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Mandibular gland secretions of meliponine worker bees: further evidence for their role in interspecific and intraspecific defence and aggression and against their role in food source signalling

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SUMMARY

Like ants and termites some species of stingless bees (Meliponini), which are very important pollinators in the tropics, use pheromone trails to communicate the location of a food source. We present data on the communicative role of mandibular gland secretions of Meliponini that resolve a recent controversy about their importance in the laying of such trails. Volatile constituents of the mandibular glands have been erroneously thought both to elicit aggressive/defensive behaviour and to signal food source location. We studied *Trigona spinipes* and *Scaptotrigona* aff. *depilis* (‘*postica*’), two sympatric species to which this hypothesis was applied. Using extracts of carefully dissected glands instead of crude cephalic extracts we analysed the substances contained in the mandibular glands of worker bees. Major components of the extracts were 2-heptanol (both species), nonanal (*T. spinipes*), benzaldehyde and 2-tridecanone (*S.* aff. *depilis*). The effect of mandibular gland extracts and of individual components thereof on the behaviour of worker bees near their nest and at highly profitable food sources was consistent. Independent of the amount of mandibular gland extract applied, the bees overwhelmingly reacted with defensive behaviour and were never attracted to feeders scented with mandibular gland extract or any of the synthetic chemicals tested. Both bee species are capable of using mandibular gland secretions for intra- and interspecific communication of defence and aggression and share 2-heptanol as a major pheromone compound. While confirming the role of the mandibular glands in nest defence, our experiments provide strong evidence against their role in food source signalling.

Key words: eusocial bees, communication, agonistic behaviour, semiochemicals, trail substance.

INTRODUCTION

Odours and pheromones are omnipresent as signals and cues and are important carriers of information in arthropods. In social insects in particular, communication critically depends on chemical signalling, with the need for efficiency growing with the degree of sociality. The highest degree of sociality in insects, as well as in any other animal, is found in species where the members of one colony together represent a highly eusocially organized superorganism (Wilson and Sober, 1989; Seeley, 1989; Wilson and Hölldobler, 2005; Reeve and Hölldobler, 2007; Gardner and Grafen, 2009). The most well known examples of superorganisms are ants, honey bees and termites. Thanks to their study we now know that an efficient communication between colony members using pheromones and other semiochemicals is indeed an essential basis for the maintenance of a superorganism. Often, the use of pheromones to recruit nestmates for collecting food or for defence is taken to illustrate the relevance of pheromones for the coordination of colony activities (Vander Meer, 1998; Wyatt, 2003). While a lot is already known about chemical communication in ants and honey bees, our knowledge is far behind and partly controversial in case of the Meliponini, the so-called stingless bees, a less well known taxonomic group of superorganismic arthropods (Camargo and Pedro, 1992; Michener, 2000). This is surprising because the meliponines are very important pollinators in tropical rain forests and also valued study objects for those seeking broad insights into tropical ecology (Roubik, 1989). In this paper we will dissect current controversy concerning the Meliponini which refers to the communicative role of their mandibular gland secretions. The mandibular glands were the first glands proposed to play an important role in meliponine communication (Lindauer and Kerr, 1958). Likewise they were reported to be important in the communication of a great number of other Hymenoptera, although sufficiently detailed studies are available for very few species.

The many species of meliponines (>400 circumtropical species) differ vastly in foraging habits and defensive ‘strategies’ and bees of the same or different sympatric species frequently compete for resources (Schwarz, 1932; Johnson and Hubbell, 1974; Roubik, 1989; Nagamitsu and Inoue, 1997; Eltz et al., 2002; Slaa, 2003; Slaa, 2006). Therefore they are particularly suited for flower ecology studies and for the study of defensive and aggressive behaviour. The behavioural differences between them have so far been mainly attributed to differences in body and/or colony size and nesting behaviour (Michener, 1974; Camargo and Pedro, 1992; Roubik, 2006). Accordingly, one finds (i) intranidal and intrasuperorganismic, (ii) internidal and intersuperorganismic, and (iii) interspecific conflicts. Analogous to the situation in ants, cleptoparasitic species (‘robber bees’) add to the diversity of meliponine bee behaviour.

In all these behaviours, efficient communication between colony members will enhance the effect of defence and aggression as well

as the efficiency of foraging. It should also be advantageous for the bees to 'understand' the signals of other colonies or even other species involved in this communication, particularly in agonistic contexts (Maynard Smith and Harper, 2003).

Like in other superorganismic hymenopterans, defensive communication rests on chemical signals in meliponines, and the actual defenders are the worker bees. Pheromones serving hymenopteran defensive communication are often produced in the mandibular glands. These are well developed in all meliponine species as well but differ considerably in size between different genera and castes (Nedel, 1960; de Cruz-Landim, 1967). Interestingly, even before the chemoecological study of mandibular gland function as the source of an alarm pheromone (Blum, 1966; Blum et al., 1970; Luby et al., 1973), Lindauer and Kerr (Lindauer and Kerr, 1958) had proposed its role in the production of scent trail substances used by some meliponine species to communicate the location of a profitable food source. Since then, the 'one gland – two functions hypothesis' has developed into a putative fact (Kerr and da Cruz, 1961), accepted in nearly all papers on meliponine communication published so far. If we assume that mandibular gland secretions do indeed induce both scent trail following to distant food sources in newly recruited worker bees and defensive/aggressive behaviour in the same workers near the nest, it would be interesting to examine how the bees accomplish the appropriate behaviour in a given situation.

While the existence of the scent trail is undisputed in several recently published works (reviewed by Nieh, 2004), the mandibular gland origin of the actual scent marks has now been brought into question (Jarau et al., 2004; Jarau et al., 2006; Schorkopf et al., 2007). Here, we therefore critically re-evaluate the communicative role of the mandibular gland contents with regard to their function in defence behaviour and food source localization.

For the present study we chose two meliponine species previously also examined in behavioural studies by Lindauer and Kerr (Lindauer and Kerr, 1958; Lindauer and Kerr, 1960) to ask the following questions. (1) Which substances are contained in the mandibular glands of worker bees? (2) Which behaviour do the mandibular gland volatiles evoke when applied close to the nest and at the food source, respectively? (3) Do the bees react differently to mandibular gland volatiles of bees from other conspecific nests or from other (sympatric) species? Whilst our results clearly confirm the role of mandibular gland secretions in nest defence, their role in food source signalling must now be considered highly unlikely.

MATERIALS AND METHODS

Research site

The experiments were carried out at the Ribeirão Preto Campus of the University of São Paulo (Brazil) between November 2004 and March 2006.

Trigona spinipes

We studied four nests of *T. spinipes* (Fabricius 1793; Apidae, Apinae, Meliponini) naturally established on campus grounds. *T. spinipes* builds external nests in the canopies of trees. Note that Lindauer and Kerr (Lindauer and Kerr, 1958; Lindauer and Kerr, 1960) used the species name *ruficrus* (Latreille 1804), a junior synonym of *spinipes*. Three of the colonies (Ts1, Ts4 and Ts8) were transferred from 'canopy level' (4–7 m) to 'ground level' (1.6–1.9 m), whilst the fourth colony (Ts2) was kept at 0.4 m after it had fallen to the ground due to several days of heavy rain.

