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# Chemical Identification, Electrophysiological and Behavioral Activities of the Pheromone of *Metamasius hemipterus* (Coleoptera: Curculionidae)

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**Abstract**—Five hydroxylated aliphatic molecules were identified as the pheromone produced by male West Indian Sugarcane Borer (WISB): 4-methyl-5-nonanol (**1**), 2-methyl-4-heptanol (**2**), 2-methyl-4-octanol (**3**), 5-nonanol (**4**) and 3-hydroxy-4-methyl-5-nonanone (**5**). Electroantennographic recordings revealed antennal responses to compounds **1**, **2**, **3** and **4**. Significant EAGs were also recorded in response to pheromone compounds of weevils belonging to the same subfamily and structurally related to the WISB pheromone. The natural pheromone elicited aggregation behavior on WISB adults in laboratory bioassays. Copyright © 1996 Elsevier Science Ltd

## Introduction

*Metamasius hemipterus* L. (Coleoptera: Curculionidae), the West Indian Sugarcane Borer (WISB) is a weevil found in every country from Florida and West Indies to Uruguay and Northern Argentina and in Africa where it was accidentally introduced.[1] It is a pest on sugarcane, bananas, Bromeliads and on several palm species: coconut, African oil palm and ornamental palm trees.[2–4] In Colombia this weevil is strongly suspected to be a vector of the red ring disease caused by the nematode *Rhadinaphelenchus cocophilus* on oil palm trees.[5] The increasing importance of *M. hemipterus* in neotropical regions prompted us to undertake the study of its pheromone communication system in order to develop a monitoring system based on synthetic attractants.

Male aggregation pheromones have been recently described in several Curculionidae species belonging to the same subfamily, Rhynchophorinae as WISB.[6–8] Four of the five male-emitted compounds were identified in *M. hemipterus* without biological data.[9] The absolute configuration of the naturally occurring major pheromone compound was determined to be (4*S*,5*S*)-4-methyl-5-nonanol.[10] In this study we present the complete pheromone identification and the electrophysiological and behavioral activities of the male pheromone.

Key words: *Metamasius hemipterus*, pheromones, monomethylated alcohols, hydroxylated aliphatic molecules, analogues, behavioral activity, EAG activity.

## Results and Discussion

### Chemical identification of the pheromone compounds

The pheromone of WISB was evidenced by collecting the volatiles emitted by the males. Five components were revealed by analyses of the male volatile extracts on GC. Mass spectra in electron impact (EI) and chemical ionization with ammonia (CI/$NH_3$) of the compounds indicated that all pheromone compounds were hydroxylated aliphatic molecules (Table 1, Fig. 1).

**Compound 1.** The pairs of 18 amu-distant ions at *m/z* 87/69 and 101/83 in the EI mass spectrum of **1** indicated an hydroxyl group at C5. EI mass spectrum data also suggested that **1** was an α-methylated alcohol. The retention time ($t_R$) of **1** was intermediary to those of 5-decanol (**11**) and both 2,6-dimethyl-5-octanol (**7**) and 2,7-dimethyl-4-octanol (**8**) suggesting a monomethylated nonanol. Two compounds were candidates as pheromone component, the 3-methyl-4-nonanol (**9**) and the 4-methyl-5-nonanol (**1**). Coinjections of compounds showed that only **1** coeluted with the natural pheromone component. The major compound (**1**) could reach 80% of the blend.

**Compound 2.** An hydroxyl group at C4 was evidenced by the presence of the pair 18 amu-distant ions at *m/z* 73/55 and 87/69 in the EI mass spectrum and by the concordance of $t_R$s on GC columns of **2** and 3-methyl-4-octanol (**10**). Compound **2** was identified as 2-methyl-4-heptanol.

