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POLLINATION BY SEXUAL MIMICRY IN *Mormolyca ringens*: A FLORAL CHEMISTRY THAT REMARKABLY MATCHES THE PHEROMONES OF VIRGIN QUEENS OF *Scaptotrigona* sp.

ADRIANA FLACH,[1] ANITA J. MARSAIOLI,[1,*] RODRIGO B. SINGER,[2] MARIA DO CARMO E. AMARAL,[2] CRISTIANO MENEZES,[3] WARWICK ESTEVAM KERR,[3] LUCIANE G. BATISTA-PEREIRA,[4] and ARLENE G. CORRÊA[4]

[1]*Chemistry Institute, State University of Campinas, PO Box 6154, 13084-971 Campinas, SP, Brazil*

[2]*Biology Institute, State University of Campinas (UNICAMP), PO Box 6109, 13084-971 Campinas, SP, Brazil*

[3]*Genetics and Biochemistry Institute, Federal University of Uberlândia, PO Box 593, 38400-902 Uberlândia, MG, Brazil*

[4]*Chemistry Department, Federal University of São Carlos, 13565-905 São Carlos, SP, Brazil*

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Abstract—The chemical composition of some volatile (2-heptanol) and nonvolatile constituents (a homologous 9-alkene/alkane series) of *Mormolyca ringens* flowers and *Scaptotrigona* sp. queen waxes (homologous 9-alkene/alkane series) and cephalic extracts (homologous series of 2-alkanols, including 2-heptanol) involved with the pseudocopulation or sexual mimicry in Orchidaceae pollination is compared. The similarity in chemical composition of flowers and insects is assigned to the chemically induced copulatory activity in *Scaptotrigona* males.

Key Words—*Mormolyca ringens*, chemical constituents, Orchidaceae, queens, *Scaptotrigona postica*, sexual mimicry, waxes.

* To whom correspondence should be addressed. E-mail: anita@iqm.unicamp.br

INTRODUCTION

Pseudocopulation or sexual mimicry is a remarkable and puzzling pollination strategy within the Orchidaceae. Flowers pollinated through pseudocopulation offer no floral reward to their pollinators and display sets of characters that prompt visitation and pollination by sexually excited male insects (mostly Hymenoptera; Kerr and Lopes, 1962; Van der Pijl and Dodson, 1966; Dressler, 1993; Ayasse et al., 2000). These flowers emit fragrances that mimic the sexual pheromones of virgin insect females (Borg-Karlson, 1990; Borg-Karlson and Tengö, 1986). Visual clues, such as coloration and floral indument of the labellum (median petal), reinforce the insect-like appearance of these flowers (Kullenberg, 1961; Van der Pijl and Dodson, 1966). In this pollination strategy, sexually excited male insects arrive at flowers and attempt copulation (normally with the median petal). As in most orchids, the pollen is packed into discrete units called pollinia. Additional flower secretions or structures hold the pollinia together. The pollinia and these secretions or structures form a complex pollen-dispersal unit or pollinarium (plural: pollinaria) (Singer and Koehler, 2004). During attempts by male insects to copulate with the flower, the pollinaria stick to the insects' body surface (Kullenberg, 1961; Van der Pijl and Dodson, 1966; Dressler, 1993). Insects laden with pollinaria then promote pollination during successive visits to other flowers by depositing the pollinia (or parts of them) on the surface of the floral stigma (Kullenberg, 1961; Van der Pijl and Dodson, 1966; Dressler, 1993).

Pollination through pseudocopulation has been particularly well documented in terrestrial orchids of the subfamily Orchidoideae from Europe, Africa, and Oceania (Kullenberg, 1961; Van der Pijl, 1995, 2001; Borg-Karlson, 1990; Borg-Karlson and Tengö, 1986; Schiestl et al., 2000; Ayasse et al., 2000, 2003). The pollination strategies of these taxa are closely associated with the biology of seasonal insects (Van der Cingel, 1995, 2001). The insects involved in pollination by pseudocopulation in orchids in the Old World are solitary bees and wasps. As a rule, these plants flower only when male insects are around, and, consequently, the males mistake the flowers for their respective female partners and promote pollination (Kullenberg, 1961; Van der Pijl and Dodson, 1966). When females finally emerge, males may be able to recognize "true females" and avoid the flowers (Van der Pijl and Dodson, 1966; but, see Ayasse et al., 2003). Consequently, pollination in these orchids is usually low (Neiland and Wilcock, 1998) and limited to a brief period of time prior to female emergence (Kullenberg, 1961; Van der Pijl and Dodson, 1966). Although a number of Neotropical orchid genera have been suggested as being pollinated by pseudocopulation (Van der Pijl and Dodson, 1966), these reports are preliminary and lack the details of literature on Afro-European or Australian taxa (Kullenberg, 1961; Borg-Karlson, 1990; Borg-Karlson and Tengö, 1986;

