous sodium hydrogen carbonate solution, and dried over sodium sulfate. Removal of the solvent under reduced pressure and purification by flash chromatography (hexane/EtOAc: 9/1) yielded product **5** (5.27 g, 18.89 mmol) (Santangelo et al. 2002). IR Max, cm^{-1}: 724, 814, 872, 984, 1028, 1075, 1139, 1204, 1352, 1466, 2859, 2938. ^{1}H NMR (200 MHz, $CDCl_3$, ppm): δ 1.38–1.93 (m, 16H); 3.32–3.56 (m, 4H); 3.68–3.93 (m, 2H); 4.56–4.59 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$, ppm): δ 19.7; 25.5; 26.0; 28.1; 28.6; 29.6; 30.8; 32.7; 33.9; 62.3; 67.5; 98.9. MS: *m/z* (%): 279 (2); 277 (2); 150 (2); 148 (2); 137 (1); 135 (1); 97 (18); 85 (100); 84 (9); 69 (9); 67 (9); 57 (12); 56 (23); 55 (37); 43 (11); 41 (20).

*2-(Heptadec-8-ynyloxy)tetrahydro-2H-pyran (**7**)*

To a stirred solution of 1-decyne (**6**) (1.3 ml, 7.39 mmol) in dry THF (5.0 ml), under argon, *n*-BuLi (2.0 ml, 3.26 M in hexane) was slowly added at −78 °C. The solution was stirred at 0 °C for 30 min and then 2-(7-bromoheptyloxy)tetrahydro-2H-pyran (**5**) (1.00 g, 3.58 mmol) in dry HMPA (0.7 ml) was added at 0 °C for 8 h. The reaction was extracted with hexane and washed with water and brine. The organic layer was separated, dried over sodium sulfate, and concentrated *in vacuo*. The crude product was purified by flash chromatography (hexane/EtOAc: 9/1) yielding compound **7** with a 91 % yield (1.09 g, 0.32 mmol) (Kang and Park 1988). IR Max, cm^{-1}: 870, 1034, 1085, 1206, 1352, 1464, 2856, 2931. ^{1}H NMR (200 MHz, $CDCl_3$, ppm): δ 0.84–0.91 (m, 3H); 1.24–1.90 (m, 28H); 2.10–2.22 (m, 4H); 3.32–3.55 (m, 2H); 3.68–3.93 (m, 2H); 4.56–4.60 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$, ppm): δ 14.2; 18.4; 18.2; 19.8; 22.7; 25.6; 26.2; 28.6; 28.9; 29.0; 29.1; 29.2; 29.2; 29.3; 29.8; 30.9, 31.9; 62.4; 67.7; 80.2; 80.4; 98.9. MS: *m/z* (%): 336 (1), 101 (13), 95 (15); 85 (100); 84 (11); 83 (10); 82 (10); 81 (22); 79 (13); 69 (10); 67 (28); 57 (11); 56 (8); 55 (26); 43 (12); 41 (17).

*8-Heptadecyn-1-ol (**8**)*

To a stirred solution of **7** (0.98 g, 2.93 mmol) in methanol (10.0 ml), *p*-toluene sulfonic acid (0.1 g) was added. The mixture was stirred at room temperature for 10 h. Methanol was removed, and the residue was diluted in ethyl ether and washed with saturated solutions of sodium hydrogen carbonate and brine. The organic layer was separated and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure and purification by flash chromatography (hexane/EtOAc: 8/2) yielded **8** in 93 % yield (0.69 g, 2.74 mmol) (Zarbin et al. 2007). IR Max, cm^{-1}: 725, 974, 1063, 1298, 1470, 2852, 2932, 3315. ^{1}H NMR (200 MHz, $CDCl_3$, ppm): δ 0.84–0.92 (m, 3H); 1.25–1.60 (m, 22H); 2.09–2.18 (m, 4H); 3.63 (t, *J* 6.5 Hz, 2H). ^{13}C NMR (50 MHz, $CDCl_3$, ppm): δ 14.1; 18.4; 18.8; 22.7; 25.7; 28.6; 28.8; 28.9; 29.0; 29.1; 29.2; 29.3; 31.9; 32.8; 63.0; 80.1; 80.4. MS: *m/z* (%): 252 (1); 152 (13); 124 (15); 121 (30); 111 (11); 110 (20); 109 (21); 108 (12); 107 (23); 98 (19); 97 (19); 96 (38); 95 (59); 94 (22); 93 (40); 91 (15); 83 (22); 82 (68); 81 (97); 80 (35); 79 (66); 77 (14); 69 (32); 68 (51); 67 (100); 66 (10); 57 (19); 56 (10); 55 (74); 53 (14); 43 (31); 41 (56).