**Table 1.** Chemical characteristics of male-produced compounds of *M. hemipterus* (underlined fragment ions correspond to base peak in mass spectra)

| Compounds | Molecular weight | Retention time (min) | Mass spectral data | |
|---|---|---|---|---|
| | | | EI | $CI/NH_3$ |
| 2-Methyl-4-heptanol (**2**) | 130 | 12.65 | 41, 43, 45, 55, 69, 73, 87, 97, 112 | 130, 148 |
| 2-Methyl-4-octanol (**3**) | 144 | 16.51 | 41, 43, 45, 57, 69, 87, 111, 126 | 144, 162 |
| 5-Nonanol (**4**) | 144 | 18.17 | 41, 43, 45, 57, 69, 87, 126 | 144, 162 |
| 4-Methyl-5-nonanol (**1**) | 158 | 20.50 | 41, 43, 45, 55, 57, 69, 83, 87, 101, 140 | 158, 176 |
| 3-Hydroxy-4-methyl-5-nonanone (**5**) | 172 | 23.70 | 41, 43, 45, 55, 57, 59, 70, 72, 85, 97, 103, 114, 115, 125, 143, 154 | 132, 115, 173, 190 |

**Compounds 3 and 4.** The same hydroxyl groups at C4 were found on **3** and **4**, according to the presence of the pair 18 amu-distant ions at *m/z* 87/69 in both EI mass spectra. However, the differences on $t_R$s and the presence of an ion at *m/z* 111 on the EI spectrum of **3** suggested that **3** was a methyl-branched alcohol and **4** a linear one. Homologous fragmentation of **2** and **3** such as GC and GC–MS data comparisons of commercial **10** and **4**, showed that **3** was 2-methyl-4-octanol and **4** was 5-nonanol.

**Compound 5.** The identification of **5** is reported here for the first time. Mass spectra by $CI/ND_3$ exhibited ions at 137 (100%), 156, 175 and 195 confirming the $M_r = 172$, expected since ions *m/z* 132, 155, 173 (100%) were present on the mass spectrum of $CI/NH_3$. Ions at *m/z* 175 and 195 revealed the presence of an exchangeable hydrogen due to an alcoholic function (in agreement with loss of water observed in the EI mass spectrum). The spectrum and $t_R$ of **5** led us to propose a $C_{10}H_{20}O_2$ formula. After obtaining a few micrograms of pure compound by micropreparative GLC, the structure of **5** was deduced from examination of its spectroscopic data.

$^1H$ NMR spectrum ($CDCl_3$, 400 Mhz) of **5** exhibited resonances for three methyl groups [δ (ppm) 0.88 (t, 6.5 Hz), 0.93 (t, 6.5 Hz), 1.1 (d, 7 Hz)], a shifted methylene group [δ (ppm) 2.45 (dt, 15, 7 Hz), 2.5 (dt, 15, 7 Hz)] and two coupled methine groups [(δ (ppm) 3.58 (m), 2.6 (qd, 6.6 Hz)], the second (δ 2.6 ppm) being also coupled with the third methyl group (δ 1.1 ppm), thus indicating the presence of a $—CH_2CH_2C(=O)CH(CH_3)CH(OH)CH_2—$ substructure. Due to the small quantity of material available, we were not able to determine from the NMR data the length of the aliphatic side chains of the molecule. However, mass spectroscopic data suggested the presence of three additional carbon atoms, allowing us to postulate two hypothetical structures, I and II (Fig. 2).

These results were confirmed and the structure of the aliphatic side chains was determined by examining the result of the aldolisation reaction of 3-heptanone with propanal (LDA, THF, −60 °C): as expected, four regio- and stereoisomers were obtained, one being identical to **5** (GC–MS and $^1H$ NMR). These results prompted us to assign structure II to compound **5**.

## Pheromone detection

**Electroantennographic (EAG) screening of pheromone analogues.** The sensitivity of WISB antennae to the pheromone components was evaluated by comparing the EAG activity of 16 pheromone analogues with that of pheromone compounds (Table 2). Olfactory responses profiles were obtained on males and females (Fig. 3).

Four of the five pheromone components (**1**, **2**, **3** and **4**) elicited high EAG amplitudes on males and females, as did all monomethylated alcohols except 3-methyl-5-nonanol. In contrast, **5** and other ketones tested had a