Scaptotrigona postica/*Scaptotrigona* aff. *depilis*

Seven out of the nine colonies studied were brought from a local meliponary and kept in wooden boxes 1.5–1.8 m above the ground. Two other colonies naturally occurred in tree trunk hollows (nest entrances at heights of 1.5 and 1.7 m) which is typical for this species.

As mentioned before, our intention was to study meliponines to which the 'one gland – two functions' hypothesis had been applied. The presumed role of the mandibular glands as a source of pheromones signalling the presence of a food source was first studied in detail for a species described as '*Trigona (Scaptotrigona) postica*' (Lindauer and Kerr, 1958; Lindauer and Kerr, 1960). However, the actual *Scaptotrigona postica* (Latreille, 1807) (Apidae, Apinae, Meliponini) does not naturally occur in the region where Lindauer and Kerr (Lindauer and Kerr, 1958) performed their, now legendary, experiments (J. M. F. Camargo, personal communication). Given these authors worked with a colony originating from the same region it seems likely that they were observing a very similar looking, common and so far undescribed species still commonly called '*postica*' in publications (Camargo and Pedro, 2007) (J. M. F. Camargo, personal communication). Unfortunately no specimens are available for reference to the work of Lindauer and Kerr (M. Lindauer, personal communication). In the present study we worked with an as yet undescribed species, which belongs to the '*Scaptotrigona depilis* group' (J. M. F. Camargo, personal communication) and which we, therefore, call *Scaptotrigona* aff. *depilis* [species *depilis* (Moure, 1942)]. Reference specimens have been added to the collection of J. M. F. Camargo at the University of São Paulo in Ribeirão Preto.

Chemical analysis – extraction of gland material

We carefully cleaned the mandibular glands from other tissues in physiological solution and under a stereomicroscope. Special care was taken not to contaminate the mandibular gland surface with the contents of other glands *via* the bath solution. The volatile contents of the glands were extracted using pentane (HPLC grade; 200 µl, 24 h at room temperature), and the extracts were reduced to 180 µl (*S.* aff. *depilis*) or 60 µl (*T. spinipes*) with a gentle stream of nitrogen for subsequent chemical analysis. For quantitative analyses, tetradecane and nonadecane served as internal standards. The relative amount of any identified substance was calculated by a comparison of its peak area with the summed area of all peaks (except peak areas smaller than 0.3% of the internal standard). Gas chromatographs (HP-GC6890A and Shimadzu GC-2010; carrier gas, hydrogen) with flame ionization detectors were used for quantitative analysis. For qualitative analyses, we used gas chromatography combined with mass spectrometry (Shimadzu GC-2010/GCMS-QP2010; carrier gas, helium). An Agilent DB-5MS column (30 m×0.25 mm, 0.25 mm thickness) was used. The temperature programme started at 50°C (5 min) and increased the temperature by 10°C per min up to 310°C (holding 310°C for 15 min). The compounds were identified by comparison of mass spectra of natural products with data from the literature (McLafferty and Stauffer, 1989; Francke et al., 2000) and those of authentic reference compounds.

Behavioural studies

At the nest entrance

To test whether a substance elicited attack behaviour we placed a clean black cotton ball [a sock stuffed with PVC foil; methodology similar to that used by Smith and Roubik (Smith and Roubik, 1983)] measuring ~10 cm in diameter as a target at a distance of 50 cm (*S.* aff. *depilis*) or 150 cm (*T. spinipes*) from the nest entrance during the night preceding the experiment. The ball either hung on a thread from a wooden broomstick or was fixed onto it. The number of

bees biting the cotton ball in the 30 s following their exposure to a test substance was taken as a measure of aggressiveness. All substances were applied either directly to the nest entrance structure (*T. spinipes*) or presented on a filter paper (about 2 cm×3 cm) fixed in front and some milllimetres below the nest entrance (*S.* aff. *depilis*). In this way the free passage of the bees was not disturbed.

The test substances were applied in turn, always following the application of a control substance (10 µl of the solvent pentane). The time interval between the application of the control and test substance was less than 2 min. This included 30 s of observation of the bees' behaviour following the application of the control substance. The first mandibular gland extract or substance to be tested on each day was chosen at random (note, following the application of pentane as a control substance). The substances tested for *S.* aff. *depilis* and *T. spinipes* are listed in Figs 1 and 2, respectively. In *T. spinipes* we also tested the labial gland substance octyl octanoate, which is known to be a trail pheromone (Schorkopf et al., 2007). In addition to the controls in which the pure solvent was applied, we included another control in which 10 µl of atmospheric air was blown towards the nest entrance.

At the feeding site

Bees leaving the feeder due to mandibular gland secretions

For *S.* aff. *depilis* we tested the effect of mandibular gland extracts and of individual synthetic chemical constituents thereof (Fig. 3) on bees visiting a feeder. Following Jarau and colleagues (Jarau et al., 2004), bees were trained to visit a small plastic dish offering a 50% w/w sugar solution at some distance from the nest (15–60 m). The gland extracts and different substances were applied on a filter paper (1 cm×1 cm) fixed 1.5 cm above the feeder dish with a pin; 10 µl of the tested substance was slowly dropped onto the filter paper. During the subsequent 10 s we counted the bees leaving the feeder. The percentage of bees leaving the feeder after application of a test substance was evaluated statistically. The substances were applied in turn but always following the application of 10 µl pentane as a control substance. The time interval between application of the control and subsequent application of the test substance was less than 30 s (including 10 s of behavioural observation after application of the control). The first substance to be tested on each day was chosen at random (note, following application of pentane as control). Only those bees that had never been tested on any of the substances (except pentane) at the feeding dish before were included in the statistical analysis.

The procedures used to study *T. spinipes* were similar to those used for *S.* aff. *depilis*. To increase the sample size (number of simultaneously tested bees), however, we changed a few aspects of the experiment and setup. We first trained more than 20 bees to visit a feeder [described by Jarau and colleagues (Jarau et al., 2000) except for the following difference: we used an unused 35 mm plastic film box to contain the 50% w/w sugar solution instead of a glass vial]. Before testing the gland extract volatiles we replaced the plastic film box containing the sugar solution with an empty one (unused, of the same brand). The latter had slits and holes cut into the plastic to allow a better diffusion of test substances (applied on a filter paper inside the vial) into the air outside the vial, thereby exposing the foragers to them. The other procedures followed those for *S.* aff. *depilis*. The chemical species tested are listed in Fig. 3.