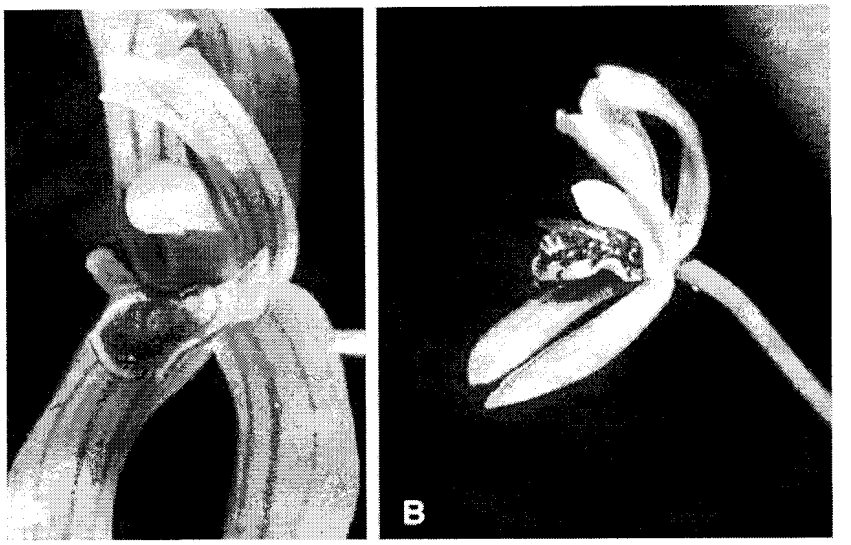

FIG. 1. (A) Flower of *Mormolyca ringens* (Orchidaceae: Maxillariinae) in frontal view. (B) *Scaptotrigona* drone attempting copulation with a flower of *M. ringens*.

Borg-Karlson, 1990; Van der Cingel, 1995; Ayasse et al., 2000, 2003; Schiestl et al., 2000; Van der Cingel, 2001). Recently, pollination through pseudocopulation has been reported for *Trigonidium obtusum* Lindl. and *Mormolyca ringens* (Lindl.) Schltr. (Figure 1A and B; both belonging to the subtribe Maxillariinae, sensu Whitten et al., 2000; Kerr and Lopes, 1962; Singer and Koehler, 2004; Singer, 2002; Singer et al., 2004). Interestingly, sexually excited drones of Meliponini pollinate both orchid species. In contrast to cases documented in the Old World, these bees are eusocial (i.e., there are castes, and there is division of labor in the hives). The hives are perennial and produce fertile individuals several times a year. In agreement, plants of *T. obtusum* and *M. ringens* produce flowers throughout most of the year (Singer, 2002; Singer et al., 2004). Gas chromatography–mass spectrometry (GC-MS) analysis of *T. obtusum* floral fragrance reveals that pentadecane is the main constituent (over 95%; Flach et al., 2004). In contrast, the fragrance of *M. ringens* contains 31 main components (Singer et al., 2004) and is visited and pollinated by *Scaptotrigona* and *Nannotrigona testaceicornis* (Apidae: Meliponini).

As part of our multidisciplinary studies on Maxillariinae orchids pollinated by pseudocopulation, we investigated the floral chemistry of *Mormolyca* and compared it with that of virgin queen bees, assuming that similar chemical components should be present in both organisms. Specifically, we wished to determine and compare the floral chemistry of *M. ringens* and the sex pheromone of virgin queens of *Scaptotrigona* sp. (Meliponini). *Scaptotrigona* is one of the two Meliponini genera recently reported to pollinate this orchid

(Singer et al., 2004; Figure 1A and B). There are around eight species of *Scaptotrigona* in Brazil (Silveira et al., 2002), but they vary in morphology and behavior and represent a taxonomically difficult group. Readers interested in the biology, taxonomic details, and phylogenetic affinities of *Scaptotrigona* bees are referred to Silveira et al. (2002).