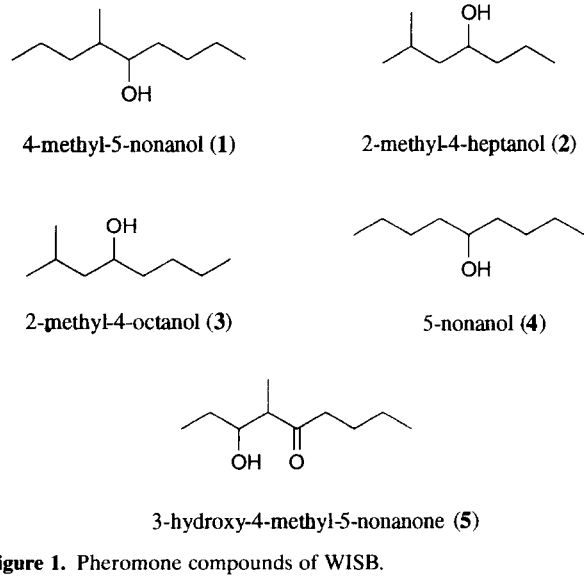


**Figure 1.** Pheromone compounds of WISB.

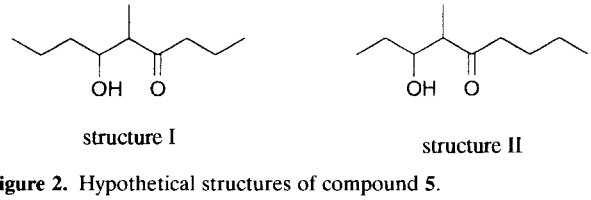


**Figure 2.** Hypothetical structures of compound 5.

**Table 2.** Compounds used as stimulus in the EAG assays

| Compound | Chemical purity | Source | Dose |
|---|---|---|---|
| Pheromone compounds | | | |
| 4-Methyl-5-nonanol (**1**) | >90% | [a] | 1–2 |
| 2-Methyl-4-heptanol (**2**) | >90% | [a] | 1–2 |
| 2-Methyl-4-octanol (**3**) | >99% | [b] | 1–2 |
| 5-Nonanol (**4**) | >99.5% | [c] | 1–2 |
| 3-Hydroxy-4-methyl-5-nonanone (**5**) | 30% | [a] | 1–2 |
| Analogue compounds | | | |
| 3-Methyl-1-pentanol | >99% | [d] | 2 |
| 5-Methyl-4-heptenol | >90% | [a] | 2 |
| 2-Methyl-5-(*E*)-hepten-4-ol (**6**) | >90% | [a] | 1–2 |
| 4-Hydroxy-6-methyl-2-heptanone | >90% | [a] | 2 |
| 2-6-Dimethyl-4-octanol (**7**) | >90% | [a] | 2 |
| 3-Methyl-4-octanol (**10**) | >90% | [c] | 2 |
| 4-Methyl-5-octanol (**14**) | >90% | [a] | 2 |
| 2-7-Dimethyl-4-octanol (**8**) | >90% | [a] | 2 |
| 2-(*E*)-Octen-4-ol | >90% | [a] | 2 |
| 3-Methyl-4-octanone | >90% | [a] | 2 |
| 4-Methyl-5-octanone | >90% | [a] | 2 |
| 2-Methyl-4-nonanol (**12**) | >90% | [a] | 2 |
| 3-Methyl-4-nonanol (**9**) | >90% | [a] | 2 |
| 3-Methyl-5-nonanol | >90% | [a] | 2 |
| 4-Methyl-5-nonanone | >90% | [a] | 2 |
| 4-Hydroxy-5-methyl-6-nonanone (**13**) | >90% | [a] | 2 |

[a]Laboratoire des Médiateurs Chimiques.
[b]ICN Biomedicals (U.S.A.).
[c]Fluka (France).
[d]Lancaster synthesis (U.K.).
1: Six doses of each compound were tested: 100 pg, 1 ng, 10 ng, 100 ng, 1 μg, 10 μg.
2: One dose of 500 ng was tested.