Choice test with feeders scented with mandibular gland extracts

We also tested the effect of the mandibular gland extracts in a simple choice test as described by Jarau and colleagues (Jarau et al., 2004). Our feeders were watch glasses with droplets of 50% (w/w) unscented sucrose solution at a distance from the nest of 48.7±36.7 m (mean±s.d., sometimes more than 70 m). The tested substances (observation period, 20 min) were 0.1 bee equivalents of mandibular gland extract taken from individuals of the same or different nests (same species), and pentane and the hypopharyngeal gland extracts as controls. We also tested 1.0, 0.1, 0.01 and 0.001 bee equivalents of the mandibular gland extract in the same way in *S.* aff. *depilis* to see how the bees' choice was affected by a decrease in the amount applied.

Nieh and colleagues (Nieh et al., 2003) tested mandibular gland extracts in *Trigona hyalinata* and found them to be both repellent and attractive for bees at a foraging site; while the mandibular gland extracts in their study had a repellent effect in the first 7 min after application, this surprisingly changed to the opposite effect after the 8th minute. Jarau and colleagues (Jarau et al., 2004) later suspected that the attractiveness of the extracts used by Nieh and colleagues (Nieh et al., 2003) originated from contamination with extracts of other cephalic glands (labial glands). We tested this assumption by choice tests using deliberately 'contaminated' mandibular gland extracts. To this end the mandibular gland was picked at its base and extracted without previous careful cleaning from other tissues ('contaminated' mandibular gland extracts).

Given the above-mentioned observations by Nieh and colleagues (Nieh et al., 2003), the effect of the mandibular gland extracts on the bees' choice behaviour was tested for different time periods following mandibular gland extract application. We studied the bees' choice behaviour (1) immediately after application of the contaminated mandibular gland extract (*S.* aff. *depilis* only) and (2) 10 min after its application (both species). Consequently the choice feeder setup was either presented to the bees immediately (as in 1) or 10 min after the application of the tested substances. The same choice tests were repeated with uncontaminated mandibular gland extracts. If contamination by other glands does not affect the attractiveness of mandibular gland extracts, the behaviour (attraction, avoidance, indifference) of the bees should not differ at identical test time points in the two tests (using uncontaminated or contaminated mandibular gland extracts). Solvent presented alone served as a control.

Tests to see whether mandibular gland secretions induce trail following in newcomers (*S.* aff. *depilis*)

When newcomers follow a conspecific scent trail to a food source they sometimes land on some of the scent marks of the trail before reaching the actual food source. It is unlikely to begin with that newcomers would first follow a scent trail of mandibular gland secretions and then avoid and flee from the same secretions at the actual food source location. However, one could argue that the bees use their mandibular gland secretions to build up the scent trail not quite to the food source itself and react differently to the same secretions when actually reaching the food source. To test this assumption we checked whether mandibular gland extracts could elicit trail-following behaviour in newcomer bees of *S.* aff. *depilis.* We laid artificial scent trails towards artificial food sources (50% w/w, unscented sucrose solution) that consisted of droplets of mandibular gland extract or its solvent (as a control) applied to the substrate in the direction of the test feeders. We adopted the trail-following assay used previously (Schorkopf et al., 2007) with minor changes; two artificial scent trails (T1 and T2; length, 5 m each) were laid, beginning at a branching point 21–35 m (median, 30 m) away from the nest and ending at one of the two test feeders. Bee numbers following T1 were statistically compared with those

following T2. Between the two feeders stood a recruitment feeder (15 foraging bees), again 5 m away from the branching point and also from each of the two test feeders. Tested scents were either mandibular gland extracts (same nests) or equal amounts of the solvent pentane. The amount of the solution forming the trails increased as they neared the respective feeders, where scent concentrations were highest. The amount of test substance increased (beginning from the branching point, 0 m) in the following order (bee equivalents dissolved in pentane): 0.0 (0 m), 0.05 (1 m), 0.1 (2 m), 0.2 (3 m), 0.3 (4 m) and 0.9 (at the feeder, 5 m). All the newcomers recruited during the 20 min experimental period landing on any of the artificial scent marks were marked with a colour, removed from the experiment and included in the statistical analysis. We also observed whether any bee circulated, inspected or otherwise followed the artificial scent trail in front of the actual food source. Every bee included in the statistics was used only once, avoiding pseudoreplication or learning effects.

Statistics

For normally distributed data of equal variance, we used the one-way ANOVA to test for the significance of differences in the percentage or number of bees that had landed on either of the two feeders presented for their choice. Tukey tests were applied for the pairwise multiple comparisons.

Non-parametric statistics were applied (1) when the general requirements for ANOVA were not met and (2) when testing for differences among the experiments on the reaction of foraging bees to mandibular gland volatiles. The Kruskal–Wallis *H*-test was applied instead, followed by the Student–Keuls test for pairwise multiple comparisons. Wilcoxon signed-rank tests were applied when testing for differences between the responses to a specific chemical species and the preceding control substance (pentane). The Mann–Whitney rank sum test was applied to test for significant differences in the bioassay which checked whether the mandibular gland secretions induce trail-following behaviour in newcomer bees.

RESULTS

Mandibular gland volatiles

Mandibular gland extracts in both *T. spinipes* and *S.* aff. *depilis* contained a variety of volatile substances (Table 1). In all cases the majority of these substances were moderately to highly volatile (volatility higher than that of 2-tridecanol). 2-heptanol was the only major compound (more than 10% of the sum of all detected peak areas) occurring in both species. *T. spinipes* had only one other major component (nonanal), while *S.* aff. *depilis* showed two additional ones (benzaldehyde and 2-tridecanone).

According to Table 1, the chemical composition of mandibular gland volatiles of *T. spinipes* differs substantially from that of *S.* aff. *depilis.* The most striking difference is the amount of volatiles contained in each individual pair of mandibular glands: the sum of all volatile peak areas (as compared with the same amount of standard substances) was about 7–30 times higher in *S.* aff. *depilis* than in *T. spinipes.* The amount of the major component 2-heptanol was even greater (10–40 times). These findings correlate with the much larger size of the mandibular glands in *S.* aff. *depilis*.

Table 1. Volatile compounds so far identified in mandibular gland extracts (GC/MS) of *Trigona spinipes* and *Scaptotrigona* aff. *depilis*, arranged according to substance class

	T. spinipes	*S.* aff. *depilis*		*T. spinipes*	*S.* aff. *depilis*
Alcohols			Ketones		
2-Heptanol*	+++	+++	2-Heptanone		++
2-Octanol*	++		2-Nonanone		+
2-Nonanol*	+	+	(Z6)-Undecen-2-one ?		+
1-Nonanol	++		2-Undecanone		++
2-Undecanol*		+	2-Dodecanone		+
2-Tridecanol*	++	++	2-Tridecenone		+
2-Pentadecanol*	+	++	2-Tridecanone	+	+++
2-Heptadecanol*		++	2-Pentadecanone		++
Docosenol**	+		2-Heptadecanone		++
Hydrocarbons			Esters		
Dodecene**	+		Undetermined butyrate	+	
Dodecene**	+		Pentyl hexanoate?		+
Dodecene**	+		2-Pentyl hexanoate ?		+
Tetradecene**	++		Hexyl hexanoate		+
Tetradecane	+	+	2-Heptyl hexanoate ?		+
Pentadecane	+		2-Heptyl hexenoate E2?		+
Hexadecene**	++		Undetermined hexanoate		+
Hexadecane	+	+	Aldehydes		
Octadecene**	++		Nonanal	+++	
Octadecane	+	+	Docosenal**	++	
Heneicosene**	+		Docosenal**	++	
Tricosene**	+		Aromatic compounds		
Pentacosene**	+		2-Phenylethanol	++	+
Terpenes and lactones			Benzaldehyde	+	+++
Citral/geranial	+		Phenylacetaldehyde	++	
Farnesol	++		Methyl benzoate	+	
Lactones					
γ-Decalactone?					