*(Z)-8-Heptadecen-1-ol (**9**)*

To a stirred solution of **8** (0.3 g, 1.19 mmol) in methanol (5.0 ml), 5 % Pd/calcium carbonate (15 mg) and quinoline (20 mg) were added. The mixture was stirred under a hydrogen atmosphere (25 psi) in a Parr® apparatus for 2 h and filtered over Celite®. The solvent was evaporated *in vacuo*, and the residue was extracted with hexane and washed with water. The organic layer was separated, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The product was purified by flash chromatography (hexane/EtOAc: 8/2), yielding **9** in 77 % yield (0.23 g, 0.90 mmol) (Overman et al. 1993). IR Max, cm^{-1}: 729, 1068, 1378, 1468, 1656, 2856, 2924, 3004, 3321, 3414. ^{1}H NMR (200 MHz, $CDCl_3$, ppm): δ 0.88 (m, 3H); 1.24–1.38 (m, 20H); 1.50–1.62 (m, 2H); 1.97–2.06 (m, 4H); 3.63 (t, *J* 6.5 Hz, 2H); 5.27–5.42 (m, 2H). ^{13}C NMR (50 MHz, $CDCl_3$, ppm): δ 14.1; 22.7; 25.8; 27.2; 27.3; 29.3; 29.4; 29.4; 29.6; 29.6; 29.7; 29.8;

32.0; 32.8; 63.1; 129.8; 130.0. MS: *m/z* (%): 236 (7); 124 (17); 123 (15); 110 (25); 109 (26); 97 (26); 96 (69); 95 (54); 83 (43); 823 (100); 81 (79); 79 (11); 71 (12); 70 (16); 69 (50); 68 (48); 67 (78); 57 (30); 56 (21); 55 (91); 54 (35); 43 (35); 41 (54).

*1-Bromo-(8Z)-heptadecene (**10**)*

To a mixture of alcohol **9** (0.15 g, 0.59 mmol) and carbon tetrabromide (0.24 g, 0.72 mmol) in dichloromethane (3.0 ml) at 0 °C, a solution of triphenylphosphine (0.23 g, 0.87 mmol) in dichloromethane (3.0 ml) was added. The reaction mixture was stirred at room temperature for 1 h, concentrated under reduced pressure, and purified by flash chromatography (hexane/EtOAc: 8/2) to yield **10** in 83 % yield (0.15 g, 0.47 mmol) (Hu et al. 2001). IR Max, cm^{-1}: 721, 1223, 1265, 1467, 2852, 2922, 3006. ^{1}H NMR (200 MHz, $CDCl_3$, ppm): δ 0.83–0.92 (m, 3H), 1.22–1.52 (m, 20H); 1.86 (m, 2H); 1.97–2.06 (m, 4H), 3.41 (t, *J* 6.8 Hz, 2H); 5.26–5.44 (m, 2H). ^{13}C NMR (50 MHz, $CDCl_3$, ppm): δ 14.2; 22.8; 27.2; 27.3; 28.2; 28.7; 29.1; 29.4; 29.4; 29.6; 29.7; 29.9; 32.0; 32.9; 34.1; 129.8; 130.2. MS: *m/z* (%): 319 (1); 318 (4); 317 (1); 316 (4); 150 (12); 148 (12); 125 (13); 111 (29); 109 (10) 98 (18); 97 (64); 96 (13); 75 (15); 85 (10); 84 (20); 83 (77); 82 (23); 81 (26); 71 (20); 70 (37); 69 (82); 68 (15); 67 (33); 57 (50); 56 (40); 55 (100); 54 (27); 43 (41); 42 (11); 41 (62).

*(Z)-1,8-Heptadecadiene (**1**)*

A solution of the bromide **10** (0.10 g, 0.31 mmol) in benzene (0.8 ml) and a 1.0 M DMSO solution of potassium *tert*-butoxide (0.8 ml) were mixed. An exothermic reaction occurred, and the temperature rose to 60 °C. After 30 min, the mixture was acidified with 1.0 M HCl and the upper layer was separated. The aqueous layer was extracted twice with hexane, and the combined extracts were dried over sodium sulfate. Then, the residue was purified by flash chromatography (eluted by hexane) yielding the diene **1** in 76 % yield (0.05 g, 0.22 mmol) (Manabe et al. 1985). IR Max, cm^{-1}: 729, 912, 992, 1458, 1641, 2853, 2923, 3001, 3079. ^{1}H NMR (200 MHz, $CDCl_3$, ppm): δ 0.80–0.95 (m, 3H); 1.18–1.46 (m, 18H); 1.90–2.13 (m, 6H); 4.87–5.06 (m, 2H); 5.26–5.44 (m, 2H); 5.71–5.92 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$, ppm): δ 14.2; 22.8; 27.2; 27.3; 28.9; 28.9; 29.4; 29.4; 29.6; 29.7; 29.9; 32.0; 33.9; 114.2; 129.8; 130.1; 139.2. MS: *m/z* (%): 236 (9); 138 (10); 124 (14); 123 (12); 110 (30); 109 (28); 97 (22); 96 (69); 95 (52); 83 (34); 82 (80); 81 (89); 79 (12); 70 (10); 69 (48); 68 (59); 67 (89); 57 (24); 56 (16); 55 (100); 54 (52); 53 (11); 43 (38); (8); 41 (76).