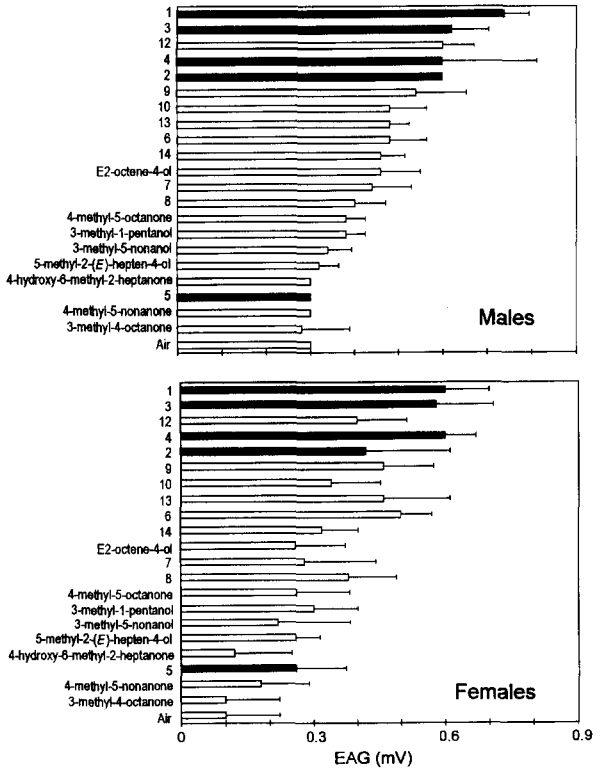


**Figure 3.** Electroantennogram screening with a series of pheromone compounds and analogues tested at the dose of 500 ng. Black bars represent the pheromone compounds.

weak EAG activity. No difference in response profile was noted between sexes.

**EAG dose–response curves.** Compounds **1**, **2**, **3**, **4**, **5** and **6** were tested over a dose range from 100 pg to 10 μg (Fig. 4). Compound **6**, the pheromone of *Rhynchophorus palmarum* was selected because of its attractiveness in the field to *M. hemipterus* adults (Calvache, personal communication). EAG responses to all compounds, but **5**, increased with their dosage. The flat dose–response curve of **5** is in good agreement with its weak activity in the screening experiment.

The highest responses were obtained from females to the highest doses of **2**, **3** and **6**. Their detection thresholds were between 1 and 10 ng of compound loading the stimulus cartridge. Compounds **1** and **4** showed a weaker activity at high dosages with a lower slope of their dose–responses curves. In the four most active compounds (**1**, **2**, **3** and **6**), the male EAG response level was lower than the female one. Saturation dosages did not seem to be reached at the highest tested dosages (10 μg) at least for compounds **1** and **6** on both sexes and for **2** on males.

Screening experiments with pheromone components and analogues demonstrated that four of the five pheromone compounds were well detected by the antennae of male and female WISB. Compound **5** had no EAG activity on males and females both in

screening and in dose–response tests, even at the highest dosages. However, no definitive conclusion can be drawn about its level of perception because of its low chemical purity. Two isomers were obtained by the chemical synthesis and the active compound only represented 15% of the loading of the stimulus cartridge. Olfactory response profiles with pheromone compounds and analogues confirmed that monomethylated alcohols were better detected by male and female antennae than ketones.

In dose–responses curves males and females had the same detection threshold to the four active pheromone compounds (**1**, **2**, **3** and **4**). In contrast, responses to high dosages of these compounds were higher in females than in males. These four EAG active compounds were detected on a wide range of doses. As opposed to minor pheromone compounds, the saturation level did not seem to be reached for **1** at the highest dose tested. Detection of a compound over a wide range of concentrations would indicate insect's capability to orient themselves to the source over distance.[11]

Compound **1**, the major constituent of the pheromone secretion which is also a pheromone component of *Rhynchophorus vulneratus* and *Rhynchophorus ferrugineus*[12] elicited high responses both on males and on females. Males and females detected other compounds structurally related to their own pheromone components but belonging to the pheromone of other Rhynchophorinae weevils. This is the case for **6** the pheromone of *Rhynchophorus palmarum*,[6] which is a sympatric species with WISB, for **10** the pheromone of *R. phoenicis*,[8] *R. vulneratus*[9,12] and for **14** the pheromone of *Rhynchophorus cruentatus*.[13]

## Biological role of the pheromone

Bioassays of the male volatile collections both on males and females were carried out in a 4-way olfactometer. Unmated females spent significantly more time in the pheromone odorized zone than in the odorless zones (Fig. 5). Mated females were not attracted by the pheromone zone. Mated and unmated males were equally attracted by their own pheromone. Considered