Quantification of single compounds relative to the sum of all detected peak areas:+++, >10%,++, >1%,+, <1%.
*Enantiomeric composition not determined in this study [determined for *Scaptotrigona* in Engels et al. (Engels et al., 1990)]. **double bond position not determined.

Behaviour

Bees attack a target upon exposure to mandibular gland extracts at the nest entrance

All colonies of both species attacked the black cotton ball 50 cm (*S.* aff. *depilis*) or 150 cm (*T. spinipes*) in front of the nest, when 0.1 bee equivalents of volatiles of the mandibular glands were released at the nest entrance structure (Figs 1 and 2), irrespective of the colony or species the mandibular gland was taken from. In contrast to this finding, bees rarely attacked the same target when air, labial gland extract, hypopharyngeal gland extract or the solvent pentane had been released in the same way. A highly significant difference (P<0.001), therefore, resulted when comparing these substances with the mandibular gland extracts. The fact that any bee attacked at all upon the release of one of the control substances is an artefact of the test procedure: even without injecting a substance into the nest entrance the movements and vibrations caused by the experimenter can cause an attack response (D.L.P.S., in preparation). All the synthetic volatiles naturally occurring in the mandibular glands and used in the bioassays caused biting and attack, albeit to different degrees. Among individual substances, 2-heptanol elicited the strongest response, but the difference between its effect and that of other compounds (Figs 1 and 2) was not always significant. Interestingly, the two enantiomers of this alcohol elicited similar responses. Although we always observed a slightly higher attack rate with *S*(+)-2-heptanol than with *R*(–)-2-heptanol, the difference between the responses was not significant.

Bees abandon the feeder when exposed to mandibular gland extracts

Bees of both species abandoned the feeder at a high rate when exposed to 0.1 bee equivalents of mandibular gland volatiles (Fig. 3). This was also observed when using mandibular glands taken from conspecific workers of other nests or even from bees belonging to the other of the two species. When the same amount of pure solvent or of labial gland extract was applied, only a few foragers left the feeder. Their number did not differ significantly (Mann–Whitney *U*-test; *S.* aff. *depilis*: N=6 trials, P>0.8; *T. spinipes*: N=6 trials, P>0.6) from that found when no substance was applied at all and air was ejected instead. Consequently, the difference in the median values among all these treatment groups (N=6 trials) was highly significant in both species (Kruskal–Wallis analysis of variance on ranks: *S.* aff. *depilis*: H=48.56, d.f.=9, $P<10^{-6}$; *T. spinipes:* H=26.97, d.f.=5, $P<10^{-4}$). The pairwise comparisons between the effect of mandibular gland extracts and air, solvent or labial gland extracts showed highly significant differences as well (P<0.001). The effect of mandibular gland extract was not nest specific for either *T. spinipes* (N=6 trials) or *S.* aff. *depilis* (N=6 trials; Kruskal–Wallis: P>0.05). According to the pairwise comparisons, *S.* aff. *depilis* reacted significantly less (P<0.05) to mandibular gland extracts taken from *T. spinipes* than to its own, whereas *T. spinipes* reacted in the same way to both mandibular extracts (P>0.05).

No preference for feeders or trails scented with mandibular gland extracts in choice tests

Scaptotrigona aff. *depilis* newcomer bees never landed on or followed artificial trails in front of the artificial feeders when the trails consisted of mandibular gland extracts or equal amounts of the solvent pentane only. Hardly any bees landed on a feeder at the end of either of the two artificial scent trails. The statistical difference between newcomer numbers arriving at the end of either of the artificial scent trails (T1, T2) was highly insignificant (Mann–Whitney: P>0.4; N=5).