METHODS AND MATERIALS

Plant Material. Fresh flowers were obtained from plants grown in the orchidarium of the Escola Superior de Agronomia Luiz de Queiroz, Universidade de São Paulo, Piracicaba, São Paulo State, Brazil. Voucher specimens (ESA 7247, ESA 16802, and ESA 1648) were deposited in the ESA and UEC herbaria. Insect voucher specimens were deposited in the Laboratório de Abelhas (Instituto de Biociências, Universidade de São Paulo, São Paulo State, Brazil).

Flower Extract. Pollinated ($N = 5$) and unpollinated ($N = 4$) flowers were extracted by immersion in bidistilled hexane (500 µl) for 24 hr. The hexane was evaporated to 50 µl under a nitrogen stream, and 1 µl was analyzed by GC-MS.

Rearing Meliponini Queens. Young larvae were transferred to artificial cells (6.6-mm diam, 8.8 mm high), where the amount of food was controlled. To obtain complete success (100% of female larvae developing into queens), the amount of food necessary to develop a worker (35.4 mg) was tripled (106 mg).

Artificial cells and their caps were made from molded *Apis mellifera* L. wax. Food for larvae was harvested from 100 natural cells from a *Scaptotrigona* colony. Artificial cells were incubated at 28°C, with ca. 75% RH that was sustained by using a saturated KCl solution. Pupae were transferred to new cells to avoid fungal infection. Ten queens were reared and manually fed every 2 hr with a syrup of honey, pollen, and water. Only queens were reared. In addition, bees were kept in captivity until they were sacrificed and excludes the possibility that these bees were fertilized.

Extraction of Cuticular Waxes from Virgin Queens. The waxes of 10 virgin queens of *Scaptotrigona* sp. were extracted by washing their abdomens with bidistilled hexane (500 µl). The solvent was reduced to 50 µl under a nitrogen stream, and 1 µl was analyzed by GC-MS.

Virgin Queens Cephalic Extract. The heads of 10 virgin queens of *Scaptotrigona* sp. were crushed and extracted with 200 µl of bidistilled ethyl acetate. After 10 min, the solution was filtrated, and the solvent was evaporated to 50 µl under a nitrogen stream, and 1 µl was analyzed by GC-MS.

GC-MS Analysis. GC analysis was carried out using a Hewlett Packard 6890 apparatus fitted with an HP-5 fused silica capillary column (30 m × 0.25 mm ×

0.25 μm). A sample volume of 1 μl was injected, and pressure programming was used to maintain a constant flow (1 ml/min) of the helium carrier gas. A Hewlett Packard 5973 mass spectrometer was used in the EI mode (ionization energy of 70 eV) and set to scan the mass range of 50–700 u at a rate of 2.94 scans/sec. The interface temperature was maintained at 280°C. The resulting data were processed using the Hewlett Packard Chemstation Software package. The temperature program was from 50 to 310°C at 4°C/min, plus 40 min at the final temperature. The injector temperature was 240°C.

Identification of Alkanes. Alkanes identification was confirmed by comparing mass spectra and retention times with those of standard alkanes and by using a Wiley275 database.

Derivatization of Alkenes. Extracts (0.3–0.5 mg) were solubilized in 50 μl of dimethyldisulfide and 10 μl of a 10% solution of iodine in ethyl ether. The reaction mixture was kept at 50°C for 12 hr, and the reaction was quenched with aqueous $Na_2S_2O_3$. The organic phase was extracted with hexane (0.5 ml) and evaporated to 50 μl under a nitrogen stream, and 1 μl of the solution was analyzed by GC-MS. The dimethyldisulfide adducts were identified, and the positions of the methylsulfide substituents were deduced from the fragmentation pattern.

Trans-Cinnamic Acid Methyl Ester. Cinnamic acid (1 mg) was treated with 200 μl of a diazomethane solution in ethyl ether at room temperature for 15 min. The solvent was evaporated and the product was analyzed by GC-MS and ^{1}H nuclear magnetic resonance (NMR). EIMS (70 eV): *m/z* (%) = 162 (M^+, 50%), 147 (2%), 131 (100%), 103 (74%), 77 (36%), 51 (12%). ^{1}H NMR (300.067 MHz, $CDCl_3$): δ = 3.81 (*s*, 3H), 6.45 (*d*, 1H, J = 15.9 Hz), 7.38 (*m*, 3H), 7.53 (*dd*, 2H), 7.70 (*d*, 1H, J = 15.9 Hz).