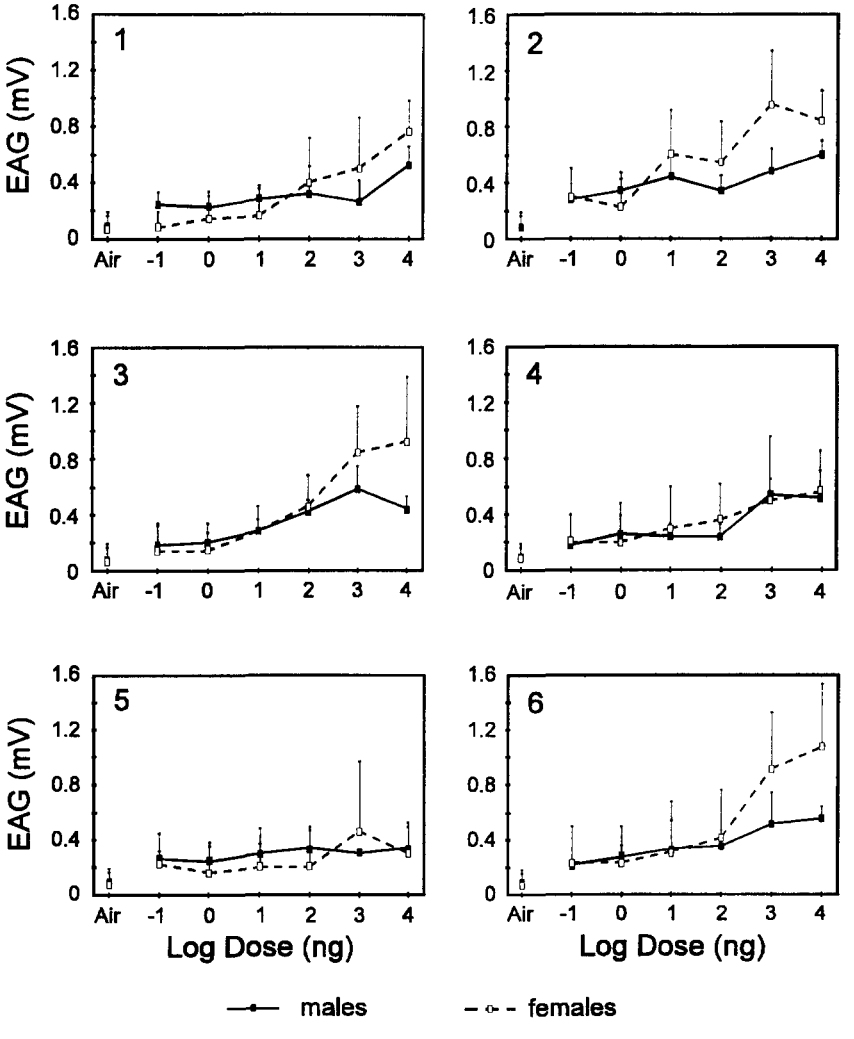


**Figure 4.** Average electroantennogram responses to stimulations with graded doses of the pheromone compounds (**1–5**) and Rhynchophorol (**6**).

as a single group (mated+unmated), males spent significantly more time in the pheromone zone. The stimulus used during the tests evoked a searching behavior to the pheromone source in *M. hemipterus*, since males and unmated females showed a significant attraction to the pheromone zone. Attracted weevils were observed walking faster toward the pheromone source, raising up the antennae. When they started to leave the pheromone zone, they immediately turned back. The insect's final choice was often to stay hidden in the arm of the pheromone zone.

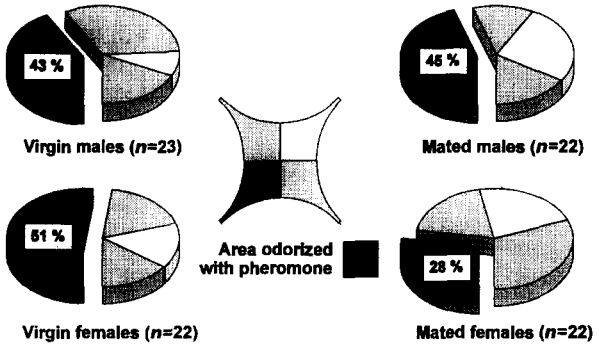


**Figure 5.** Mean percentage of time spent by WISB in odorized area with natural pheromone (2 MDE) and in odorless zones, in a four-way olfactometer.