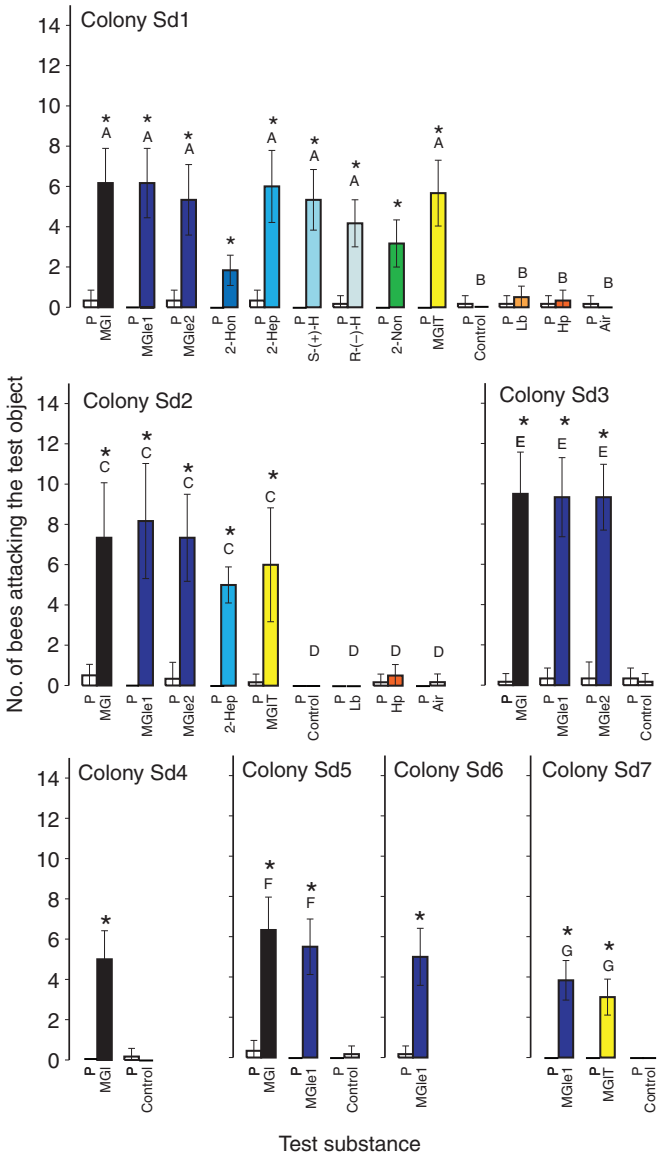

Fig. 1. Number of workers of *Scaptotrigona* aff. *depilis* attacking a black cotton ball at a distance of 50 cm from the nest within 30 s of release of the test substances at the nest entrance. Test substances: (i) 0.1 bee equivalents of the mandibular gland extracts of nestmates (MGl) or conspecific bees of other colonies (MGle1, MGle2); (ii) corresponding amounts of 2-heptanone (2-Hon), 2-heptanol (racemate; 2-Hep), *S*(+)2-heptanol [S-(+)-H], *R*(–)2-heptanol [R-(–)-H], 2-nonanol (racemate; 2-Non), solvent (as control), labial glands (Lb), hypopharyngeal glands (Hp) and air. Also tested: 0.1 bee equivalent of *T. spinipes* mandibular glands (MGlT). P, additional control with pentane preceding each of the above-mentioned substances. *Significant difference (α=0.05) in bee numbers between P and the substance tested subsequently. Every test was repeated 6 times in each of the seven colonies. Columns with the same letter at the top (except the preceding controls, P, which were not included in the ANOVA) represent values that do not differ significantly from each other (α=0.05) (means ± s.d.).

Neither *Trigona* nor *Scaptotrigona* foragers preferred feeders scented with pure mandibular gland extracts to feeders to which only solvent had been applied (Figs 4 and 5). *Scaptotrigona* bees even avoided feeders scented with mandibular gland extract. When decreasing the amount of mandibular gland extract applied to the feeder, its obvious repellent effect decreased as well (Fig. 5A). When

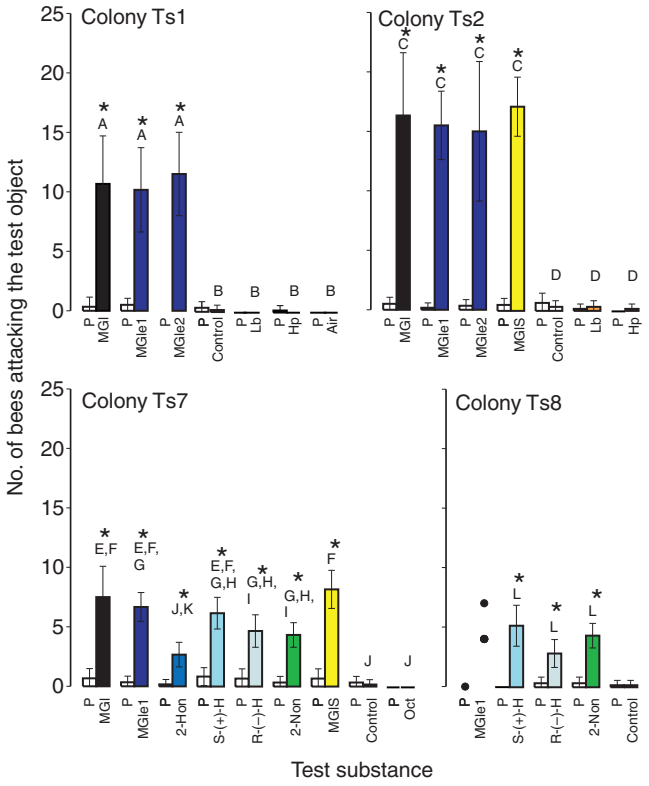

Fig. 2. Number of workers of *T. spinipes* attacking a black cotton ball at a distance of 150 cm from the nest within 30 s following the release of the test substance at the nest entrance. Test substances: (i) 0.1 bee equivalents each of the mandibular gland extracts of nestmates or conspecific bees of other colonies; (ii) corresponding amounts of *S*(+)2-heptanol, *R*(–)2-heptanol, 2-nonanol (racemate), solvent (as control), labial and hypopharyngeal glands, air and octyl octanoate (Oct). Also tested: 0.1 bee equivalents of *Scaptotrigona* mandibular glands (MGlS) and a corresponding amount of 2-heptanone. *Significant difference (α=0.05) in bee numbers between P and the substance tested subsequently. Every test was repeated 6 times in each of the seven colonies (except colony Ts8 where MGle1 and its preceding control were both only tested twice). Data for four different nests are shown (colonies Ts1, Ts2, Ts7 and Ts8). Columns with the same letter at the top (except the preceding controls, P, which were not included in the analysis of variance) represent values that do not differ significantly from each other (α=0.05) (means ± s.d.). For other abbreviations see legend of Fig. 1.

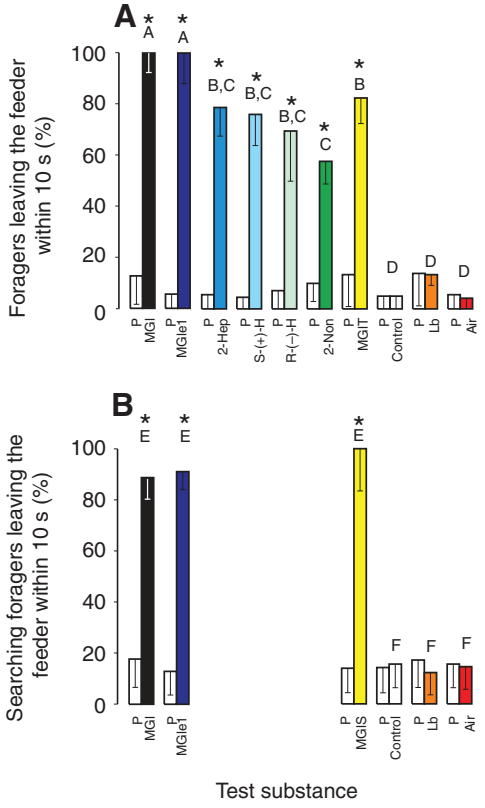

Fig. 3. Percentage of foragers leaving the feeder within 10 s following the application of scent (open bars) or control substance (solvent pentane); data are medians (bars; six trials each) and the corresponding 95% confidence intervals (negative direction). Substances were tested on feeding foragers of *S.* aff. *depilis* (A) and food-searching foragers of *T. spinipes* (B): 0.1 bee equivalents of mandibular gland extracts from individuals of either the same or other nests, and from individuals of the sympatric species *T. spinipes* or *S.* aff. *depilis*. 2-heptanol (racemate and pure enantiomers) and 2-nonanol (racemate) were only applied in case of *Scaptotrigona*. Control: equal amounts of solvent (pentane), labial gland extract of the same nest or air. Pentane (solvent) was applied preceding (time interval <30 s) each application of the above-mentioned chemicals. *Significant difference (α=0.05) in bee numbers between P and the substance tested subsequently. For other abbreviations see legend of Fig. 1.