Trans-Cinnamic Acid Ethyl Ester. Cinnamic acid (2 mg) in ethanol (200 μl) and sulfuric acid (10 μl) were heated to reflux for 12 hr. The reaction mixture was extracted with dichloromethane (2 × 1 ml) and washed with aqueous $NaHCO_3$, after which the solvent was evaporated and the product was analyzed by GC-MS. EIMS (70 eV): *m/z* (%) = 176 (M^+, 34%), 147 (20%), 131 (100%), 103 (44%), 77 (25%), 51 (10%). ^{1}H NMR (300.067 MHz, $CDCl_3$): δ = 1.34 (*t*, 3H), 4.26 (*q*, 2H), 6.44 (*d*, 1H, J= 16.2 Hz), 7.38 (*m*, 3H), 7.52 (*dd*, 2H), 7.69 (*d*, 1H, J = 16.1 Hz).

Electrophysiology. N. testaceicornis male antennae (N = 5) were excised (pulled from the head) with forceps, and a few segments were cut off at the base and the tip (Bjostad, 1998). Each antenna was then fixed between two stainless-steel electrodes by pushing the base and tip into droplets of an electrically conductive gel (Spectra 360® electrode gel, Parker, Orange, NJ, USA) applied onto the metal electrodes.

Electroantennogram (EAG) recordings were carried out by using a Syntech EAG system (Hilversum, The Netherlands). An antenna was stimulated by

subjecting it to puffs (0.3 sec) of purified and humidified air (1.2 l/min) delivered through a Pasteur pipette, containing a filter paper strip (ca. 0.8 cm), impregnated with 5 µl of test solution. Hexane and dichloromethane were used as control stimulus: crude floral extract and volatiles were captured by dynamic *headspace*. Control stimulation was made before and after each test compound of each EAG experiment. Test compounds were applied randomly at intervals of 90 sec. The Syntech EAG software calculated the normalized values automatically. The mean normalized responses of the different compounds were submitted to ANOVA for statistical analysis and were compared by using Tukey's test ($P < 0.05$).

RESULTS AND DISCUSSION

Monitoring Mormolyca Floral Chemistry. While studying *M. ringens* floral chemistry, we observed that pollinated flowers attracted neither *Scaptotrigona* (Singer et al., 2004) nor *Nannotrigona*, the alternative pollinator. We assigned this phenomenon to floral chemistry changes in pollinated and unpollinated flowers.

Monitoring (Table 1, Figure 2) of the hexane extracts of unpollinated (Figure 2A) and pollinated (Figure 2B) flowers by GC-MS showed that the floral chemistry changed as soon as pollination occurred. The total relative abundance of alkene/alkane ratio for unpollinated flowers is 0.8, and in pollinated flowers, the ratio is lower (0.3). Additionally, the 9,17-octadecadienal and C-25 and C-27 alkenes were less abundant in pollinated flowers (1.60, 2.02, and 1.15%, respectively; Table 1) than in unpollinated flowers (7.88, 10.20, and 9.7% respectively; Table 1), and the males were no longer attracted to the pollinated flowers (Singer et al., 2004).

This analysis was repeated five times with different flowers, and the same results were always obtained. To determine the position of unsaturation, we derivatized the floral extract with dimethyldisulfide and iodine, as described by Buser et al. (1983). The fragmentation patterns of all the *bis*-methylsulfide derivatives in the total ion chromatogram were similar with major fragments at *m/z* 173 and at *m/z* 243 + n14, which was consistent with a homologous series of 9-alkenes ranging from C-23 to C-31, all of them possessing odd carbon numbers. The remaining constituents (peaks 1–6) were identified by coinjection with standards. Schiestl and Ayasse (2002) analyzed flower extracts of *Ophrys fusca* and *O. bilunulata* and detected alkenes ranging from C-25 to C-29, but with unsaturations at C-7, C-9, C-11, or C-12. In *M. ringens*, however, the double bond was always found at C-9.