Both males and females of *M. hemipterus* were attracted to the male pheromone. As defined by Borden,[14] an aggregation pheromone is a substance produced by members of either or both sexes inducing members of both sexes to aggregate. Thus, the male volatiles constitute a true aggregation pheromone. This is the first demonstration of pheromonal communication in this weevil. Pheromone chemistry and behavior elicited by the components are similar to those observed on closely related Rhynchophorinae (Fig. 6): *R. palmarum*,[6,7,9] *R. phoenicis*,[8] *R. cruentatus*[13] and *R. vulneratus*.[12] In the laboratory WISB pheromone was attractive alone, without any combination with host–plant volatiles. In the field insects are permanently exposed to host–plant volatiles eliciting synergistic effects with the pheromone. This common phenomenon reported in many Coleoptera species[11] also occurs in WISB.[15]

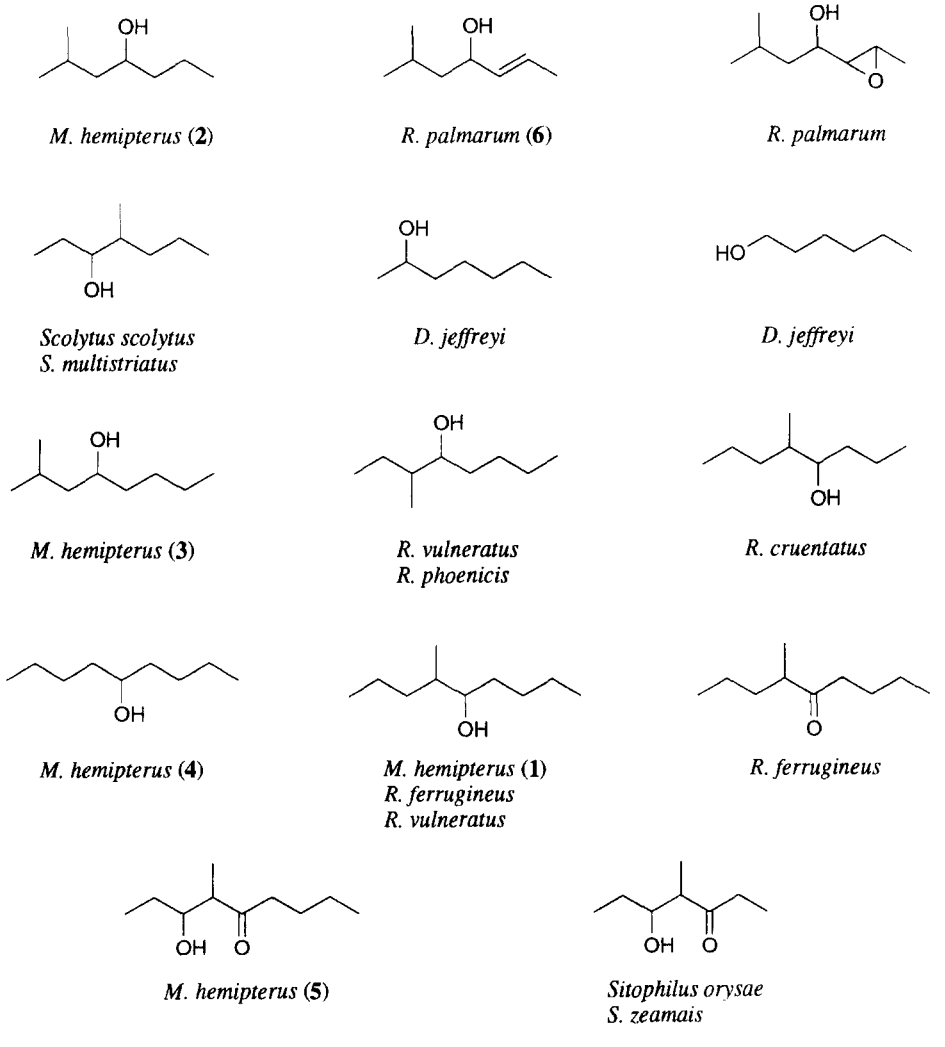


**Figure 6.** Pheromone compounds of Scolytidae beetles and Rhynchophorinae weevils structurally related to the pheromone compounds of WISB.

A sexual secondary function of the pheromone could be suggested since mated females did not respond to the male volatiles in contrast to virgin females which were significantly attracted by the male pheromone ($p < 0.05$). In preliminary tests performed in the laboratory, at a short distance from a weaker pheromone source (0.5 male day equivalent) in a windless olfactometer, females but not males were attracted to male volatiles. The response of mated and unmated males to their pheromone was similar, suggesting a polygamous character of the species as in *Sitophilus zeamais* (Coleoptera: Curculionidae).[16]

Several roles of the aggregation pheromone in nature were proposed.[11,17] This pheromone may serve to aggregate conspecifics around alimentary sources. It may also serve in recruitment of a sexual partner (aggregated insects easily find a sexual partner). The pheromone could trigger off aggregation at a suitable place (safe place) as a means to escape harmful effects of the environment (predators). Aggregation pheromones are powerful tools for insects among many others which ensure them success in the colonizing new habitats.