applying as little as 0.001 bee equivalents, neither a repellent nor an attractive effect was seen (ANOVA, $F_{1,10}$, P>0.6). With 1.0 bee equivalent of mandibular extract often no bee landed on either of the choice feeders (distance from each other, 20 cm) during the first experimental minute. Bees approaching the setup then flew agitatedly in circles and up and down. Obviously the amount of behaviourally active volatiles applied was sufficient to cause an 'agitating' effect within a radius of at least 0.5 m around the feeder. When we applied 1.0 bee equivalent of gland extracts 'contaminated' with labial gland at the test feeder, *Scaptotrigona* bees still avoided landing on it (Kruskal–Wallis, P<0.05, N=34 bees) during the first 5 min after application (Fig. 5B). Interestingly, the opposite effect was observed 10 min after application of the same extract. The bees then preferred the 'contaminated' mandibular gland extract (ANOVA, $F_{1,10}$, P<0.005, N=103 bees) and the number of individuals landing on both feeders per time unit also increased significantly (mean difference at 0–5 and 10–15 min=69, ANOVA, $F_{1,10}$, P<0.001). The change from a repellent to an attractive effect was never seen when using 'uncontaminated' (without salivary gland) mandibular gland extract. Instead, even after 10 min a repellent effect was close to being significant (ANOVA, $F_{1,10}$, P>0.06). In contrast to the case in *S.* aff. *depilis* we did not observe a statistically significant repellent effect during mandibular gland extract choice tests in *T. spinipes* (Figs 4 and 5). However, the ('uncontaminated') mandibular gland extracts never acted as an attractant in any choice test in *T. spinipes* either. But why didn't it repel the bees? The explanation is as follows: choice tests in *T. spinipes* lasted for 20 min. The repellent effect evidently is very strong according to the percentage of bees that abandon the feeder during the first seconds following the application of the mandibular gland extract in the setup described above (see Fig. 3). We conclude that the amount of effective mandibular gland compounds causing avoidance and repellent effects quickly decreases with time. This conclusion is supported by the mandibular gland analysis: both major

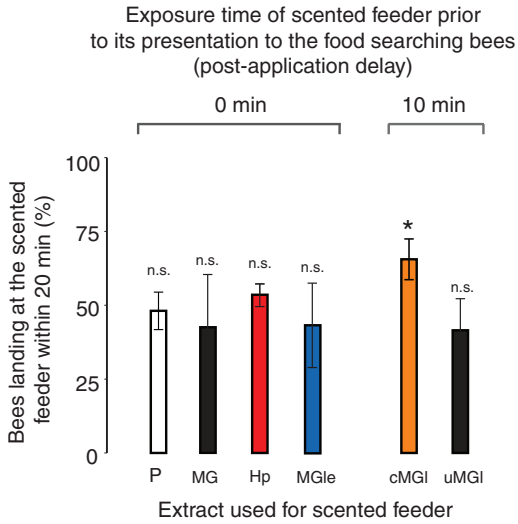

Fig. 4. Percentage of recruited newcomers of *T. spinipes* landing on the feeder scented with one of the test substances in choice tests with the pure solvent (pentane) in the second feeder. Test substances: (i) extracts (0.1 bee equivalents) of hypopharyngeal glands, mandibular glands of the same colony and of conspecific bees of a foreign colony; (ii) pure solvent (pentane) serving as control, and contaminated (cMGl) or uncontaminated (uMGl) extracts of the mandibular gland. The feeders scented with cMGl or uMGl were presented after a post-application delay of 10 min (see Materials and methods). Each test was repeated at least 6 times. *Significant difference (P<0.05) between the choice tests shown; n.s., no significant difference (P>0.05). For other abbreviations see legend of Fig. 1.

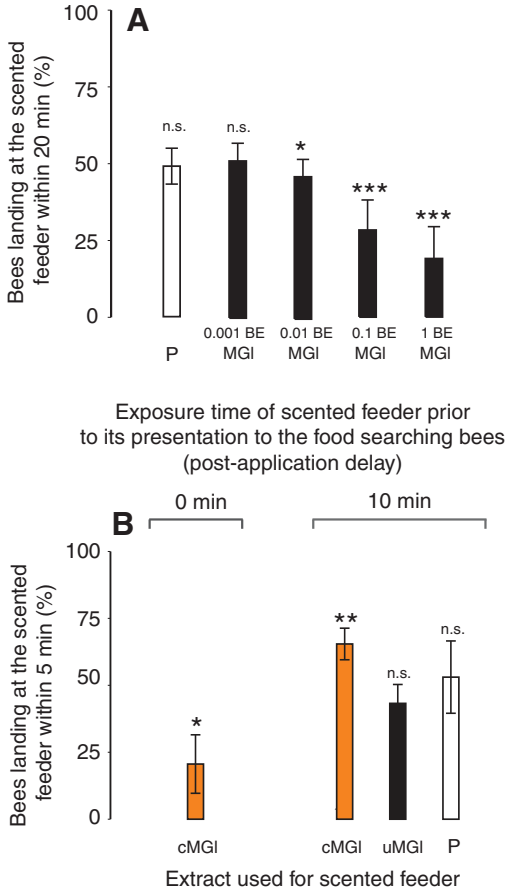

Fig. 5. Percentage of *S.* aff. *depilis* bees in choice tests. Bees had to choose between a feeder treated with test substance (in pentane solvent) or a control feeder treated with solvent (equal amount of pentane). (A) Effect of different amounts of mandibular gland extract. The test substance feeder was scented with 0.001, 0.01, 0.1 or 1 bee equivalents (BE) of mandibular gland extract or pentane for control. (B) Choice tests for the effect of labial gland contamination of mandibular gland extracts. The test substance feeder was scented with 1 bee equivalent of 'contaminated' (cMGl) or 'uncontaminated' (uMGl) mandibular gland extract or pentane for control. n.s., no significant difference; asterisks indicate significant difference (*P<0.05, **P<0.01, ***P<0.001) between scented and unscented (solvent) feeder. For other abbreviations see legend of Fig. 1.

compounds of the mandibular gland secretions (2-heptanol and nonanal) in *T. spinipes* are highly volatile. Their evaporation is expected to be considerable within the first 20 min. In contrast, octyl octanoate, the most abundant substance of the salivary glands (Schorkopf et al., 2007), which is likely to have caused the attractive effect in the 10–30 min period after the application of the contaminated mandibular gland extracts, is much less volatile. We assume that octyl octanoate therefore is still present after 10 min.

DISCUSSION

Signals eliciting defensive behaviours are thought to significantly contribute to the inclusive fitness in group living or colonial animals (Maynard Smith and Harper, 2003). It therefore is not surprising that pheromones inducing defensive/aggressive behaviours are almost always present in eusocially organized insect societies (Wyatt, 2003). Trail pheromones, on the other hand, which induce trail following of recruits to food sources far away from the nest, are less frequently found but still common in several taxa of termites and hymenopterans. Among flying workers of the Hymenoptera, however, they seem to be found only in the Meliponini.