Olfactometer Bioassays

Behavioral responses of *O. discicolle* to natural and synthetic compounds were tested in a Y-tube olfactometer using humidified, charcoal-filtered air flowing at 2.5 L/min. The olfactometer consisted of a Y-shaped glass tube (4×40 cm) with two 20 cm arms. The odor sources were placed at the ends of the arms. Each odor source consisted of a piece of filter paper (2×2 cm) impregnated with the synthetic compounds, volatiles collected from insects and/or, food source, or hexane.

A beetle was introduced into the base of the olfactometer, and its behavior was observed for 10 min. A positive response was defined when the insect walked against the airflow more than 5 cm into an arm towards the odor source or the control and remained there for more than 2 min. No response was defined when the insect did not leave the main tube. Each insect was counted as one datapoint and was tested only once. The odor source was replaced after every test. The insects that did not choose either arm were excluded from the statistical analysis. The olfactometer was moved after every three tests to exclude influences by light sources, cleaned with ethanol, and left to dry for 5 min. In each bioassay, 30 insects of each sex were tested. The data were analyzed using a *chi-square* test with the BioEstat program (version 5.0) (Ayres et al. 2003).

Five behavior experiments were conducted for each sex to determine the responses to the following odors: T1 - odors from males *vs.* hexane, T2 - odors from carcass *vs.* hexane, T3 - odors from males plus carcass *vs.* hexane, T4 - odors from males *vs.* odors from carcass, and T5 - odors from males plus carcass *vs.* odors from carcass.

Three experiments were conducted to determine the biological activity of the synthetic major and minor male-specific compounds. Females were tested with the synthetic major [(*Z*)-1,8-heptadecadiene] plus odors from carcass, with the synthetic minor (1-heptadecene) male-specific compound plus odors from carcass, and with a blend of the two male-specific compounds at a ratio of 94:6 plus odors from carcass. The odors from carcass were used as the control of these three experiments.

The amounts of synthetic materials used were 480 ng of the major (*Z*)-1,8-heptadecadiene (**1**), and 32 ng of the minor 1-heptadecene **2**, similar to the amounts that were detected in the natural extracts used in the bioassays.

Results and Discussion

Comparison of the chromatograms obtained in the analysis of volatile collections from male and female *O. discicolle* adults showed the presence of two male-specific compounds (Fig. 1). The Kovats indices (KI) were calculated for three different columns (**1**: 1674/RTX-5, 1882/RTX-Wax, and 1673/EC-1; **2**: 1695/RTX-5, 1871/RTX-Wax, and 1692/EC-1). The ratio between compounds **1** and **2** was calculated

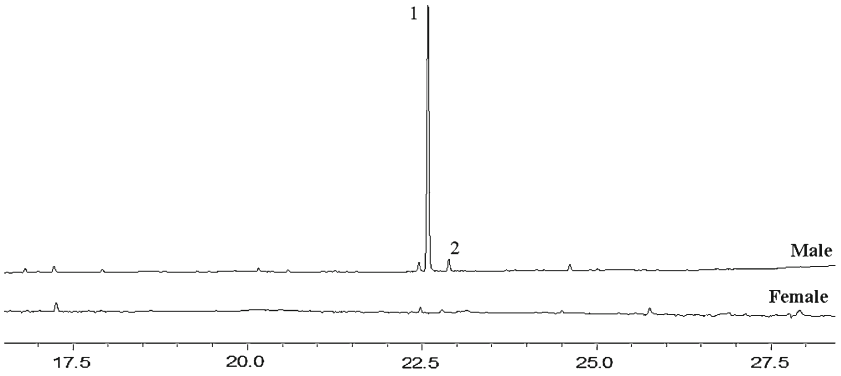


Fig. 1 Comparison of gas chromatograms obtained from extracts of volatile collections from *Oxelytrum discicolle* males and females, showing the male-specific major (1) and minor (2) compounds

to be 94:6, respectively, based on the areas of the GC peaks detected by FID.

The biological activity of the extract containing the male-specific compounds was tested for males and females (Fig. 2). In **T1**, we tested the extract of volatile collections *versus* hexane, and neither males (P=0.466) nor females (P=0.716) revealed a preference. Recently, von Hoermann and co-workers (2012) observed that neither sex of *Dermestes maculatus* was attracted to volatiles released by males, but rather the combination of the odor of males and the odor of a pig carcass (alimentary source) was strongly attractive to females.

Based on these results, the attractiveness of an extract containing the volatiles of a rat carcass in the bloated phase was tested *vs.* hexane (**T2**). Tests showed that males preferred the carcass odor (73 %, P=0.011), while females did not show any preference (P=0.466, Fig. 2), suggesting that males are responsible for exploring the environment and finding the food source.