Chemical identification of the pheromone and the determination of its aggregative role opens the way for synthesizing field pheromone attractants in order to control *M. hemipterus* populations.

## Experimental Section

### Pheromone collections

The pheromone used both for chemical identification and as a stimulus in olfactometer tests was isolated by volatile collections on 15 wild males from French West Indies. They were placed in a cylindrical glass jar (6 × 22 cm) with moist filter paper and without food to avoid volatile contamination in the pheromone collection. Volatiles were collected daily at an airflow of 400 mL/min, in a room maintained at 25–28 °C, during the last 6 h of photophase, the period of pheromone release.[18] The released weevil volatiles were trapped on cartridges containing 0.5 mg of Supelpak™-2 (16–50 mesh) and were removed every 3 days, i.e. every 45 male day equivalents (MDE). Total weevil volatiles were eluted from Supelpak™-2 with 6 mL of dichloromethane (HPLC, SDS) during 6 h at −30 °C and concentrated at 535 μL under nitrogen flow (150 mL/min).

### Chemical identification

**Analysis conditions.** Pheromone extracts in dichloromethane were analyzed by GC and GC–MS. GC analyses were conducted on two gas chromatographs, a Carlo Erba Fractovap 2900 and a HP 5890, equipped with split-splitless injectors (225 °C) and a flame ionization detector at 240 °C. Chromatographs were equipped with a FFAP–CB (Chrompack, Middelburg, The Netherlands) polar phase column (WCOT 25 m × 0.32 mm i.d.), programmed from 35 to 55 °C at 20 °C/min, 8 min at 55 °C and from 55 to 220 °C at 5 °C/min. A CPSil-8CB apolar phase column temperature programmation was from 35 to 60 °C at 35 °C/min, 5 min at 60 °C and from 60 to 220 °C at 5 min. Helium was the carrier gas at 0.8 bar pressure. The extracts were analyzed by GC–MS on a Girdel 32 chromatograph linked to a Nermag R10–10C quadrupole analyzer piloted by a SIDAR acquisition system. The chromatograph was equipped with Ross injector (180 °C) and a nonpolar fused silica capillary column (25 m × 0.32 mm i.d.) WCOT CPSil-8CB from Chrompack (Middelburg, The Netherlands) with helium as carrier gas at a 0.3 bar pressure and operated for 3 min at 100 °C and from 100 to 220 °C at 5 °C/min.

Micropreparative gas chromatography was carried out using a Girdel 300 chromatograph equipped with a Ross injector (180 °C), a flame ionization detector (220 °C) and a fraction collector.[19] A 15 m × 0.53 mm ID apolar fused silica capillary column WCOT DB-1 (J&W Scientifics, U.S.A.) was operated isothermally at 80 °C with helium at a 0.3 bar pressure.

Spectral data were obtained in electron impact at 70 eV and chemical ionization at 92.5 eV using ammonia and deuteriated ammonia as reactant gases. The IR spectra were recorded by GC–FTIR spectrometry coupling.

**Reference compounds.** They were obtained from commercial sources and by synthesis in our laboratory: **3** from ICN Biomedicals (U.S.A.), **9** and **4** from Fluka (France). Compound **8** was prepared by catalytic microhydrogenation of synthetic ipsdienol (Borregaard); **1**, **2**, **7**, **9** and **11** were synthesized by reacting adequate aldehydes with Grignard reagents.[9]

**Synthesis of 4-methyl-5-nonanone (5): aldolisation of 3-heptanone and propanal.** A solution of *N,N*-diisopropylamine (4.65 g, 46 mmol) was prepared in 50 mL of tetrahydrofuran at −78 °C. To this solution was added 28.75 mL of *n*-butyllithium (1.6 N in hexane, 46 mmol). The resulting solution was allowed to warm to room temperature for 1 h, then recooled to −60 °C and 5.2 g (50 mmol) of 3 heptanone in 10 mL of tetrahydrofuran were added, maintaining this mixture at −60 °C for 20 min. The resulting mixture was recooled to −78 °C and 3 g (51 mmol) of propionaldehyde in 10 mL of tetrahydrofuran were added dropwise. The reaction was stirred 0.5 h and then hydrolyzed with a solution of ammonium chloride, extracted with diethyl ether and washed with brine. The organic layer was dried on magnesium sulfate and concentrated to give 7.9 g (45 mmol, 100%) of a mixture of four regio- and stereoisomers.