Meliponines also differ from the other hymenopterans in regard to their sting apparatus, which is atrophied in both workers and queens (Abdalla and Cruz-Landim, 2001). In general, meliponines actively defend their nest by biting their offenders. We can only speculate on why the Meliponini have given up their stings as defensive organs. Neither do we know intermediate stages of sting reduction nor the evolutionary forces (e.g. robbers, predators) at work in early meliponines. Ants, phorid flies and cleptoparasitic insects may have represented very frequent and harmful enemies in the evolutionary past, as they do today (Nogueira-Neto, 1997). If indeed true, stings possibly were not as efficient in the defence against these intruders as other mechanisms like biting or sticky resin deposition near the nest entrance. Considering the lack of a functional sting it would seem useful to have the glands secreting the defensive substances near to the mandibles, the only effective mechanical weapon of meliponine worker bees.

The present paper indeed demonstrates semiochemicals in the secretions of the meliponine mandibular glands and their use for colony defence and aggressive communication at food sources. An additional function of the mandibular gland secretions as a repellent/deterrent against predators or resource competitors like ants seems possible but has not been shown yet. One case where a repellent function could turn out to be highly relevant is the food competition observed at honeydew resources. *T. spinipes* collects honeydew from hemipteran species such as *Aethalion reticulatum* Linnaeus 1767, a behaviour we frequently observed during our research. Sympatrically occurring ant species of the genus *Camponotus* (Castro, 1975) intensely forage honeydew from the same species during the day as well as during the night. However,

when *T. spinipes* discovers a profitable *A. reticulatum* site it somehow manages to oust the ants, which will only return to the site after dawn when the bees have already left (Castro, 1975) (J. M. F. Camargo, personal communication). During agonistic encounters *T. spinipes* frequently assumes an aggressive posture with open and ready-to-bite mandibles. We hypothesize that mandibular secretions are released during such encounters.

Another hint of a deterrent function of mandibular gland secretions in some meliponines comes from a recent study in Asian meliponines. Ants chose to feed on meliponine individuals 'washed' in hexane or chloroform in preference to unwashed individuals (Lehmberg et al., 2008). Although the authors of this study suggest that plant terpenes on the bees' cuticles are responsible for the deterrent effects, the compounds actually responsible were not identified.

The existence of a powerful chemical defence originating from the mandibular gland against vertebrate predators has already been shown for the meliponine genus *Oxytrigona* (Roubik et al., 1987). Some of the substances of *S.* aff. *depilis* and *T. spinipes* listed in the present paper (Table 1) have already been reported to be effective repellents in other insects (Eisner et al., 2005). Benzaldehyde, reported as repellent in ants (Eisner et al., 1978; Eisner et al., 2005) as well as in honey bees (Townsend, 1963; Crane, 1990), is one candidate for a substance with a specific eco-ethological significance in *Scaptotrigona*. Similarly 2-heptanone and 2-heptanol are known as 'alarm' or repellent substances in many hymenopterans, including honey bees (Free, 1987; Wongsiri et al., 2006) and several ant species (Vander Meer et al., 1998). A recently accepted patent (US patent number 6,071,973) by Vander Meer and colleagues (Vander Meer et al., 2000) lists several ant repellents (tested ant species: *Solenopsis invicta*) among which are 2-heptanone, 2-heptanol, 2-octanol, 2-nonanone and 2-nonanol. The latter five are also found in the mandibular gland secretions analysed in the present study (Table 1).

Defence rather than scent path marking

According to our data the mandibular glands of meliponine worker bees produce semiochemicals that elicit defensive and aggressive behaviour but not trail following to food sources. In this regard two points seem to be relevant. (1) The majority of the main volatile components (Table 1) found in the mandibular glands of the species studied in the present work quickly diffuse into the air. This favours prompt communication serving defensive or aggressive actions. High volatility appears unsuitable, however, for scent trails leading to a food source: the recruitment of newcomers takes some time and most of the highly volatile substances are likely to have already largely evaporated. (2) In both *T. spinipes* and *S.* aff. *depilis* mandibular gland volatiles elicit defensive/aggressive action both close to the nest and at the food source (Figs 1–3). At the food source the bees were never attracted to mandibular gland extract or to individual chemical components contained in it, irrespective of the concentration applied (Figs 4 and 5). The same was true for artificial scent paths tested in *S.* aff. *depilis.*

For many ants and some termites, details of the glandular sources of trail and alarm pheromones already exist (Kaib, 1999; Kaib, 2000; Wyatt, 2003). In the ants both communicative systems appear to have evolved several times and independently in different taxonomic groups (Hölldobler and Wilson, 1990; Billen, 2006). Different glands play an important role in the two functions. In most studied ant species communicative volatiles secreted from the mandibular glands apparently support defensive or aggressive actions, often even on the trail to or at food sources (Maschwitz, 1964; Leuthold and Schlunegger, 1973; Hölldobler and Wilson, 1990). In contrast we know of no study conclusively showing that the mandibular glands of worker ants play a substantial role in trail laying to food or nest sites.

Pheromones and allomones eliciting aggression and defence

The experiments showing that mandibular gland secretions release aggressive or defensive behaviour not only among nestmates but also among conspecifics of different colonies or even individuals of different species imply that the bouquet of volatiles of the mandibular gland secretions contains both pheromones and allochemicals.

2-heptanol and 2-heptanone

2-heptanol and the corresponding ketone 2-heptanone are interpreted as key pheromone or allochemical substances of meliponine mandibular glands, eliciting defensive or aggressive behaviour in worker bees. 2-heptanol was the only substance identified as a major component in the mandibular glands of bees from all colonies of both species studied. We therefore suggest that it serves as both an intraspecific and an interspecific key 'defence' allomone. Several authors have already attributed an 'alarm' pheromone function to 2-heptanol and its ketone both in meliponines [M. S. Blum and W. E. Kerr, unpublished, cited in Kerr (Kerr, 1969); M. S. Blum, W. E. Kerr, F. Padovani and R. E. Doolittle, unpublished, cited in Blum and Brand (Blum and Brand, 1972)] (Luby et al., 1973; Weaver et al., 1975; Keeping et al., 1982; Smith and Roubik, 1983; Johnson et al., 1985) and in other hymenopterans (Free, 1987; Vander Meer et al., 1998). Kerr and colleagues (Kerr et al., 1981) described an increase in the number of departing worker bees in *T. spinipes* by 20–30% and found *S.* aff. *depilis* to be 'disorganized' at their nest entrance after a small cotton ball treated with 2-heptanol was put in the nest entrance. Note that M. S. Blum and W. E. Kerr [unpublished, cited by Kerr (Kerr, 1969); no quantitative data shown] had previously observed an attack response when using 2-heptanone at the nest entrance. However, in 1981 Kerr and colleagues (Kerr et al., 1981) were still convinced that 2-heptanol was highly attractive to foragers of *T. spinipes*. Engels and colleagues (Engels et al., 1987) observed a strong alarm response in *S.* aff. *depilis* inside the hive when placing a disc scented with 2-heptanol into the brood or storage area.