TABLE 1. COMPOUNDS IDENTIFIED IN HEXANE EXTRACTS OF UNPOLLINATED AND POLLINATED FLOWERS OF *MORMOLYCA RINGENS* AND CUTICULAR WAXES FROM VIRGIN QUEENS OF *SCAPTOTRIGONA* SP

Peak no. in Figure 1	Compound	Unpollinated flower (%)	Pollinated flower (%)	Virgin queen bees (%)	Method of identification
1	Cinnamic acid methyl ester	1.35	1.00	–	MS, standard
2	Cinnamic acid	2.23	0.01	–	MS, standard
3	Cinnamic acid ethyl ester	1.20	0.80	–	MS, standard
4	Palmitic acid	4.62	3.20	–	MS, standard
5	Heneicosane	0.30	–	0.99	RI, MS, standard
6	Linoleic acid	3.50	2.61	–	MS, standard
7	9,17-Octadecadienal	7.88	1.60	–	MS, Wiley275 database
8	9-Tricosene	1.05	1.40	3.13	MS, DMDS derivative
9	Tricosane	4.50	3.10	24.34	RI, MS, standard
10	Tetracosane	1.36	1.11	0.68	RI, MS, standard
11	9-Pentacosene	10.20	2.02	1.34	MS, DMDS derivative
12	Pentacosane	10.40	13.50	26.15	RI, MS, standard
13	Hexacosane	1.21	1.60	0.66	RI, MS, standard
14	9-Heptacosene	9.70	1.15	7.99	EM, DMDS derivative
15	Heptacosane	14.20	28.00	21.80	RI, MS, standard
16	Octacosane	1.25	2.02	–	RI, MS, standard
17	9-Nonacosene	10.10	16.20	8.38	MS, DMDS derivative
18	Nonacosane	11.40	20.10	2.65	RI, MS, standard
19	9-Hentriacontene	3.45	0.28	0.69	MS, DMDS derivative
20	Hentriacontane	0.10	0.30	1.20	RI, MS, standard
Total		100.0	100.0	100.0	