When testing the extract of insect volatiles plus carcass volatiles *vs.* hexane (**T3**), only females preferred this combination (70 %, P=0.028, Fig. 2), whereas males revealed no preference (P=0.466), indicating that the male-specific compounds most likely act as sex pheromones.

In **T4**, when presented with the extract of insect volatiles *vs.* carcass volatiles, males showed a preference for carcass volatiles (68 %, P=0.040), and females did not (P=0.274, Fig. 2). This bioassay supported the results of **T2** and the role of males in finding food.

To confirm the activity of the volatiles released by males as a sex attractant, we tested the attractiveness of the insect released volatiles plus carcass volatiles *vs.* carcass volatiles

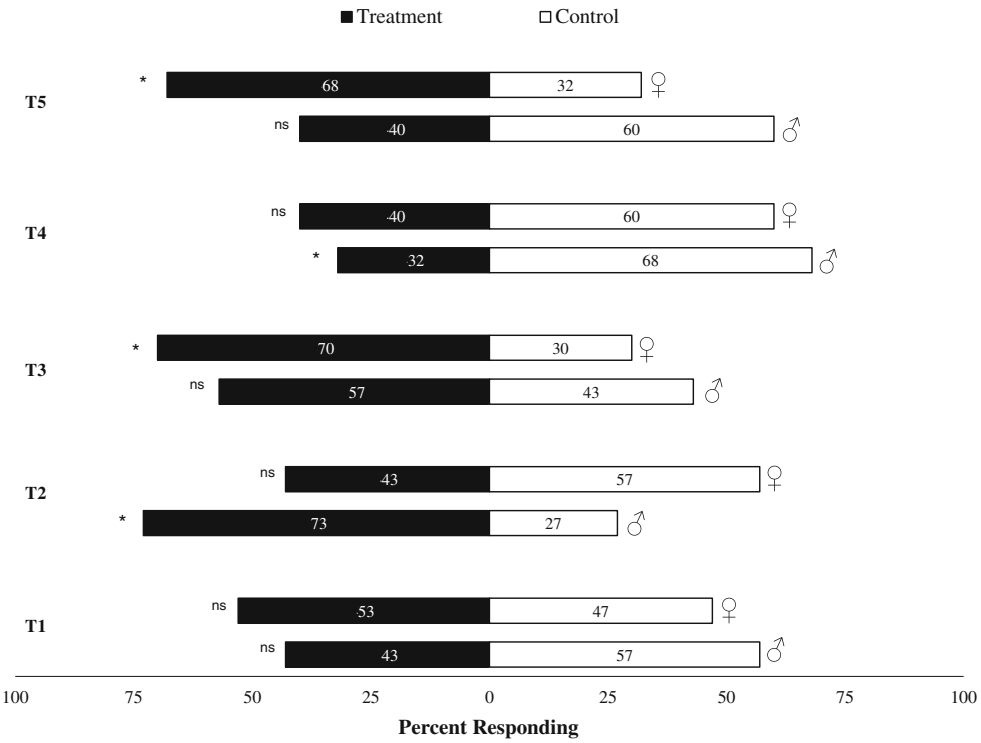


Fig. 2 Responses of males (♂) and females (♀) of *Oxelytrum discicolle* to the volatiles collected from males and from a rat carcass (bloated phase). Statistics: chi-square test (* P<0.05)