Similarly, according to Cruz-López and colleagues (Cruz-López et al., 2007), 2-heptanone releases defence behaviour in worker bees of *Oxytrigona mediorufa* using the experimental protocol of Smith and Roubik (Smith and Roubik, 1983). Barrera-Gordillo and colleagues (Barrera-Gordillo, 2005) (R. Barrera-Gordillo and L. Cruz-López, unpublished data) obtained similar results with 2-heptanol and other compounds in *Scaptotrigona mexicana.*

The composition of the mandibular gland 2-heptanol regarding the relative share of its two enantiomers is still unknown. However, both meliponine species studied by us reacted to both enantiomers (the difference between the attack responses was not significant). In cephalic secretions of *S.* aff. *depilis* [referred to as *S. postica* Latreille 1811 by Engels and colleagues (Engels et al., 1990)] only the *S*(+) enantiomer of 2-heptanol was found (Engels et al., 1990). The same applies to all other 2-alcohols of the cephalic secretions analysed by these authors. We conclude that both species do react to both enantiomers even if indeed only one of them [*S*(+)] is actually secreted by the mandibular glands.

Octyl octanoate: no 'mandibular gland substance'

Our analyses of numerous mandibular glands in *T. spinipes* did not provide evidence for the presence of octyl octanoate. Kerr and

colleagues (Kerr et al., 1981) assumed this ester to be one of the main components of the mandibular glands and suggested its role in defence behaviour. However, a significant role of octyl octanoate was indeed demonstrated for the communication of a profitable food source in *T. spinipes*, as was its occurrence in the salivary glands (e.g. cephalic labial glands) of the same species (Schorkopf et al., 2007). The same could be true for *T. silvestriana* of Central America and Mexico, where Johnson and colleagues (Johnson et al., 1985) found similar components in head extracts and possibly mistakenly attributed octyl octanoate to the mandibular glands. When testing octyl octanoate on a filter paper 25 cm upwind of the nest entrance of *T. silvestriana* these authors observed 'erratic flights' of worker bees at the nest entrance which they interpreted as a 'weaker [alarm] response' compared with the strong responses elicited by 2-heptanol and 2-nonanol, other compounds of the cephalic extract. The same effect of octyl octanoate was never observed during our experiments with *T. spinipes*, nor could it be elicited by the salivary gland extracts, which contain octyl octanoate (Schorkopf et al., 2007). Johnson and colleagues (Johnson et al., 1985) were among the first to assume that octyl octanoate is used for scent trail laying (to a food source) in *T. spinipes* when discussing the paper by Kerr and colleagues (Kerr et al., 1981).

A similar paradigm change: the female-attracting scent trails of male bumble bees

More than 30 years ago a similar change of paradigm was necessary regarding bumble bees. Their mandibular glands were mistakenly believed to produce scent trail substances used by male bumble bees to attract conspecific females (Haas, 1952). According to a review (Blum and Brand, 1972) even 20 years later the scientific community was convinced of the 'mandibular gland hypothesis'. Due to the studies of Kullenberg and colleagues (Kullenberg, 1973; Kullenberg et al., 1973) and those following them, we now know that it is actually the labial glands that produce volatiles attracting females. In fact it may have been the misinterpretation of evidence by Haas (Haas, 1952) that misled Lindauer and Kerr (Lindauer and Kerr, 1958), who referred to this work when proposing that mandibular gland secretions are used for marking a scent trail by meliponines.

Intranidal and internidal communication on the intraspecific and interspecific level

According to our data at least some meliponine species are capable of exchanging aggressive signals between individuals not only of the same (intranidal communication) but also of different conspecific nests (internidal communication). In addition these species can chemically communicate aggression to a sympatric species (interspecific communication) of a different genus both at the nest (Figs 1 and 2) and at a food source (Fig. 3). Johnson (Johnson, 1980) had already shown that in *Trigona fulviventris* flight and defensive postures followed the application of synthetic mandibular gland components (a mixture of 2-heptanol, 2-nonanol, 2-nonanone, 2-tridecanol, 2-pentadecanone and 2-heptadecanone, then believed to represent trail-marking compounds) of a competing meliponine {*Scaptotrigona* [*Trigona* in Johnson (Johnson, 1980)] *pectoralis*}. Such communication abilities are in agreement with our findings showing that both *S.* aff. *depilis* and *T. spinipes* bees use 2-heptanol as a major pheromone compound of their mandibular gland secretions. According to the present study the reaction of *S.* aff. *depilis* to the same amount of mandibular gland extract (0.1 bee equivalents) of the sympatric species *T. spinipes* is less pronounced than that to extract of its own glands (Fig. 3). The reasons may be as follows: (1) some volatiles necessary to induce an identical response in *S.* aff. *depilis* are missing in *T. spinipes* glands; (2) no behaviourally relevant volatile is missing but the amount of semiochemicals is too small in *T. spinipes* to elicit a response in *S.* aff. *depilis.* With the data at hand we can only argue for reason 2: 2-heptanol is found in much larger absolute amounts in *S.* aff. *depilis* than in *T. spinipes*.

By rejecting the 'one gland – two functions' hypothesis (Lindauer and Kerr, 1958; Kerr and Cruz, 1961) we do not deny the possibility that mandibular gland secretions are involved in behaviours other than defence and aggression. However, our new data do argue against their function as scent trail markings, which has often been postulated but was never convincingly proven in any species of the Meliponini.

It seems Lindauer and Kerr (Lindauer and Kerr, 1958; Lindauer and Kerr, 1960) themselves already communicated an argument against their mandibular gland hypothesis when writing (Lindauer and Kerr, 1960): 'With certain species of Meliponini, the capture [of newcomers landing on the tested feeder; added remark] must be made very carefully: the bees must not be seized with forceps or touched with any other object, for they then secrete through the mouth a liquid with a characteristic scent, which frightens off both marked bees and newcomers, so that no more bees land on the feeding table; a closer investigation of this phenomenon is still needed'. Such an investigation had not been carried out until the present study.

We dedicate this paper to the late Martin Lindauer and Warwick Estevam Kerr, the pioneers of meliponine communication research, which now has reached its 50th anniversary. Financial support came from the Austrian Science Fund FWF to F.G.B. (P17530). The input of Robert Twele and Wittko Francke, University of Hamburg, Germany, in the structure assignment of volatiles is gratefully acknowledged. We thank João M. F. Camargo, Geusa de Freitas and Jairo de Souza (University of São Paulo) for their help in finding appropriate bee colonies. J. M. F. Camargo and S. R. de Menezes Pedro helped to verify the identity of the species used for this study and provided us with important biogeographical and taxonomic information about them. Izabel C. C. Turatti and Norberto P. Lopes (University of São Paulo) generously offered access to their chromatographic equipment. We thank two anonymous reviewers for their comments and suggestions. Last but not least the continued hospitality of Ronaldo Zucchi in Ribeirão Preto is greatly appreciated. The present research complies with the current Brazilian environmental laws, SISBIO 65469826, Nr. 15200/1.

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