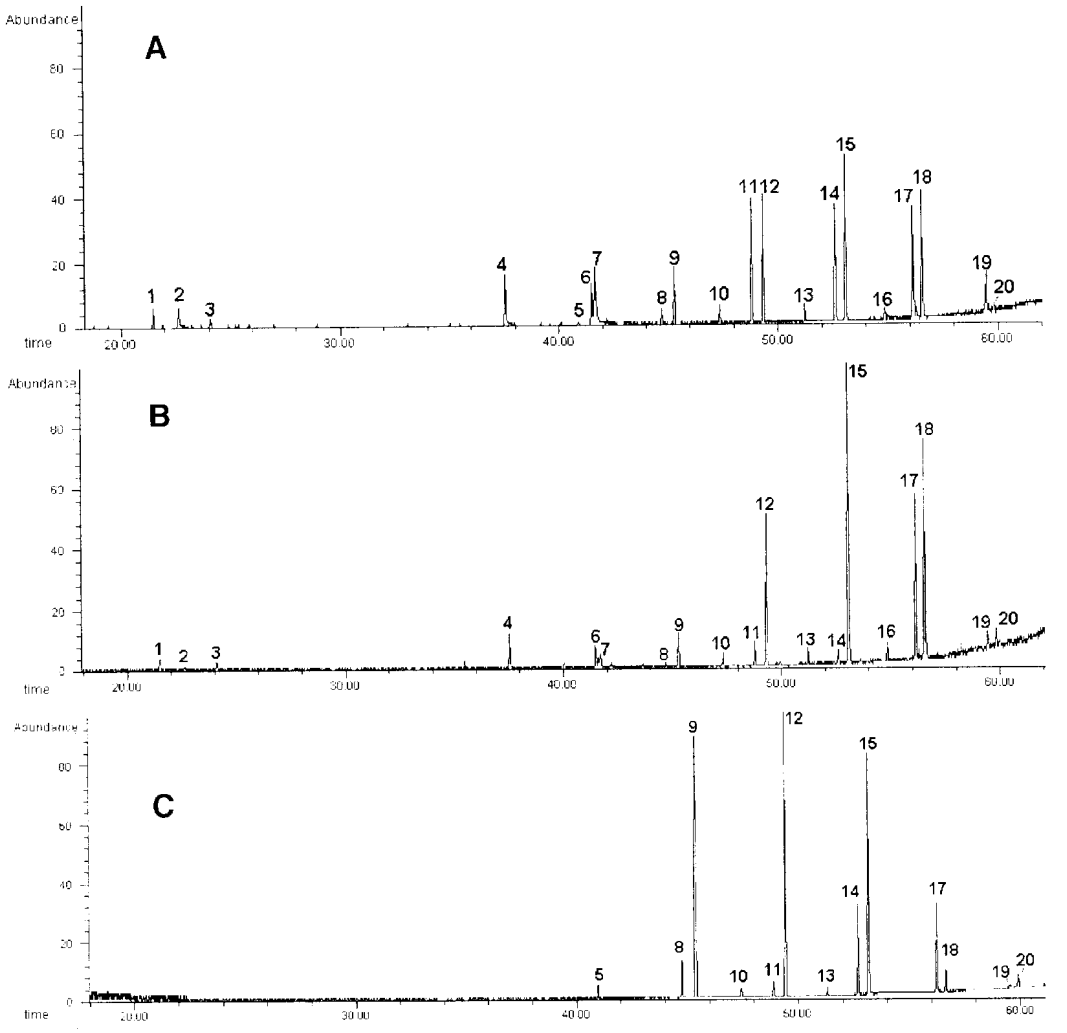

FIG. 2. Total ion chromatogram of hexane extracts from unpollinated (A) and pollinated (B) flowers of *M. ringens* and cuticular waxes from virgin queens of *Scaptotrigona* sp. (C) Identification of numbered compounds in Table 1.

Monitoring Scaptotrigona *sp. Virgin Queens (bee) and* M. ringens *(Orchidaceae) Chemistry.* The attraction of *Scaptotrigona* sp. and *Nannotrigona* males to *M. ringens* flowers and the induction of pseudocopulation suggested that the flowers had chemical compositions similar to virgin queen bees that were cultivated for investigation. This was based on the previous knowledge that in all Meliponini genera (except for Melipona), workers and queen castes are differentiated by a quantitative feeding system (Kerr, 1948). Thus, larvae ingesting moderate amounts of food will develop into workers, while larvae ingesting large amounts of food will develop into queens (Camargo, 1972b). Therefore, we used the technique of Camargo (1972a) and Menezes et al. (2004) to

TABLE 2. 2-ALKANOLS IDENTIFIED IN ACETATE CEPHALIC EXTRACTS OF VIRGIN QUEENS OF *Scaptotrigona* SP

Compound	Relative abundance (%)
Heptan-2-ol	28.3
Nonan-2-ol	25.6
Undecan-2-ol	19.1
Tridecan-2-ol	27.0
Total	100.0

produce queens "in vitro." We were more successful in rearing *Scaptotrigona* sp. than *N. testaceicornis*, thus, the chemistry from different *Scaptotrigona* sp. virgin queen's (all originated from the same hive) bodies and heads ($N = 10$ individuals) was investigated. GC-MS analysis of the cephalic acetate extracts revealed the presence of a mixture of 2-alkanols (Table 2). A similar mixture of 2-alkanols was reported by Engels et al. (1997) in the volatile fraction of the cephalic secretions of receptive virgins of *S. postica*. These authors also demonstrated that the 2-alkanols acted as long-range attraction signals (Engels et al., 1997). Because we also detected the same 2-alkanol mixture, we concluded that the virgin queen bees we were dealing with were receptive to males.

In addition to an attractive, long-range "perfume," virgin queen bees also require additional short-range chemical signals to prompt male sexual behavior (Schiestl et al., 2000). However, this additional short-range precopulatory signal has not yet been identified for *Scaptotrigona*. We hypothesized that the short-range attractive components were located on the abdominal surface. To examine this possibility, female bees ($N = 10$) were washed with hexane, and the extract was analyzed by GC-MS. This analysis revealed a homologous series of alkanes and alkenes. The latter showed an odd series C-23 to C-31 with unsaturation at position 9, identical to that seen in the *M. ringens* hexane extract, but in a slightly different alkane/alkene ratio.