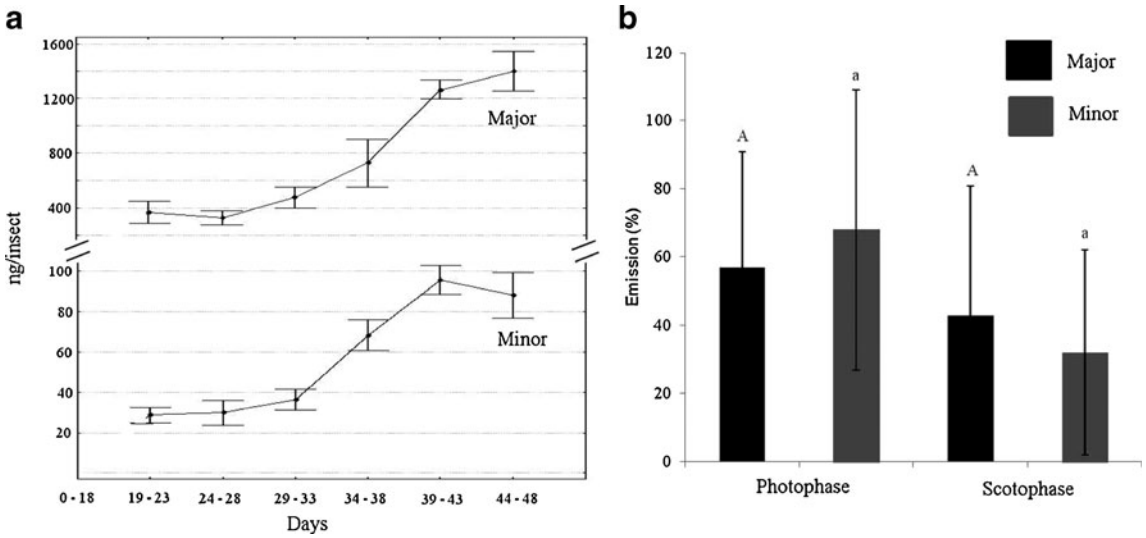


Fig. 3 **a** Emission dynamics of the male-specific compounds (major and minor) of *Oxelytrum discicolle*. **b** Emission of the male-specific compounds released by males of *O. discicolle* during photophase and scotophase (N=6). Columns followed by the same letter do not differ. Statistics: t test (* P<0.05)

(**T5**). Females preferred the combination (68 %, P=0.048), while males showed no preference (P=0.274, Fig. 2).

The dynamics of emission of the male-specific compounds (1–48 d) revealed that after 18 d of age, adults started to release both components. The major compound reached a maximum value of 1,400±188 ng/insect (44–48 d) and the minor compound 96±13 ng/insect (39–43 d) (Fig. 3a). Insects emitted these compounds during both photophase and scotophase (Fig. 3b), with no difference between the two phases (P>0.05).

For structure assignments of *O. discicolle* sex pheromones, the extracts of volatiles collected from males were analyzed by GC/MS and GC/FT-IR. The infrared spectrum of the major compound (**1**) (Fig. 4a) showed bands characteristic of a

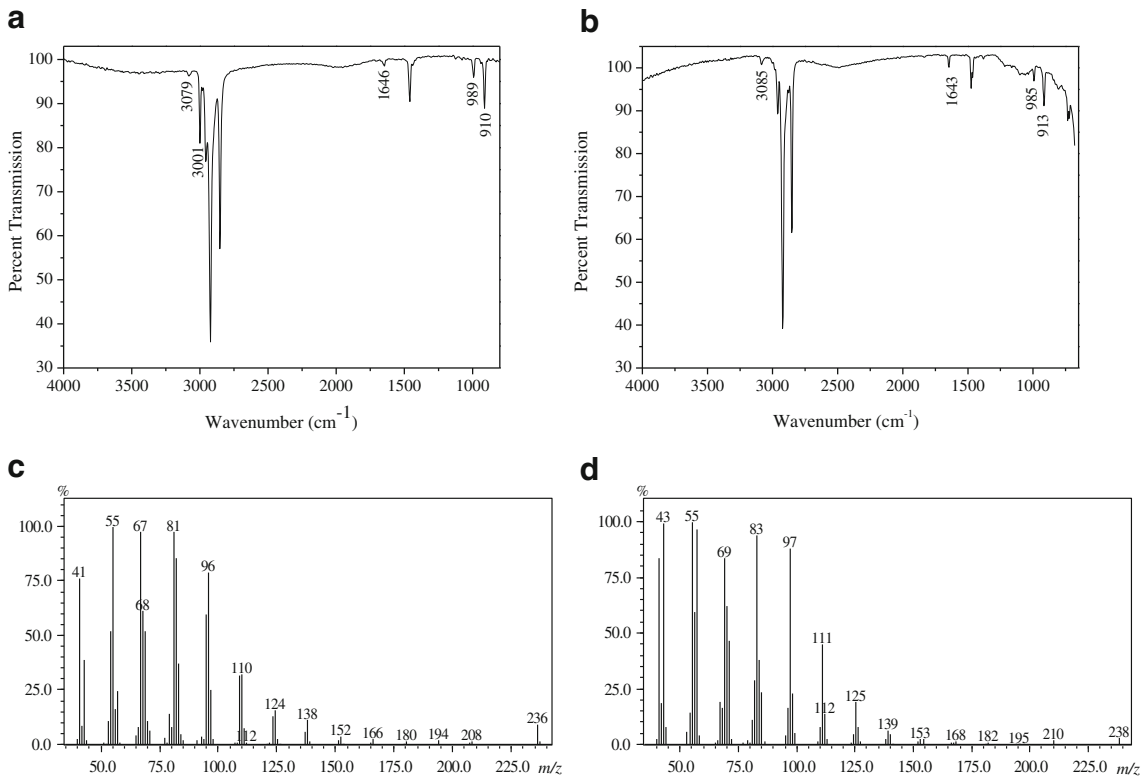


Fig. 4 Infrared and electron impact mass spectra of the male-specific major (**a** and **c**) and minor (**b** and **d**) compounds