### Electrophysiology

**EAG recordings.** Electrophysiological measurements were carried out on unmated male and female adults

between 22 and 42 days after the adult emergence. The whole insect was restrained in a styrofoam block. Head and antennae were maintained with entomological needles. The left antenna was removed to implant the reference electrode and the recording electrode was inserted in a small hole (∅ 20 μm) made with a sharpened tungsten needle on the tip of the antennal club of the right antenna. Both electrodes were glass capillaries filled with Roeder's solution (NaCl: 9.0 g/L, KCl: 0.2 g/L, glucose: 4.36 g/L) connected to a Microprobe 725 WPI preamplifier through chlorinated silver wires. Electroantennogram responses were filtered (dc to 300 Hz) and displayed on a Tektronix 5510 digitizing oscilloscope.

**Odorous stimuli.** Chemicals used as olfactory stimuli are listed in the Table 2. Most compounds were synthesized in the Laboratoire des Médiateurs Chimiques. Absolute purities were at least 90%, controlled by GC stimulus compounds were diluted in *n*-pentane (99% for traces analyses, SDS, France) and delivered as 1 μL sample deposited on 1 $cm^2$ filter paper inserted into a glass cartridge (Pasteur pipette). A cartridge with a clean filter paper was used as control. During electroantennogram tests, glass cartridges were oriented toward the preparation at 5 cm from the antenna. A permanent flow of humidified pure air (2.6 L/min) was blown out over the antenna. A stimulation consisted in an air-puff at 0.5 L/min during 0.5 s. An interval of 4 min separated two consecutive stimulations in order to avoid sensory adaptation.

For dose–response studies, six doses (Table 2) of each compound were presented at random, but in ascending order of concentration. Approximately 2 min after the last stimulation a control stimulation was carried out to verify the absence of any contamination in the stimulation set-up. In the screening assay doses were of 500 ng of each compound. Five males and five females were tested in dose–response curves as in screening tests.

## Behavioral tests

**Insects.** Experimental adults were laboratory-reared from strains originating in the French West Indies and in Colombia. In the laboratory, weevils (larvae and adults) were fed with fresh sugarcane and were maintained at $27 \pm 2$ °C, $85 \pm 10$% relative humidity under a 12:12 light–dark regime.

**Olfactometer.** The response of *M. hemipterus* weevils to conspecific male volatiles was tested in a four-armed olfactometer.[20] The square exposure chamber dimensions were $32 \times 32 \times 1.2$ cm high. Four odor fields were created with a vacuum pump/compressor sucking up air through the central hole on the floor of the chamber. The airflow rate was maintained at 400 mL/min/arm. Each arm was connected to a 50 mL glass vial. The center of the chamber's floor was used as a starting test zone. Light intensity on the olfactometer was controlled at 2 lux. A video camera (Cohu 4722) was positioned over the olfactometer in order to record each experiment on VHS video system (Panasonic AG 7330).

**Bioassay.** Pheromone response of four groups of insects were compared: mated males, mated females, unmated males and unmated females (22 replicates/group). Weevils were preconditioned in the assay room 1 h before the test by keeping them individually on moist filter paper. Experiments were conducted during the 2 h following the offset of the photophase, in a darkened room maintained at $27 \pm 2$ °C. The pheromone stimulus obtained by volatile collections was delivered as a 5 μL sample (2 MDE) on a filter paper ($1 \times 1$ cm), inserted into one of the 50 mL glass vials. The other three vials kept empty provided standard clean air. Insects were tested individually and the stimulus was replaced at each replicate. The test started when the insect left the central hole of the chamber's floor and lasted 3 min. The stimulus zone rotated 90° daily (after each set of 8 replicates) eliminating bias inherent to the stimulus position in the olfactometer.

Bioassay data were analysed with a laboratory written software that recorded time spent per odor field during the 3 min of test, discarding the time spent by the insect hidden on one of the olfactometer arms. Data were subjected to ANOVA on the transformed variable by the square root of $X+0.5$.[21] Means of percent time spent per odor field were compared by Newman–Keuls test with a significant level of $\alpha = 0.05$.

## Acknowledgments

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