Working with *Ophrys* (Orchidaceae) and *Andrena* bees, Schiestl and Ayasse (2002) have demonstrated that these two organisms have similar alkane/alkene constituents, not identical in abundance; the alkane/alkene mixture (present in both virgin bees and orchid labella) was demonstrated to be responsible for the pseudocopulatory behavior in the *Andrena* bees–*Ophrys* system (Schiestl et al., 2000). Extrapolating these results to the *Mormolyca* × *Scaptotrigona* system, we suggest that the homologous 9-alkane/alkene series present in the flowers and virgin queen bees may be responsible for the chemically induced copulatory activity in males.

Changes in Floral Chemistry. As indicated above, floral chemistry undergoes a compositional change following pollination (a decrease in the relative alkene/alkane abundance, assumed to be the short-range floral attractant), so that

the flower no longer attracts *Scaptotrigona* males. Although the relative floral alkene/alkane composition and abundance were similar in *M. ringens* and in *Scaptotrigona* sp. virgin queen bees, we were intrigued by the lack of similarity in the long-range attraction chemicals detected in the cephalic extracts of *Scaptotrigona* sp. (homologous series of 2-alkanols). A detailed examination of the floral hexane extract revealed no 2-alkanol. Because we considered that this negative result might reflect an inappropriate sampling method, we trapped the emitted volatiles of an unpollinated flower by using dynamic headspace methodology (Singer et al., 2004). At first, 2-alkanol was not detected, but a closer analysis of the total ion chromatogram for specific ions revealed the presence of 2-heptanol. We concluded that male attraction does not require large quantities of the active substance, and that, in this case, 2-heptanol may have been the active principle responsible for attracting *S. postica* males.

Based on these results, and on a similar phenomenon (Ayasse et al., 2000), we suggest that the 2-alkanols (specifically, 2-heptanol) act as long-range chemical signals, whereas homologous odd 9-alkene/alkane series act as short-range signals and induce copulation in *Scaptotrigona* sp. To confirm, *in vivo*, the pollinators' response to floral chemistry, we collected as many male bees as possible, all flying around the *M. ringens* flowers. However, many did not survive long enough to reach the analytical laboratory, and only the remaining specimens, i.e., *N. testaceicornis* drones, were investigated by electroantennography (EAG). Mean depolarization achieved by *N. testaceicornis* male antennae in response to the crude floral extracts and volatile components of *M. ringens* is

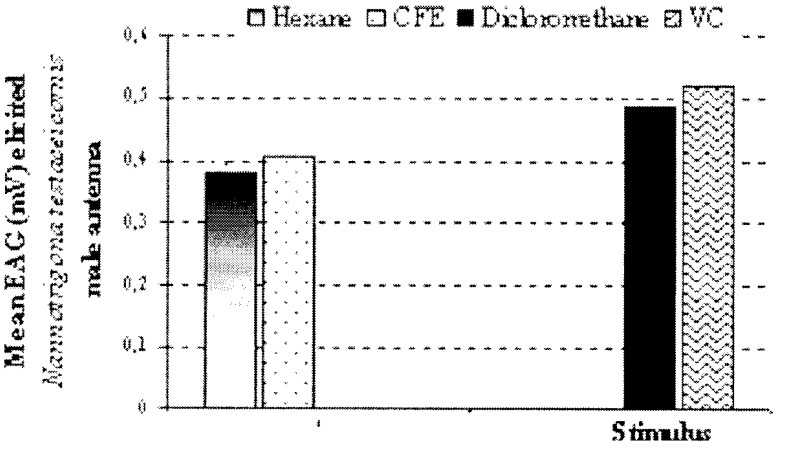

FIG. 3. Mean values (±SD, in mV) of electroantennography (EAG) responses of *Nannotrigona testaceicornis* male antenna to crude floral extracts (CFE) and volatile components (VC) of *M. ringens*. Mean values marked with the same letter are not significantly different at $P < 0.05$ based on Tukey's test ($N = 5$). Hexane, 100%; CFE, 1 mg/ml; dichloromethane, 100%; VC, not determined.

shown in Figure 3. Both extracts elicited EAG responses statistically different from controls. These results indicated that *N. testaceicornis* recognized the chemical components present in both extracts via antennae chemoreceptors and suggested that this bee uses *M. ringens* volatiles to find these specific flowers. EAG provided conclusive data on the selective antennae depolarization by the orchid floral constituents compared with the control (solvents).

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