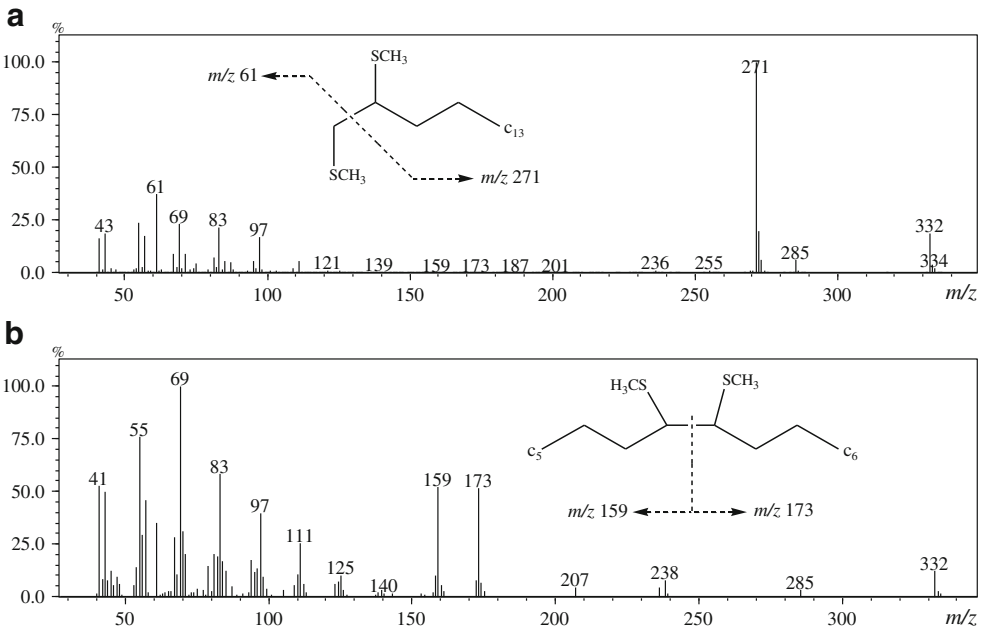


Fig. 5 Electron impact mass spectra of DMDS adducts, showing the position of the double bonds of the monounsaturated compounds. **a** adduct formed from the terminal double bond. **b** adduct formed from the double bond at carbon 8

hydrocarbon between 2800 cm^{-1} and 3000 cm^{-1}, and bands at 3079 cm^{-1} (out-of-phase $=CH_2$ stretch), 3001 cm^{-1} (*Z* =CH stretch), 1646 cm^{-1} (carbon-carbon double bond), 989 cm^{-1} ("*trans*" CH wag) and 910 cm^{-1} (Z =CH wag) (Attygalle 1994; Attygalle et al. 1994,1995; Nyquist 1984; Pouchert 1989; Smith 1999), suggesting the existence of a terminal double bond and a Z-configured double bond.

The infrared spectrum of the minor compound (**2**) (Fig. 4b) also showed the characteristic bands of hydrocarbons, but only one band characteristic of a double bond at 3085 cm^{-1}. The bands at 1643 cm^{-1}, 985 cm^{-1}, and 913 cm^{-1} supported the existence of a single terminal double bond in the molecule.

The mass spectra of both compounds were similar, exhibiting molecular ions at *m/z* 236 for **1** (Fig. 4c) and *m/z* 238 for **2** (Fig. 4d). Based on the information from the mass spectra and infrared spectra, the empirical formulas were proposed to be $C_{17}H_{32}$ for compound **1** and $C_{17}H_{34}$ for compound **2**.

To prove the presence of double bonds, the natural products were submitted to catalytic hydrogenation over Pd/C, resulting in a single product. The resulting product showed an M^+ at *m/z* 240, as a result of insertion of four hydrogens in compound **1,** and two hydrogens in **2**. The fragmentation pattern of the hydrogenated product strongly suggested it to be *n*-heptadecane, which was confirmed by co-injection with an authentic sample.

Determination of the position of double bonds using only GC/MS analysis was not possible without the use of derivatization. One of the best methods available involved the addition of dimethyl disulfide (DMDS) to the double bond

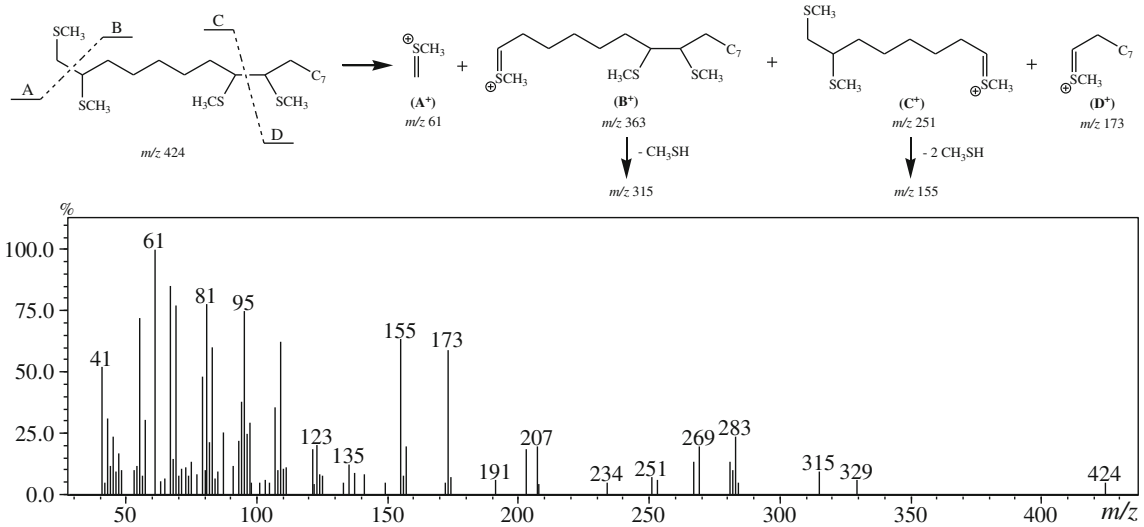
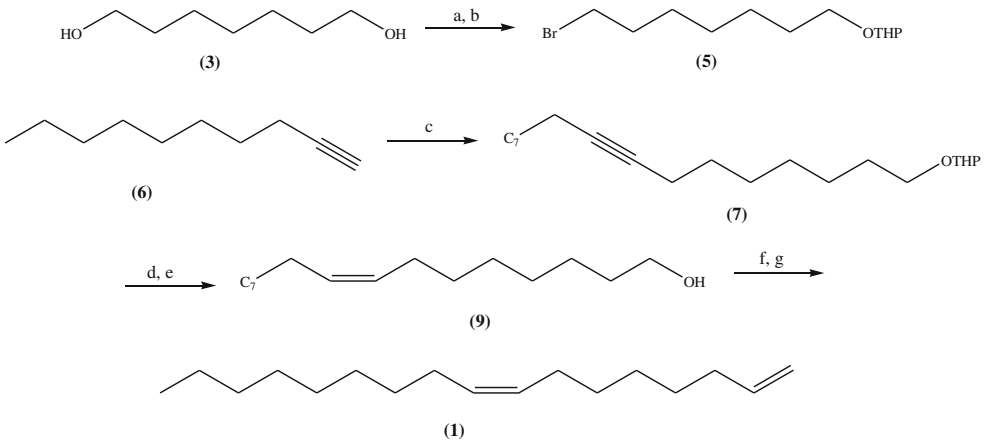


Fig. 6 Fragmentation scheme and EI-MS of the DMDS adduct of the natural diene

Scheme 1 Synthesis of (*Z*)-1,8-heptadecadiene. **a** HBr 48 %, 84 %, **b** DHP, *p*TSA, 90 %, **c** *n*-BuLi, **5**, 91 %, **d** *p*TSA, 93 %, **e** H_2, Lindlar, 77 %, **f** CBr_4, Ph_3P, 83 %, **g** *t*-BuOK, 76 %



followed by GC/MS analysis of adducts. However, the application of this method to polyunsaturated compounds was not straightforward, and the interpretation of mass spectra can be complicated without a standard for comparison. One way to apply the DMDS method to polyenes was to first carry out a partial reduction, employing diimine (produced from) hydrazine to produce monoenes, followed by DMDS reaction (Jham et al. 2005).

The proposed strategy was carried out, and two products were observed after the reaction of diene **1** with diimine. The mass spectrum of the first adduct showed intense fragments at *m/z* 61 and *m/z* 271, associated with the terminal double bond (Fig. 5a). The mass spectrum of the second adduct revealed fragments at *m/z* 159 and *m/z* 173, indicating a double bond for this adduct with an unsaturation at carbon 8 (Fig. 5b). However, knowing the natural product to be a diene with a terminal double bond, this second double bond could have been located at carbons 8 or 9.

To determine unambiguously the correct position of the second double bond in compound **1**, a direct DMDS derivatization was conducted with the extract of natural volatiles. Through mass spectral analysis of the resulting adduct, it was confirmed that the double bonds are located at carbons 1 and 8; the terminal position of one double bond was again confirmed by the fragments at *m/z* 315 (*m/z* 363–48 mass units) and at *m/z* 61, while the fragments at *m/z* 251, *m/z* 173 and *m/z* 155 (*m/z* 251–96 mass units) characterized the position of the second double bond of molecule at carbon 8. The addition of four thiomethyl groups to the final product resulted in a molecular ion at *m/z* 424 (Fig. 6).

Based on these data and the results of the GC/FT-IR analyses, (*Z*)-1,8-heptadecadiene was proposed as the major compound (**1**). Because of the total absence of the absorption at 980–965 cm^{-1} (a characteristic band diagnostic of the *trans*=CH wag, Attygalle 1994) it was possible to affirm that even small amounts of the *trans*-isomer was not present under the same GC peak. In addition, after the DMDS derivatization of the monoenes obtained by partial reduction of major component **1** with diimine, only one DMDS adduct resulting from an internal double bond could be detected by GC/MS analysis, confirming the existence of only one isomer.

To confirm this, a seven-step synthesis was developed to obtain **1**, according to Scheme 1.

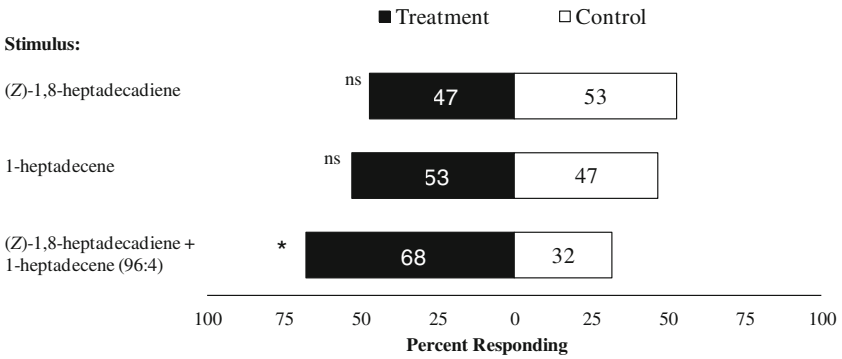


Fig. 7 Results of Y-tube bioassays testing the attraction of *Oxelytrum discicolle* females to synthetic (*Z*)-1,8-heptadecadiene (**1**) plus odors collected from a carcass, to synthetic 1-heptadecene (**2**) plus odors collected from a carcass, and to a blend of the two synthetic compounds **1** and **2** (94:6) plus odors collected from a carcass. The odors from carcass were used as the control of these experiments. The amounts of material used were 480 ng of the major (*Z*)-1,8-heptadecadiene (**1**), and 32 ng of the minor 1-heptadecene **2.** Statistics: *chi-square test* (* $P<0.05$)

As the initial step, a monobromination of the 1,7-heptanediol (**3**) was achieved to yield 7-bromoheptan-1-ol (**4**) at an 84 % yield (Chong et al. 2000), which was subsequently protected with DHP to give ether **5** in 90 % yield (Santangelo et al. 2002). The anion from 1-decyne (**6**), generated with *n*-BuLi, was alkylated with bromide **5** in 91 % yield providing the intermediate **7** (Kang and Park 1988), which was deprotected to yield alcohol **8** in 93 % yield (Zarbin et al. 2007). This intermediate was stereoselectively hydrogenated over $Pd/CaCO_3$ (Lindlar's reagent) to form (*Z*)-8-heptadecen-1-ol (**9**) in 77 % yield (Overman et al. 1993). The primary alcohol **9** was converted to the bromide **10** in 83 % yield (Hu et al. 2001), which upon elimination of HBr produced the desired diene **1** in 76 % yield (Manabe et al. 1985). The overall yield of the synthesis was 31 %.

The synthetic (*Z*)-1,8-heptadecadiene (**1**) was co-injected with the extract of volatiles of males, and co-elution with the major male-specific compound in three different GC columns was observed. Additionally, the mass and infrared spectra of the synthetic compound perfectly matched that of the natural compound. In addition, by co-injection of the same extract with an authentic sample of 1-heptadecene (**2**), the identity of the minor compound also was confirmed.

A Y-tube olfactometer was used to verify the biological activity of the synthetic male-specific compounds (Fig. 7). In the first test, only the major male-specific compound (**1**) plus extract of carcass volatiles was tested against the control (extract of carcass volatiles), and the females were not attracted to this compound (47 % for the treatment, 53 % for the control, $N=30$, $P=0.716$). In the second test, only the minor male-specific compound (**2**) plus extract of carcass volatiles was tested against the control (carcass extract), and the females again were not attracted to this compound (53 % for the treatment, 47 % for the control, $N=30$, $P=0.716$). In the third and final bioassay, a mixture of the two synthetic compounds (at the same ratio as found in the volatile extracts of males) plus extract of carcass volatiles against the control (extract of carcass volatiles) was tested. The results showed that females were significantly attracted to the mixture of compounds (68 % to the treatment, 32 % to the control, $N=30$, $P=0.040$). These data confirmed that both male-produced compounds work as sex pheromones of the species.

In summary, we determined the male-produced sex pheromones released by the carrion beetles *O. discicolle* as 1-heptadecene and (*Z*)-1,8-heptadecadiene. Moreover, we observed that males are responsible for food foraging and then release pheromonal components that call females, which are attracted only by the combination of the food volatiles and the volatiles released by males, exhibiting a synergism. Therefore, it is expected that *O. discicolle* males arrive sooner than females on cadavers. With further studies, the time that it takes between male arrival and female arrival may be accounted for in the PMI. Additionally, the knowledge that the insects take 18 days to begin pheromone production may be used to differentiate between males of the first wave that colonize the corpse, from those that are offspring of the first wave and remain in the corpse until they become adults. It is also possible to estimate the age of the first male adults reared on the corpse, using the time-lapse between beetle capture and start of pheromone production. The results contribute both to the understanding of the chemical ecology of *O. discicolle* and to its potential to improve the accuracy of PMI estimation.

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